

# CD4<sup>+</sup>CD25<sup>+</sup> Immunoregulatory T Cells Suppress Polyclonal T Cell Activation In Vitro by Inhibiting Interleukin 2 Production

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## Summary

Peripheral tolerance may be maintained by a population of regulatory/suppressor T cells that prevent the activation of autoreactive T cells recognizing tissue-specific antigens. We have previously shown that CD4<sup>+</sup>CD25<sup>+</sup> T cells represent a unique population of suppressor T cells that can prevent both the initiation of organ-specific autoimmune disease after day 3 thymectomy and the effector function of cloned autoantigen-specific CD4<sup>+</sup> T cells. To analyze the mechanism of action of these cells, we established an in vitro model system that mimics the function of these cells in vivo. Purified CD4<sup>+</sup>CD25<sup>+</sup> cells failed to proliferate after stimulation with interleukin (IL)-2 alone or stimulation through the T cell receptor (TCR). When cocultured with CD4<sup>+</sup>CD25<sup>-</sup> cells, the CD4<sup>+</sup>CD25<sup>+</sup> cells markedly suppressed proliferation by specifically inhibiting the production of IL-2. The inhibition was not cytokine mediated, was dependent on cell contact between the regulatory cells and the responders, and required activation of the suppressors via the TCR. Inhibition could be overcome by the addition to the cultures of IL-2 or anti-CD28, suggesting that the CD4<sup>+</sup>CD25<sup>+</sup> cells may function by blocking the delivery of a costimulatory signal. Induction of CD25 expression on CD25<sup>-</sup> T cells in vitro or in vivo did not result in the generation of suppressor activity. Collectively, these data support the concept that the CD4<sup>+</sup>CD25<sup>+</sup> T cells in normal mice may represent a distinct lineage of "professional" suppressor cells.

Key words: suppressor T cells • interleukin 2 • autoimmune disease • self-tolerance • interleukin 2 receptor  $\alpha$  chain (CD25)

It is widely accepted that the development of autoimmune disease involves a breakdown in the mechanisms that control self- versus nonself-discrimination. The primary mechanism that leads to tolerance to self-antigens is thymic deletion of autoreactive T cells. However, some autoreactive T cells may escape thymic deletion or recognize antigens expressed only extrathymically. T cell anergy (1) and T cell ignorance/indifference (2) have been proposed as the primary mechanisms used to control these potentially harmful populations. However, anergic or ignorant T cell populations have the potential to be activated when their target self-antigens are released into the lymphoid system during the course of an infection or when they are activated by cross-reactive epitopes present on infectious agents (3). Thus, these "passive" mechanisms for self-tolerance may not be sufficient to completely control potentially pathogenic T cells. Over the past 10 years, evidence has accumulated for an "active" mechanism of immune suppression in which a distinct subset of cells suppresses the activation of autoreactive T cells that have escaped the passive mechanisms of tolerance (4).

A number of organ-specific autoimmune diseases can be induced in rodent strains that do not normally develop autoimmunity by procedures that interfere with normal T cell maturation or by rendering the animals partially T cell deficient (5). In general, a defined subset of T cells from syngeneic healthy donors can prevent the development of autoimmunity on transfer to lymphopenic recipients, indicating that the normal immune system contains immunoregulatory T cells that can prevent the activation of autoreactive T cells (6). For example, Powrie et al. have shown that colitis can be induced in immunodeficient SCID mice by transfer of the CD45RB<sup>high</sup> subset of CD4<sup>+</sup> T cells from normal mice, but not by the CD45RB<sup>low</sup> population (7). The CD45RB<sup>low</sup> population, when transferred together with the CD45RB<sup>high</sup> population, completely inhibited development of the disease. Evidence for the existence of regulatory T cells has also been obtained in both the Bio-Breeding rat and nonobese diabetic mouse strains that spontaneously develop autoimmune diabetes (8, 9). Immunoregulatory T cells are also likely to be responsible for the relative resistance of mice that express a transgenic TCR

specific for a peptide from myelin basic protein to the development of experimental allergic encephalomyelitis, whereas mice that express the same TCR on a recombination activating gene (RAG)-deficient ( $^{-/-}$ ) background are highly susceptible (10).

Although many of these studies have demonstrated that the immunoregulatory T cells are present in the subpopulation of CD4<sup>+</sup> cells that express activation/memory markers, a more detailed phenotypic characterization of the suppressor population is lacking. Studies using two different model systems have suggested that a potent CD4<sup>+</sup> immunoregulatory T cell population that can be defined by expression of the IL-2R  $\alpha$  chain (CD25) is responsible for the prevention of certain autoimmune diseases. In the first model system (11, 12), genetically susceptible mice that were thymectomized on day 3 of life (d3Tx)<sup>1</sup> developed organ-specific autoimmune disease involving one or more organs. The disease process was mediated by CD4<sup>+</sup> T cells; however, CD4<sup>+</sup> T cells from normal adult mice could inhibit the development of disease in the d3Tx animals if they were transferred by day 14 of life. Furthermore, the inhibitory activity was completely contained in the minor (10%) subset of CD4<sup>+</sup> T cells that coexpressed CD25 (13, 14). In the second model, when CD4<sup>+</sup>CD25<sup>+</sup> T cells were depleted from CD4<sup>+</sup> T cells isolated from peripheral lymphoid tissues of normal adult mice and the remaining CD4<sup>+</sup>CD25<sup>-</sup> cells injected into *nu/nu* mice recipients, the recipients developed a high incidence of organ-specific autoimmune disease (13, 15). Again, cotransfer of populations enriched in CD4<sup>+</sup>CD25<sup>+</sup> prevented the induction of disease by the CD4<sup>+</sup>CD25<sup>-</sup> population. We have also recently demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> T cells can inhibit the capacity of a cloned line of autoantigen-specific effector cells to transfer disease to *nu/nu* recipients (16). Thus, the CD4<sup>+</sup>CD25<sup>+</sup> population can inhibit both the induction and effector function of autoreactive T cells.

Although the existence of immunoregulatory T cell populations has been amply documented, the activity of these suppressor populations has been measured in vivo in model systems that require weeks to months of assessment of disease activity. Therefore, it has proven difficult to determine their mechanism of action, antigen specificity, or cellular targets. In this report, we demonstrate that the population of CD4<sup>+</sup>CD25<sup>+</sup> T cells present in the peripheral lymphoid system of normal mice is a potent inhibitor of polyclonal T cell activation in vitro. Suppression is mediated by a cytokine-independent, cell contact-dependent mechanism that requires activation of the suppressor cell via the TCR. The CD4<sup>+</sup>CD25<sup>+</sup> cells inhibit the induction of IL-2 production in the responder CD4<sup>+</sup>CD25<sup>-</sup> population, and suppression can be overcome by the addition of exogenous IL-2 or enhancement of endogenous IL-2 production.

<sup>1</sup>Abbreviations used in this paper: AC, accessory cell; d3Tx, thymectomized on day 3 of life; FasL, Fas ligand; RT, reverse transcriptase.

## Materials and Methods

**Mice.** Female BALB/c and C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). C57BL/10 mice were obtained from Taconic Farms (Germantown, NY). DO.11.10-TCR transgenic SCID mice were bred in our own facilities (14). IL-4 $^{-/-}$  mice (BALB/c background) were originally obtained from N. Noben-Trauth (NIAID, National Institutes of Health) (17) and were bred in our facilities. IL-10 $^{-/-}$  mice were originally obtained from R. Kuhn and W. Muller (University of Koln, Koln, Germany) and backcrossed in our facilities onto the C57BL/10 (N7) background. BALB/c mice were thymectomized on day 3 of life and screened for the presence of antigastric autoantibodies as previously described (18).

**Media, Reagents, and Antibodies.** All cells were grown in RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), 2 mM l-glutamine, 10 mM Hepes, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (all from Biofluids), and 50  $\mu$ M 2-ME (Sigma Chemical Co., St. Louis, MO). PMA, ionomycin, and Con A were purchased from Sigma Chemical Co. Biotin-conjugated anti-CD25 (7D4), FITC-conjugated streptavidin, PE-conjugated anti-CD45RB (16A), PE-conjugated anti-CD62L (Mel-14), PE-conjugated anti-CD69, PE-conjugated anti-CD5, anti-CD28, anti-CD40 (HM40-3), anti-B7-2 (GL1), anti-CTLA-4, anti-IL-2 (S4B6), anti-IL-4 (11B11), anti-IL-10 (JES5-2A5), or (SXC-1 and SXC-2) and anti-IFN- $\gamma$  (XMG1.2) were purchased from Pharmingen (San Diego, CA). Anti-TGF- $\beta$  was purchased from R&D Systems (Minneapolis, MN). PE-conjugated anti-CD4 was purchased from Becton Dickinson (Mountain View, CA). Anti-CD3 (2C11) was purified from ascites and used at 1–3  $\mu$ g/ml or purchased from Pharmingen and used at 0.5  $\mu$ g/ml. Human rIL-2 was purchased from Peprotech (Rocky Hill, NJ). Flow cytometry analysis was analyzed using Cell Quest<sup>®</sup> software (Becton Dickinson).

**Cell Purification.** LNs (axillary, inguinal, superficial cervical, mandibular, and mesenteric) were harvested from 8–10-wk-old female mice. They were mashed through a wire mesh into HBSS/5% FCS to prepare single cell suspensions. LN preparations were then enriched for T cells on T cell columns (R&D Systems). To purify CD4<sup>+</sup>CD25<sup>+</sup> cells, the enriched T cells were incubated with biotin-conjugated anti-CD25 (15  $\mu$ g/10<sup>8</sup> cells) in PBS/2% FCS for 15 min at 4°C, washed, incubated with FITC-conjugated streptavidin (15  $\mu$ g/10<sup>8</sup> cells) in PBS/2% FCS for 15 min at 4°C, and washed. The cells were then incubated with anti-FITC microbeads (Miltenyi Biotec, Inc., Auburn, CA) for 15 min at 4°C and magnetic separation was performed with a VS<sup>+</sup> positive selection column according to the suggested protocol (Miltenyi). The retained cells were eluted from the column as purified CD4<sup>+</sup>CD25<sup>+</sup> cells. The flow-through was then passed over CD4<sup>+</sup> subset columns (R&D Systems) to obtain CD4<sup>+</sup>CD25<sup>-</sup> cells. The purity of CD4<sup>+</sup>CD25<sup>+</sup> cells typically ranged from 88 to 95%. For some experiments, CD4<sup>+</sup>CD25<sup>+</sup> cells were purified from LN cells by flow cytometry using PE-conjugated anti-CD4 and FITC-conjugated anti-CD25 on a FACStar<sup>®</sup> Cell Sorter (Becton Dickinson); the resultant purity was 99.5%. T cell-depleted spleen suspensions were prepared by first lysing the erythrocytes with ACK lysis buffer, followed by treatment with anti-Thy 1.2 culture supernatants (HO-13.4) and rabbit complement for 45 min at 37°C.

**Proliferation Assays.** CD4<sup>+</sup>CD25<sup>-</sup> cells were cultured in 96-well plates (0.2 ml) with T cell-depleted spleen cells as accessory cells (ACs) (3,000 R irradiated), 0.5  $\mu$ g/ml anti-CD3 (or coated with 10  $\mu$ g/ml anti-CD3 for plate-bound), and the indicated numbers

of CD4<sup>+</sup>CD25<sup>+</sup> cells for 72 h at 37°C/7% CO<sub>2</sub>. Cultures were pulsed with [<sup>3</sup>H]TdR for the last 6 h of culture. Transwell™ experiments were carried out in 24-well plates (0.8 ml) with CD4<sup>+</sup>CD25<sup>-</sup> cells, AC, and 0.5 μg/ml anti-CD3 in the presence or absence of CD4<sup>+</sup>CD25<sup>+</sup> cells in the Transwell™ (Corning Costar, Cambridge, MA).

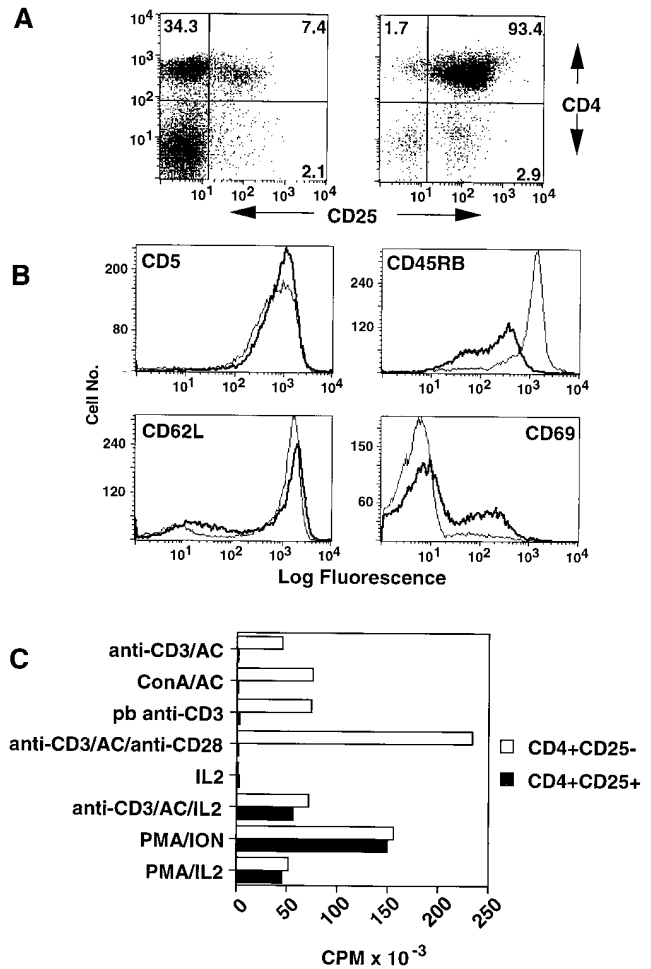
**Reverse Transcriptase PCR Reactions.** CD4<sup>+</sup>CD25<sup>-</sup> cells or CD4<sup>+</sup>CD25<sup>+</sup> cells were purified and were left unstimulated or stimulated for 15 h with T cell-depleted spleen and 0.5 μg/ml anti-CD3. RNA was purified with RNazol B (Tel-test, Friendswood, TX) and cDNA was reverse transcribed from 3 μg of RNA using Superscript II (GIBCO BRL, Gaithersburg, MD). PCR reactions were carried out with Ready To Go® PCR beads (Pharmacia Biotech AB, Piscataway, NJ) using 1 μl of cDNA reaction. β-actin, IL-2, IL-4, IL-10, IFN-γ, and TNF-α primers were purchased from Clontech (Palo Alto, CA). Fas ligand (FasL) upper: 5'-CTGGTGGCTCTGGTTGGAAT-3' and lower: 5'-GTTTAGGGGCTGGTTGTTGC-3' were synthesized by Bio-Synthesis (Lewisville, TX).

**Cytokine ELISA and Northern Blot Analysis.** Cultures for Northern blot analyses and ELISAs were carried out in 24-well plates (0.8 ml) with 5 × 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>-</sup> cells/well, 5 × 10<sup>5</sup> AC, and 0.5 μg/ml anti-CD3 in the presence or absence of 2.5 × 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>+</sup> cells. For ELISAs, supernatants were taken at the indicated times and IL-2 was quantified as previously described (19) using JES6-1A12 as the capture antibody and JES6-5H4 as the detection antibody. The lower limit of detection was 78 pg/ml. For Northern blot analysis, RNA was purified after 16 h, using RNazol B (Tel-test). Northern blots were performed with 5 μg of RNA using the IL-2 PCR fragment or β-actin PCR fragment (using Clontech primers) as a probe. The PCR fragment was labeled with [<sup>32</sup>P]dCTP using an oligolabeling kit (Pharmacia Biotech AB). The final washing conditions were 0.1 × SSC, 0.1% SDS at 65°C.

## Results

**Phenotypic and Functional Characterization of CD4<sup>+</sup>CD25<sup>+</sup> Cells.** The CD4<sup>+</sup>CD25<sup>+</sup> population typically represented 5–8% of the total LN population or 10–15% of CD4<sup>+</sup> cells (Fig. 1 A, left). This population could be isolated easily to levels of 90–95% purity (Fig. 1 A, right) with the magnetic anti-FITC microbead procedure (see Materials and Methods), and the labeled cells could then be analyzed directly on the FACS® or placed into culture. When compared with CD4<sup>+</sup>CD25<sup>-</sup> T cells, the CD4<sup>+</sup>CD25<sup>+</sup> cells were similar in their pattern of expression of CD5, had a slightly higher proportion of CD62L<sup>low</sup> cells, and had a higher proportion of CD69<sup>+</sup> cells (Fig. 1 B). They had an unusual pattern of expression of CD45RB, and were composed primarily of cells that expressed intermediate and low levels. Thus, although modestly enriched in cells that express activation/memory cell markers, the CD4<sup>+</sup>CD25<sup>+</sup> population contained a significant proportion of cells with a naive/resting phenotype. All of the CD4<sup>+</sup>CD25<sup>+</sup> cells expressed TCR-α/β at a level similar to that of the CD4<sup>+</sup>CD25<sup>-</sup> population; the percentage of cells in both populations that expressed a given Vβ was also identical (data not shown).

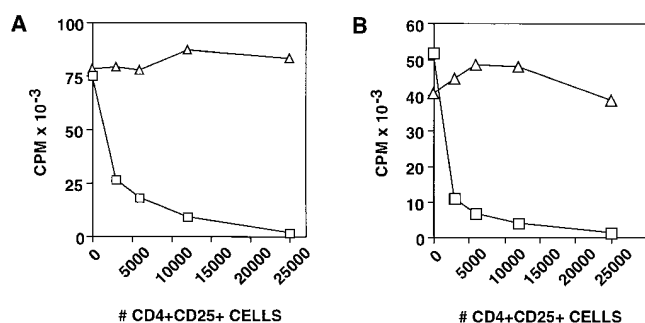
Although their constitutive expression of CD25 raised the possibility that the CD4<sup>+</sup>CD25<sup>+</sup> population might be



**Figure 1.** Characterization of CD4<sup>+</sup>CD25<sup>+</sup> cells. (A) Total lymph node cells (left) or purified CD4<sup>+</sup>CD25<sup>+</sup> cells (right) from BALB/c mice were stained with PE-conjugated anti-CD4 and biotin-conjugated anti-CD25 followed by FITC-conjugated streptavidin. (B) Purified CD4<sup>+</sup>CD25<sup>-</sup> cells or purified CD4<sup>+</sup>CD25<sup>+</sup> cells were double stained for CD25 and the indicated surface molecules. (C) Proliferative responses of CD4<sup>+</sup>CD25<sup>+</sup> cells. Cells (5 × 10<sup>4</sup>) were incubated with the indicated agents (10 ng/ml PMA, 1 mM ionomycin, 3 ng/ml IL-2, 3.0 μg/ml soluble anti-CD3, 3.0 μg/ml Con A, 10 μg/ml anti-CD28, or wells coated with 10 μg/ml anti-CD3) in the presence of AC (5 × 10<sup>4</sup>). Results are expressed as the mean of triplicate cultures.

hyperresponsive to stimulation with IL-2 and/or via the TCR, they were completely unresponsive to stimulation with high concentrations of IL-2, soluble anti-CD3, plate-bound anti-CD3, and Con A in the presence of T cell-depleted spleen cells as AC (Fig. 1 C). Moreover, they were also unresponsive to stimulation with anti-CD3 and optimal concentrations of anti-CD28. However, the addition of IL-2 did restore responsiveness to soluble anti-CD3 to levels similar to those observed with the CD4<sup>+</sup>CD25<sup>-</sup> population. In addition, the CD4<sup>+</sup>CD25<sup>+</sup> responded normally when stimulated with the TCR-independent stimuli, phorbol ester and calcium ionophore or phorbol ester and IL-2.

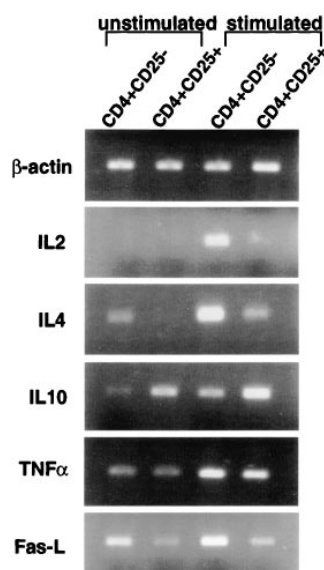
**CD4<sup>+</sup>CD25<sup>+</sup> Cells Suppress the Responses of CD4<sup>+</sup>CD25<sup>-</sup> T Cells.** When the CD4<sup>+</sup>CD25<sup>+</sup> population was



**Figure 2.** CD4<sup>+</sup>CD25<sup>+</sup> cells suppress the proliferation of CD4<sup>+</sup> cells. (A) CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) were incubated with plate bound anti-CD3 (triangles) or with 1.0  $\mu\text{g/ml}$  soluble anti-CD3 (squares) in the presence of AC ( $5 \times 10^4$ ) and the indicated number of CD4<sup>+</sup>CD25<sup>+</sup> cells. (B) CD4<sup>+</sup>CD25<sup>+</sup> cells do not suppress antigen-specific CD4<sup>+</sup> cells from TCR transgenic mice. CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) were purified from DO.11.10 SCID mice on a BALB/c background and stimulated with 0.5  $\mu\text{M}$  ovalbumin peptide (amino acid 323–339; triangles) or 0.5  $\mu\text{g/ml}$  anti-CD3 (squares) in the presence of AC ( $5 \times 10^4$ ) and the indicated number of CD4<sup>+</sup>CD25<sup>+</sup> cells. Results are expressed as the mean of triplicate cultures.

cocultured with CD4<sup>+</sup>CD25<sup>-</sup> cells, marked suppression of the response to stimulation with soluble, but not plate-bound, anti-CD3 was observed (Fig. 2 A). Moreover, responses to Con A in the presence of AC were similarly inhibited and the suppression was not restricted by the MHC as CD4<sup>+</sup>CD25<sup>+</sup> T cells from BALB/c mice suppressed the response of CD4<sup>+</sup>CD25<sup>-</sup> T cells from C57BL/6 mice (data not shown). In multiple experiments of this type, significant suppression (>70%) of the response to soluble anti-CD3 was observed at a final ratio of suppressors/responders of 1:4 and complete suppression was seen at ratios of 1:2. Suppression was not overcome by increasing the concentration of soluble anti-CD3 (data not shown). Most importantly, suppression required activation of the CD4<sup>+</sup>CD25<sup>+</sup> population, as the CD25<sup>+</sup> cells were unable to suppress the antigen-specific responses of CD4<sup>+</sup>CD25<sup>-</sup> T cells from mice that expressed an antiovalbumin transgenic TCR, but could readily suppress the response of the same cells to anti-CD3 (Fig. 2 B).

*The Suppressive Effects of CD4<sup>+</sup>CD25<sup>+</sup> Cells Are Cytokine Independent and Cell Contact Dependent.* It seemed likely that one mechanism by which the CD4<sup>+</sup>CD25<sup>+</sup> T cells could mediate suppression was by the secretion of suppressor cytokines. Supernatants derived from anti-CD3-stimulated CD4<sup>+</sup>CD25<sup>+</sup> contained low levels of IL-10, but contained no detectable levels of IL-2, IL-4, or IFN- $\gamma$  (data not shown). When the CD4<sup>+</sup>CD25<sup>+</sup> cells were examined for cytokine gene expression by reverse transcriptase (RT)-PCR, IL-2 mRNA was not detected, but it was readily detectable when CD4<sup>+</sup>CD25<sup>-</sup> cells were stimulated with anti-CD3 (Fig. 3). This result is consistent with the failure of the CD4<sup>+</sup>CD25<sup>+</sup> T cells to proliferate when stimulated with anti-CD3. Activated CD4<sup>+</sup>CD25<sup>+</sup> cells did not express significant amounts of IL-4 mRNA, but the levels of IL-10 mRNA were increased in both unstimulated and anti-CD3-stimulated CD4<sup>+</sup>CD25<sup>+</sup> populations when com-

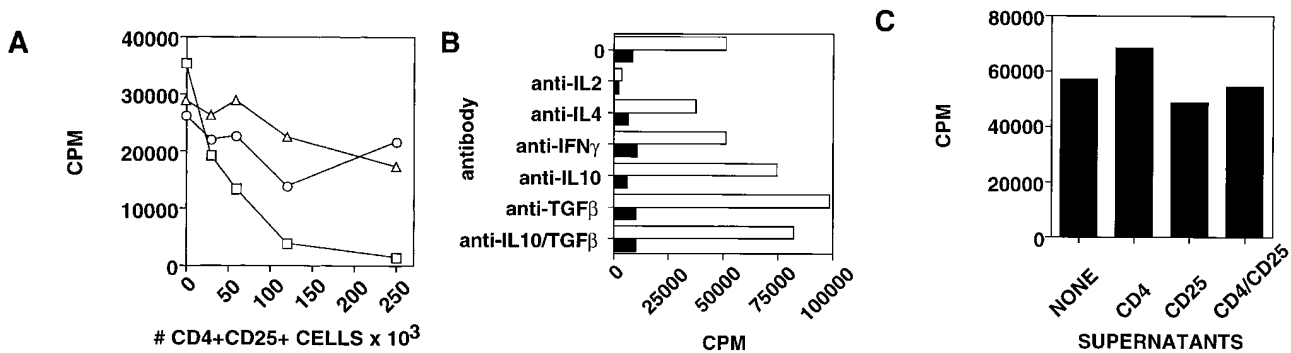


**Figure 3.** PCR analysis of CD4<sup>+</sup>CD25<sup>+</sup> cells. RNA was purified from  $6\text{--}8 \times 10^6$  CD4<sup>+</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD25<sup>+</sup> cells either unstimulated or stimulated with an equivalent number of AC and 0.5  $\mu\text{g/ml}$  anti-CD3 for 15 h. RNA was reverse transcribed and primers for the indicated genes were used to amplify the cDNA. The number of cycles for each primer set are as follows:  $\beta$ -actin, 20 cycles; IL-2, 25 cycles; IL-4, IL-10, TNF- $\alpha$ , and FasL, 30 cycles.

pared with CD4<sup>+</sup>CD25<sup>-</sup> cells. FasL message was lower in resting CD4<sup>+</sup>CD25<sup>+</sup> cells and was not induced by stimulation, whereas TNF- $\alpha$  was upregulated comparably in both CD25<sup>+</sup> and CD25<sup>-</sup> populations. These results are in contrast to the results of Asano et al. (13), in which unstimulated CD4<sup>+</sup>CD25<sup>+</sup> cells expressed IL-2, IL-4, and TGF- $\beta$ , as well as IL-10.

The IL-10 ELISA and RT-PCR data raised the possibility that IL-10 might mediate the suppressive activity of the CD4<sup>+</sup>CD25<sup>+</sup> cells. However, when CD4<sup>+</sup>CD25<sup>+</sup> were separated from CD4<sup>+</sup>CD25<sup>-</sup> cells by culture in a Transwell<sup>TM</sup>, suppression was not observed (Fig. 4 A). Furthermore, the addition of neutralizing antibodies to IL-10 as well as other known suppressive cytokines alone or in combination also failed to abrogate suppression (Fig. 4 B). As it remained possible that an unknown suppressor cytokine was operative in this model, supernatants were collected from stimulated CD4<sup>+</sup>CD25<sup>+</sup> cells or stimulated CD4<sup>+</sup>CD25<sup>-</sup> cells coincubated with CD4<sup>+</sup>CD25<sup>+</sup> cells and tested for their capacity to suppress the responses of freshly explanted CD4<sup>+</sup>CD25<sup>-</sup> cells (Fig. 4 C); however, no suppression was seen. Lastly, to definitively rule out the involvement of IL-4 or IL-10 in this system, CD4<sup>+</sup>CD25<sup>+</sup> T cells were purified from mice genetically deficient in these cytokines. CD4<sup>+</sup>CD25<sup>+</sup> cells from both of the knockout mice were comparable to controls in their ability to suppress (Fig. 5). Collectively, these studies demonstrate that CD4<sup>+</sup>CD25<sup>+</sup> cells do not mediate their suppressive effects by secretion of a soluble suppressor factor.

*CD4<sup>+</sup>CD25<sup>+</sup> Cells Suppress IL-2 Production by CD4<sup>+</sup>CD25<sup>-</sup> Cells.* As an initial approach to an analysis of the cell surface molecules that might be involved in mediating cell contact-dependent suppression in this in vitro model, we added various antibodies to cell surface antigens that could be targets of the CD4<sup>+</sup>CD25<sup>+</sup> cells. We focused our efforts on the two major pathways involved in the interac-



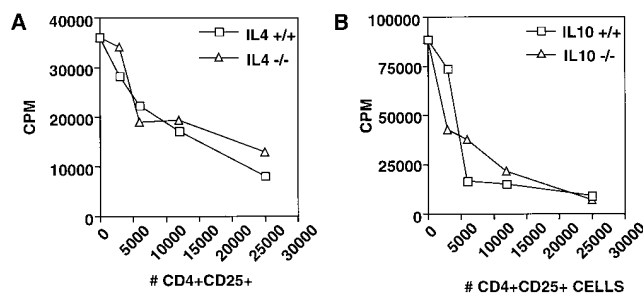
**Figure 4.** A soluble factor does not mediate CD4<sup>+</sup>CD25<sup>+</sup> induced suppression. (A) CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^5$ ) were cultured in 24-well plates in the presence of 3.0  $\mu\text{g/ml}$  soluble anti-CD3 and AC ( $5 \times 10^5$ ). The indicated number of CD4<sup>+</sup>CD25<sup>+</sup> cells was added directly to the culture (squares) or to the transwell in the absence (triangles) or presence (circles) of AC ( $5 \times 10^5$ ). (B) CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) were cultured with AC ( $5 \times 10^4$ ), 0.5  $\mu\text{g/ml}$  anti-CD3, and 10  $\mu\text{g/ml}$  of the indicated antibodies in the absence (white bars) or presence (black bars) of CD4<sup>+</sup>CD25<sup>+</sup> cells ( $2.5 \times 10^4$ ). (C) Supernatants were collected after a 48-h stimulation with soluble anti-CD3 and AC ( $5 \times 10^4$ ) from CD4<sup>+</sup>CD25<sup>-</sup> cells alone ( $5 \times 10^4$ ), CD4<sup>+</sup>CD25<sup>+</sup> cells alone ( $5 \times 10^4$ ), or CD4<sup>+</sup>CD25<sup>-</sup> ( $5 \times 10^4$ ) cells cocultured with CD4<sup>+</sup>CD25<sup>+</sup> cells ( $2.5 \times 10^4$ ). Supernatants (0.1 ml) were then added to CD4<sup>+</sup> cells ( $5 \times 10^4$ ) stimulated with anti-CD3 and AC ( $5 \times 10^4$ ). Results are expressed as the mean of triplicate cultures.

tion between CD4<sup>+</sup> cells and AC, namely CD28-CTLA-4/B71/2 and the CD40/CD40L pathways. As previously reported (20), addition of either anti-CTLA-4 or anti-CD40 had modest enhancing effects on the responses of the CD4<sup>+</sup>CD25<sup>-</sup> cells, but had no effects on the suppression of the responses by the CD4<sup>+</sup>CD25<sup>+</sup> cells (Fig. 6). Anti-B7-2 completely inhibited stimulation of the CD4<sup>+</sup>CD25<sup>-</sup> population, as B7-2 is the major costimulatory molecule expressed by resting T cell-depleted spleen cells that were used as AC. Most importantly, the addition of anti-CD28 or exogenous IL-2 not only markedly enhanced the responses of the CD4<sup>+</sup>CD25<sup>-</sup> cells, but almost completely abrogated the suppressive effects due to CD4<sup>+</sup>CD25<sup>+</sup> cells.

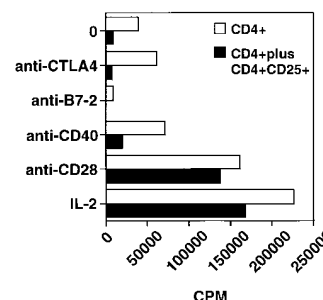
The neutralization of suppression by the addition of IL-2 or anti-CD28 to the cocultures of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> populations, as well as the ability of exogenous IL-2 to overcome the nonresponsiveness of the CD4<sup>+</sup>CD25<sup>+</sup> cells when cultured alone (Fig. 1), raised the possibility that the CD25<sup>+</sup> population was exerting its inhibitory effects by binding IL-2 and functioning as an "IL-2

sink". We initially examined IL-2 production in anti-CD3-stimulated cultures of CD4<sup>+</sup>CD25<sup>-</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, and the mixture of the two populations by ELISA. IL-2 was readily detectable in the supernatants of CD4<sup>+</sup>CD25<sup>-</sup> cells cultured alone, undetectable in the supernatants of CD4<sup>+</sup>CD25<sup>+</sup> cells, and markedly reduced in the supernatants of the coculture (Fig. 7 A). This result is consistent with the possibility that the CD4<sup>+</sup>CD25<sup>+</sup> population was blocking the production of IL-2 by the CD4<sup>+</sup>CD25<sup>-</sup> cells, but it did not rule out the possibility that the CD4<sup>+</sup>CD25<sup>+</sup> cells inhibited proliferation by sequestering IL-2. Therefore, we directly examined the induction of IL-2 mRNA by Northern blot analysis. IL-2 mRNA was readily detected when the CD4<sup>+</sup>CD25<sup>-</sup> cells were cultured alone, but was undetectable when the CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> were cocultured (Fig. 7 B). Although the level of  $\beta$ -actin mRNA was slightly lower in the cocultures, longer exposure of this blot did not reveal IL-2 mRNA. In other experiments, a faint band for IL-2 mRNA could sometimes be seen. In any case, these results strongly support the view that the CD4<sup>+</sup>CD25<sup>+</sup> population exerts its inhibitory effects by blocking the induction of IL-2 production by the CD4<sup>+</sup>CD25<sup>-</sup> cells at the level of RNA transcription.

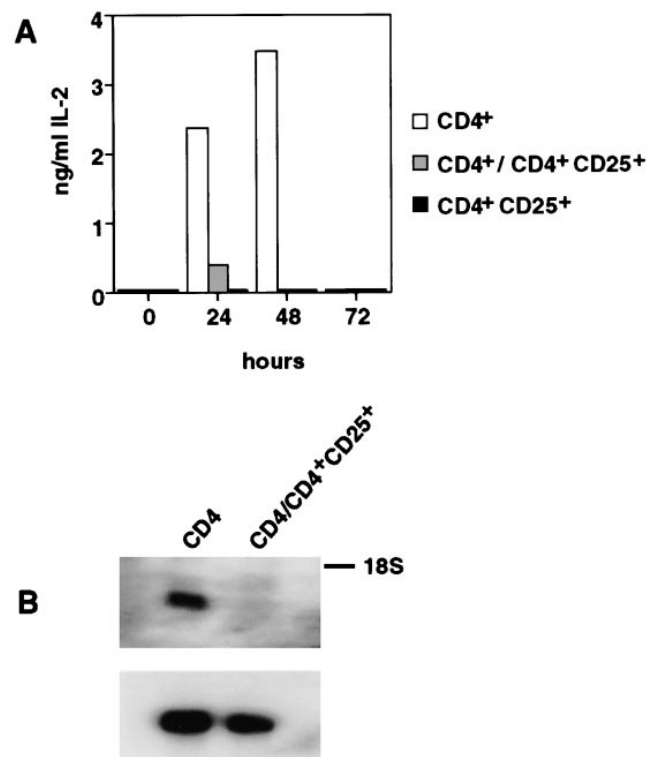
*The Induction of CD25 on CD25<sup>-</sup> Cells Does Not Lead to the Generation of Suppressor Cells.* We and others have shown that the CD4<sup>+</sup>CD25<sup>+</sup> population is solely responsible for



**Figure 5.** CD4<sup>+</sup>CD25<sup>+</sup> cells from IL-10<sup>-/-</sup> and IL-4<sup>-/-</sup> mice suppress proliferation. CD4<sup>+</sup>CD25<sup>+</sup> cells were purified from (A) IL-4<sup>-/-</sup> and BALB/c control mice or (B) IL-10<sup>-/-</sup> and C57BL/10 mice, and the indicated numbers were cocultured with CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) from control mice stimulated with 0.5  $\mu\text{g/ml}$  anti-CD3 and AC ( $5 \times 10^4$ ). Results are expressed as the mean of triplicate cultures.



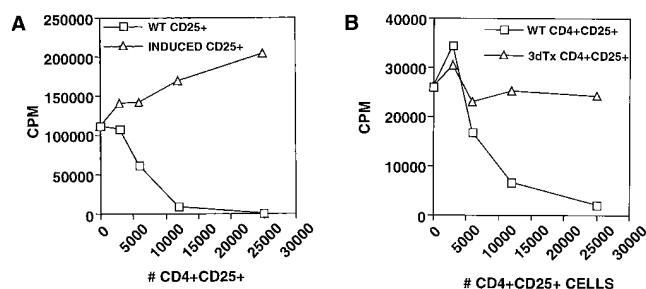
**Figure 6.** Abrogation of suppression by IL-2 or anti-CD28. CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) were cultured in the presence or absence of CD4<sup>+</sup>CD25<sup>+</sup> cells ( $2.5 \times 10^4$ ) and 10  $\mu\text{g/ml}$  of the indicated antibodies or 3 ng/ml IL-2. Results are expressed as the mean of triplicate cultures.



**Figure 7.** CD4<sup>+</sup>CD25<sup>+</sup> cells suppress IL-2 production. (A) CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ), AC ( $5 \times 10^4$ ), and 0.5  $\mu$ g/ml anti-CD3 were cultured in the presence or absence of CD4<sup>+</sup>CD25<sup>+</sup> cells ( $2.5 \times 10^4$ ), supernatants were taken at the indicated times and IL-2 was quantified. Each sample was tested in triplicate. (B) RNA was purified from CD4<sup>+</sup> cells stimulated in the presence or absence of CD4<sup>+</sup>CD25<sup>+</sup> cells after 16 h using RNazol B (Tel-test). RNA was separated by gel electrophoresis, transferred to nitrocellulose and probed for IL-2 message (top) or  $\beta$ -actin message (bottom) by using an IL-2 PCR fragment or a  $\beta$ -actin PCR product as a probe.

inhibition of the induction of autoimmunity after 3dT<sub>x</sub> (13, 14). In addition, we have also demonstrated that the induction of CD25 expression on CD25<sup>-</sup> T cells derived from mice that express a transgenic TCR on a SCID background was not sufficient to suppress the induction of autoimmune disease after 3dT<sub>x</sub> in vivo. This result raised the possibility that the CD4<sup>+</sup>CD25<sup>+</sup> cells present in normal mice represent a unique cell population. To extend these studies to our in vitro model, we purified CD4<sup>+</sup>CD25<sup>-</sup> from normal mice, cultured them for 24 h in the presence of Con A to induce CD25 expression, and then mixed them with freshly isolated CD4<sup>+</sup>CD25<sup>-</sup> cells. Although >80% of the Con A-stimulated CD25<sup>-</sup> cells expressed CD25 (data not shown), no suppression of the proliferative responses of the CD4<sup>+</sup>CD25<sup>-</sup> cells was observed (Fig. 8 A). This result demonstrates that the mere expression of CD25 is insufficient to mediate suppression.

It has been postulated that the induction of autoimmunity after 3dT<sub>x</sub> is secondary to a deficiency of the CD4<sup>+</sup>CD25<sup>+</sup> population that develops later during ontogeny (13). Paradoxically, a greater percentage (25–30 versus 10%) of CD4<sup>+</sup> T cells in 3dT<sub>x</sub> mice express CD25 compared with



**Figure 8.** (A) CD4<sup>+</sup>CD25<sup>+</sup> cells were purified from BALB/c mice while CD4<sup>+</sup>CD25<sup>-</sup> cells were obtained by cell sorting and were cultured with AC and Con A for 24 h and were then used as induced CD4<sup>+</sup>CD25<sup>+</sup> cells. Induced or control CD4<sup>+</sup>CD25<sup>+</sup> cells were then cocultured with 0.5  $\mu$ g/ml anti-CD3 and AC. (B) CD4<sup>+</sup>CD25<sup>+</sup> cells were purified from 3dT<sub>x</sub> mice or BALB/c control mice and cocultured with CD4<sup>+</sup>CD25<sup>-</sup> cells from control mice stimulated with 0.5  $\mu$ g/ml anti-CD3 and AC. Results are expressed as the mean of triplicate cultures.

normal mice (18). To rule out the possibility that the in vivo induction of CD25 would render cells suppressive, CD4<sup>+</sup>CD25<sup>+</sup> cells were purified from 3dT<sub>x</sub> mice between 4 and 6 mo of age and added to cultures of CD4<sup>+</sup>CD25<sup>-</sup> cells from normal BALB/c mice. No suppression was observed in these cocultures (Fig. 8 B). These results again support the view that the CD4<sup>+</sup>CD25<sup>+</sup> cells present in normal mice are a unique suppressor population and that the CD4<sup>+</sup>CD25<sup>+</sup> in the 3dT<sub>x</sub> mice are autoimmune effector cells.

## Discussion

The role of immunoregulatory or suppressor T cells has been well documented in the immunologic literature over the past 25 years. However, a great deal of controversy remains as to their lineage, antigen specificity, and mechanism of action. The concept of antigen-specific suppressor factors that acted in a complex cascade has not been validated by biochemical and molecular studies (21). Many of the older in vivo studies on the activity of suppressor cells have been reinterpreted as being secondary to alterations in the Th1–Th2 balance that was induced by different modes of antigen delivery. Most recent data on the importance of suppressor cells has been derived from their involvement in mediating transplantation tolerance and in preventing the induction of autoimmune disease. The induction of autoimmune disease after 3dT<sub>x</sub> has been considered for many years to be secondary to a deficiency of T suppressor cells that normally develop after day 3 of life. The suppressor cells in this model belong to the minor subpopulation of CD4<sup>+</sup> T cells that coexpress CD25 and are capable of suppressing the induction of autoimmunity after 3dT<sub>x</sub>, the induction of autoimmunity induced by transfer of CD4<sup>+</sup>CD25<sup>-</sup> T cells to *nu/nu* recipients, and the capacity of activated autoantigen-specific T cell clones to induce disease in *nu/nu* recipients.

The goal of our study was to develop an in vitro model system in which the function of these potent CD4<sup>+</sup>CD25<sup>+</sup>

T cells could be analyzed. We found that the CD4<sup>+</sup>CD25<sup>+</sup> cells were themselves completely nonresponsive to stimulation by TCR-derived signals in the presence or absence of costimulation. More importantly, they could adoptively suppress the responses of CD4<sup>+</sup>CD25<sup>-</sup> cells in coculture studies. Although suppression in many model systems *in vivo* and *in vitro* is mediated by the secretion of one of the suppressor cytokines (IL-4, IL-10, or TGF- $\beta$ ), the mechanism of suppression by CD4<sup>+</sup>CD25<sup>+</sup> cells appeared to be mediated by a contact-dependent mechanism. No suppression was seen when the suppressors were separated from the responders by a semipermeable membrane, and supernatants from stimulated CD4<sup>+</sup>CD25<sup>+</sup> cells could not transfer suppression. Neutralizing antisuppressor cytokine mAbs alone or in combination also failed to abrogate suppression. Finally, to rule out the involvement of the newly described (22) T regulatory 1 population that exerts bystander suppression by the secretion of IL-10, we demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> cells from both IL-4<sup>-/-</sup> and IL-10<sup>-/-</sup> donors were as effective as T cells from wild-type mice in mediating suppression *in vitro*. We have also shown that these same CD4<sup>+</sup>CD25<sup>+</sup> cells from cytokine-deficient mice are capable of mediating suppression of autoimmune disease *in vivo* when they are transferred to d3Tx mice (McHugh, R., A.M. Thornton, and E.M. Shevach, unpublished observations). Although we were unable to neutralize suppression *in vitro* with anti-TGF- $\beta$ , it still remains possible that TGF- $\beta$  may play some role in the action of these cells *in vivo*, as TGF- $\beta$ <sup>-/-</sup> mice develop spontaneous inflammatory disease of a variety of organs and have an increase in Th1 inflammatory cytokines (23). The potential relationship of the CD4<sup>+</sup>CD25<sup>+</sup> population to the CD45RB<sup>low</sup> cells whose ability to protect animals from inflammatory bowel disease could be reversed by anti-TGF- $\beta$  (24) and with TGF- $\beta$ -producing suppressor populations (the so-called Th3 cells) induced by oral delivery of antigen (25, 26) remains to be determined.

The requirement for cell contact to observe suppression raised the possibility that the CD4<sup>+</sup>CD25<sup>+</sup> population might be mediating suppression by actually killing the responder T cell population by the Fas/FasL pathway. However, no evidence for cell death was observed in the cocultures and identical numbers of viable cells were recovered in the presence or absence of the CD4<sup>+</sup>CD25<sup>+</sup> cells after 24 h of culture (data not shown). Furthermore, we could not detect mRNA by RT-PCR for the FasL after stimulation of the CD4<sup>+</sup>CD25<sup>+</sup> cells with anti-CD3, whereas it was readily induced in the CD4<sup>+</sup>CD25<sup>-</sup> population. Another potentially trivial mechanism by which the CD25<sup>+</sup> population might be mediating inhibition was by passively absorbing IL-2 produced by the responder cells. We definitively ruled out this possibility by demonstrating that the suppressor cells almost completely inhibited IL-2 gene transcription and hence IL-2 production in the responder T cell population. Inhibition of IL-2 gene transcription has also been observed with suppressor T cell populations isolated from animals that have been recently subjected to to-

tal lymphoid irradiation (TLI; reference 27). No information is available on the phenotype of the suppressor cells in that model, but spleen cells from TLI-treated animals contain increased numbers of CD4<sup>-</sup>CD8<sup>-</sup>CD3<sup>low</sup> cells. Although the binding of IL-2 by the CD4<sup>+</sup>CD25<sup>+</sup> cells appeared to play no role in suppression, it is still possible that the expression of CD25 may be related to the functional capacity of these cells to inhibit the development of autoimmune effector cells as IL-2<sup>-/-</sup>, IL-2R  $\beta$ -chain (CD122)<sup>-/-</sup>, and CD25<sup>-/-</sup> mice develop multiple manifestations of inflammatory autoimmune diseases (28–31). These studies suggest that IL-2 itself may either directly or indirectly play an important role in the development and/or function of this unique population of CD25<sup>+</sup> suppressor cells.

There are a number of unique aspects of suppression in this *in vitro* model which may offer some insight into the physiological function of the CD4<sup>+</sup>CD25<sup>+</sup> cells *in vivo*. First, suppression *in vitro* required that the suppressor population be exposed and presumably activated via the TCR since the antigen-specific response of naive T cells from TCR transgenic mice was not suppressed by the CD4<sup>+</sup>CD25<sup>+</sup> cells, whereas the responses of the same cell mixture to anti-CD3 stimulation were completely suppressed. Second, the responses to soluble anti-CD3 in the presence of normal T cell-depleted spleen cells were easily suppressed, whereas the responses to plate-bound anti-CD3 were unaffected. This result may be secondary to a qualitatively distinct activating signal by plate-bound mAb, but is also consistent with the possibility that the target of the suppressor population is actually the AC rather than the responding T cell. Lastly, suppression could be overcome in the coculture studies by the addition of IL-2 or by enhancing endogenous IL-2 production by the addition of anti-CD28 to the cultures. It thus appears that the induction of the IL-2R on the responder population is not blocked by the addition of the suppressor population, and we have confirmed this by FACS<sup>®</sup> analysis (data not shown). Again, this observation is consistent with the possibility that the AC is the target of the suppressor cell; however, as of yet we have not been able to demonstrate that the suppressor cells interfered with the delivery of either positive or negative costimulatory signals. The addition of anti-CTLA-4 did not reverse suppression (Fig. 6), and CD4<sup>+</sup>CD25<sup>+</sup> cells from either CD28<sup>-/-</sup> or CD40L<sup>-/-</sup> mice were fully capable of inhibiting anti-CD3 stimulation *in vitro* (data not shown).

It should also be noted that the nonresponsiveness of the purified CD4<sup>+</sup>CD25<sup>+</sup> to stimulation with anti-CD3 could be overcome by the addition of exogenous IL-2. It is possible that the anergic state of the CD4<sup>+</sup>CD25<sup>+</sup> population was broken by the addition of IL-2. Alternatively, as the CD4<sup>+</sup>CD25<sup>+</sup> population is heterogeneous in expression of the membrane markers we have studied (Fig. 1 B), the response seen in the presence of IL-2 may be secondary to CD25<sup>+</sup> conventional memory T cells that “contaminate” the suppressor cell population. Surprisingly, the addition of anti-CD28 had no effect on the proliferative responses of

the CD4<sup>+</sup>CD25<sup>+</sup> population, while abrogating the suppression in the cocultures of the CD25<sup>+</sup> and CD25<sup>-</sup> cells. In the latter case, the anti-CD28 may directly stimulate the CD25<sup>-</sup> cells to produce IL-2, whereas in the former the vast excess of suppressors may still be capable of inhibiting anti-CD28-induced IL-2 production by the few conventional memory cells present in the CD25<sup>+</sup> pool.

Sakaguchi et al. (32) have proposed that any CD25<sup>+</sup> cell, rather than a distinct, functional CD4<sup>+</sup>CD25<sup>+</sup> subset, may mediate suppression. We have previously shown that the induction of CD25 on a homogeneous population of CD25<sup>-</sup> T cells derived from the TCR-transgenic SCID mouse was insufficient to prevent the induction of post-3dTX autoimmunity in vivo. Similarly, suppression of proliferation was not seen when CD25<sup>+</sup> cells, generated by in vitro mitogen stimulation of CD25<sup>-</sup> cells, were added to fresh CD25<sup>-</sup> populations. Not surprisingly, CD25<sup>+</sup> T cells derived from 3dTx animals were also unable to mediate suppression. In both of these latter situations, enhancement of proliferation was usually observed. We have been unable to detect any differences in the level of expression of the  $\alpha/\beta$ -TCR between CD25<sup>+</sup> and CD25<sup>-</sup> cells; the pattern of V $\beta$  usage was also identical in both populations. One caveat in the interpretation of these results is the potential heterogeneity of the CD25<sup>+</sup> population. Studies are now in progress to determine whether the suppressive activity of the CD25<sup>+</sup> cells can be localized to a smaller subpopulation (e.g., CD69<sup>+</sup>, CD62L<sup>low</sup>, CD45RB<sup>low or int</sup>). It is possible that restrictive TCR usage might be observed if suppressor activity can be localized to a more defined subpopulation of CD25<sup>+</sup> cells. Our data support the concept that the CD4<sup>+</sup>CD25<sup>+</sup> may represent a distinct lineage of professional suppressor cells.

One of the major drawbacks of this in vitro model is that as yet we have been unable to separate the requirements for activation of the suppressor populations from those of the responder populations and this has hindered our further analysis of the antigen specificity (if any) of the suppressor cells and their cellular targets. Several studies (33, 34) have

presented evidence that the CD4<sup>+</sup>CD25<sup>+</sup> population in the 3dTx model recognizes the same set of autoantigens as the autoantigen-specific effector pool, but these findings have not been confirmed (35). No data are available as to the antigen-specificity of the regulatory cells in the models where lymphopenic animals are reconstituted with CD45RB<sup>low</sup> cells although it has been proposed that the T cells that mediate dominant tolerance recognize the autoantigen or peptides derived from it (36) and that both the effector and suppressor repertoires are generated in the thymus. No studies have suggested that receptor-based immunoregulation (37, 38) is operative in these model systems. Although our in vitro studies on polyclonal activation do not address the issue of the antigen-specificity of suppressor cells, they are most consistent with a model in which the suppressor and effector populations compete at the AC surface for antigen and/or costimulatory signals. To a certain extent, our results resemble the observations of Lombardi et al. (39), who have shown that one mechanism by which anergic antigen-specific T cell clones inhibit proliferative responses of normal T cell clones in cocultures is by competition for antigen or costimulatory signals generated at the surface of the AC. Cobbold et al. (40) have also proposed that the suppressor populations induced by nondepleting anti-CD4 mAbs in a model of infectious transplantation tolerance are alloantigen-specific cells that are incapable of differentiating into effectors, but function by inhibiting the delivery of antigen/costimulatory signals to the effector population. One possibility is that the CD4<sup>+</sup>CD25<sup>+</sup> cells are themselves specific for ubiquitously expressed autoantigens. Such cells would have escaped negative selection in the thymus because they would have downregulated their TCR signaling properties and their capacity to differentiate into effector cells. However, they could compete for costimulatory signals with low affinity autoreactive precursor cells on the surface of the same AC. The in vitro model system described in this report should facilitate identification of the antigen specificity and the mechanism of suppression of these potent regulatory cells.

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