International Immunology, Vol. 10, No. 12, pp. 1969–1980

Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state

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Keywords: anergy, autoimmune disease, CD25, self-tolerance, suppression

Abstract

Elimination of $CD25^+$ T cells, which constitute 5–10% of peripheral $CD4^+$ T cells in normal naive mice, leads to spontaneous development of various autoimmune diseases. These immunoregulatory $CD25^+CD4^+$ T cells are naturally unresponsive (anergic) *in vitro* to TCR stimulation, and, upon stimulation, suppress proliferation of $CD25^-CD4^+$ T cells and $CD8^+$ T cells. The antigen concentration required for stimulating $CD25^+CD4^+$ T cells to exert suppression is much lower than that required for stimulating $CD25^-CD4^+$ T cells to proliferate. The suppression, which results in reduced IL-2 production by $CD25^-CD4^+$ T cells, is dependent on cellular interactions on antigen-presenting cells (and not mediated by far-reaching or long-lasting humoral factors or apoptosis-inducing signals) and antigen non-specific in its effector phase. Addition of high doses of IL-2 or anti-CD28 antibody to the *in vitro* T cell stimulation culture not only breaks the anergic state of $CD25^+CD4^+$ T cells, but also abrogates their suppressive activity simultaneously. Importantly, the anergic/suppressive state of $CD25^+CD4^+$ T cells appeared to be their basal default condition, since removal of IL-2 or anti-CD28 antibody from the culture milieu allows them to revert to the original anergic/suppressive state. Furthermore, transfer of such anergy/suppression-broken T cells from normal mice produces various autoimmune diseases in syngeneic athymic nude mice. These results taken together indicate that one aspect of immunologic self-tolerance is maintained by this unique $CD25^+CD4^+$ naturally anergic/suppressive T cell population and its functional abnormality directly leads to the development of autoimmune disease.

Introduction

Self-reactive T cells mediate various autoimmune diseases in humans and animals (reviewed in 1). T cells with high-affinity TCR for intrathymically expressed self-antigens may be clonally deleted in the thymus. It is obscure, however, how the normal immune system controls the self-reactive T cells having escaped the thymic clonal deletion or those reactive with extrathymically expressed self-antigens (2–4). One of the possible mechanisms of the control would be T cell-mediated suppression of the activation/expansion of self-reactive T cells in the periphery, although there is much controversy regarding the role of T cell-mediated suppression in immunologic tolerance (reviewed in 5–8). Supporting the contribution of this mechanism to self-tolerance, there are accumulating demonstrations that autoimmune disease can be produced in normal rodents by simply eliminating a subpopulation of peripheral $CD4^+$ T cells and reconstitution of the eliminated population

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Transmitting editor: S.-i. Nishikawa

Received 17 August 1998, *accepted* 10 September 1998

can prevent the autoimmune development (9–15). For example, elimination of CD25 (IL-2 receptor α chain)-expressing T cells, which constitute 5–10% of $CD4^+$ T cells and <1% of $CD8^+$ T cells in normal naive mice and in normal humans (14–19), produced various autoimmune diseases immunopathologically similar to human counterparts (such as autoimmune gastritis, thyroiditis and insulin-dependent diabetes mellitus) (14,15). This indicates that an abnormality in the T cell-mediated control of self-reactive T cells may directly lead to development of autoimmune disease in genetically susceptible hosts. To further elucidate this mechanism of self-tolerance, we have investigated *in vitro* how a particular subpopulation of $CD4^+$ T cells, e.g. $CD25^+CD4^+$ T cells, physiologically controls other T cells, including self-reactive T cells.

We show in this report that peripheral $CD25^+CD4^+$ T cells in normal naive mice exhibit virtually no proliferative responses to TCR stimulation *in vitro* and, upon stimulation, suppress activation/proliferation of other T cells. Based on this finding, we address the following key issues regarding the cellular and molecular basis of this unresponsiveness (anergy) and suppression. Where and how do the $CD25^+CD4^+$ T cells suppress other T cells? Do they require antigen stimulation to exert suppression? If so, do they specifically suppress the T cells with the same antigen specificity or non-specifically any T cells? Can this T cell anergy and suppression be functionally broken, and thereby autoimmune disease be induced?

Methods

Mice

Eight-week-old BALB/c or BALB/c *nu*/+ mice and 6-week-old BALB/c nude (*nu*/*nu*) mice were purchased from Japan SLC (Shizuoka, Japan). DO11.10 transgenic mice were a gift of Dr D. Y. Loh (Roche Japan, Kamakura, Japan) (20). The transgene constructs for BOG1 transgenic mice (M. Iwata *et al.*, manuscript in preparation) were made by ligating genomic DNA fragments of TCR α or β chains from a T cell hybridoma BOG8 specific for an ovalbumin (OVA 271–285) peptide in an $I\text{-}A^d$-restricted manner to pCαE or C41/45 construct (21), as previously reported (22,23). The founder mouse was backcrossed 6 times to BALB/c mice and used in the present experiments. All these mice were maintained in our animal facility and cared for in accordance with the institutional guideline for animal welfare.

Preparation of lymphocytes

Lymph node and spleen cell suspensions prepared from 8-week-old BALB/c mice, DO11.10 or BOG1 transgenic mice were stained with FITC-conjugated anti-CD25 (7D4) (24) and phycoerythrin (PE)-conjugated anti-CD4 (H129.19) or PE-conjugated anti-CD45RB (16A) (25) (purchased from PharMingen, San Diego, CA), and sorted by a FACS (Epics Elite, Coulter Electronics, Miami, FL). Purity of sorted $CD25^+CD4^+$ T cells or $CD25^-CD4^+$ T cells was >95 and ~99% respectively. To sort $CD25^+$ or $CD25^-$ $CD45RB^{lo}CD4^+$ T cells, $CD4^+$ T cells were first enriched from spleen and lymph node cells by removing B cells, $CD8^+$ T cells and adherent cells by panning on antibody-coated plastic dishes, as previously described (14).

Assessment of the expression of TCR V_α and V_β families by RT-PCR

PCR primers for detecting cDNA coding for particular TCR V_α or V_β families were synthesized according to the sequences used by J.-L. Casanova *et al.* (26). RNA extraction, cDNA synthesis and PCR were also performed according to their methods from 1×10^6 cells purified by FACS.

In vitro proliferation assay

Lymph node and spleen cells (2.5×10^4) sorted as described above, and erythrocyte-depleted, mitomycin C (MMC)-treated BALB/c or CB.17-SCID spleen cells (5×10^4) as antigen-presenting cells (APC) were cultured for 3 days in 96-well round-bottom plates (Corning Coster, Cambridge, MA) in RPMI 1640 medium supplemented with 10% FCS (Gibco/BRL, Gaithersburg, MD), penicillin (100 U/ml), streptomycin (100 μg/ml) and 50 mM 2-mercaptoethanol. In some experiments (Figs 4 and 6), CD86 cDNA-transfected P815 cells (27), a gift from Dr M. Azuma (National Center for Pediatric Diseases), were fixed with 1% paraformaldehyde for 15 min or MMC-treated and used as APC. Anti-CD3 antibody (145-2C11) (Cederlane, Hornby, Ontario, Canada) at a final concentration of 10 μg/ml, concanavalin A (Con A) at 1.0 μg/ml or OVA peptides at various concentrations was added to the culture for stimulation. Anti-CD28 antibody (37.51) (28) was purchased from PharMingen. Incorporation of [^{3}H]thymidine (1 μCi/well) by proliferating lymphocytes during the last 6 h of the culture was measured. Recombinant murine IL-2 (rIL-2) with 3.89×10^6 U/mg of IL-2 activity, a gift of Shionogi (Osaka, Japan), was added to the culture at the final concentration of 100 U/ml. Monoclonal anti-IL-4 antibody (11B11) or anti-IL-10 antibody (JES5-2A5), purchased from PharMingen, or polyclonal anti-transforming growth factor (TGF)-β antibody, purchased from R & D Systems (Minneapolis, MN), was used at 10 μg/ml in cell culture. The concentration of the antibodies was sufficient for neutralizing 100 pg/ml of IL-4, 1000 pg/ml of IL-10 and 200 pg/ml of TGF-β. Antibodies for Fas ligand (FasL) (MFL-1) (29), a gift of Dr. H. Yagita (Juntendo University) or tumor necrosis factor (TNF)-α (R & D Systems) were used at 10 μg/ml. In transmembrane culture, 2.5×10^4 $CD25^-CD4^+$ T cells with 5×10^4 APC in the inner well and 2.5×10^5 $CD25^+$ or $CD25^-CD4^+$ T cells with 5×10^6 APC in the outer well were stimulated with anti-CD3 antibody in 24-well transwell culture plates (Corning Coster). For the last 6 h of 3 day cultures, cells in the inner wells were pulsed with [^{3}H]thymidine after transfer to 96-well plates.

Cytokine assay

IL-2 activity was assessed by bioassay utilizing the CTLL-2 T cell line (30). The ELISA for IL-4 or IL-10 (Endogen, Cambridge, MA) or TGF-β (Seikagaku, Tokyo, Japan) was performed according to the manufacturer's instructions. RT-PCR of the IL-2 gene transcript was performed as previously described (15).

In vivo transfer of activated spleen cells

Spleen and lymph node cells of 8-week-old BALB/c *nu*/+ mice were stimulated with Con A (2.5 μg/ml) (Sigma, St Louis, MO) and rIL-2 (50 U/ml), or Con A alone, for 3 days, and 3×10^7 blastic cells (90% of which were $CD3^+$) were i.v. transferred to BALB/c athymic nude mice, which were histologically and serologically examined 3 months later (14).

Histology and serology

For immunohistochemical staining, cryostat sections of the stomachs were stained with monoclonal anti-parietal cell autoantibody, with immunoperoxidase-conjugated affinity-purified mouse anti-rat IgG (Jackson ImmunoResearch, Philadelphia, PA) as the secondary reagent (31). Stomachs and other organs were fixed with 10% formalin and processed for hematoxylin & eosin staining. Serum titers of autoantibodies specific for the gastric parietal cells were assessed by ELISA (32). Gastritis was graded 0 to 2+ depending on macroscopic and histological severity; 0 = the gastric mucosa was histologically intact; 1+ = gastritis with histologically evident destruction of parietal cells and cellular infiltration of the gastric mucosa; 2+ = severe destruction of the gastric mucosa accompanying the formation of giant rugae due to compensatory hyperplasia of mucous-secreting cells (31,32).

Results

$CD25^+CD4^+$ T cells are unresponsive to TCR stimulation and suppressive to other T cells

The $CD25^+CD4^+$ T cell population purified by a FACS, to our surprise, exhibited virtually no proliferative response to *in vitro* stimulation with anti-CD3 antibody or Con A, which activates T cells by aggregating TCR molecules (33) (Fig. 1A). In contrast, the purified $CD25^-CD4^+$ T cells showed significantly (2- to 3-fold) higher responses than the unseparated $CD4^+$ T cells. Furthermore, the $CD25^+CD4^+$ T cells suppressed the responses of the $CD25^-CD4^+$ T cells in a dose-dependent fashion when the two populations were mixed in various ratios and stimulated with anti-CD3 antibody or Con A. They also suppressed the proliferative responses of $CD8^+$ T cells. In these experiments, the attachment of anti-CD25 mAb (7D4, rat IgM) (24) to the cell surface CD25 molecules during and after cell sorting did not seem to be responsible for rendering $CD25^+CD4^+$ T cells unresponsive and suppressive, since $CD45RB^{lo}CD4^+$ T cells, one-third of which were $CD25^+$ (Fig. 1B) (14), purified by utilizing anti-CD45RB mAb (25) showed a similar hyporesponsiveness to anti-CD3 stimulation and a similar suppressive activity on $CD25^-CD4^+$ T cells. Further dissection of the $CD45RB^{lo}CD4^+$ T cells into the $CD25^+$ or $CD25^-$ population revealed that $CD25^+CD45RB^{lo}CD4^+$ T cells were unresponsive and suppressive but $CD25^-CD45RB^{lo}CD4^+$ T cells were not, indicating that the hyporesponsiveness and the suppressive activity of the $CD45RB^{lo}CD4^+$ population could be attributed to $CD25^+CD4^+$ T cells included in it. Taken together, the $CD25^+CD4^+$ population in normal naive mice appears to be naturally unresponsive to TCR stimulation and suppress the activation/proliferation of other T cells.

V_α/V_β TCR repertoire of $CD25^+CD4^+$ T cells

To characterize this unresponsive/suppressive $CD25^+CD4^+$ population in terms of the TCR repertoire or clonality, the composition of T cells transcribing particular V_α or V_β TCR gene segments was compared between the $CD25^+$ or $CD25^-$ $CD4^+$ population by RT-PCR (Fig. 2). Judging from the spectrum and intensity of the bands of individual V_α or V_β PCR products in agarose gels, there appeared to be no significant differences in the V_α/V_β TCR repertoire, indicating that the $CD25^+CD4^+$ population is not mono- or oligoclonal and may not be significantly skewed in the TCR repertoire. The result, together with the profile of cell surface markers (14), indicates that this population is distinct from $NK1.1^+$ T cells, another $CD4^+$ population with immunoregulatory activity (34). Indeed, the $CD25^+CD4^+$ population in C57BL/6 mice was $NK1.1^-$ (J. Shimizu, unpublished data).

$CD25^+CD4^+$ T cells suppress antigen-specific immune responses and require TCR stimulation to exert suppression

To determine whether $CD25^+CD4^+$ T cells control antigen-specific immune responses as well, we analyzed the functions of $CD25^+CD4^+$ T cells in TCR transgenic mice expressing transgenic TCR of known antigen specificity (Fig. 3A). Two strains of TCR transgenic mice, DO11.10 and BOG1, express different transgenic αβ TCR specific for different OVA peptides (OVA-P) [OVA-P(323–339) or OVA-P(271–285) respectively] in an $I\text{-}A^d$-restricted fashion (20,22). Both strains harbored $CD25^+CD4^+$ T cells constituting ~5% of peripheral $CD4^+$ T cells as observed in non-transgenic mice (see Fig. 1). These $CD25^+CD4^+$ T cells were unresponsive to the respective OVA peptides *in vitro* and potently suppressed antigen-specific proliferative responses of co-cultured transgenic $CD25^-CD4^+$ cells in a cell dose-dependent manner (Fig. 3A).

This non-proliferative $CD25^+CD4^+$ population in TCR-transgenic mice as well as normal mice, on the other hand, seemed to require activation signals via TCR to exert suppression, since stimulation with OVA peptides induced strong suppressive activity in $CD25^+CD4^+$ T cells from DO11.10 transgenic mice, but not in those from non-transgenic littermates, whereas polyclonal stimulation with anti-CD3 antibody efficiently induced the suppressive activity in both (Fig. 3B). Indeed, anti-CD3 stimulation increased the cell size and the CD25 expression level of the non-proliferative $CD25^+CD4^+$ population from non-transgenic mice, whereas stimulation of the same population with OVA peptides did not (data not shown).

Specificity of $CD25^+CD4^+$ T cell-mediated suppression

$CD25^-CD4^+$ T cells from BOG1 or DO11.10 specifically responded to OVA-P(271–285) or OVA-P(323–339) respectively, without cross-reactions, and responded well to the mixtures of the two peptides without competitively inhibiting each other's response (Fig. 4A and B). With these two strains, we attempted to determine whether $CD25^+CD4^+$ T cells stimulated by a specific antigen specifically suppress $CD25^-CD4^+$ T cells with the same antigen specificity or non-specifically suppress those with other antigen specificities as well (Fig. 4C). The concentration of each peptide for stimulating $CD25^-CD4^+$ T cells was chosen to elicit an

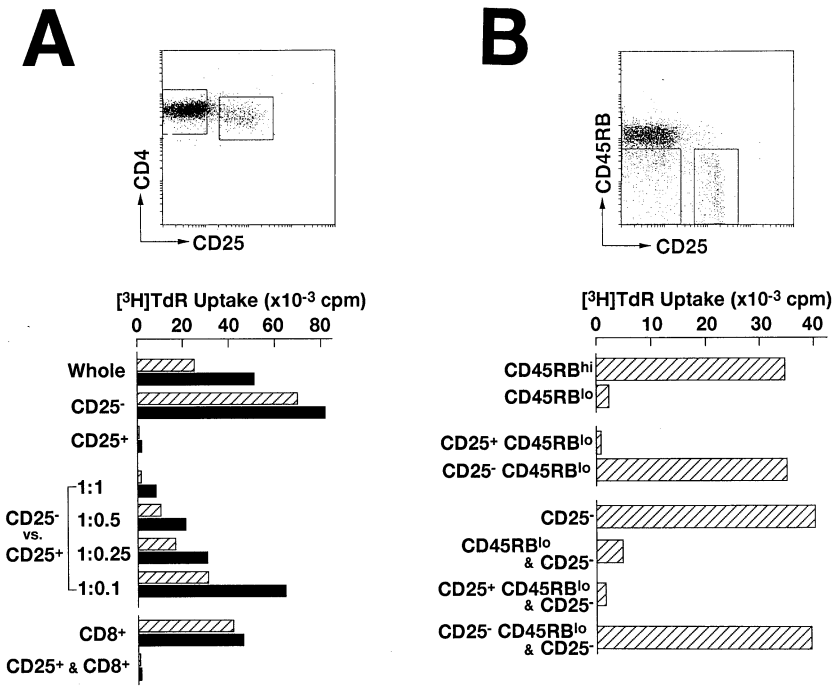

Fig. 1. $CD25^+CD4^+$ T cells are unresponsive to TCR stimulation and suppressive on $CD25^-CD4^+$ T cells. (A) $CD25^+$ or $CD25^-CD4^+$ T cells (enclosed in figure) purified by FACS (to >95% purity) from BALB/c spleen and lymph node cells, or these two populations mixed at various ratios, were cultured for 3 days with anti-CD3 mAb (hatched bars) or Con A (black bars), along with MMC-treated spleen cells as APC. $CD8^+$ T cells purified by FACS were similarly stimulated alone or after being mixed with an equal number of $CD25^+CD4^+$ T cells. (B) $CD45RB^{lo}$ or $CD45RB^{high}$ $CD4^+$ cells (designated as $CD45RB^{lo}$ or $CD45RB^{hi}$ respectively) purified from BALB/c spleen and lymph node cells were cultured with anti-CD3 antibody. Purified $CD25^+$ or $CD25^-CD45RB^{lo}$ cells (enclosed in figure) of the $CD4^+$ population, or the mixtures of either population with $CD25^-CD4^+$ T cells at an equal ratio, were similarly cultured. A representative result of more than five independent experiments is shown in (A) and (B). In these experiments (including those shown in Figs 3–9 below), background counts in the wells with APC only were <2000 c.p.m. The means of duplicate cultures are shown in each figure, and the standard errors of the mean were all within 10% of the mean in Fig. 1 and 3–9.

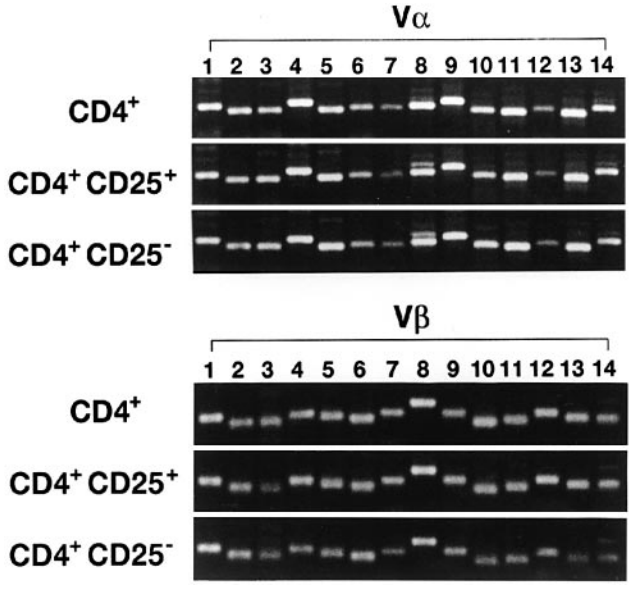

Fig. 2. Usage of TCR V_α or V_β gene families by $CD25^+$ or $CD25^-$ $CD4^+$ T cells. Transcription of particular V_α or V_β TCR genes was assessed by RT-PCR for 1×10^6 $CD25^+$ or $CD25^-CD4^+$ T cells or unseparated $CD4^+$ T cells purified by FACS from spleens and lymph nodes of 2-month-old BALB/c mice. A representative result of three independent experiments is shown.

equivalent degree of responses in each $CD25^-CD4^+$ T cell population [i.e. 0.1 mM of OVA-P(323–339) and 0.6 μM of OVA-P(271–285)]. DO11.10-derived $CD25^+CD4^+$ T cells stimulated with the peptide mixture markedly suppressed the responses of BOG1-derived $CD25^-CD4^+$ T cells depending on the concentration of OVA-P(323–339) (to which DO11.10 responded, but BOG1 did not) in the mixture, whereas stimulation of the culture with OVA-P(271–285) alone (to which BOG1 responded, but DO11.10 did not) did not elicit suppression. Likewise, BOG1-derived $CD25^+CD4^+$ T cells stimulated with the mixed peptides suppressed the responses of DO11.10-derived $CD25^-CD4^+$ T cells, but failed to exert suppression when stimulated with OVA-P(323–339) alone. A similar result was obtained with a combination of DO11.10 mice and 3A9 transgenic mice expressing transgenic TCR specific for hen egg lysozyme in an $I\text{-}A^k$-restricted manner (data not shown). It was noted that the concentration of the peptides required for eliciting suppression was 10- to 100-fold lower than that required for triggering proliferation of $CD25^-CD4^+$ T cells. For example, the concentration of OVA-P(323–339) required for DO11.10-derived $CD25^+CD4^+$ T cells to elicit significant suppression was <0.001 μM (Fig. 4C), whereas the peptide concentration needed for triggering proliferation of DO11.10-derived $CD25^-CD4^+$ T cells was ~0.1 μM (Fig. 4A). A similar difference was also observed with BOG1 mice. Taken together, these results indicate that, upon stimulation of $CD25^+CD4^+$ T cells with a specific

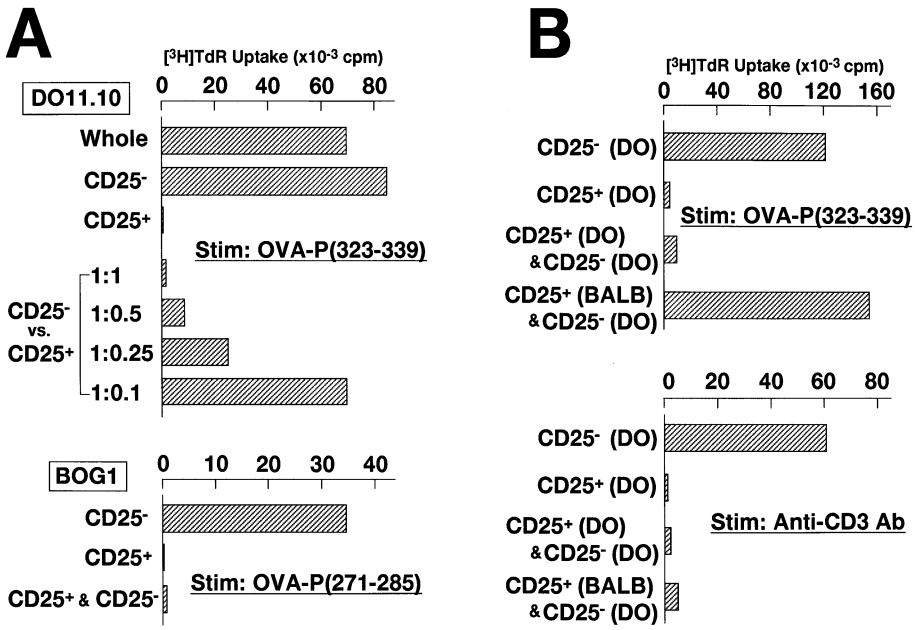

Fig. 3. Suppressive activity of $CD25^+CD4^+$ T cells from TCR transgenic mice. (A) $CD25^+$ or $CD25^-CD4^+$ T cells, or two populations mixed at various ratios, purified from the spleens of DO11.10 or BOG1 transgenic mice, were cultured for 3 days with 0.3 μM OVA-P(323–339) or 0.6 μM OVA-P(271–285) respectively. (B) $CD25^+CD4^+$ T cells purified from DO11.10 transgenic mice (DO) (which are on BALB/c background) or non-transgenic littermates (BALB), or either population mixed at an equal ratio with $CD25^-CD4^+$ T cells from transgenic mice, were cultured for 3 days with OVA-P(323–339) or anti-CD3 antibody. A representative result of three independent experiments is shown in (A) and (B).

antigen, the target of their suppression may not be confined to the T cells specific for the same antigen and that much lower concentration of antigen can stimulate $CD25^+CD4^+$ T cells to exert suppression than the antigen concentration required for the activation/proliferation of $CD25^-CD4^+$ T cells.

The mechanism of $CD25^+CD4^+$ T cell-mediated suppression

In the kinetic study of cell proliferation, IL-2 formation and IL-2 gene transcription, $CD25^-CD4^+$ T cells co-cultured with $CD25^+CD4^+$ T cells under anti-CD3 stimulation constantly exhibited a low degree of proliferation on day 2, followed by reduced proliferation and IL-2 transcription on day 3 (Fig. 5). It contrasted with $CD25^-CD4^+$ T cells that showed vigorous proliferation, active IL-2 production (~100 U/ml) and active IL-2 gene transcription from the beginning or $CD25^+CD4^+$ T cells that showed undetectable levels of proliferation, IL-2 production (<1 U/ml) and IL-2 gene transcription during the assay. Thus, $CD25^+CD4^+$ T cells, upon stimulation, appeared to potently suppress proliferation of $CD25^-CD4^+$ T cells even if the latter have already received activation signals from APC, although it remains to be determined whether reduced IL-2 gene transcription in $CD25^-CD4^+$ T cells is the immediate consequence of the suppressive signal from $CD25^+CD4^+$ T cells or an indirect consequence of inhibited proliferation.

To determine the molecular basis of the suppression, we first examined the possible role of immunosuppressive cytokines, such as IL-4, IL-10 and TGF-β, since the peripheral $CD25^+CD4^+$ population of normal naive BALB/c mice indeed transcribed IL-4, IL-10 and TGF-β gene more actively than the $CD25^-CD4^+$ population as previously shown (15). However, addition of anti-IL-4, anti-IL-10 or anti-TGF-β mAb (or their combination) to the anti-CD3 stimulation culture at sufficient concentrations to neutralize the cytokines was unable to abrogate the suppression (Fig. 6A). IL-4, IL-10 and TGF-β were also undetectable by ELISA in the culture supernatants of anti-CD3- or Con A-stimulated $CD25^+CD4^+$ T cells: <15 pg/ml for IL-4, <40 pg/ml for IL-10 and <160 pg/ml for TGF-β; and these supernatants were not suppressive when added to the culture of anti-CD3-stimulated $CD25^-CD4^+$ T cells (Fig. 6B). Furthermore, $CD25^+CD4^+$ T cells, even when outnumbering $CD25^-CD4^+$ T cells 10-fold, failed to suppress the proliferation of $CD25^-CD4^+$ cells across a 0.45 μm pore size membrane, contrasting with marked suppression when the two populations were on the same side of the membrane (Fig. 6C). It is thus unlikely that the suppression is mediated by far-reaching and long-lasting humoral factors secreted by $CD25^+CD4^+$ T cells. Failure of suppression across the membrane would also make it implausible that the suppression is due to absorption of IL-2 by $CD25^+CD4^+$ T cells and resulting deprivation of IL-2 available for $CD25^-CD4^+$ T cells.

$CD25^+CD4^+$ T cells in normal mice did not appear to induce apoptosis in $CD25^-CD4^+$ T cells and thereby control them, since the number of viable (i.e. propidium iodide non-stained) $CD25^-CD4^+$ T cells was comparable with that of $CD25^+CD4^+$ T cells after 3 days of anti-CD3-stimulated co-culture (data not shown), and addition of antibodies blocking FasL or TNF-α failed to annul the suppression (Fig. 6D). Furthermore, no detectable expression of CD80/CD86 on anti-CD3-stimulated $CD25^+CD4^+$ T cells (T. Takahashi, unpublished data) would make it unlikely that the suppression was mediated by a negative signal through the CTLA-4 molecules, if any, expressed on $CD25^-CD4^+$ T cells (35).

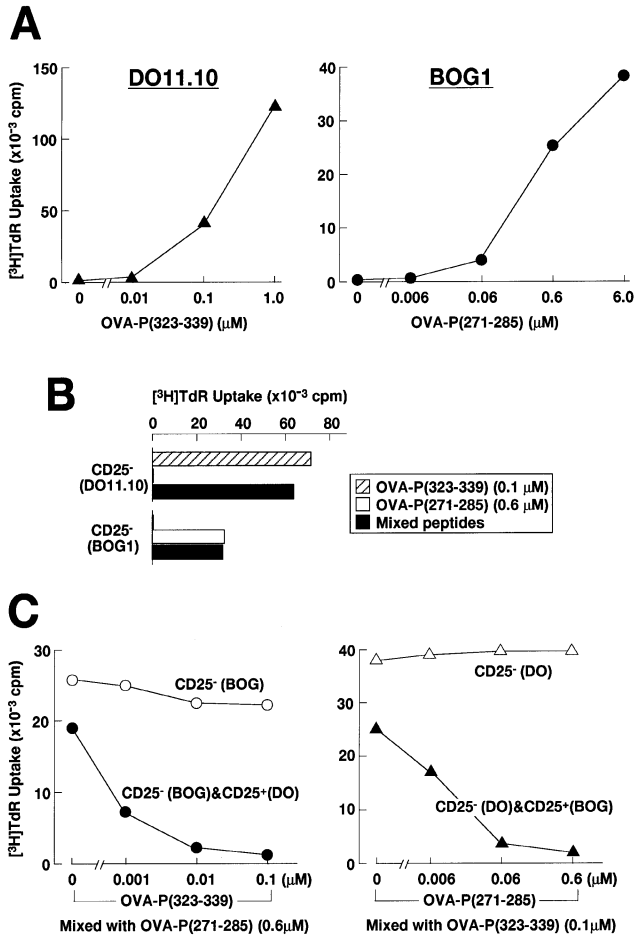

Fig. 4. Antigen specificity of $CD25^+CD4^+$ T cell-mediated suppression. (A) $CD25^-CD4^+$ T cells purified from the spleens of DO11.10 or BOG1 mice were stimulated with various concentrations of OVA peptides for 3 days. (B) $CD25^-CD4^+$ T cells from DO11.10 mice or BOG1 mice were stimulated for 3 days with indicated concentration of OVA-P(323–339), OVA-P(271–285) or the mixtures of these two peptides. (C) $CD25^-CD4^+$ T cells from DO11.10 or BOG1 mice were mixed with an equal number of $CD25^+CD4^+$ T cells from BOG1 or DO11.10 mice respectively, and stimulated for 3 days with the mixtures of OVA-P(271–285) and OVA-P(323–339) at various concentrations as indicated. A representative result exhibiting the means of duplicate cultures of more than three independent experiments is shown in these figures.

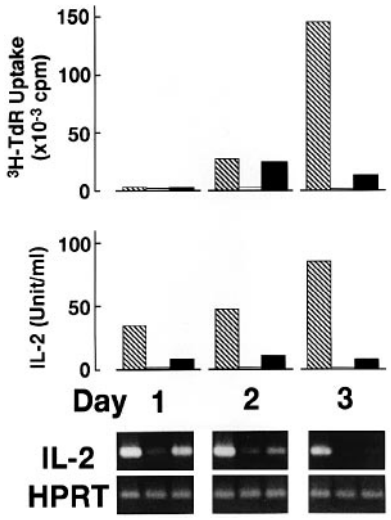

Fig. 5. IL-2 formation and IL-2 gene transcription in $CD25^+$ or $CD25^-$ $CD4^+$ T cells, or the mixture of the two populations. $CD25^-CD4^+$ T cells (hatched bars), $CD25^+CD4^+$ T cells (open bars), or the mixtures of the two populations at an equal ratio (black bars) were stimulated with anti-CD3 antibody and assessed on various days for cell proliferation as shown in Fig. 1(A) for IL-2 concentration of the culture supernatants by bioassay, and for transcription of IL-2 gene by RT-PCR with transcription of HPRT gene as control. Paraformaldehyde-fixed CD86-transfected P815 cells (see Fig. 7 below) were used as APC to avoid contamination of IL-2 message from APC in RT-PCR. A representative result of three independent experiments is shown.

On the other hand, the suppression was significantly reduced by increasing the number of splenic APC or P815 mastocytoma cells transfected with CD86 cDNA, suggesting that a cognate interaction among three elements ($CD25^+CD4^+$ T cells, $CD25^-CD4^+$ T cells and APC) may be required for suppression (Fig. 7A and B). This effective suppression on the CD86-transfectants, which were also expressing class II MHC, ICAM-1 and CD2, but no CD40, indicates that CD40–CD40 ligand interaction may not be required for the suppression. Furthermore, fixation of the transfectants with paraformaldehyde did not affect the degree of suppression, indicating that APC do not need to be viable for the suppression.

These results collectively indicate that $CD25^+CD4^+$ T cells interact with $CD25^-CD4^+$ T cells on the surface of APC in a cell-contact manner, leading to reduced IL-2 formation and proliferation of the latter.

Effects of IL-2 or anti-CD28 antibody on the unresponsiveness and suppressive activity of $CD25^+CD4^+$ T cells

Given the inhibition of IL-2 formation in $CD25^-CD4^+$ T cells (Fig. 5) and the findings by others that exogenous IL-2 can break T cell unresponsiveness (36,37), we examined the effect of IL-2 on the unresponsiveness of $CD25^+CD4^+$ T cells and their suppressive activity. In the presence of exogenously added rIL-2 at the concentration >10 U/ml, stimulation with anti-CD3 antibody, or with OVA peptides in the case of utilizing DO11.10 TCR transgenic mice, elicited proliferation not only in $CD25^+CD4^+$ T cells but also the mixtures of $CD25^+CD4^+$ T cells and $CD25^-CD4^+$ T cells in an IL-2 dose-dependent fashion (Fig. 8A and B). Likewise, addition of anti-CD28 mAb, which was reported to prevent the induction of anergy in T cell clones (28), broke the unresponsiveness of $CD25^+CD4^+$ T cells and elicited proliferation of the cell mixtures stimulated with anti-CD3 antibody in BALB/c mice (Fig. 8C) or OVA peptides in DO11.10 transgenic mice (Fig. 8D). The suppression was easily abrogated at low concentrations of anti-CD28 antibody, whereas higher antibody concentrations were required for triggering proliferation of $CD25^+CD4^+$ T cells. This indicates that proliferation of the cell mixtures at high anti-CD28 concentrations is due to proliferation of both $CD25^+CD4^+$ T cells and $CD25^-CD4^+$ T cells included in the mixtures—not due to proliferation of either population alone. Thus, high doses of IL-2 and anti-CD28 antibody not only

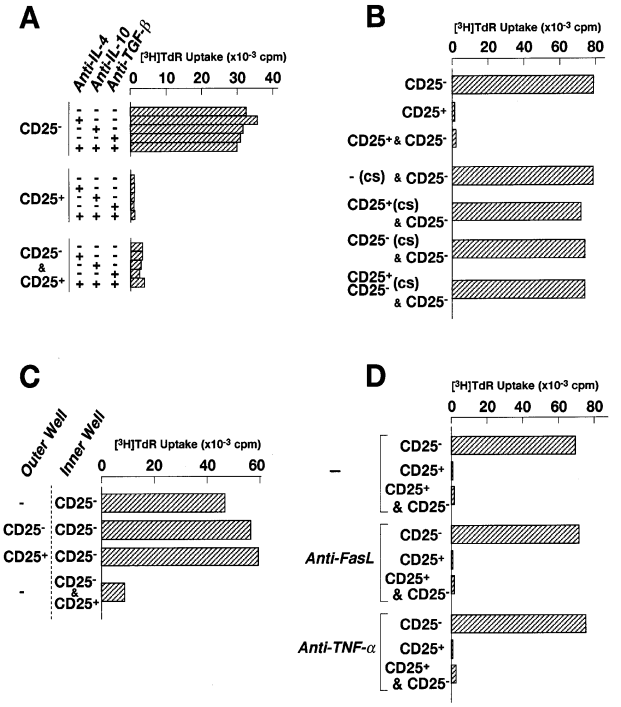

Fig. 6. No significant role of humoral factors or apoptosis in $CD25^+CD4^+$ T cell-mediated suppression. (A) Neutralizing mAb for IL-4, IL-10 or TGF-β, or a combination of these antibodies, were added on day 0 to the 3 day culture of anti-CD3-stimulated $CD25^-$ or $CD25^+CD4^+$ T cells, or the mixtures of these at an equal ratio. (B) Culture supernatants (cs) of $CD25^+CD4^+$ T cells [$CD25^+$ (cs)], $CD25^-CD4^+$ T cells [$CD25^-$ (cs)] or APC alone [–(cs)] stimulated for 3 days with anti-CD3 antibody were added to the culture of $CD25^-CD4^+$ T cells as 75% of the culture volume and stimulated with anti-CD3 antibody. (C) In transmembrane culture, $CD25^-CD4^+$ T cells or a mixture of $CD25^+CD4^+$ T cells and $CD25^-CD4^+$ T cells at an equal ratio (inner well) were separated from 10 times the number of $CD25^+$ or $CD25^-CD4^+$ T cells or APC only (outer well) by a 0.45 μm pore size membrane and stimulated with anti-CD3 antibody for 3 days. (D) Anti-FasL antibody or anti-TNF-α antibody was added at concentrations sufficient to block FasL or neutralize TNF-α respectively to the culture of $CD25^+$ or $CD25^-CD4^+$ T cells or the mixtures of the two populations, which were stimulated with anti-CD3 antibody for 3 days. A representative result exhibiting the means of duplicate cultures of three independent experiments is shown.

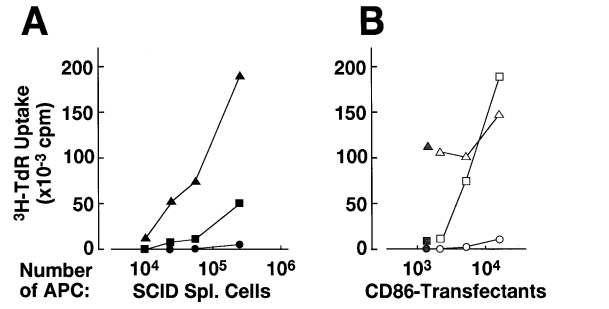

Fig. 7. Role of APC in the $CD25^+CD4^+$ T cell-mediated suppression. (A) $CD25^+CD4^+$ T cells (circle), $CD25^-CD4^+$ T cells (triangle) or mixtures of the two populations at an equal ratio (square) were stimulated with anti-CD3 antibody in the presence of graded numbers of MMC-treated spleen cells from CB.17-SCID mice (closed symbols), or (B) CD86 cDNA-transfected P815 cells treated with MMC (gray symbols) or graded numbers of the transfectants fixed with paraformaldehyde (open symbols). A representative result exhibiting the means of duplicate cultures of three independent experiments is shown.

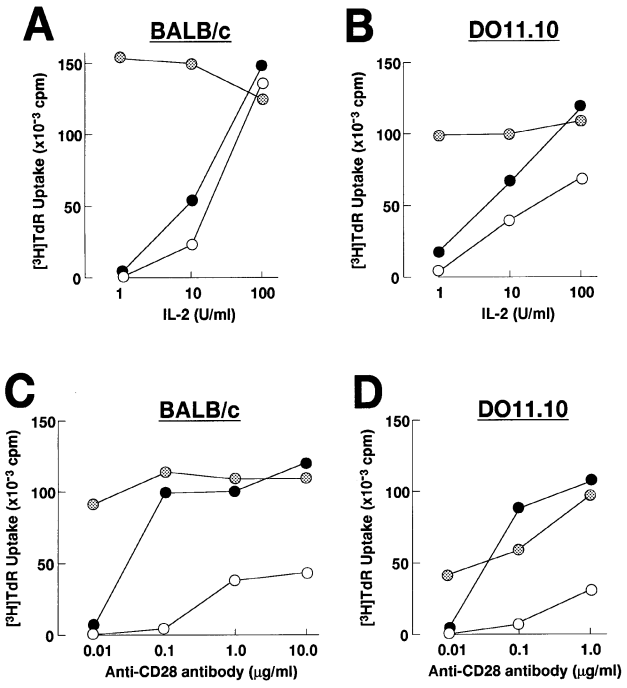

Fig. 8. Breaking unresponsiveness and abrogating suppressive activity of $CD25^+CD4^+$ T cells by T cell stimulation and exogenous IL-2 or anti-CD28 antibody. $CD25^+CD4^+$ T cells (open circle), $CD25^-CD4^+$ T cells (gray circle) or the two populations mixed at an equal ratio (closed circle) were prepared from normal BALB/c mice (A and C) or DO11.10 transgenic mice (B and D) and stimulated with anti-CD3 antibody or OVA peptides respectively, with graded concentrations of rIL-2 (A and B) or anti-CD28 antibody (C and D). A representative result of five independent experiments is shown in (A) and (B), and of three independent experiments in (C) and (D).

break the unresponsiveness of $CD25^+CD4^+$ T cells but also abrogate their suppressive activity.

When these proliferating $CD25^+CD4^+$ T cells stimulated with anti-CD3 antibody and rIL-2 or anti-CD28 antibody were harvested on day 7 and re-stimulated with anti-CD3 antibody along with fresh APC, but without exogenous rIL-2 or anti-CD28 antibody, they showed no proliferative response and strongly suppressed the responses of freshly co-cultured $CD25^-CD4^+$ T cells (Fig. 9A and B). This indicated that, upon removal of IL-2 or anti-CD28 antibody, the $CD25^+CD4^+$ T cells reverted to the original unresponsive/suppressive state.

Induction of autoimmune disease by breaking the anergic/suppressive state of $CD25^+CD4^+$ T cells

The *in vitro* analyses described above raise the possibility that autoimmune disease may develop in normal animals if the breakage of the anergy/suppression of $CD25^+CD4^+$ T cells leads to activation of self-reactive T cells from $CD25^-$ dormant states. To test this, spleen and lymph node cell suspensions from euthymic BALB/c *nu*/+ mice were stimulated with Con A and exogenous rIL-2 (or Con A alone) for 3

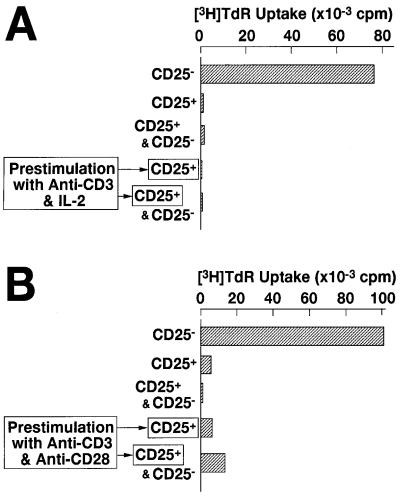

Fig. 9. Reversal of $CD25^+CD4^+$ T cells to the unresponsive/suppressive state after removing IL-2 or anti-CD28 antibody. $CD25^+CD4^+$ T cells pre-stimulated with anti-CD3 antibody and rIL-2 (A) or anti-CD28 antibody (B) for 7 days were washed and re-stimulated with anti-CD3 antibody along with fresh APC, but without exogenous rIL-2 or anti-CD28 antibody, for 3 days. These pre-stimulated $CD25^+CD4^+$ T cells were also mixed with freshly prepared $CD25^-CD4^+$ T cells at an equal ratio and stimulated. A representative result of three independent experiments is shown.

days and then transferred to BALB/c athymic nude (*nu*/*nu*) mice (14). The majority (92%, $n = 12$) of the nude mice transferred with Con A/IL-2-stimulated T cells indeed developed histologically evident autoimmune gastritis with massive infiltration of inflammatory cells and high titers of anti-parietal cell autoantibodies in the circulation (for histology and autoantibodies, see 7,29) (Fig. 10A). Immunohistochemical staining of the affected gastric mucosa by anti-parietal cell autoantibodies showed a specific loss of and damage to parietal cells (Fig. 10B, C). The gastritis was thus immunopathologically similar to autoimmune gastritis with pernicious anemia in humans (38). Some (30%) of the nude mice also developed histologically evident oophoritis with anti-oocyte autoantibodies and 17% of the mice had thyroiditis with anti-thyroglobulin autoantibodies (9,31,32). Few autoimmune diseases developed in the nude mice transferred with the same number of T cells stimulated with Con A alone. The IL-2 activity in the culture supernatants of spleen cells stimulated with Con A alone for 3 days was 30 times less than the dose of rIL-2 added to the Con A/IL-2 culture, being insufficient for breaking unresponsiveness of $CD25^+CD4^+$ T cells *in vitro* (data not shown). Thus, breakage of the unresponsiveness/suppression in $CD25^+CD4^+$ T cells could elicit autoimmune diseases similar to those produced by direct removal of $CD25^+CD4^+$ T cells (14,15).

Discussion

We showed in this report that $CD25^+CD4^+$ T cells present in the periphery of normal naive mice are naturally anergic, if one defines anergy as a reversible anti-proliferative state (39). This natural anergy is closely linked with the suppressive activity in $CD25^+CD4^+$ T cells, as illustrated by the simultaneous abrogation of suppression upon breaking their anergic state. It remains to be determined, however, whether every T cell in the $CD25^+CD4^+$ population is anergic and suppressive or the unresponsiveness of the $CD25^+CD4^+$ population as a whole is due to a suppression exerted by a small number of suppressive T cells included in the $CD25^+CD4^+$ population, as hyporesponsiveness of the $CD45RB^{lo}CD4^+$ population could be attributed to suppressive $CD25^+CD4^+$ cells included in it (Fig. 1B). Even if the latter is the case and the anergic/suppressive $CD4^+$ population can be further reduced to a smaller subpopulation included in the $CD25^+CD4^+$ population, such a subpopulation itself may well be unresponsive (unless it is too small to detect its proliferation), since the $CD25^+CD4^+$ population freshly prepared (Fig. 1) or expanded once or several times *in vitro* with Con A and IL-2 (Fig. 9) showed virtually no proliferation upon TCR stimulation irrespective of their high responsiveness as suppressive cells to low concentration of antigens (Fig. 4). Furthermore, the T cell lines and clones we have so far established *in vitro* from $CD25^+CD4^+$ T cells are all anergic (J. Shimizu *et al.*, unpublished data). It is hence likely that the normal immune system harbors a unique $CD4^+$ T cell population that is naturally anergic and suppressive.

IL-4, IL-10 and/or TGF-β play roles in T cell-mediated suppression in various models of autoimmune or inflammatory disease (40–46). In the present study, *in vitro* neutralization of these cytokines with specific antibodies did not exhibit any significant effects on the $CD25^+CD4^+$ T cell-mediated suppression. $CD25^+CD4^+$ T cells did not appear to induce apoptosis in $CD25^-CD4^+$ T cells either, although it was reported that the CD25 gene knockout mice developed autoimmunity possibly due to abnormality in apoptosis (47,48). On the other hand, no suppressive activity in the culture supernatants of $CD25^+CD4^+$ T cells (or the cell mixtures) and the failure to elicit suppression across a factor-permeable but cell-impermeable membrane, together with the dependency of the suppression upon the number of APC *in vitro*, suggest that cognate interactions among $CD25^+CD4^+$ T cells, $CD25^-CD4^+$ T cells and APC may be required for the suppression (49,50). Furthermore, no significant effects of parafolmaldehyde fixation of APC on the degree of suppression indicates that the suppression is not exerted through modifying intracellular antigen processing by APC or their *de novo* synthesis of cell surface molecules or cytokines. Based on these findings and the antigen non-specific property of the $CD25^+CD4^+$ T cell-mediated suppression, the possible mechanism of the suppression would be: (i) on APC, $CD25^+CD4^+$ T cells may directly deliver to $CD25^-CD4^+$ T cells a negative signal for activation/proliferation; (ii) $CD25^+CD4^+$ T cells, which are expressing various adhesion molecules (such as ICAM-1, LFA-1 and CD2) at higher levels than $CD25^-CD4^+$ T cells (12), may physically interfere with the interaction of the latter with APC (e.g. by competing for co-stimulatory molecules, such as CD80/86), although no significant difference was observed between the two populations in the expression levels of CD28 (T. Takahashi *et al.*, unpublished data); or (iii) $CD25^+CD4^+$ T cells may chemically modify functions of cell surface molecules already expressed on APC and thereby interfere with activation/proliferation of $CD25^-CD4^+$ T cells on APC. It is currently under investigation

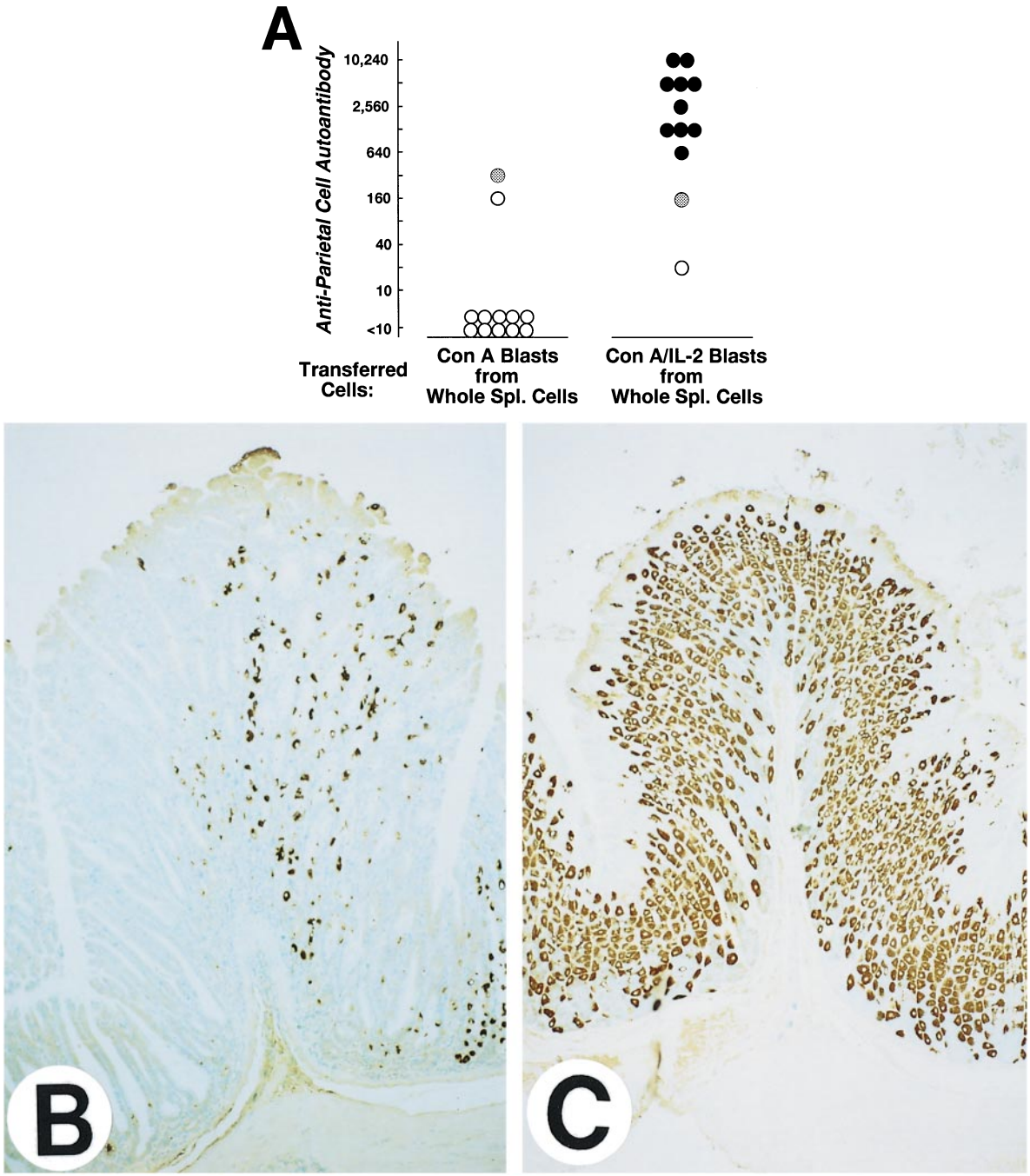

Fig. 10. Induction of autoimmune disease in athymic nude mice by transferring T cells stimulated with Con A and IL-2. (A) Spleen and lymph node cells of BALB/c *nu*/+ mice were stimulated with Con A and rIL-2, or Con A alone, for 3 days, and 3×10^7 blastic cells (90% of which were $CD3^+$) were i.v. transferred to BALB/c athymic nude mice. The recipients were histologically examined 3 months later and titers of anti-parietal cell autoantibdoies were assessed by ELISA. The gastric lesion was histologically graded as macroscopically and histologically evident gastritis (closed symbols), histologically evident gastritis (gray symbols) or intact gastric mucosa (open symbols). (for details of histological grading of gastritis, see 31,32.) The gastric mucosa of a gastritis-bearing mouse after transfer of Con A/IL-2-stimulated cells (B) or intact gastric mucosa of a mouse transferred with Con A-stimulated cells (C) was stained by immunohistochemistry with anti-parietal cell autoantibody (original magnification: ×250). Note loss and damage of parietal cells in (B), but not in (C).

which of these plausible mechanisms directly or indirectly leads to reduced IL-2 formation and non-proliferation of $CD25^-$ $CD4^+$ cells. It also remains to be determined how high doses of IL-2 or anti-CD28 antibody abolish the $CD25^+CD4^+$ T cell-mediated suppression, i.e. either by abrogating the suppressive activity of $CD25^+CD4^+$ T cells or by stimulating $CD25^-$

$CD4^+$ T cells to overwhelm suppressive signals from $CD25^+CD4^+$ T cells, or both.

Specific antigens as well as polyclonal T cell activators can stimulate $CD25^+CD4^+$ T cells to exert suppression, as shown with OVA-TCR transgenic mice (Fig. 3 and 4). It does not necessarily mean, however, that any $CD4^+$ T cells specific for non-self antigens can become $CD25^+CD4^+$ anergic/suppressive T cells. In an accompanying study, we show that anergic/suppressive $CD25^+CD4^+CD8^-$ thymocytes are generated in the thymus of TCR-transgenic mice as well as non-transgenic normal mice, but not in RAG-2 gene-deficient TCR transgenic mice, which lack endogenous rearrangements of TCR genes, and that the $CD25^+CD4^+$ T cell/thymocyte population in TCR transgenic mice harbored a higher proportion of T cells expressing endogenous TCR α chains than the $CD25^-CD4^+$ population (M. Itoh *et al.*, submitted). These findings suggest that $CD25^+CD4^+$ T cells exhibiting suppressive activity upon OVA stimulation may be expressing dual TCR, one composed of the transgenic $\alpha\beta$ chains, and the other composed of an endogenous α chain and the transgenic β chain (31,51,52); while the former recognizes OVA peptide, the latter might contribute to rendering $CD25^+CD4^+$ T cells anergic and suppressive through interacting with self-peptides in association with class II MHC molecules in the thymic selection process. Thus, the $CD25^+CD4^+$ T cells in the thymus and periphery may not be mere activated T cells, but constitute a functionally distinct subpopulation of T cells (15).

The degree of the $CD25^+CD4^+$ T cell-mediated suppression is dependent on the number of $CD25^+CD4^+$ T cells (Figs 1 and 3) and their suppressive activity, which depends on the concentration of the antigens to which they respond (Fig. 4). The physiological number (5–10% of $CD4^+$ T cells) and activity of $CD25^+CD4^+$ T cells suffice to inhibit autoimmune development (Fig. 10) (14,15), while allowing other T cells to respond to antigen-specific or non-specific TCR stimuli *in vitro* (Figs 1 and 3) and *in vivo* (14), although the suppression is antigen non-specific in its effector phase. For the following reasons, the anergic/suppressive $CD25^+CD4^+$ T cells responsible for maintaining self-tolerance may be potentially self-reactive in antigen specificity and being continuously stimulated by tissue-specific or ubiquitous self-antigens, although they themselves may be harmless and show no significant changes in TCR repertoire because of their anergic non-proliferative property. First, judging from the finding that $CD25^+CD4^+$ T cells require TCR stimulation to exert suppression, the autoimmune-preventive/anergic $CD25^+CD4^+$ T cells should also require TCR stimulation to suppress self-reactive T cells. Second, the requirement of a cognate interaction between $CD25^+CD4^+$ T cells and other T cells on the surface of APC (and no contribution of far-reaching humoral factors to the suppression) indicates that the autoimmune-preventive/anergic $CD25^+CD4^+$ T cells should be guided to the same APC (or its very vicinity) on which self-reactive $CD4^+$ or $CD8^+$ T cells are activated. Third, the majority of $CD25^+CD4^+$ T cells in normal naive mice in a specific pathogen-free condition appear to be in an 'activated' state (i.e. $CD5^{high}$, $CD45RB^{lo}$, ICAM-1^{high}, LFA-1^{high} and partially $CD69^{high}$) (14), suggesting that the autoimmune-preventive/anergic $CD25^+CD4^+$ T cells may be continuously stimulated in the internal environment. Furthermore, the antigen concentration required for stimulating $CD25^+CD4^+$ T cells to exert suppression was much lower than the concentration required for triggering other T cells to proliferate, indicating that the autoimmune-preventive/anergic $CD25^+CD4^+$ T cells could be stimulated by self-antigens even at too low antigen concentration to stimulate self-reactive T cells. This possible self-reactivity and high antigen sensitivity of anergic/suppressive $CD25^+CD4^+$ T cells would make it possible for them to be guided to the APC presenting self-antigens, easily stimulated there and able to control stably the activation/expansion of $CD25^-CD4^+$ or $CD8^+$ self-reactive T cells on the same APC.

One of the unique characteristics of $CD25^+CD4^+$ T cells is that the anergic/suppressive state is apparently their basal default condition, as illustrated by the finding that removal of IL-2 or anti-CD28 antibody from the culture milieu allowed the anergy-broken and non-suppressive $CD25^+CD4^+$ T cells to revert to the original anergic state and to reacquire their suppressive activity. It contrasts with artificially induced T cell anergy, which will never spontaneously return to an anergic state once it is broken (53–57). It is of note, on the other hand, that autoimmune disease could be induced by breaking the anergic/suppressive state of $CD25^+CD4^+$ T cells for a limited period, as illustrated by the development of autoimmune disease in *nu*/*nu* mice transferred with T cells treated *in vitro* with Con A and IL-2 for 3 days (Fig. 10). This indicates that transient breakage of the anergic/suppressive state of $CD25^+CD4^+$ T cells may suffice to elicit autoimmune disease in genetically susceptible animals if a sufficient number of $CD4^+$ pathogenic self-reactive T cells are allowed to expand and/or differentiate to autoimmune effector T cells before suppressive activity recovers in the $CD25^+CD4^+$ T cells.

In conclusion, we have shown that naturally anergic and suppressive T cells are present in the normal immune system as a functionally and phenotypically distinct subpopulation of T cells and actively preventing autoimmune disease by suppressing activation/expansion of self-reactive T cells. Further analysis of this T cell-mediated mechanism of self-tolerance would contribute to our understanding of the cause and mechanism of autoimmune disease and help in devising new strategies for treating or preventing it. Manipulation of the $CD25^+CD4^+$ T cell population would also make it possible to suppress or enhance immune responses to non-self antigens in general.

Acknowledgements

We thank Drs D. Y. Loh (Hoffmann La-Roche), T. Saito (Chiba University), K. Iwabuchi (Hokkaido University) and O. Kanagawa (Washington University) for providing us transgenic mice; Dr M. Azuma (National Center for Peditric Diseases) for CD86 transfectants; Drs H. Yagita and K. Okumura (Juntendo University) for anti-FasL antibody; Dr T. Tanaka (Osaka University) for anti-IL-2 receptor β antibody; Dr I. Foerster (University of Cologne) for discussion; and Ms E. Moriizumi for immunohistochemistry and histology. This work was supported by grants-in-aid from the Ministry of Education, Science, Sports and Culture, the Ministry of Human Welfare, and the Organization for Pharmaceutical Safety and Research of Japan. This work was presented at the International Kyoto T Cell Conference, 2–4 October 1997, and the Annual Meeting of the Japanese Society for Immunologists [*Proc. Jpn Soc. Immunol.* 27:p116 and p143 (abstr.)], 29–31 October 1997, and International ThymOz Meeting in Australia, 25–28 March 1998.

Abbreviations

APC	antigen-presenting cell
Con A	concanavalin A
FasL	Fas ligand
MMC	mitomycin C
OVA	ovalbumin
PE	phycoerythrin
TGF	transforming growth factor
TNF	tumor necrosis factor

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