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## **CD4<sup>+</sup>CD25<sup>+</sup> T Cells Inhibit Both the Induction and Effector Function of Autoreactive T Cells and Represent a Unique Lineage of Immunoregulatory Cells**

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# CD4<sup>+</sup>CD25<sup>+</sup> T Cells Inhibit Both the Induction and Effector Function of Autoreactive T Cells and Represent a Unique Lineage of Immunoregulatory Cells

Elisabeth Suri-Payer, Anna Z. Amar, Angela M. Thornton, and Ethan M. Shevach<sup>1</sup>

Thymectomy of susceptible strains of mice on day 3 of life results in a spectrum of organ-specific autoimmunity that can be prevented by reconstitution of the thymectomized animals early in life with normal adult lymphocytes. The effectors and suppressors of autoimmunity in this model have been convincingly shown to be CD4<sup>+</sup> T cells. It has been demonstrated recently that the regulatory CD4<sup>+</sup> T cells that prevent disease coexpress CD25. We have further characterized the population of CD4<sup>+</sup>CD25<sup>+</sup> immunoregulatory cells and demonstrated that they can suppress not only the induction of disease post-thymectomy, but can also efficiently suppress disease induced by cloned autoantigen-specific effector cells. Furthermore, the CD4<sup>+</sup>CD25<sup>+</sup> T cells appear to be members of a unique lineage of regulatory T cells, as the induction of CD25 expression on a monospecific population of T cells derived from TCR transgenic SCID mice did not result in suppression of post-thymectomy autoimmunity. In addition, the TCR transgenic SCID mice were highly susceptible to autoimmune disease induced by the cloned line of autoantigen-specific effectors, while normal mice were relatively resistant. The capacity of the cloned line to transfer disease to *nu/nu* recipients could be inhibited by normal spleen cell populations containing CD4<sup>+</sup>CD25<sup>+</sup> cells and by purified CD4<sup>+</sup>CD25<sup>+</sup> cells. Although the target Ag(s) and mechanism of action of the CD4<sup>+</sup>CD25<sup>+</sup> T cells remain to be determined, it is likely that they also play an important role in modulating other autoimmune diseases that are mediated by activation of "ignorant" self-reactive T cells present in the normal peripheral lymphocyte pool. *The Journal of Immunology*, 1998, 160: 1212–1218.

Organ-specific autoimmune diseases, such as gastritis, oophoritis, orchitis, thyroiditis, and pancreatitis, have been commonly induced in susceptible mouse strains by thymectomizing pups at day 3 of life (d3Tx)<sup>2</sup> (1–4). The autoimmune response is characterized by a mononuclear infiltrate into the target organ, T lymphocyte activation, and autoantibody production directed against specific tissue Ags. The best-studied model in this respect is gastritis, which develops in 60% of neonatally thymectomized BALB/c mice (1, 2, 5) and very closely resembles autoimmune gastritis in humans (pernicious anemia) (6). Lymphocytic infiltration (starting with CD4<sup>+</sup> T cells) can be detected in the gastric mucosa and submucosa as early as 3 to 4 wk after thymectomy (5, 7, 8). The target autoantigen is the H/K ATPase, the proton pump of the gastric parietal cells. Serum of patients and mice contain Ab directed against the H/K ATPase  $\alpha$ - and  $\beta$ -chains (6, 9), and CD4<sup>+</sup> T cells isolated from the lymph node (LN) draining the stomach of mice with gastritis proliferate in response to this enzyme (5, 10). In addition, transgenic (Tg) mice expressing the H/K ATPase  $\beta$ -chain under control of the MHC class II promotor do not develop gastritis after d3Tx, but still develop disease in other organs (11).

A similar spectrum of organ-specific autoimmunity can be induced by a variety of other manipulations of the lymphoid system

(reviewed in Ref. 12). Organ-specific autoimmunity develops in T cell-deficient recipients after transplantation of neonatal thymi as well as transfer of neonatal spleen cells or adult thymocytes (2, 13–15). Second, cyclosporin treatment of neonatal BALB/c mice induces autoimmune destruction in a variety of organs (16). Third, autoimmunity can also be induced in adult animals by repeated total body irradiation (17) or adult thymectomy, followed by cyclophosphamide treatment or irradiation (18, 19). Although several distinct mechanisms may be involved in the generation of autoimmunity in these models, one common feature of these as well as other experimental models of autoimmunity (20, 21) is that autoreactive T cells are selectively activated in animals with T cell lymphopenia.

Two fundamentally different models have been proposed to explain this association between lymphopenia and autoimmunity. In both models, normal adult mice contain autoreactive T cells. In the "empty space" model (22), the paucity of T cells in the peripheral lymphoid organs permits the expansion of the precursors of autoreactive T cells because the T lymphopenic environment facilitates the interaction of autoreactive T cells with professional APC. Such activated autoreactive T cells could then migrate to nonlymphoid organs and induce organ-specific autoimmunity. The second model (23) proposes that the lymphopenic state results in the selective absence of a critical population of regulatory T cells that continuously suppress the activation of autoreactive T cells. Strong support for the existence of such regulatory T cells has been obtained recently by the demonstration that Ab- and complement-mediated depletion of the minor (10%) subpopulation of CD4<sup>+</sup> T cells in normal adult peripheral lymphoid tissues that coexpress CD4 and C25 generates a population of CD4<sup>+</sup>CD25<sup>-</sup> T cells that are potent inducers of autoimmunity when injected into *nu/nu* mice (24); furthermore, the induction of autoimmunity can be prevented by cotransfer of the CD4<sup>+</sup>CD25<sup>+</sup> population (24). However, interpretation of the results of this study is complicated by

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<sup>2</sup> Abbreviations used in this paper: d3Tx, thymectomized on day 3 of life; LN, lymph node; PCAb, anti-parietal cell antibody; PE, phycoerythrin; Tg, transgenic.

the requirement to transfer the disease inducing population to a T cell-deficient environment for autoimmunity to become manifest. Thus, the empty space of the recipient mice may still provide an important component to disease pathogenesis.

In this work, we further characterize the capacity of CD4<sup>+</sup>CD25<sup>+</sup> cells to inhibit organ-specific autoimmunity. We demonstrate that this minor subpopulation of T cells is not only able to suppress the induction of autoimmune effectors after d3Tx, but is also capable of suppressing disease induced by cloned autoantigen-specific T effector cells. Second, we show that the CD4<sup>+</sup>CD25<sup>+</sup> cells are members of a unique lineage of professional immunoregulatory T cells. Finally, we provide strong evidence that the absence of CD4<sup>+</sup>CD25<sup>+</sup> cells is primarily responsible for the susceptibility of lymphopenic animals to develop autoimmunity, as Tg mice that contain CD4<sup>+</sup> T cells that express only a single TCR and very low numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells are highly susceptible to disease following transfer of autoantigen-specific T cell clones, while normal mice are relatively resistant. Taken together, these results provide an impetus for further studies to examine the critical role of suppressor T cells in controlling susceptibility to autoimmune disease in experimental animals and humans.

## Materials and Methods

### Animals and thymectomy

BALB/c and BALB/c-timed pregnant mice were purchased from National Cancer Institute (Frederick, MD). The day of birth was taken as day 0 of age. Three-day-old pups were thymectomized, as described (14). BALB/c *nu/nu* and C.B-17 SCID mice were purchased from Charles River Laboratories (Raleigh, NC) and Taconic (Germantown, NY), respectively. BALB/c DO.11.10 TCR-Tg animals (25) were received from Dr. M. Jenkins (University of Minnesota Medical School, Minneapolis, MN). To obtain mice containing T cells expressing exclusively the Tg TCR and no endogenous TCR chains, the DO.11.10 mice were bred to C.B-17 SCID mice and housed in sterile cages. Only mice of the F<sub>2</sub> generation that were devoid of B cells and exclusively expressed the DO.11.10 TCR clonotype (KJ1-26<sup>+</sup>) (25) were used for the experiments.

### Flow cytometry

Cells ( $1 \times 10^5$  to  $1 \times 10^6$ ) were first incubated with Fc block (PharMingen, San Diego, CA) to prevent nonspecific binding, and then reacted with FITC anti-TCR V $\alpha$ 2, PE anti-TCR V $\beta$ 2, FITC or PE anti-CD4, FITC anti-CD3 (2C11), PE anti-CD25 (3C7), or isotype-matched control Ab (all from PharMingen). To determine the percentage of T lymphocytes bearing the DO.11.10 TCR in Tg mice, blood lymphocytes were incubated with KJ1-26 supernatant (1/10 dilution) (25) and then reacted with FITC goat anti-mouse IgG F(ab')<sub>2</sub> (Biosource, Camarillo, CA) and PE anti-CD4. Viable cells (FSC/SSC or 7-aminocoumarin D (Sigma Chemical Co., St. Louis, MO) exclusion of dead cells) were then analyzed on a FACScan using CellQuest software (Becton Dickinson, Mountain View, CA).

### Preparation of cell populations for the treatment of d3Tx animals

Spleen and LN were harvested from 6- to 8-wk-old BALB/c mice or from 6- to 12-wk-old (DO.11.10  $\times$  SCID)F<sub>2</sub> mice. Single cell suspensions were prepared by mashing the organs through a wire mesh into 5% FCS/HBSS. Spleen cells were depleted of erythrocytes by a 1-min treatment with ammonium-chloride-lysing buffer. LN and spleen cells were pooled in 5% FCS/HBSS, an aliquot of cells was incubated with PE anti-CD4 and FITC anti-CD3 mAb, and the percentage of CD4<sup>+</sup> cells was determined in a FACScan. The remaining cells of both groups were washed and resuspended in PBS at  $5 \times 10^7$  CD4<sup>+</sup> cells/ml each. Two hundred microliters of lymphocyte suspension ( $1 \times 10^7$  CD4 T cells, 2–3  $\times 10^7$  total cells) were injected i.p. into 10 day-old d3Tx animals. A control group of d3Tx mice received PBS only. To activate the TCR-Tg T cells, some of the recipients were immunized with OVA (100  $\mu$ g) in 50  $\mu$ l of CFA or with CFA alone i.p. 1 day after cell transfer. To prepare CD25<sup>+</sup> cells, BALB/c lymphocytes were incubated with rat anti-mouse CD25 mAb 7D4 (10<sup>7</sup> cells in 125  $\mu$ l 2% FCS/HBSS + 1.3  $\mu$ l 7D4 ascites) for 30 min on ice, followed by low-tox M rabbit complement (Cedarlane, Hornby, Ontario)

for 40 min at 37°C. Aliquots of treated and control lymphocytes were reacted with PE-conjugated anti-CD25 mAb 3C7 and FITC anti-CD4 mAb and analyzed by FACS to verify complete depletion of CD25<sup>+</sup> cells. CD25<sup>+</sup> or untreated spleen cells ( $2 \times 10^7$ , each containing 25% CD4<sup>+</sup> cells) were then injected i.p. into 10-day-old d3Tx mice in 200  $\mu$ l PBS.

### Detection of anti-parietal cell Ab (PCAb) and histologic evaluation of autoimmune gastritis

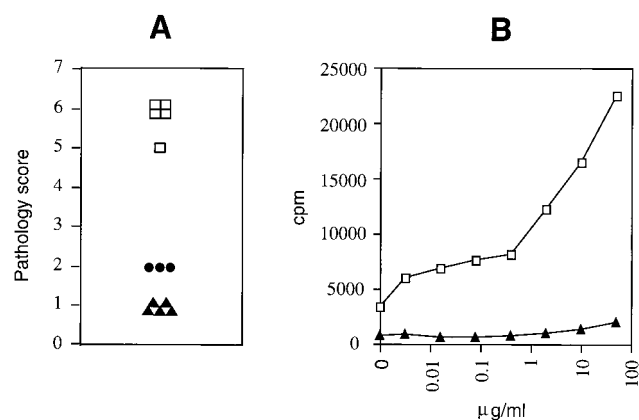
PCAb were detected by immunofluorescence on cryostat sections of normal BALB/c stomach, as described (14). For histologic evaluation of gastritis, stomachs were removed, fixed in Bouin's solution, sectioned, and stained with hematoxylin and eosin (American Histolabs, Rockville, MD). Cellular infiltrates and tissue damage were read "blind" by two investigators and scored as follows: Grade 0.5 to 1.5, normal gastric mucosa that contained a few lymphocytes scattered throughout the submucosa. Grade 2, small aggregates containing three to four layers of cells in the mucosa or sparse infiltrates of cells in the submucosa covering ~5% of the section. Grade 3, frequent and larger infiltrates extending into the mucosa. Grade 4, infiltrates spanning half to the entire width of the mucosa. Grade 5, partial or complete (grade 6) obliteration of parietal and chief cells with hyperplasia of mucous and epithelial cells.

### Preparation of gastric microsomes and recombinant H/K ATPase-expressing insect cell membranes

H/K ATPase-enriched rabbit gastric microsomes were prepared as described previously (5). The protein concentration of the microsomes was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) and ranged from 1.5 to 4.5 mg/ml. Baculovirus expressing the H/K ATPase  $\alpha$ - and  $\beta$ -chains (DLZBAS $\alpha$ ) was obtained from Dr. De Pont (University of Nijmegen, Nijmegen, The Netherlands) (26). For production of viral stocks, 70% confluent insect cell monolayers (SF21, a gift of Dr. S. Chen-Kiang, Cornell University School of Medicine, New York, NY) or  $2 \times 10^6$  cells/ml in spinner flasks were infected with a multiplicity of infection of 0.5, and infection was allowed to proceed for 4 to 5 days. The culture medium was harvested, cells were pelleted, and the supernatant containing approximately  $1 \times 10^7$  plaque-forming units was filtered twice through 0.2  $\mu$ m and stored at 4°C. For H/K ATPase production,  $2 \times 10^6$ /ml SF21 cells were infected in spinner flasks at 5 to 10 multiplicity of infection. The cells were harvested on day 3, resuspended at  $1 \times 10^7$  cells/ml in homogenization buffer (5 mM Tris-HCl, pH, 7.5, 0.25 M sucrose, 2 mM EDTA, and 1 mM PMSF), and homogenized three times for 1 min on ice using an OMNI tissue homogenizer (PGC Scientifics, Gaithersburg, MD). Lysates were cleared of cell debris and bigger organelles by 10-min centrifugation at  $600 \times g$ , followed by  $4000 \times g$ . Cell membranes were then pelleted in 4-ml polycarbonate tubes for 30 min at  $100,000 \times g$  (TLA 100.3 rotor; Beckman Instruments, Palo Alto, CA) and resuspended in 1 ml storage buffer (50 mM HEPES, 1 mM EDTA, 1 mM PMSF, and 1  $\mu$ M leupeptin). The protein concentration and the H/K ATPase content of the microsomes and insect cell membranes were determined as previously described (5).

### Preparation of gastric LN cells, proliferation assay, and generation of H/K ATPase-reactive T cell lines/clones

Gastric LN from *nu/nu* mice injected with CD25<sup>+</sup> spleen cells 3 mo earlier were pooled; single cell suspensions were prepared and stimulated in vitro with various concentrations of gastric microsomes; and Ag proliferation was measured as described previously (5). H/K ATPase-reactive T cell lines were generated from gastric LN cells of d3Tx animals, as described (5). Established cell lines were cloned by limiting dilution. To test the specificity of the cell lines/clones,  $4 \times 10^4$  T cells were incubated with  $5 \times 10^5$  irradiated BALB/c spleen cells (3000 rad) and graded concentrations of gastric microsomal Ag or recombinant H/K ATPase. Cultures were pulsed with [<sup>3</sup>H]TdR for the last 16 h of the 40-h incubation, and results are expressed as mean cpm of triplicate wells. To induce gastritis, cells were harvested 3 days after restimulation. T cell blasts were counted, and 1 to  $25 \times 10^6$  blasts were injected i.v. in 200  $\mu$ l PBS into various recipient animals. In some experiments, normal BALB/c spleen cells ( $50$ – $100 \times 10^6$ ) or 1 to  $2 \times 10^6$  CD4<sup>+</sup>CD25<sup>+</sup> (~85% pure) were coinjected with the T cell blasts ( $5 \times 10^6$ ). The CD4<sup>+</sup>CD25<sup>+</sup> population was purified from normal LN cells by enriching for CD4<sup>+</sup> T cells using T cell purification columns (R&D Systems, Minneapolis, MN), followed by CD8<sup>+</sup> T cell depletion using anti-CD8<sup>+</sup> magnetic beads (Miltenyi, Auburn, CA). CD4<sup>+</sup>CD25<sup>+</sup> cells were then purified by incubation with biotin anti-CD25 (PharMingen), followed by streptavidin-FITC (PharMingen), both at 15  $\mu$ g/10<sup>8</sup> cells. The cells were then labeled with anti-FITC magnetic microbeads (Miltenyi) and purified on a positive selection column, according to the manufacturer's suggested protocol.



**FIGURE 1.** Induction of autoimmune gastritis and H/K ATPase-reactive T cells by CD25<sup>-</sup> spleen cells. **A**, Spleen cells from BALB/c mice were treated with anti-CD25 mAb and C and injected i.p. into 6-wk-old BALB/c *nu/nu* mice. Mice were killed 12 wk later, their PCAb titer was measured, and the grade of inflammation and PC cell destruction were determined in hematoxylin and eosin (H&E) sections of stomach. Each symbol depicts one mouse. Mice with strong lymphocytic infiltration and PC destruction (grade 5–6) also display high PCAb titers (>1:1000, □). The mice with less severe infiltration have lower Ab titer (1:50–1:500, ●), and Ab-negative mice did not develop gastritis (▲). Control *nu/nu* mice in our colony never showed any signs of gastritis (data not shown). **B**, Gastric LN cells from *nu/nu* mice with pathologic evidence of gastritis proliferate in response to H/K ATPase (□), while LN cells from untreated *nu/nu* mice do not respond to this Ag (▲).

## Results

### BALB/c mice contain H/K ATPase-reactive T cells

We have demonstrated previously (5) that the effector cells that mediate autoimmune gastritis in d3Tx mice recognize the proton pump of gastric parietal cells, H/K ATPase. The ability to transfer autoimmune gastritis with CD4<sup>+</sup>CD25<sup>-</sup> T cells, but not with unseparated CD4<sup>+</sup> T cells, from adult BALB/c mice to *nu/nu* recipients (24), as well as the capacity to induce gastritis in adult BALB/c animals by manipulations of peripheral lymphoid tissues (17–19) strongly suggest that normal adult BALB/c mice also contain H/K ATPase-reactive cells. We therefore treated spleen/LN cells from adult BALB/c animals with anti-CD25 mAb and C and injected the remaining cells into *nu/nu* mice. Twelve weeks after the injection of the CD25<sup>-</sup> lymphocytes, 61% of the *nu/nu* recipients had developed gastritis (Fig. 1A). When the gastric LN cells of the animals with significant disease were stimulated with the H/K ATPase-enriched preparation of rabbit microsomes (Fig. 1B),

a vigorous Ag-proliferative response was observed that resembled in magnitude that seen with gastric LN cells from d3Tx animals (see Ref. 5). Unseparated LN cells from normal mice do not respond to H/K ATPase (Ref. 5 and data not shown). This result shows that normal BALB/c mice contain H/K ATPase-reactive T cells and that these cells can be activated and expanded in the recipient *nu/nu* mice.

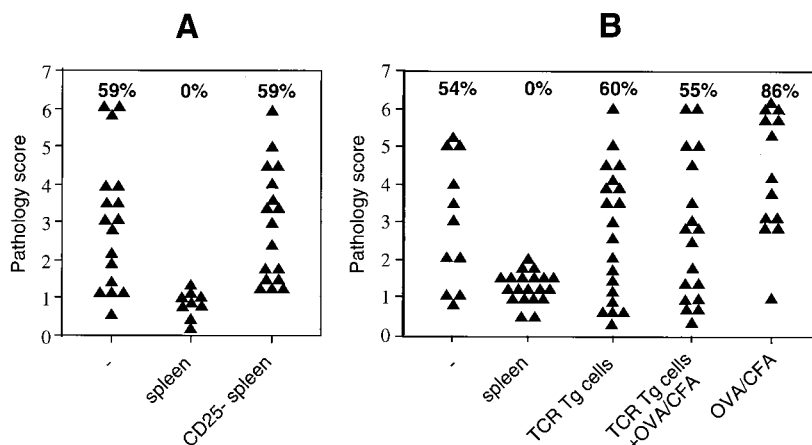
### CD25<sup>+</sup> cells are efficient in preventing autoimmune gastritis after d3Tx

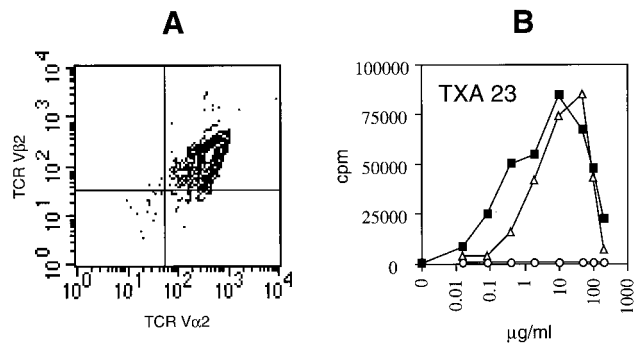
It has been proposed (27) that the CD4<sup>+</sup>CD25<sup>+</sup> cells may be deficient in neonatal mice after thymectomy and that their absence is responsible for the organ-specific autoimmunity observed. To directly test whether CD25<sup>+</sup> cells from adult mice are capable of inhibiting the induction of gastritis after d3Tx, we compared the capacity of unseparated normal spleen/LN cells from adult mice or spleen/LN cells depleted of CD25<sup>+</sup> cells to inhibit the development of gastritis post-d3Tx. When d3Tx animals were injected on day 10 with spleen/LN cells, the development of gastritis was totally abrogated (Fig. 2A). In contrast, injection of an equal number of spleen/LN cells that had been depleted of CD25<sup>+</sup> cells neither diminished nor enhanced the incidence or severity of post-d3Tx-induced gastritis (Fig. 2A). These data strongly suggest that CD25<sup>+</sup> cells are critical in inhibiting the initiation of gastritis post-d3Tx. Similar results have been reported by Asano et al. (27).

### The CD4<sup>+</sup>CD25<sup>+</sup> cells represent a unique lineage of immunoregulatory T cells

The inability of CD25<sup>-</sup> spleen/LN cells to prevent the development of gastritis strongly suggests that the defect in the d3Tx mice is not secondary to the empty space generated by the removal of the thymus (22), as the lymphoid system in the reconstituted animals should have been repopulated equally by both populations of cells used in these studies. However, it is not clear from these studies whether the CD25<sup>+</sup> population is derived from normal CD4<sup>+</sup>CD25<sup>-</sup> cells that have been activated *in vivo* in response to normal antigenic stimulation or whether the CD4<sup>+</sup>CD25<sup>+</sup> are a unique population of professional immunoregulatory cells. To address this question, we bred BALB/c DO.11.10 TCR/Tg mice to C.B-17 SCID mice for two generations and selected mice of the F<sub>2</sub> generation in which all CD4<sup>+</sup> T cells expressed the DO.11.10 clonotypic TCR directed against chicken OVA peptide 323–339 and which contained no B220<sup>+</sup> cells. It should initially be noted that the percentage of CD4<sup>+</sup> cells that express CD25 is much lower in these TCR-Tg/SCID mice (2.7 ± 0.8%, range 1.5–4.1%, *n* = 11) compared with the percentage in several strains of

**FIGURE 2.** CD25<sup>+</sup> T cells from normal BALB/c mice, but not from TCR-Tg/SCID mice, prevent the induction of gastritis post-d3Tx. BALB/c pups were thymectomized on day 3 and were left untreated or reconstituted i.p. on day 10 with either **A**, normal spleen cells or CD25<sup>-</sup> normal spleen cells, or **B**, normal spleen cells or spleen cells from TCR-Tg/SCID mice. Some of the untreated animals and some of the recipients of the TCR-Tg T cells were then immunized with OVA in CFA. Mice were scored positive for gastritis if they had a gastritis score above 2 and a PCAb titer of 1:1000; the percentage of positive mice is indicated on the top of each panel. Each symbol (▲) represents one mouse, and most data are pooled from two independent experiments of 8 to 10 mice each.





**FIGURE 3.** TXA-23 cells express  $V\alpha 2/V\beta 2$  and react with the H/K ATPase. *A*, TXA-23 cells were stained with FITC anti- $V\beta 2$  and PE anti- $V\alpha 2$ . *B*, TXA-23 cells were stimulated with rabbit microsomes enriched for H/K ATPase (■), with membranes from insect cells transfected with rat H/K ATPase  $\alpha$ - and  $\beta$ -chains ( $\Delta$ ), or with control insect cell membranes ( $\circ$ ); [ $^3$ H]TdR incorporation was determined after 96 h of culture.

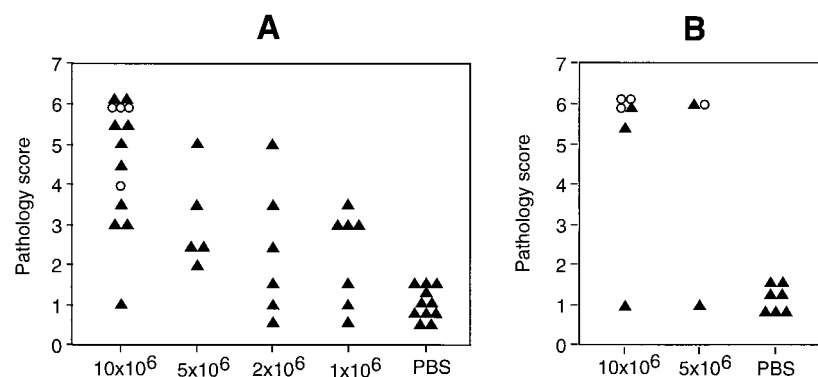
normal mice that we and others have studied ( $\sim 10\%$ ) (23 and data not shown). As none of the Tg T cells from these mice can recognize the H/K ATPase and thereby induce gastritis by themselves, we attempted to inhibit the development of autoimmunity in d3Tx mice by injection of the TCR-Tg T cells. Reconstitution of the d3Tx mice on day 10 with  $CD4^+$  Tg T cells did not inhibit gastritis, while reconstitution with the same number of T cells from conventional BALB/c mice completely prevented the development of disease (Fig. 2*B*). However, as the frequency of the  $CD25^+$  cells differed markedly between the two populations, it is possible that the absolute number of  $CD25^+$  cells present in the Tg population may not have been high enough to prevent gastritis. To rule out this possibility, we reconstituted d3Tx mice with the TCR-Tg cells and immunized the mice with OVA/CFA. Although this protocol resulted in expression of  $CD25$  on up to 50% of the Tg T cells (data not shown), no inhibition of gastritis was seen in the reconstituted, immunized d3Tx mice (Fig. 2*B*). As the injection of CFA alone blocks the development of diabetes in nonobese diabetic mice (28), control unreconstituted d3Tx mice were injected with OVA/CFA. In fact, such animals had a higher incidence of gastritis than untreated d3Tx mice, but this difference was not statistically significant ( $p = 0.1$ ). Taken together, these studies demonstrate that the mere induction of expression of  $CD25$  on a high percentage of T cells *in vivo* is insufficient to inhibit the induction of autoimmune gastritis.

#### *CD25<sup>+</sup> cells inhibit gastritis induced by H/K ATPase-reactive effector T cells*

Autoimmune diseases may potentially be blocked either by preventing the primary activation of autoreactive T cells or by pre-

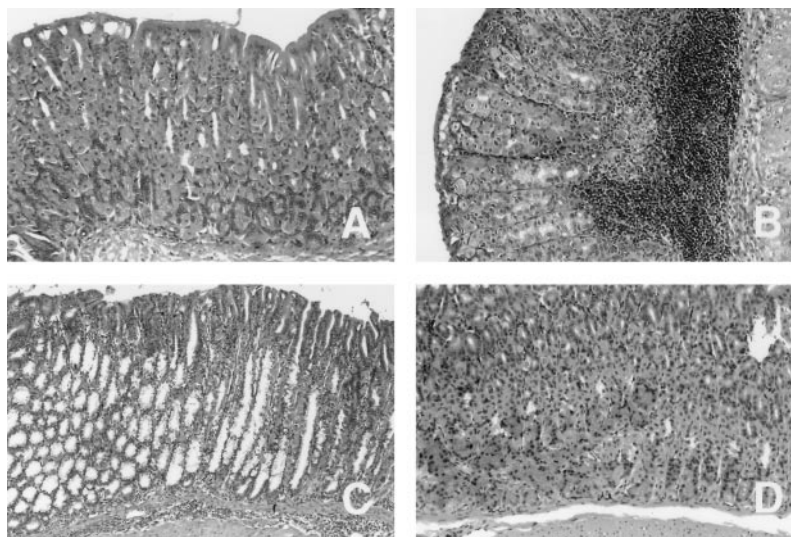
venting autoreactive effector T cells from inducing tissue destruction. As the  $CD25^+$  cells prevented infiltration of the gastric mucosa, tissue destruction, and PCAb production, it is quite likely that the  $CD25^+$  cells prevented the initial activation of autoreactive T cells after d3Tx. To evaluate whether the  $CD25^+$  cells can also prevent gastritis by interfering with the function of effector T cells, we generated H/K ATPase-reactive T cell lines from d3Tx animals. Two of these cell lines, TXA-23 and TXA-15.7, uniformly expressed  $CD4$  and the  $V\alpha 2/V\beta 2$  TCR (Fig. 3*A* and data not shown) and proliferated when stimulated with H/K ATPase-enriched rabbit microsomes and rat H/K ATPase expressed on insect cell membranes (Fig. 3*B* and data not shown). The cell lines were then cloned by limiting dilution and tested for their ability to induce gastritis in T cell-deficient *nu/nu* and SCID recipients. The recipients were killed 8 wk after cell transfer, and gastritis was evaluated histologically. Marked destruction of gastric parietal cells was observed in all recipients that received as few as 5 to  $10 \times 10^6$  cloned  $V\alpha 2/V\beta 2$  T cells (Figs. 4 and 5). It should be mentioned that in contrast to *nu/nu* recipients of T cells from d3Tx mice, the *nu/nu* recipients of the cloned lines did not develop PCAb (data not shown), presumably because the lines produce a Th1 pattern of cytokines (IFN- $\gamma$  and TNF- $\alpha$ , data not shown) that is not adequate to provide B cell help. Thus, PCAb are not necessary for tissue destruction.

While *nu/nu* mice were susceptible to disease induced by TXA-23 cells, normal BALB/c mice were almost completely resistant, although some of the animals injected with  $25 \times 10^6$  cells demonstrated a minimal lymphocytic infiltrate (Fig. 6*A*). To distinguish between the possibility that the failure of TXA-23 to induce disease in normals was secondary to the presence of immunoregulatory  $CD4^+CD25^+$  cells or whether the transferred cells were inhibited from homing to spleen/LN or to the gastric mucosa secondary to competition by normal host T cells, we transferred TXA-23 cells into TCR-Tg/SCID mice. Interestingly, all of the TCR-Tg mice developed severe gastritis (Fig. 6*B*). Although these results are most consistent with the possibility that the failure of TXA-23 cells to transfer disease to normal mice is secondary to the presence of  $CD4^+CD25^+$  cells, the total number of  $CD4^+$  T cells recovered from TCR-Tg/SCID mice is frequently only 30% of  $CD4^+$  T cells recovered from normal mice. Thus, it remained possible that the susceptibility of the TCR-Tg/SCID mice to disease was secondary to a quantitative deficiency of normal  $CD4^+$  T cells. To directly test the inhibitory capacity of the  $CD4^+CD25^+$  T cells, TXA-23 cells ( $5 \times 10^6$ ) were mixed with spleen cells ( $50$ – $100 \times 10^6$ ) from BALB/c mice and injected into *nu/nu* mice. Marked inhibition of the development of disease was seen when the normal spleen cells were cotransferred (Fig. 7*A*). As it is impossible to test whether  $CD4^+CD25^-$  cells from normal spleen can inhibit disease, as they by themselves are capable of inducing



**FIGURE 4.** H/K ATPase-reactive cell lines induce gastritis after transfer to BALB/c *nu/nu* (*A*) or C.B-17 SCID (*B*) mice. TXA-23 ( $\blacktriangle$ ) or TXA-15.7 ( $\circ$ ) blasts were injected *i.v.* 3 days after restimulation with H/K ATPase. Symbols represent single mice from two to three independent experiments killed at 8 wk after cell transfer. None of the recipient mice developed PCAb.

**FIGURE 5.** Morphology of the gastric mucosa of untreated *nu/nu* mice (A), of *nu/nu* mice receiving  $1 \times 10^6$  (B) or  $10 \times 10^6$  BALB/c mice receiving  $10 \times 10^6$  H/K ATPase-reactive T cells (D). Mice were killed 8 wk after cell transfer, and stomach sections were stained with H&E. A and D show normal fundic glands with well-differentiated surface epithelial cells, parietal cells, and chief cells (pathology grade 1). Lymphocyte clusters were observed in the mucosa of the *nu/nu* mice that received  $1 \times 10^6$  TXA-23 cells (B, pathology grade 4), while severe destruction of the mucosal architecture, disappearance of parietal and chief cells, as well as hyperplasia of mucous and endocrine cells were observed in *nu/nu* mice that received  $10 \times 10^6$  TXA-23 cells (C, pathology grade 6).



gastritis (Fig. 1 and Ref. 24), CD4<sup>+</sup>CD25<sup>+</sup> cells were purified from normal spleen and cotransferred with TXA-23 cells into *nu/nu* recipients. Unfortunately, we could only purify sufficient numbers of cells to treat relatively few animals, but most of the *nu/nu* mice receiving the mixture of TXA-23 cells and CD25<sup>+</sup> cells did not develop gastritis (Fig. 8), thereby directly demonstrating that the function of autoreactive effector T cells can be blocked by CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells in the absence of CD4<sup>+</sup>CD25<sup>-</sup> T cells.

## Discussion

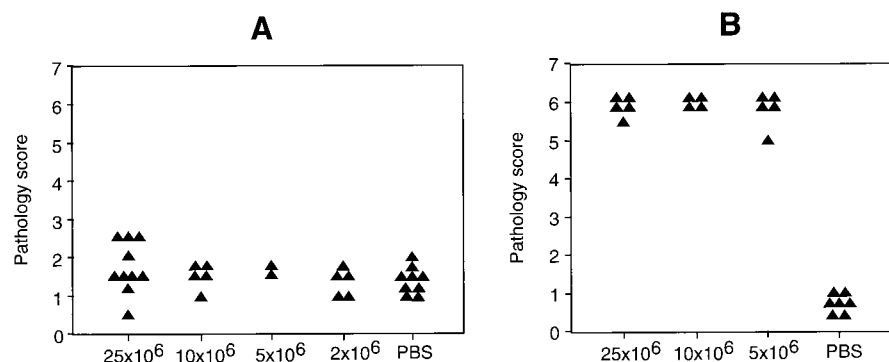
Self-reactive T cells are frequently not deleted in the thymus and are not tolerized in peripheral lymphoid tissues, but rather persist in the periphery in a state of ignorance (29). It also has been shown convincingly that the state of ignorance can be broken due to the release of the autoantigen in an immunogenic form (30); alternatively, cells with the latent potential to recognize autoantigens may be activated by cross-reactive epitopes present on relatively benign infectious agents (31, 32). Yet, the overall incidence of autoimmune disease is fortunately relatively low, which suggests that other immunoregulatory mechanisms must be needed to prevent the activation of the autoreactive T cell repertoire.

The induction of organ-specific autoimmune disease post-d3Tx has proven to be a useful model system for the analysis of the role of immunoregulatory T cells in the development of autoimmunity. In susceptible strains of mice, a high incidence of disease develops that can uniformly be prevented by reconstitution of the d3Tx animals with normal adult T lymphocytes by day 10 to 14 of life. The effectors (15, 23, 24) and suppressors (16, 33–35) of autoimmune

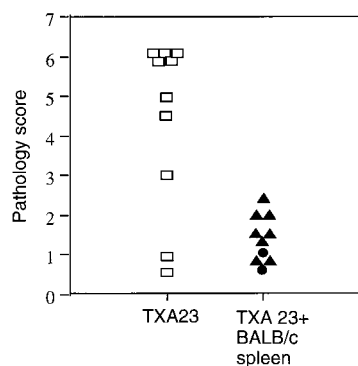
disease in this model have both been shown convincingly to be CD4<sup>+</sup> T cells, while CD8<sup>+</sup> T cells or B cells appear to play minimal roles in mediating the pathology of or protection from autoimmune disease.

One major problem in the analysis of the immunoregulatory T cell population in the d3Tx and other models of autoimmunity is the lack of specific markers. Although the immunoregulatory cells have been defined as expressing high levels of CD5 (33, 34) or low levels of CD45RB (20, 21), those populations comprise ~80% and 30 to 40% of CD4<sup>+</sup> T cells, respectively. A recent major advance has been made by Sakaguchi et al. (24, 27), who demonstrated that when the minor (~10%) subpopulation of CD4<sup>+</sup> cells that coexpressed CD25 was depleted from suspensions of adult peripheral lymphoid tissues, the remaining CD4<sup>+</sup>CD25<sup>-</sup> cells induced a syndrome of organ-specific autoimmunity upon transfer to *nu/nu* recipients that resembled that seen post-d3Tx. However, it has not yet been determined whether the CD4<sup>+</sup>CD25<sup>+</sup> population is a unique lineage of professional suppressor cells or whether any activated T cells that expressed CD25 could exhibit immunoregulatory functions. Furthermore, a possible contribution of the T lymphopenic environment could also not be addressed, as these studies were all performed by transferring the potential autoimmune effector cells to immunodeficient mice.

The results of the studies presented in this work have resolved several of these important questions and clearly define the CD4<sup>+</sup>CD25<sup>+</sup> T cells as members of a potentially unique lineage of regulatory T cells. First, the CD4<sup>+</sup>CD25<sup>+</sup> cells are the critical



**FIGURE 6.** H/K ATPase-reactive TXA-23 cells (▲) do not induce gastritis upon transfer into normal BALB/c mice (A), but readily induce disease upon transfer to TCR-Tg/SCID mice (B). Experimental protocol was identical to that described in the legend to Figure 4.



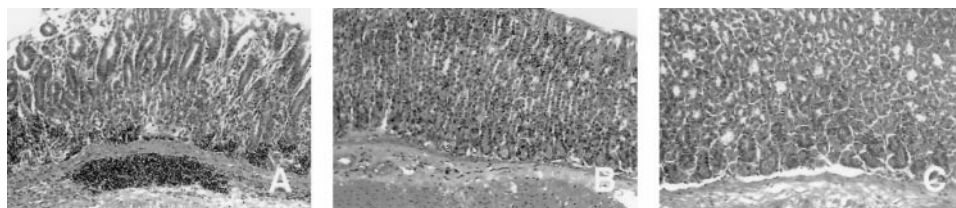
**FIGURE 7.** Normal spleen cells inhibit the disease induced by transfer of TXA-23 cells. TXA-23 blasts ( $5 \times 10^6$ , □) were injected alone or coinjected with normal BALB/c spleen cells ( $50 \times 10^6$ , ▲, or  $100 \times 10^6$ , ●) into *nu/nu* recipients. All animals were killed after 8 wk, and the extent of gastritis was determined, as in Figure 4.

population that prevents the development of autoimmunity post-d3Tx, as reconstitution of d3Tx animals with spleen cell populations depleted of  $CD25^+$  cells failed to prevent disease. It thus appears that the regulatory cell population that controls the capacity of normal  $CD4^+CD25^-$  T cells to induce autoimmunity upon transfer to *nu/nu* mice is identical to the one that controls the development of autoimmunity post-d3Tx. Although this result appears to rule out the possibility that the prevention of autoimmunity in this model is simply a result of filling up the empty space of the d3Tx recipient, it remained possible that  $CD4^+CD25^-$  cell population may add more putative autoreactive precursor cells to the d3Tx animal, thus pushing the balance of filler vs autoreactive cells to the latter. We directly addressed this question by reconstituting the d3Tx animals with T cells from a TCR-Tg/SCID animal that could recognize neither the autoantigen nor the autoreactive T cells themselves in an anti-idiotypic fashion (36); however, the cells from the TCR-Tg/SCID animal, although inert, could still compete for APC, cytokines, and space. Although the T cells from the TCR-Tg/SCID were completely incapable of suppressing disease, interpretation of this experiment was also complicated because the TCR-Tg/SCID  $CD4^+$  T cell population contained only ~30% of the  $CD25^+$  cells present in the  $CD4^+$  population from normal mice. This result by itself suggested that the  $CD4^+CD25^+$  population in normal mice was a unique lineage that was reduced greatly in the TCR-Tg/SCID. Furthermore, activation of the TCR-Tg/SCID cells with Ag in adjuvant, while effective in inducing expression of  $CD25$  on greater than 50% of the  $CD4^+$  cells, did not result in prevention of disease. These results are most consistent with the concept that the active immunoregulatory population contained within the  $CD4^+CD25^+$  population in normal mice represents a lineage of cells with unique immuno-

regulatory properties. The small number of  $CD4^+CD25^+$  cells in the TCR-Tg/SCID population may express  $CD25$  as a result of activation with cross-reacting environmental Ags.

Irrespective of the mechanism whereby the immunoregulatory cells suppress disease, it has been widely assumed that they would function by inhibiting the generation of autoreactive effectors from their precursors. In most studies (34), including our own (unpublished observations), the d3Tx animals have to be reconstituted by day 10 to 14 of life to prevent disease. We were therefore surprised to observe that large numbers of cloned  $CD4^+$  H/K ATPase-specific T cells failed to transfer disease to normal mice, while *nu/nu* and SCID mice were susceptible to disease induction by approximately 25-fold fewer cells. As it seemed likely that the immunoregulatory  $CD4^+CD25^+$  cells were responsible for prevention of disease in the normal mouse, we attempted to induce disease in the TCR-Tg/SCID mice by transfer of the H/K ATPase-reactive clone. In fact, the TCR-Tg/SCID mice developed more severe gastritis than the *nu/nu* or SCID mice, which suggests that nonspecific activation of the TCR-Tg/SCID T cells by the transferred cloned T cells actually enhanced the inflammatory process (37), which in turn may have led to additional cytokine production by the host cells. These results support the view that TCR-Tg/SCID mice do not possess the regulatory  $CD4^+CD25^+$  cells needed to control autoimmunity. Furthermore, it appears likely that the  $CD4^+CD25^+$  population is also responsible for the resistance of normal mice to the transfer of disease by the pathogenic clone, as the addition of normal spleen cells or highly purified  $CD4^+CD25^+$  to the autoreactive effector T cells markedly suppressed their capacity to transfer disease into *nu/nu* recipients. The absence of  $CD4^+CD25^+$  T cells may explain the high incidence of spontaneous encephalitis in mice transgenic for an anti-myelin basic protein TCR on a  $RAG^{-/-}$  background (38), while mice with the same TCR on a conventional background exhibit a low incidence of spontaneous disease.

A number of important questions remain to be addressed about the  $CD4^+CD25^+$  population, including the nature of the physiologic ligand recognized by their TCR (if any), whether they must be activated via the TCR to exert their suppressive functions, and how they mediate their suppressive effects. A number of studies have suggested that they may recognize the same Ag as the autoimmune effector cells, i.e., in the BALB/c d3Tx model, the H/K ATPase. However, these studies were based on the observation that female cells were less efficient at suppressing autoimmune disease in male mice than cells from adult males (4), and more recent studies (33) have not been able to reproduce these findings. In addition, intrathymic injection of the autoantigen (gastric parietal cells) did not lead to the deletion/inactivation of suppressor cells in the spleen (35), while it did prevent the development of autoimmune effectors. A second possible target for the TCR of the



**FIGURE 8.** Purified  $CD4^+CD25^+$  T cells inhibit the induction of gastritis induced by TXA-23 cells. Morphology of the gastric mucosa of *nu/nu* mice injected with  $5 \times 10^6$  TXA-23 cells alone (A), TXA-23 cells and  $50 \times 10^6$  normal BALB/c spleen cells (B), or TXA-23 cells and  $1 \times 10^6$  purified  $CD4^+CD25^+$  T cells from normal BALB/c mice. BALB/c *nu/nu* recipients of TXA-23 cells alone manifested severe gastritis with partial parietal cell destruction (pathology score 5). Marked inhibition of gastritis was seen when BALB/c spleen cells or when purified  $CD4^+CD25^+$  T cells were coinjected with TXA-23 cells (pathology score 1.5).

CD4<sup>+</sup>CD25<sup>+</sup> is the TCR of the autoimmune effector cells. Suppression by CD4<sup>+</sup> anti-Id-specific T cells has been described in experimental allergic encephalomyelitis (36), and a similar mechanism may be operative in gastritis (39). A variation of this model is that the immunoregulatory cells are anti-ergotypic (40) and become activated by the recognition of activation markers on the effector T cells. Finally, it is possible that this population is auto-reactive and is activated by ubiquitously expressed Ags; they could then mediate bystander suppression by the secretion of suppressor cytokines (IL-4, IL-10, or TGF- $\beta$ ) or could even exert suppression by a contact-dependent mechanism at the level of the autoimmune effector cells (Fas/Fas ligand) or at the level of the APC perhaps by competing for costimulatory molecules.

With the exception of the model that proposes that the suppressor cells are specific only for autoantigens, one problematic attribute of the suppressor population is how they discriminate between immune responses against autoantigens and foreign Ags. Perhaps they only suppress responses to autoantigens because they are generally of lower affinity at the level of TCR recognition. We strongly believe that the potent activity of the CD4<sup>+</sup>CD25<sup>+</sup> cells is not only confined to autoimmunity observed after d3Tx and that studies to enhance their suppressive effects may prove to be fruitful in other models of organ-specific autoimmunity or in transplantation immunology (24); conversely, studies to inhibit their effects may be useful in augmenting the immune responses to tumor-related Ags that now represent well-studied autoantigens.

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