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Angela M. Thornton and Ethan M. Shevach

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# Suppressor Effector Function of CD4<sup>+</sup>CD25<sup>+</sup> Immunoregulatory T Cells Is Antigen Nonspecific

Angela M. Thornton and Ethan M. Shevach<sup>1</sup>

CD4<sup>+</sup>CD25<sup>+</sup> T cells represent a unique population of “professional” suppressor T cells that prevent induction of organ-specific autoimmune disease. *In vitro*, CD4<sup>+</sup>CD25<sup>+</sup> cells were anergic to stimulation via the TCR and when cultured with CD4<sup>+</sup>CD25<sup>-</sup> cells, markedly suppressed polyclonal T cell proliferation by specifically inhibiting the production of IL-2. Suppression was cytokine independent, cell contact dependent, and required activation of the suppressors via their TCR. Further characterization of the CD4<sup>+</sup>CD25<sup>+</sup> population demonstrated that they do not contain memory or activated T cells and that they act through an APC-independent mechanism. CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from TCR transgenic (Tg) mice inhibited responses of CD4<sup>+</sup>CD25<sup>-</sup> Tg T cells to the same Ag, but also inhibited the Ag-specific responses of Tg cells specific for a distinct Ag. Suppression required that both peptide/MHC complexes be present in the same culture, but the Ags could be presented by two distinct populations of APC. When CD4<sup>+</sup>CD25<sup>+</sup> T cells were cultured with anti-CD3 and IL-2, they expanded, remained anergic, and in the absence of restimulation via their TCR, suppressed Ag-specific responses of CD4<sup>+</sup>CD25<sup>-</sup> T cells from multiple TCR transgenics. Collectively, these data demonstrate that CD4<sup>+</sup>CD25<sup>+</sup> T cells require activation via their TCR to become suppressive, but once activated, their suppressor effector function is completely nonspecific. The cell surface molecules involved in this T-T interaction remain to be characterized. *The Journal of Immunology*, 2000, 164: 183–190.

The development of autoimmune disease involves a breakdown in the mechanisms that control self vs non-self discrimination. The primary mechanism that leads to self tolerance is thymic deletion of autoreactive T cells, but thymic deletion is not perfect and autoreactive T cells do escape to the periphery. Cells that escape thymic deletion are then subject to mechanisms of peripheral tolerance including T cell anergy (1) and T cell ignorance/indifference (2). However, anergy can be reversible and ignorant T cell populations have the potential to be activated when their target self-Ags 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 other mechanisms of tolerance (4).

A variety of organ-specific autoimmune diseases can be induced in rodent strains that are not normally susceptible by interfering with normal T cell maturation or by causing a partial T cell deficiency (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. (7) 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. 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 (NOD) mouse strains that spontaneously develop autoimmune diabetes (8, 9). CD4<sup>+</sup> T cells that express TCRs encoded by endogenous  $\alpha/\beta$ -chain genes are also likely to be responsible for the relative disease resistance of mice that express a transgenic (Tg)<sup>2</sup> TCR specific for a peptide from myelin basic protein (10).

Studies using two different model systems have demonstrated that a potent CD4<sup>+</sup> immunoregulatory T cell population can be defined by expression of the IL-2R  $\alpha$ -chain (CD25). In the first model system (11, 12), genetically susceptible mice that were thymectomized on day 3 of life (d3Tx) 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 within 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. In addition, we have also 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>

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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<sup>1</sup> Address correspondence and reprint requests to Dr. Ethan M. Shevach, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 10, Room 11N311, 10 Center Drive, MSC 1892, Bethesda, MD 20892-1892. E-mail address: ems1@box-e.nih.gov

<sup>2</sup> Abbreviations used in this paper: Tg, transgenic; d3Tx, thymectomized on day 3 of life; HA, hemagglutinin; PCC, pigeon cytochrome c.

population can inhibit both the induction and effector function of autoreactive T cells.

We have previously developed an *in vitro* model system for suppressor T cell function and have demonstrated that the CD4<sup>+</sup>CD25<sup>+</sup> T cell population present in normal mice was a potent inhibitor of polyclonal T cell activation (17). Suppression was mediated by a cytokine-independent, cell contact-dependent mechanism that required activation of the CD4<sup>+</sup>CD25<sup>+</sup> cells via the TCR. The CD4<sup>+</sup>CD25<sup>+</sup> cells inhibited the induction of IL-2 production in the responder CD4<sup>+</sup>CD25<sup>-</sup> population. Although the responses to soluble anti-CD3 in the presence of normal T-depleted spleen cells were easily suppressed, the responses to plate-bound anti-CD3 were unaffected and suppression could be overcome by the addition of exogenous IL-2 or by enhancing endogenous IL-2 production by the addition of anti-CD28 to the cultures.

In this report, we extend our *in vitro* studies of the function of the CD4<sup>+</sup>CD25<sup>+</sup> population and demonstrate that they appear to be a homogeneous population of suppressors that do not contain memory or activated T cells. Although our previous studies suggested that the target of the suppressor population was actually the APC rather than the responding T cell, we now demonstrate that CD4<sup>+</sup>CD25<sup>+</sup> cells act through an APC-independent mechanism. In addition, we show that CD4<sup>+</sup>CD25<sup>+</sup> T cells present in the peripheral lymphoid tissues of TCR Tg mice can inhibit Ag-specific proliferation of TCR Tg cells specific for the same Ag. However, once the CD4<sup>+</sup>CD25<sup>+</sup> TCR Tg T cells are activated by their cognate Ag, their suppressor effector function is completely Ag nonspecific. Furthermore, we have generated short-term cell lines by stimulation of CD4<sup>+</sup>CD25<sup>+</sup> cells with anti-CD3 and IL-2. Such cell lines remained anergic when stimulated with anti-CD3, but exhibited enhanced Ag nonspecific suppressor cell function that no longer required engagement of their TCR. Collectively, these studies are most compatible with a model in which CD4<sup>+</sup>CD25<sup>+</sup> cells require activation with specific Ag to develop their suppressive activity; however, once stimulated, they are competent to suppress in an Ag-independent manner.

## Materials and Methods

### Mice and cell lines

Female BALB/c and C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). Hemagglutinin (HA) TCR Tg (18) and pigeon cytochrome *c* (PCC) TCR Tg (19) mice were maintained at Taconic (Germantown, NY) under National Institute of Allergy and Infectious Diseases contract. HNT TCR Tg mice were obtained from D. Lo (The Scripps Institute, La Jolla, CA) (20) and were bred in our facilities. P815 cells transfected with B7-2 were obtained from L. Lanier (21)

### Media, reagents, and Abs

All cells were grown in RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (all from Biofluids, Rockville, MD), and 50 µM 2-ME (Sigma, St. Louis, MO). Biotin-anti-CD25 (7D4), FITC-streptavidin, PE-anti-CD45RB (16A), PE-anti-CD62L (Mel-14), PE-anti-CD69, FITC-anti-CD69, PE anti-CD38, PE anti-B7-2, FITC-anti-B220, FITC-anti-K<sup>b</sup>, PE-anti-K<sup>b</sup> FITC-anti-Fas, and purified anti-CD3 (2C11) were purchased from PharMingen (San Diego, CA). PE-anti-CD4 was purchased from Becton Dickinson (Mountain View, CA). Tricolor-anti-CD4 was purchased from Caltag (Burlingame, CA). Human rIL-2 was purchased from Peprotech (Rocky Hill, NJ). Flow cytometry analysis was analyzed using CellQuest software (Becton Dickinson). HA<sub>110-119</sub>, PCC<sub>88-104</sub>, and HNT<sub>126-138</sub> peptides were synthesized and purified by HPLC by the Laboratory of Molecular Structure, Peptide Synthesis Laboratory (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and used at 32 µM, 0.1 µM, and 1 µg/ml final concentration, respectively.

### Cell purification

CD4<sup>+</sup>CD25<sup>+</sup> cells were purified as previously described (17). 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 by flow cytometry on a FACStar Cell Sorter (Becton Dickinson). T-depleted spleen cells (TAS) were used as APC and were prepared by first lysing the erythrocytes with ACK lysis buffer, followed by treatment with anti-Thy 1.2 culture supernatant (HO-13.4) and rabbit C for 45 min at 37°C. The cells were then irradiated at 3000 rad. LPS-TAS were made by treating TAS with 10 µg/ml LPS for 48 h. Cells were fixed in 0.5% paraformaldehyde for 30 min at 37°C. To generate IL-2-treated CD4<sup>+</sup>CD25<sup>+</sup> cells, CD4<sup>+</sup>CD25<sup>+</sup> cells (typically 1–2 × 10<sup>6</sup>), purified by cell sorting, were cultured with an equivalent number of APC, 0.5 µg/ml anti-CD3, and 5 ng/ml IL-2 for 3 days and were then split and cultured in medium containing IL-2 for an additional 3–4 days.

### Proliferation assays

CD4<sup>+</sup>CD25<sup>-</sup> cells (5 × 10<sup>4</sup>) were cultured in 96-well plates (0.2 ml) with APC (5 × 10<sup>4</sup>), 0.5 µg/ml anti-CD3, 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. All experiments were set up in triplicate.

### Cell cycle analysis

CD4<sup>+</sup>CD25<sup>-</sup> cells purified from C57BL/6 mice were cultured with an equivalent number of APC from BL/6 mice and 0.5 µg/ml anti-CD3 in the absence or presence of CD4<sup>+</sup>CD25<sup>+</sup> cells (2.5 × 10<sup>4</sup>) purified from BALB/c mice. The cells were cultured for 96 h and stained and processed as described (22).

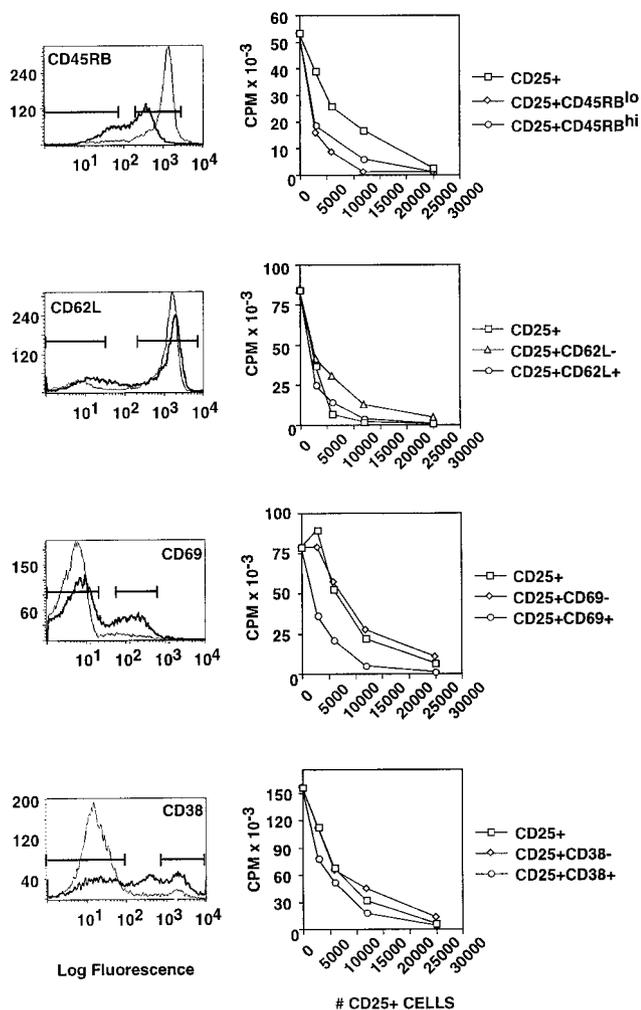
## Results

### The CD4<sup>+</sup>CD25<sup>+</sup> population does not contain conventional activated/memory T cells

Because the CD4<sup>+</sup>CD25<sup>+</sup> comprise 10% of the CD4<sup>+</sup> T cells in normal mouse peripheral lymphoid tissues, it remained possible that this population contained activated T cells that had been induced to express CD25 by exposure to environmental Ags *in vivo*. In addition, the CD4<sup>+</sup>CD25<sup>+</sup> population had an unusual pattern of expression of membrane markers that are associated with memory/activated cells. Notably, the CD4<sup>+</sup>CD25<sup>+</sup> population lacked the CD45RB<sup>high</sup> subset of T cells and consisted only of CD45RB<sup>int</sup> and CD45RB<sup>low</sup> cells. On the other hand, the CD4<sup>+</sup>CD25<sup>+</sup> population only contained a modest increase in CD62L<sup>low</sup> cells (35% vs 25%) and CD69<sup>+</sup> (35% vs 8%) T cells when compared with the CD4<sup>+</sup>CD25<sup>-</sup> population (Fig. 1). Approximately, 60% of the CD25<sup>+</sup> cells expressed CD38, which has recently been defined by Read et al. (23) as a marker for T cells with *in vitro* immunosuppressive activity very similar to the CD4<sup>+</sup>CD25<sup>+</sup> cells. We therefore attempted to identify a population within the CD4<sup>+</sup>CD25<sup>+</sup> pool that might have enhanced or diminished immunosuppressive functions based on the differential expression of one of the activation/memory markers. However, as shown in Fig. 1, any subpopulation of T cells that expressed CD25 was a potent inhibitor of the proliferative response of CD4<sup>+</sup>CD25<sup>-</sup> T cells to anti-CD3. Although minor differences were observed between some of the subpopulations in different experiments, no consistent subpopulation with an altered suppressor function could be identified. It should be pointed out that the assay used in this study could readily detect a population which lacked suppressor activity as we have previously shown that CD25<sup>+</sup> T cells from d3Tx animals, that lack suppressor function *in vivo*, that failed to suppress *in vitro*, and that induction of CD25 expression on CD25<sup>-</sup> cells by TCR stimulation also failed to result in the induction of suppressor function (17).

### CD4<sup>+</sup>CD25<sup>+</sup> cells do not prevent the induction or the delivery of costimulatory signals

Our previous studies suggested that CD4<sup>+</sup>CD25<sup>+</sup> cells might inhibit proliferation of CD4<sup>+</sup>CD25<sup>-</sup> cells by acting upon the APC



**FIGURE 1.** The suppressive effect of  $CD4^+CD25^+$  cells cannot be further subdivided. Lymph node (LN) cells from BALB/c mice were stained with tricolor-anti-CD4 and biotin-anti-CD25 followed by streptavidin-FITC and one of the following: PE-anti-CD45RB, PE-anti-CD62L, PE-anti-CD69, or PE-anti-CD38, and the various subsets of cells were purified by cell sorting.  $CD4^+CD25^-$  cells ( $5 \times 10^4$ ) were incubated with APC ( $5 \times 10^4$ ), anti-CD3, and the indicated numbers of either  $CD4^+CD25^+$  cells or  $CD4^+CD25^+$  cells further subdivided based on the FACS profiles shown to the left.  $CD4^+CD25^+$  cells are shown in the bold line, whereas  $CD4^+CD25^-$  cells are shown in the lighter line. Results of the proliferation assays are shown as the mean of triplicate cultures.

either by inhibiting the induction of costimulatory molecules or by competing for costimulatory signals. We first investigated the possibility that  $CD4^+CD25^+$  cells inhibited the induction of costimulatory molecules by examining CD86 expression by flow cytometry. As shown in Fig. 2A, CD86 was not expressed on APC cultured alone, but was up-regulated on most B220-positive cells after 48 h of culture with purified  $CD4^+$  T cells and anti-CD3. When  $CD4^+CD25^+$  cells were added, CD86 expression was not affected. In addition, up-regulation of CD40 and ICAM-1 expression was not inhibited in the presence of the  $CD25^+$  cells (data not shown). To further confirm that  $CD4^+CD25^+$  cells did not inhibit proliferation of  $CD4^+CD25^-$  cells by inhibiting the induction of expression or functional activation of other molecules involved in costimulation or cell adhesion on the APC,  $CD4^+CD25^-$  cells were stimulated with APC that were already fully competent to provide costimulation. Following stimulation of T-depleted spleen cells with LPS for 48 h, nearly 100% of the B220 positive cells

expressed CD86 at high levels (data not shown). When these LPS-activated T-depleted spleen cells were used as APC,  $CD4^+CD25^+$  cells were still capable of inhibiting proliferation, regardless of whether the APC were irradiated or fixed (Fig. 2B). Similar results were seen when P815 cells stably transfected with CD86 were used as APC. These results also argue against the possibility that the  $CD25^+$  T cells inhibit accessory cell function by preventing the up-regulation or activation of cell interaction molecules other than CD86.

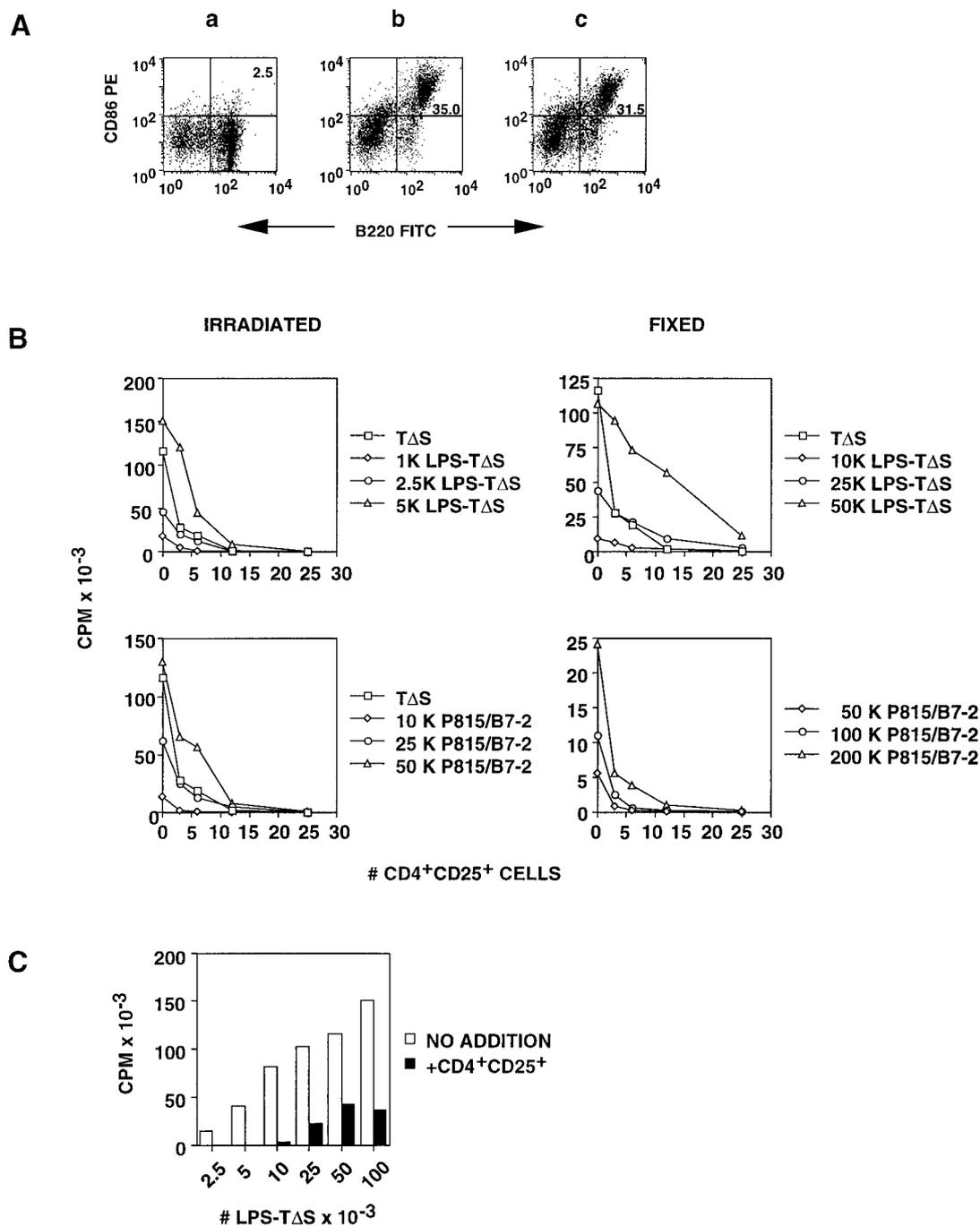
We next examined whether  $CD4^+CD25^+$  cells competed for the delivery of costimulation by examining whether suppression could be overcome by an excess of activated APC. We cultured a fixed number of  $CD25^-$  responder cells with a fixed number of  $CD25^+$  suppressor cells and varied the number of LPS-activated T-depleted spleen cells as APC (Fig. 2C). Significant proliferative responses were observed at the lowest concentration of APC (2500/well), and this response was almost completely inhibited by the  $CD25^+$  cells. Although the proliferative responses were enhanced by addition of higher numbers of APC, suppression only decreased from 95% to 75%. Thus, it is very unlikely that  $CD4^+CD25^+$  cells compete for the delivery of costimulatory molecules.

#### *CD4<sup>+</sup>CD25<sup>+</sup> cells induce cell cycle arrest*

Our previous studies demonstrated that  $CD4^+CD25^+$  cells did not appear to kill the responding  $CD4^+CD25^-$  cells (17). Although the  $CD4^+CD25^+$  cells inhibited the induction of IL-2 mRNA synthesis by  $CD4^+CD25^-$  cells, it was unclear what stage of the T cell activation process was arrested by the  $CD4^+CD25^+$  cells. As suppression of the response to anti-CD3 could be seen with histoincompatible combinations of suppressors and responders,  $CD4^+CD25^-$  cells from C57BL/6 mice were cocultured with  $CD4^+CD25^+$  cells from BALB/c mice and activation markers were examined by flow cytometry on the  $K^b$ -positive responding cells. Following 24 h of stimulation with anti-CD3, up-regulation of CD25 and CD69 on the responder cells was observed in the absence or presence of  $CD4^+CD25^+$  cells (Fig. 3A). Although the expression of CD25, CD69, and Fas continued to increase over the next 48 h of culture in the absence of  $CD4^+CD25^+$  cells, no further up-regulation of these markers was seen on responders cultured in the presence of  $CD4^+CD25^+$  cells. Furthermore, in the presence of  $CD4^+CD25^+$  cells, the responders did not blast, as indicated by a lack of increase in forward scatter, and did not progress into the M or S phases of the cell cycle (Fig. 3B).

#### *Ag-specific CD4<sup>+</sup>CD25<sup>+</sup> T cells can be isolated from TCR Tg mice on a conventional background*

In our initial studies, the requirement for activation of the suppressor cell via the TCR was apparent as  $CD4^+CD25^+$  cells from normal BALB/c mice could suppress the anti-CD3, but not the Ag (OVA), response of  $CD4^+$  T cells from DO11.10 TCR Tg mice. Therefore, we were unable to separate the activation signals for induction of suppressor cell function from the requirements of responder T cell activation because anti-CD3 was used to stimulate both cell populations (17). Although we failed to identify  $CD4^+CD25^+$  in TCR Tg SCID mice (14), Takahashi et al. (24) have shown that  $CD4^+CD25^+$  cells are present in the TCR Tg mice on a conventional background; furthermore, these cells suppressed the proliferative responses of  $CD4^+CD25^-$  Tg cells specific for the same peptide. We confirmed these results with  $CD4^+CD25^+$  T cells isolated from mice expressing a Tg TCR specific for peptide 110–119 of influenza HA.  $CD4^+CD25^+$  cells from the HA TCR Tg mice inhibited the proliferation of HA TCR Tg  $CD4^+CD25^-$  T cells stimulated with anti-CD3 (Fig. 4A) as



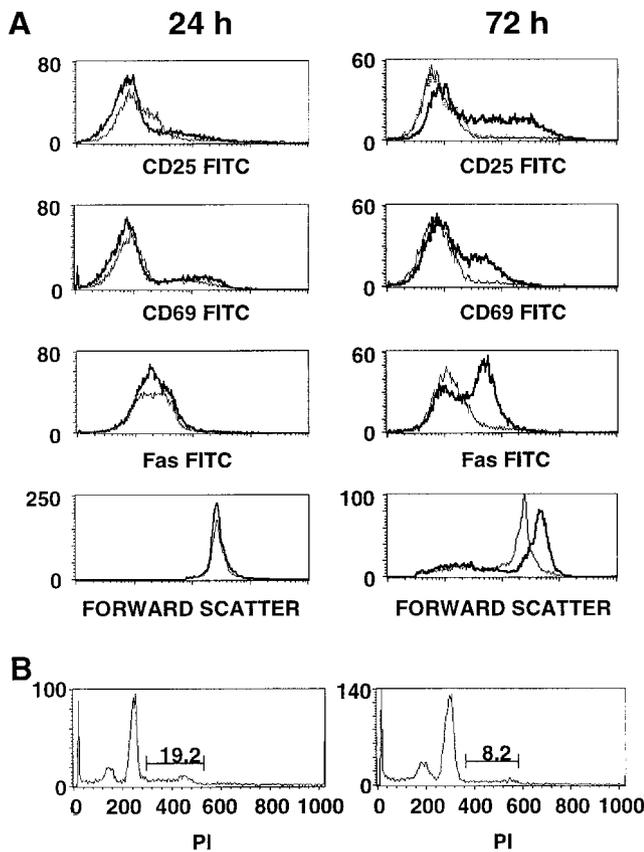
**FIGURE 2.** CD4<sup>+</sup>CD25<sup>+</sup> cells do not inhibit the induction of costimulatory molecules. **A**, CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) were cultured with APC ( $5 \times 10^4$ ) and anti-CD3 in the presence or absence of CD4<sup>+</sup>CD25<sup>+</sup> cells ( $2.5 \times 10^4$ ) for 48 h. The cells were then stained with FITC-anti-B220 and PE-anti-CD86. **Aa**, APC cultured alone. **Ab**, CD4<sup>+</sup>CD25<sup>-</sup> cells with APC. **A**, CD4<sup>+</sup>CD25<sup>-</sup> cultured with APC and CD4<sup>+</sup>CD25<sup>+</sup> cells. **B**, CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) were cultured with anti-CD3 and the indicated numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells. LPS-treated TΔS (*upper panels*) or P815 cells transfected with B7-2 (*lower panels*) were used as APC and were either irradiated (*left panels*) or fixed with paraformaldehyde (*right panels*). **C**, CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) were cultured with anti-CD3 and the indicated numbers of LPS-TΔS in the presence (■) or absence (□) of CD4<sup>+</sup>CD25<sup>+</sup> cells ( $2.5 \times 10^4$ ).

well as HA peptide (Fig. 4B), whereas CD4<sup>+</sup>CD25<sup>+</sup> cells from normal BALB/c donors inhibited only the anti-CD3 response. Inhibition could not be overcome by increasing or decreasing the peptide concentration (data not shown).

#### Suppressor effector function is Ag nonspecific

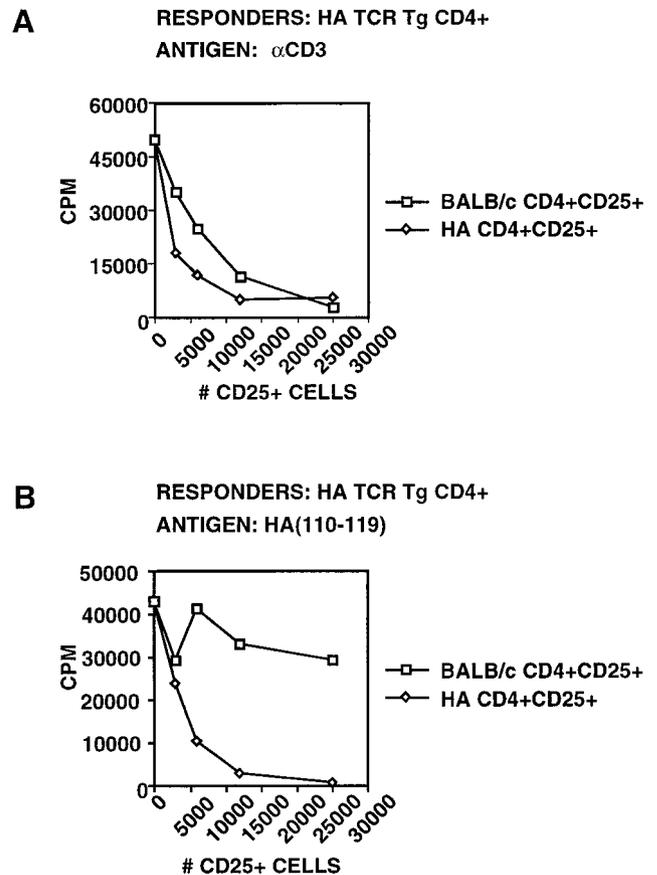
Although the above results suggested that suppression might be Ag specific, they were also compatible with the possibility that specific Ag was required for activation of the CD4<sup>+</sup>CD25<sup>+</sup> cells, but

once activated, suppressor effector function would be nonspecific. We used two distinct approaches to investigate this possibility and to determine whether activation of the suppressor and effector required that their target Ags be presented on the surface of the same APC. In the first approach, we took advantage of our ability to separate the activation signals required for suppressor cell function and those required for responder cell function by mixing CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from mice expressing a Tg TCR specific for one peptide MHC complex (HA<sub>110-119</sub>/I-E<sup>d</sup>) with



**FIGURE 3.** Responding CD4<sup>+</sup>CD25<sup>-</sup> cells are initially activated. *A*, CD4<sup>+</sup> cells from C57BL/6 mice were cultured with APC and anti-CD3 in the absence (bold line) or presence of CD4<sup>+</sup>CD25<sup>+</sup> cells (light line). FACS analysis was performed at 24 h and at 72 h. All histograms were gated on K<sup>b</sup> positive cells. *B*, CD4<sup>+</sup> cells from C57BL/6 mice were cultured with APC and anti-CD3 in the absence (left panel) or presence (right panel) of CD4<sup>+</sup>CD25<sup>+</sup> cells. Cells were stained at 48 h with FITC-anti-K<sup>b</sup> and fixed. The cells were then stained with propidium iodide and analyzed by flow cytometry. Histograms were gated on K<sup>b</sup>-positive cells. The percentage of cells in M/S phase on unstimulated cells was ~7%.

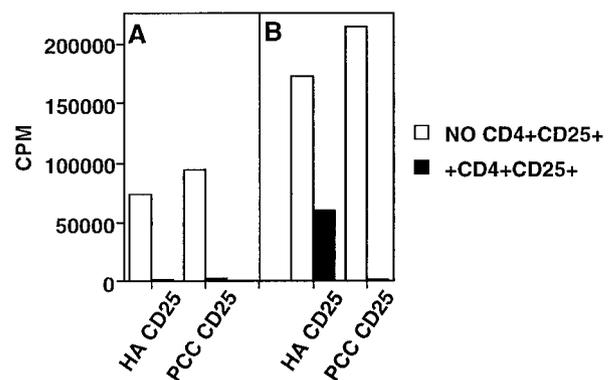
CD4<sup>+</sup>CD25<sup>-</sup> T cells from TCR Tg mice which recognized a distinct peptide MHC complex PCC<sub>88-104</sub>/I-E<sup>k</sup>. When CD4<sup>+</sup>CD25<sup>-</sup> from HA TCR Tg mice were stimulated with HA peptide in the presence of a fixed ratio of CD4<sup>+</sup>CD25<sup>+</sup> cells (1:0.5) from the HA TCR Tg mice, the HA response was inhibited by >95%. More importantly, HA-specific responses could be inhibited to the same extent in the presence of CD4<sup>+</sup>CD25<sup>+</sup> T from PCC TCR Tg mice and PCC (Fig. 5A). Inhibition of the HA-specific response by PCC CD4<sup>+</sup>CD25<sup>+</sup> cells was dependent upon the presence of PCC peptide and APC that expressed I-E<sup>k</sup> (data not shown). Conversely, the response to PCC of cells from PCC TCR Tg mice could be inhibited by both CD4<sup>+</sup>CD25<sup>+</sup> cells from PCC TCR Tg mice and by CD4<sup>+</sup>CD25<sup>+</sup> cells from HA TCR Tg mice (Fig. 5B). Again, suppression of the PCC specific response by HA CD4<sup>+</sup>CD25<sup>+</sup> cells was dependent upon the presence of HA peptide and APC which expressed I-E<sup>d</sup> as cpm in the absence of HA peptide were 164,725. Although CD4<sup>+</sup>CD25<sup>+</sup> cells from PCC TCR Tg could suppress Ag-specific proliferation of cells from HA TCR Tg mice by >95%, CD4<sup>+</sup>CD25<sup>+</sup> cells from HA TCR Tg mice consistently suppressed the PCC response by ~65% at the fixed ratio of 1:0.5. However, >95% suppression could be achieved when the ration was increased to 1:1 (data not shown). Ag-specific responses were suppressed to the same extent when APC were obtained from the



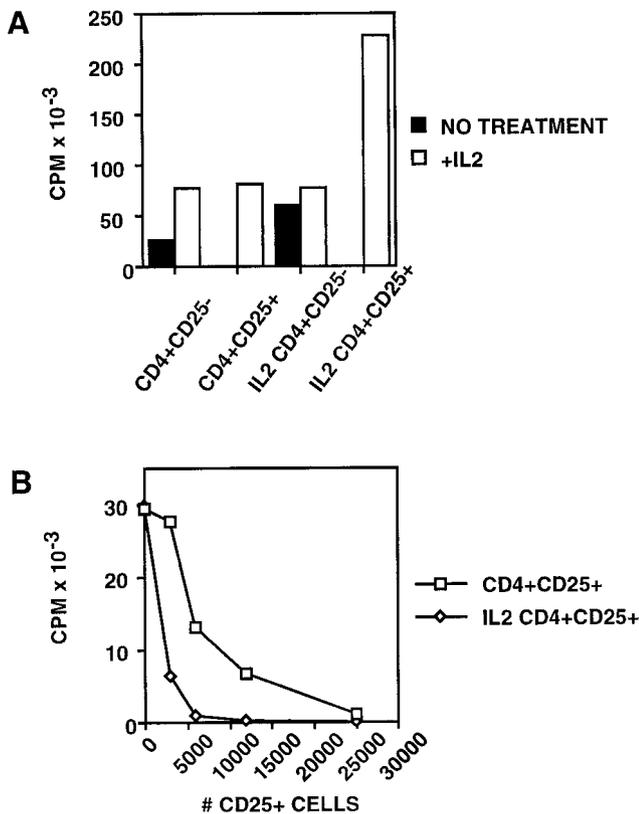
**FIGURE 4.** CD4<sup>+</sup>CD25<sup>+</sup> cells require stimulation through their TCR to suppress. CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) from HA TCR Tg mice were cultured with APC and anti-CD3 (*A*) or 32  $\mu$ M HA (*B*) peptide. The cells were cocultured with the indicated number of CD4<sup>+</sup>CD25<sup>+</sup> cells purified from BALB/c or HA TCR Tg mice.

parental strains or from F<sub>1</sub> H-2<sup>k/d</sup> mice (data not shown). Thus, suppression does not require that the target Ags be presented on the same APC.

In the second approach, we took advantage of the previous observations of Takahashi et al. (24) that CD4<sup>+</sup>CD25<sup>+</sup> cells can be expanded by stimulation with anti-CD3 and IL-2. When these cells were restimulated with anti-CD3 alone, they remained anergic but



**FIGURE 5.** CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) from HA TCR Tg mice (*A*) or PCC TCR Tg mice (*B*) were cultured with APC ( $5 \times 10^4$  each of H-2<sup>d</sup> and H-2<sup>b</sup>) and both peptides in the absence (□) or presence (■) of the indicated CD4<sup>+</sup>CD25<sup>+</sup> cells ( $2.5 \times 10^4$ ). CD4<sup>+</sup>CD25<sup>+</sup> cells from PCC TCR Tg mice were purified by cell sorting.



**FIGURE 6.** CD4<sup>+</sup>CD25<sup>+</sup> cells cultured in IL-2 remain anergic and suppressive and become potent suppressors. **A**, Freshly isolated CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells or activated CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells ( $5 \times 10^4$ ) were incubated with APC ( $5 \times 10^4$ ) and anti-CD3 in the absence or presence of 5 ng/ml IL-2. **B**, CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) were incubated with APC ( $5 \times 10^4$ ), anti-CD3, and the indicated number of freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> cells or activated CD4<sup>+</sup>CD25<sup>+</sup> cells.

were still capable of suppressing the responses of freshly isolated CD4<sup>+</sup>CD25<sup>-</sup> cells. We also observed that CD4<sup>+</sup>CD25<sup>+</sup> cells that had been stimulated with anti-CD3 and IL-2 for 7 days were anergic when restimulated with anti-CD3 alone, but proliferated in response to anti-CD3 and IL-2 (Fig. 6A). In contrast, CD4<sup>+</sup>CD25<sup>-</sup> cells that were cultured in the same manner proliferated vigorously when restimulated with anti-CD3 alone. More importantly, when the cultured CD4<sup>+</sup>CD25<sup>+</sup> cells were mixed with fresh CD4<sup>+</sup>CD25<sup>-</sup> responders, they exhibited markedly enhanced suppressor activity (Fig. 6B). As few as  $3 \times 10^3$  cultured CD4<sup>+</sup>CD25<sup>+</sup> cells could suppress proliferation by 75%, whereas  $12 \times 10^3$  freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> cells were required to produce the same degree of suppression.

In the previous experiments, the IL-2 cultured CD4<sup>+</sup>CD25<sup>+</sup> were reactivated with anti-CD3 when mixed with CD4<sup>+</sup>CD25<sup>-</sup> responders. Because we can now separate the signals needed for activation of the suppressor and responder, it was therefore of interest to examine whether the cultured CD4<sup>+</sup>CD25<sup>+</sup> T cells from normal BALB/c mice could suppress Ag-specific responses of T cells from the TCR transgenics in the absence of restimulation via their TCR. Surprisingly, when CD4<sup>+</sup>CD25<sup>+</sup> from BALB/c mice were cultured for 7 days with anti-CD3 and IL-2, washed, and then cocultured with freshly isolated CD4<sup>+</sup>CD25<sup>-</sup> cells from different TCR Tg mice, marked suppression of Ag-specific responses was seen. The magnitude of suppression of the Ag-specific responses was similar to the suppression of the anti-CD3 responses, where the suppressors were reactivated through their TCR. Similar non-

specific suppression was seen when CD4<sup>+</sup>CD25<sup>+</sup> cells from the HA TCR Tg were cultured with anti-CD3 and IL-2 for 7 days and then added to the other TCR Tg responses. Taken together, these studies demonstrate that following activation of the CD4<sup>+</sup>CD25<sup>+</sup> T cells with anti-CD3 and IL-2, their effector function is completely Ag nonspecific and MHC unrestricted.

## Discussion

A subpopulation (~10%) of CD4<sup>+</sup> T cells that expresses CD25 can be identified in normal lymphoid tissues and exhibits potent immunoregulatory functions *in vivo*. Transfer of CD4<sup>+</sup>CD25<sup>-</sup> T cells to immunodeficient recipients results in the development of organ-specific autoimmunity. Furthermore, the CD4<sup>+</sup>CD25<sup>+</sup> population suppresses the induction of autoimmunity following d3Tx and can also suppress the induction of autoimmune disease by autoreactive T cell clones. We and others have previously shown that the CD4<sup>+</sup>CD25<sup>+</sup> population is both anergic and suppressive *in vitro*. The goal of the present studies was to further characterize the *in vitro* suppressive activity of this unique cell population in an attempt to define their cellular target and the mechanism by which they mediate their suppressive effect.

Our previous studies suggested that the APC was the cellular target for the CD4<sup>+</sup>CD25<sup>+</sup> population. CD4<sup>+</sup>CD25<sup>+</sup> T cells only suppressed APC-dependent responses and suppression of T cell proliferation could be overcome by addition of IL-2 or enhancement of IL-2 production by costimulation with anti-CD28. Collectively, these observations suggested that the suppressor population was inhibiting the generation or delivery of costimulatory signals required for IL-2 production. Potent suppression was also observed at low ratios of responders to suppressors (4:1), which was also consistent with the APC as the primary target. One possibility was that the suppressor population acted on the APC to prevent the up-regulation of expression of cell surface molecules involved in costimulation (CD80/CD86). However, in the coculture studies presented here, the up-regulation of expression of CD80/CD86 proceeded normally in the presence of the CD4<sup>+</sup>CD25<sup>+</sup> cells. It also remained possible that the suppressor population deactivated the APC in some unknown manner to prevent the delivery of the costimulatory signal or to prevent the activation of cell surface molecules that mediate the physical interactions involved in intercellular cooperation (25). However, the CD4<sup>+</sup>CD25<sup>+</sup> population continued to exert potent suppressor activity when activated, fixed APC were used as accessory cells. As it is highly unlikely that paraformaldehyde fixed APC would transduce a membrane mediated signal, APC deactivation does not appear to be the mechanism by which the CD4<sup>+</sup>CD25<sup>+</sup> T cells function. Lastly, we considered a simple competition model in which the suppressor population would bind to the APC and prevent the effector cell from receiving a costimulatory signal or a signal for cell adhesion. However, when an excess of viable activated APC were added to cultures, the magnitude of suppression was only slightly diminished. In contrast to the failure of activated APC to overcome suppression, we have previously shown (17) that the addition of anti-CD28 readily abrogates suppression. At present, we do not have an adequate explanation for this discrepancy, but it is likely that the delivery of a direct costimulatory signal to the responder T cell by a stimulatory mAb is more potent than the delivery of costimulation via an activated APC.

Although expression of the CD25 Ag has greatly facilitated the isolation of the suppressor T cell population, CD25 expression can be induced on all T cells following activation, and it seemed likely that the freshly explanted CD25<sup>+</sup> cells might be composed of a mixture of suppressor cells and effector cells activated *in vivo*. We

have previously shown that the CD25<sup>+</sup> population is heterogeneous for expression of a number of memory/activation markers. Our attempts to subdivide the CD25<sup>+</sup> population into suppressors and effectors by the differential expression of these memory/activation markers (CD45, CD62L, CD69, and CD38) were uniformly unsuccessful. Any cell population that expressed CD25 was a potent suppressor of anti-CD3-induced T cell proliferation *in vitro*. We have not as yet evaluated these same subpopulations for suppressor function *in vivo*. Studies by Powrie and colleagues (7) have demonstrated that the population of CD4<sup>+</sup>CD45RB<sup>low</sup> cells is a potent suppressor of autoimmune inflammatory bowel disease *in vivo*. Recently, Read et al. (23) have split the CD4<sup>+</sup>CD45RB<sup>low</sup> population into two subpopulation based on the differential expression of CD38. The CD4<sup>+</sup>CD45RB<sup>low</sup>CD38<sup>+</sup> population appears to be very similar to the CD4<sup>+</sup>CD25<sup>+</sup> population in its capacity to suppress T cell activation *in vitro*, whereas the CD4<sup>+</sup>CD45RB<sup>low</sup>CD38<sup>-</sup> population appears to contain memory T cells that can easily be activated upon stimulation *in vitro* and do not mediate suppression *in vitro*. Read et al. (23) have not reported the properties of CD25<sup>-</sup>CD38<sup>+</sup> cells and we have thus far been unsuccessful in isolating this population. As both CD25<sup>+</sup>CD38<sup>+</sup> and CD25<sup>+</sup>CD38<sup>-</sup> were indistinguishable in our assays, expression of CD25 may therefore be the most specific marker of T cells with immunoregulatory function *in vitro*.

Our previous studies on the *in vitro* activity of the CD4<sup>+</sup>CD25<sup>+</sup> T cells were all performed with anti-CD3 as a polyclonal stimulus of T cell activation. Although we have ruled out deactivation of the APC or competition for APC-derived costimulation as the mechanisms for suppressor T cell function, in the absence of an assay for Ag-specific suppression, it has been difficult to exclude competition for Ag/MHC as the basic mechanism operating in this model. We had previously shown that the CD4<sup>+</sup>CD25<sup>+</sup> population is reduced by 90–95% in mice which express a single Tg TCR (14). However, as first shown by Takahashi et al. (24), CD4<sup>+</sup>CD25<sup>+</sup> T cells in mice that express a Tg TCR on a conventional background can be identified although they are present at less than 50% of the level present in wild-type mice. The specificity of the CD4<sup>+</sup>CD25<sup>+</sup> T cells which can be isolated from TCR Tg mice is most likely determined by the endogenous TCR  $\alpha$ -chain and not by the Tg  $\alpha$ -chain as TCR Tg mice on a SCID background lack the suppressor CD4<sup>+</sup>CD25<sup>+</sup> lineage. When CD4<sup>+</sup>CD25<sup>+</sup> T cells from Tg mice were stimulated with their target peptide Ag, they suppressed the response of CD4<sup>+</sup>CD25<sup>-</sup> Tg T cells specific for the same peptide. However, CD4<sup>+</sup>CD25<sup>+</sup> T cells specific for one peptide/MHC complex could suppress the response of CD4<sup>+</sup>CD25<sup>-</sup> T cells specific for a distinct peptide/MHC complex and vice versa. Cross-suppression did not require the presentation of both complexes on the surface of the same APC as CD4<sup>+</sup>CD25<sup>+</sup> specific for one peptide/MHC complex would suppress the response of CD4<sup>+</sup>CD25<sup>-</sup> T cells specific for a second peptide/MHC complex even when the Ags were presented by two distinct populations of APC.

These studies suggested that once activated via the TCR, suppressor effector function was completely Ag nonspecific. To address this issue, we stimulated CD4<sup>+</sup>CD25<sup>+</sup> T cells with anti-CD3 and IL-2 for 7 days. As reported previously, CD4<sup>+</sup>CD25<sup>+</sup> T cells can be expanded quite efficiently by this protocol and the stimulated cells remained anergic and suppressive (24). In fact, their capacity to suppress anti-CD3 stimulation of CD4<sup>+</sup>CD25<sup>-</sup> T cells was enhanced 4- to 6-fold when compared with freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> cells (Fig. 6). More importantly, in contrast to freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> cells, activated CD4<sup>+</sup>CD25<sup>+</sup> cells were powerful suppressors of the responses of CD4<sup>+</sup>CD25<sup>-</sup> Tg T

Table I. Activated CD4<sup>+</sup>CD25<sup>+</sup> cells inhibit Ag-specific responses<sup>a</sup>

Responder	Activated CD4 <sup>+</sup> CD25 <sup>+</sup>	% Suppression	
		Expt. 1	Expt. 2
BALB/c CD4 <sup>+</sup>	BALB/c	98	98
HA CD4 <sup>+</sup>	BALB/c	83	91
HNT CD4 <sup>+</sup>	BALB/c	69	84
PCC CD4 <sup>+</sup>	BALB/c	65	95
BALB/c CD4 <sup>+</sup>	HA TCR tg	97	97
HA CD4 <sup>+</sup>	HA TCR tg	99	98
HNT CD4 <sup>+</sup>	HA TCR tg	74	87
PCC CD4 <sup>+</sup>	HA TCR tg	66	97

<sup>a</sup> Responder CD4<sup>+</sup>CD25<sup>-</sup> cells ( $5 \times 10^4$ ) were incubated with anti-CD3 (in the case of BALB/c cells) or the appropriate peptide and APC ( $5 \times 10^4$ ), in the presence or absence of activated CD4<sup>+</sup>CD25<sup>+</sup> cells (experiment 1;  $1.25 \times 10^4$ ; experiment 2,  $2.5 \times 10^4$ ). Activated CD4<sup>+</sup>CD25<sup>+</sup> cells were generated from BALB/c or HA TCR Tg mice by stimulating with anti-CD3 and IL-2 for 1 wk. Data are expressed as percent suppression as compared to responder cells stimulated in the absence of IL-2-treated CD4<sup>+</sup>CD25<sup>+</sup> cells.

cells to a variety of Ags (Table I). There were no apparent MHC restrictions on this *in vitro* suppressor activity. The potent suppressor activity of the activated CD4<sup>+</sup>CD25<sup>+</sup> cells (70–80% suppression at ratios of responder:suppressor of 16–20:1) is actually problematic in terms of understanding the mechanism of suppression. As we reported for the freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> T cells, suppression was also observed when the IL-2-activated CD4<sup>+</sup>CD25<sup>+</sup> cells were separated from the responders by a semi-permeable membrane and suppression could not be reversed by a mixture of mAbs specific for suppressor cytokines (data not shown). Nevertheless, it is very difficult to exclude the possibility that suppression is mediated by a short-acting as yet uncharacterized suppressor molecule. Alternatively, activation of the CD4<sup>+</sup>CD25<sup>+</sup> population during *in vitro* culture may result in the induction of a membrane molecule that can rapidly and efficiently induce inhibition of IL-2 production and cell cycle arrest by repeated hits on the responder population.

Although regulatory T cell populations with a number of cell surface phenotypes and distinct profiles of activity have been described over the past few years, there are a number of principles that have emerged from studies in both mice and rats that allow a number of conclusions to be drawn about their origin and potential target Ags. First, regulatory T cell populations are generated during T cell differentiation in the thymus (26). CD4<sup>+</sup>CD25<sup>+</sup> T cells have been identified in the mouse thymus that resemble in their functional properties the CD4<sup>+</sup>CD25<sup>+</sup> T cells we and others have characterized in peripheral lymphoid tissues; these thymocytes can also suppress the induction of autoimmune disease *in vivo* (27). Regulatory T cells isolated from rat thymus are more potent suppressor populations *in vivo* than regulatory T cells isolated from peripheral tissues (28). Maintenance of regulatory T cells in the periphery requires the presence of tissue-specific Ags and IL-2. Seddon and Mason (29) have recently demonstrated that regulatory T cell populations in the rat that are capable of suppressing autoimmune thyroiditis cannot be detected in the peripheral lymph nodes and spleen of athyroid rats, but are present in the thymus. CD4<sup>+</sup>CD25<sup>+</sup> T cells cannot be detected in the thymus or the peripheral lymphoid tissues of IL-2-deficient mice (26). These observations suggest that regulatory T cells may require continuous restimulation by exposure to their target Ags in the periphery. It remains to be determined whether such restimulation involves IL-2-mediated cell division or merely survival. Taken together, we would propose that following the induction of autoimmune damage by autoimmune effector cells, regulatory T cells that recognize

organ-specific Ags are attracted to the involved organ, are restimulated by their target Ag, and mediate suppression. Suppression may be mediated by the production of suppressor cytokines in the target organ with bystander suppression of the effector cells or suppression may be mediated by direct cell-cell contact of the suppressors with the effectors. Although our *in vitro* studies suggest that the regulatory and suppressor cells need not recognize the same APC, the most efficient pathway for delivery of a cell contact mediated suppressor signal would be by the process of "linked-suppression" (30, 31), in which the two populations recognize two distinct antigenic epitopes on the surface of the same APC.

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