

# Human CD25<sup>+</sup>CD4<sup>+</sup> T Suppressor Cell Clones Produce Transforming Growth Factor $\beta$ , but not Interleukin 10, and Are Distinct from Type 1 T Regulatory Cells

Megan K. Levings,<sup>1</sup> Romina Sangregorio,<sup>1</sup> Claudia Sartirana,<sup>1</sup>  
Anna Lisa Moschin,<sup>1</sup> Manuela Battaglia,<sup>1</sup> Paul C. Orban,<sup>2</sup>  
and Maria-Grazia Roncarolo<sup>1,3</sup>

<sup>1</sup>San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), <sup>2</sup>San Raffaele Scientific Institute, and  
<sup>3</sup>Università Vita-Salut San Raffaele, Via Olgettina 58, Milan 20132, Italy

## Abstract

T regulatory (Tr) cells are essential for the induction of peripheral tolerance. Several types of Tr cells exist, including CD4<sup>+</sup> T cells which express CD25 constitutively and suppress immune responses via direct cell-to-cell interactions, and type 1 T regulatory (Tr1) cells, which function via secretion of interleukin (IL)-10 and transforming growth factor (TGF)- $\beta$ . The relationship between CD25<sup>+</sup>CD4<sup>+</sup> T cells and Tr1 cells remains unclear. Here, we demonstrate at the clonal level that Tr1 and CD25<sup>+</sup>CD4<sup>+</sup> T cells are two distinct subsets of regulatory cells with different cytokine production profiles. Furthermore, CD25<sup>-</sup>CD4<sup>+</sup> T cells can be rendered anergic by IL-10 and differentiated into Tr1 cells in the absence of CD25<sup>+</sup>CD4<sup>+</sup> T cells. Cloned human CD25<sup>+</sup>CD4<sup>+</sup> T cell populations are heterogeneous and only a subset of clones continues to express high levels of CD25 and is suppressive. The intensity of CD25, cytotoxic T lymphocyte antigen (CTLA)-4, and glucocorticoid-induced tumor necrosis factor (TNF) receptor expression correlates with the suppressive capacity of the T cell clones. None of the CD25<sup>+</sup>CD4<sup>+</sup> T cell clones with suppressive function produce IL-10, but all produce TGF- $\beta$ . Suppression mediated by CD25<sup>+</sup>CD4<sup>+</sup> T cell clones is partially dependent on TGF- $\beta$ , but not on constitutive high expression of CD25. Together these data indicate that naturally occurring human CD25<sup>+</sup>CD4<sup>+</sup> T cells are distinct from IL-10-producing Tr1 cells.

Key words: suppressor T lymphocytes • IL-10 • TGF- $\beta$  • interleukin 2 receptor  $\alpha$  chain • Tr1

## Introduction

Active suppression by T regulatory (Tr)\* cells is essential for induction of tolerance to both self and foreign antigens in vivo. Various subsets of Tr cells have been described and much effort has been focused on understanding their ontogeny, function, and mechanisms of action. Within the CD4<sup>+</sup> T cell subset, at least three different types of cells with suppressive function may exist: CD25<sup>+</sup>CD4<sup>+</sup> T cells (1, 2), type 1 T regulatory (Tr1) cells (3), and Th3 cells (4). These cell types appear to be distinguishable based on their cytokine production profile, and their ability to suppress immune responses via direct cell-to-cell interactions or secretion of immunosuppressive cytokines. However, the re-

lationship between these different types of CD4<sup>+</sup> Tr cells is unclear (3, 5, 6).

Tr1 cells were initially defined in studies of CD4<sup>+</sup> T cells which had been activated in the presence of IL-10 and rendered anergic (7). Analysis of these anergic T cell populations at the clonal level showed that they contained a subset of T cell clones (Tr1 cells) which possessed a unique cytokine production profile (8). The Tr1 cells produced high levels of IL-10 and TGF- $\beta$ , moderate amounts of IFN- $\gamma$  and IL-5, but little or no IL-2 or IL-4. These data indicate that IL-10-energized CD4<sup>+</sup> T cells contained the precursors of Tr1 cells, and that IL-10 was a critical factor for their differentiation. Importantly, Tr1 cells were shown to be involved in down-regulation of immune responses in vitro and in vivo, via the production of the immunosuppressive cytokines IL-10 and TGF- $\beta$  (3).

Another subset of CD4<sup>+</sup> Tr cells (Th3 cells) was identified in studies of oral tolerance. In mice that were orally

Address correspondence to Maria-Grazia Roncarolo, San Raffaele Telethon Institute for Gene Therapy, Via Olgettina 58, Milan 20132, Italy. Phone: 39-02-2643-4702; Fax: 39-02-2643-4668; E-mail: m.roncarolo@hsr.it

\*Abbreviations used in this paper: GITR, glucocorticoid-induced TNF receptor; NGF, nerve growth factor; MFI, mean fluorescence intensity; Tr, T regulatory.

tolerized to myelin basic protein, Th3 cells suppressed induction of EAE via a TGF- $\beta$ -dependent mechanism (9). In addition, oral treatment of multiple sclerosis patients with myelin basic protein (MBP) and proteolipid protein (PLP) resulted in the generation of MBP or PLP-specific Th3 cells that secreted TGF- $\beta$  (10). Th3 cells also produced variable amounts of IL-10 and IL-4 (4), and IL-4 was shown to be a key factor for the differentiation of Th3 cells, in contrast to Tr1 cells (11, 12).

In addition to Tr1 and Th3 cells, CD4<sup>+</sup> T cells that constitutively express the IL-2R $\alpha$  (CD25) possess potent suppressive properties in vitro and in vivo (1, 2). CD25<sup>+</sup>CD4<sup>+</sup> T cells are generated in the thymus, and are thought to arise via "altered negative selection" by self-peptides (6). Although CD25<sup>+</sup>CD4<sup>+</sup> T cells require activation via the TCR to exert their regulatory function, once activated, their suppressive activity is antigen nonspecific, thus, Tr1 and Th3 cells, these cells mediate bystander suppression in vivo (4, 8, 13). The suppressive activity of CD25<sup>+</sup>CD4<sup>+</sup> T cells is related to their ability to inhibit IL-2 production and promote cell-cycle arrest in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, via a mechanism which remains to be defined, but requires direct cell-to-cell contact and may involve signals through CTLA-4 and/or glucocorticoid-induced TNF receptor (GITR; references 14–17).

CD25<sup>+</sup>CD4<sup>+</sup> T suppressor cells have recently been identified in human peripheral blood and thymus (18–24). Like their mouse counterparts, human CD25<sup>+</sup>CD4<sup>+</sup> T cells are anergic, and strongly suppress the proliferative responses of both naive and memory CD25<sup>-</sup>CD4<sup>+</sup> T cells in response to alloantigens or polyclonal activation. Pure populations of human CD25<sup>+</sup>CD4<sup>+</sup> T cells contain both CD45RO<sup>+</sup> and CD45RO<sup>-</sup> cells, but the suppressive activity is enriched in the CD45RO<sup>+</sup> memory fraction (18).

The role of cytokines in the suppressive effects mediated by CD25<sup>+</sup>CD4<sup>+</sup> Tr cells, and thus their relationship to Tr1 and Th3 cells, is highly controversial. Some studies failed to detect production of IL-10 and/or TGF- $\beta$  by human CD25<sup>+</sup>CD4<sup>+</sup> T cells (21, 24), whereas others clearly showed secretion of these cytokines in response to a variety of stimuli (19, 20, 22). Interestingly, when activated by alloantigens, CD25<sup>+</sup>CD4<sup>+</sup> T cells produced IL-10, TGF- $\beta$ , low levels of IFN- $\gamma$ , but no IL-4 or IL-2, a profile of cytokine production which was remarkably similar to that of Tr1 cells (19). Nevertheless, neither IL-10 nor TGF- $\beta$  seemed to be directly required for their in vitro suppressive effects (18, 19, 23, 24). Furthermore, although mouse CD25<sup>+</sup>CD4<sup>+</sup> T cells secreted more IL-10 than CD25<sup>-</sup>CD4<sup>+</sup> T cells (25), their suppressive function appeared to be independent of IL-10, as CD25<sup>+</sup>CD4<sup>+</sup> T cells isolated from mice genetically deficient for IL-10 retained their suppressive capacity in vitro and in vivo in a model of autoimmune gastritis (2, 26). In addition, in vitro studies failed to show reversal of suppression by anti-TGF- $\beta$  mAbs in the presence or absence of anti-IL-10 mAbs (26, 27). Finally, CD25<sup>+</sup>CD4<sup>+</sup> T cells from TGF- $\beta$ 1<sup>-/-</sup> mice were fully suppressive in vitro, and target cells which were ge-

netically altered to be unresponsive to TGF- $\beta$  were susceptible to suppression mediated by CD25<sup>+</sup>CD4<sup>+</sup> T cells (28).

These data, suggesting that IL-10 and TGF- $\beta$  are dispensable, are hard to reconcile with recent studies in the mouse indicating that naturally occurring CD25<sup>+</sup>CD4<sup>+</sup> T cells prevent inflammatory bowel disease via an IL-10 and/or TGF- $\beta$ -dependent mechanism (16, 29). In addition, unlike their wild-type counterparts, CD25<sup>+</sup>CD45RB<sup>low</sup>CD4<sup>+</sup> T cells from IL-10-deficient mice fail to protect from a CD45RB<sup>high</sup>CD4<sup>+</sup> T cell-induced wasting disease (30). Furthermore, Nakamura and colleagues have recently proposed that TGF- $\beta$  produced by CD25<sup>+</sup>CD4<sup>+</sup> T cells, and bound to their cell surface, is the major mechanism by which murine CD25<sup>+</sup>CD4<sup>+</sup> T cells suppress T cell responses (31). Recently, membrane-bound TGF- $\beta$  has also been reported to contribute to the suppressive activity of human CD25<sup>+</sup>CD4<sup>+</sup> thymocytes (32).

The large body of controversial data, mostly regarding the cytokines produced by CD25<sup>+</sup>CD4<sup>+</sup> T cells and their role in suppression function, together with the observation that human CD25<sup>+</sup>CD4<sup>+</sup> T cells and Tr1 cells appeared remarkably similar in many aspects (3), prompted us to reexamine the potential relationship between IL-10- and TGF- $\beta$ -producing Tr1 cells and naturally occurring CD25<sup>+</sup>CD4<sup>+</sup> T suppressor cells at the clonal level.

## Materials and Methods

**Induction of Anergy by IL-10.** Total PBMCs, or PBMCs depleted of CD25<sup>+</sup> cells by incubation with anti-CD25-coupled microbeads (Miltenyi Biotech) and passage over an LD-depletion column (Miltenyi Biotech) were used as responders. Depleted cells were routinely 97–100% CD25<sup>-</sup> as assessed by FACS<sup>®</sup> analysis. Primary MLRs were performed as described previously (7) by culture of  $5 \times 10^5$  responder cells together with  $5 \times 10^5$  irradiated (6,000 RADS) CD3-depleted stimulator cells in 1 ml of X-vivo 15 medium (Biowhittaker), supplemented with 10% FCS (Mascia Brunelli), 1% pooled AB human serum (Biowhittaker), and 100 U/ml penicillin/streptomycin (Bristol-Myers Squibb), in 24-well plates, and in the absence or presence of IL-10 (100 U/ml, a kind gift from Schering-Plough, Kenilworth, NJ). For analysis of primary proliferation, parallel cultures were performed in 96-well round bottom plates ( $10^5$  responder cells plus  $10^5$  stimulator cells in 200  $\mu$ l) and after 4 d, wells were pulsed for 16 h with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine (Amersham Biosciences). For secondary MLRs, cells were harvested after 10 d, washed, and  $10^5$  cells/well were replated alone, or in the presence of  $10^5$  cells/well of freshly prepared irradiated and CD3-depleted stimulator cells. After 2 d, wells were pulsed for 16 h with [<sup>3</sup>H]thymidine.

**Differentiation of Tr1 Cells.** CD4<sup>+</sup> T cells were purified from PBMCs by negative selection using the Untouched CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotech), according to the manufacturer's instructions. CD25<sup>+</sup> cells were depleted as described above, and the resulting CD4<sup>+</sup>CD25<sup>-</sup> T cells were routinely 97–100% pure. CD4<sup>+</sup>CD25<sup>-</sup> T cells were activated by culture with irradiated (7,000 RAD) L-cell transfectants expressing hCD32 (FC $\gamma$ RII), hCD58 (LFA-3) and hCD80 (33), and 100 ng/ml of anti-CD3 (OKT3; Orthoclone) in X-vivo 15 medium supplemented with 10% FCS, 1% human serum, 100 U/ml penicillin/streptomycin, rhIL-2 (100 U/ml; Chiron Corp.), and rhIL-15 (1 ng/ml; R&D

Systems) in the absence or presence of IL-10 (100 U/ml) and rhIFN- $\alpha$  2b (5 ng/ml; PeproTech), as described previously (33). At day 7, T cells were collected, washed, counted, and restimulated under identical conditions for an additional 7 d. At day 14 of *in vitro* culture, cells were collected, washed, counted, and analyzed for their profile of cytokine production and proliferative capacity.

Intracellular cytokines were detected by flow cytometry as described previously (33). Briefly,  $10^6$  T cells/ml were stimulated with immobilized anti-CD3 (10  $\mu$ g/ml) and soluble anti-CD28 (1  $\mu$ g/ml) mAbs (BD Biosciences) in complete medium. After 3 h of activation, brefeldin A (10  $\mu$ g/ml; Sigma-Aldrich) was added. After a total of 6 h of activation, T cells were collected, washed, fixed, and permeabilized. Permeabilized T cells were incubated with PE-labeled anti-hIL-4, anti-hIL-2 or anti-hIL-10, and FITC-coupled anti-hIFN- $\gamma$  (all antibodies were obtained from BD Biosciences) and analyzed using a FACScan™.

Polarized T cells were tested for their ability to suppress the proliferation of naive CD4<sup>+</sup> T cells to alloantigens (33). Autologous CD4<sup>+</sup> T cells, which were cryopreserved at the start of the experiment, were cultured (50,000 cells/well) together with irradiated, allogeneic CD3-depleted PBMCs (50,000 cells/well), in the absence or presence of polarized T cells (100,000, 50,000, or 25,000 cells/well), in a final volume of 200  $\mu$ l of complete medium. After 4 d, wells were pulsed for 16 h with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine.

**Cloning of Human CD25<sup>+</sup>CD4<sup>+</sup> T Cells.** CD4<sup>+</sup> T cells were purified from PBMCs by positive selection with anti-CD4-coupled microbeads (Miltenyi Biotech). After isolation of CD4<sup>+</sup> T cells, CD25<sup>+</sup> cells were stained with FITC-coupled anti-CD4 and PE-coupled anti-CD25 mAbs (BD Biosciences) and CD25<sup>+</sup> and CD25<sup>-</sup> cells were purified by FACS®-sorting on a FACStar™. CD25<sup>+</sup>CD4<sup>+</sup> and CD25<sup>-</sup>CD4<sup>+</sup> T cells were subsequently cloned at 1 cell/well in 96-well round bottom plates in the presence of an allogeneic feeder-cell mixture consisting of  $5 \times 10^5$  PBMCs/ml (irradiated 6,000 RADS),  $5 \times 10^4$  JY cells/ml (irradiated 10,000 RADS), and 0.05  $\mu$ g/ml PHA (Roche). All cultures were performed in X-Vivo 15 medium supplemented with 5% pooled human serum, and 100 U/ml penicillin/streptomycin. After 3 d, IL-2 (40 U/ml) was added. After 8 d, one 96-well plate from each cloning was pulsed overnight with [<sup>3</sup>H]thymidine in order to determine the total number of wells with proliferating cells (the cloning efficiency). At day 14, growing wells were picked and restimulated with an allogeneic feeder-cell mixture consisting of  $10^6$  irradiated PBMCs/ml,  $10^5$  irradiated JY cells/ml, 0.1  $\mu$ g/ml PHA, and 100 U/ml IL-2. Clones were split as necessary, and restimulated as above every 14 d. The medium was replenished every 3–5 d. Clones were used for experiments between days 10 and 14 after restimulation (i.e., in the resting phase).

**Proliferation and Suppression of T Cells.** To analyze the proliferative capacity of T cell clones in response to polyclonal activation, 96-well round-bottom plates (Costar) were coated overnight at 4°C with anti-CD3 mAbs (10  $\mu$ g/ml) in 0.1 M Tris, pH 9.5, and washed three times with PBS. T cell clones were plated at an initial density of  $2 \times 10^5$  cells/ml (40,000 cells/well) in a final volume of 200  $\mu$ l of medium in the absence or presence of IL-2 (100 U/ml). To test the proliferative response to cytokines, T cell clones were plated as described above in the absence or presence of IL-2 (100 U/ml), IL-15 (10 ng/ml, R&D Systems), or IL-2 and IL-15. To test for the capacity of T cell clones to suppress the proliferation of autologous CD4<sup>+</sup> T cells, CD4<sup>+</sup> T cells were purified from autologous PBMCs by positive selection

(Miltenyi Biotech) and stimulated with anti-CD3 mAbs which had been immobilized on plastic (1  $\mu$ g/ml) or bound to allogeneic CD3-depleted PBMCs (irradiated 6,000 RADS; 0.5  $\mu$ g/ml). CD4<sup>+</sup> T cells (40,000 cells/well) were cultured alone, or in the presence of a 1:1 ratio of T cell clones in a final volume of 200  $\mu$ l of complete medium in 96-well round-bottom plates. In some cultures anti-IL-10R (10  $\mu$ g/ml, 3F9; BD Biosciences) and/or anti-TGF- $\beta$  (10  $\mu$ g/ml, 1D11; R&D Systems) mAbs were added. After the indicated time, wells were pulsed for 16 h with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine. Cells were harvested, and counted in a scintillation counter.

**Retroviral Constructs and Transduction of T Cell Clones.** The Moloney murine leukemia virus-based vector encoding the human CD25 (LCD25 $\Delta$ N) under control of the LTR and the low affinity nerve growth factor (NGF) receptor under control of the SV40 promoter was generated from the LXS $\Delta$ N vector (34). Stable Am12-based cell lines producing LCD25 $\Delta$ N or LXS $\Delta$ N were established. Transduction of T cell clones was performed 48 h after restimulation by two successive rounds of infection on retronectin-coated plates as described previously (35). Transduced cells were purified after staining with anti-NGFR mAbs and sorting by FACS®. After expansion, T cell clones which were transduced with control vectors, or vectors encoding hCD25 were used in suppression assays as described above.

**ELISAs.** T cell clones ( $10^6$  cells/ml) were stimulated with immobilized anti-CD3 mAbs (10  $\mu$ g/ml) and anti-CD28 (1  $\mu$ g/ml), and supernatants were collected after 24 h for IL-2, 48 h for IL-4, IL-5, IL-10, and IFN- $\gamma$  and 72 h for TGF- $\beta$ . Levels of TGF- $\beta$  in acidified supernatants were determined by capture ELISA according to the manufacturer's instructions (R&D Systems). Levels of IL-2, IL-4, IL-5, IL-10, and IFN- $\gamma$  were determined either by capture ELISA (BD Biosciences) or by the cytometric bead array kit (CBA; BD Biosciences), according to the manufacturer's instructions. A direct comparison of capture ELISA and CBA demonstrated that the two methods were highly comparable in terms of the amount of cytokine detected in the supernatant. The limits of detection were as follows: IL-2, 20 pg/ml; IL-4, 20 pg/ml; IL-5, 20 pg/ml, IL-10, 20 pg/ml; IFN- $\gamma$ , 60 pg/ml; TGF- $\beta$ , 60 pg/ml.

**FACS® Staining.** Immunofluorescence analysis was performed on resting T cell clones. Analysis of the expression of intracellular CTLA-4 was performed as described previously (19). Expression of GITR was determined by staining with an anti-GITR mAb (R&D Systems) followed by a secondary goat anti-mouse Ab coupled to PE (Caltag). For detection of membrane-bound TGF- $\beta$ , CD4<sup>+</sup> T cells were purified from PBMCs by negative selection (Miltenyi Biotech), and were stained with anti-CD25 mAbs coupled to PE (BD Biosciences). CD25<sup>+</sup> and CD25<sup>-</sup>CD4<sup>+</sup> T cells were then purified either by FACS® sorting, or following incubation with anti-PE-coupled microbeads by positive selection (Miltenyi Biotech). Purified T cells, or total PBMCs, were subsequently stained with a biotinylated-chicken-polyclonal IgY anti-TGF- $\beta$  Ab (R&D Systems; reference 31). After washing, cells were stained with streptavidin TRI-color (Caltag), and analyzed by FACS®.

**TCR V $\beta$  Spectratyping.** Total RNA was extracted from CD25<sup>+</sup>CD4<sup>+</sup> T cell clones with Eurozol (Euroclone) and 1  $\mu$ g of RNA was reverse transcribed into cDNA. V $\beta$  spectratyping was performed as described previously (36). Fluorescent PCR products were analyzed using a Fluorimager and ImageQuant software (Molecular Dynamics).

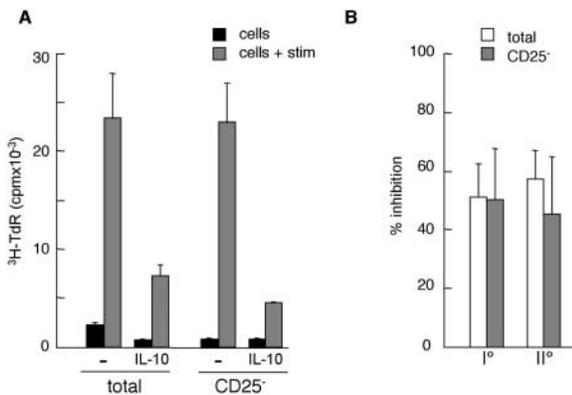
**Statistical Analysis.** All analysis for statistically significant differences was performed with Student's paired *t* test. P values <0.05

were considered significant. All cultures were performed in triplicate and error bars represent the SD unless otherwise indicated.

## Results

**Induction of Anergy by IL-10 Does Not Require CD25<sup>+</sup>CD4<sup>+</sup> T Cells.** We have previously shown that activation of T cells in the presence of IL-10 not only inhibits the primary proliferative response, but also results in the induction of long-lasting antigen-specific unresponsiveness (7). Importantly, IL-10-anergized cells contained the precursors of Tr1 cells, as cloning under conditions which reversed the anergic state resulted in the isolation of Tr1-cell clones which suppressed immune responses *in vitro* and *in vivo* (8). To investigate whether CD25<sup>+</sup>CD4<sup>+</sup> T cells may represent the precursors of Tr1 cells, we first examined whether these cells were required for IL-10-mediated inhibition of primary proliferative responses to alloantigens or induction of unresponsiveness in secondary responses. MLRs were performed in parallel with either total PBMCs or PBMCs which had been depleted of CD25<sup>+</sup> cells, as responder cells. Depletion of CD25<sup>+</sup> cells resulted in a purity of CD25<sup>-</sup> cells >97%. No significant differences in the ability of IL-10 to inhibit proliferation in primary responses or induce unresponsiveness in secondary MLRs, were observed between cultures performed in the presence or absence of CD25<sup>+</sup>CD4<sup>+</sup> T cells (Fig. 1, A and B).

**In Vitro Differentiation of IL-10-producing Tr1 Cells from CD25<sup>-</sup>CD4<sup>+</sup> T Cells.** Activation of naive CD4<sup>+</sup> T cells from cord blood or peripheral blood with anti-CD3 mAbs, cross-linked onto L-cells expressing human CD32, CD58, and CD80, in the presence of IL-10 and IFN- $\alpha$ , resulted in T cell lines containing IL-10-producing cells which suppressed antigen-specific responses *in vitro* (33).

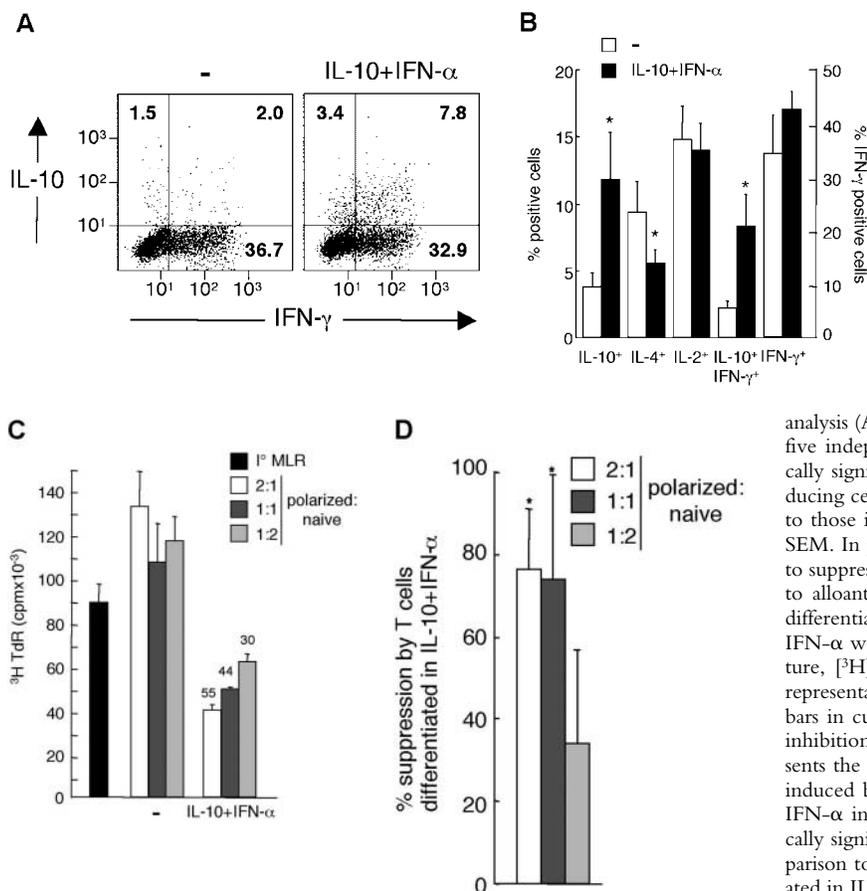


**Figure 1.** IL-10-induced anergy does not depend on the presence of CD25<sup>+</sup>CD4<sup>+</sup> T cells. Total PBMCs or PBMCs depleted of CD25<sup>+</sup> cells were stimulated with irradiated CD3-depleted allogeneic APCs for 10 d in the absence or presence of IL-10 (100 U/ml). After 10 d, cells were harvested and left unstimulated (cells) or restimulated with the same allogeneic APCs in the absence of IL-10 (cells + stim). After 48 h of culture, [<sup>3</sup>H]thymidine was added for an additional 16 h. One representative secondary MLR out of four performed shown in panel A. In B, the average percent inhibition of proliferation induced by IL-10 in primary (I°) and secondary (II°) MLRs performed with total or CD25<sup>-</sup> PBMCs as responder cells from four independent experiments is shown.

To further investigate whether CD25<sup>+</sup>CD4<sup>+</sup> T cells may represent the precursors of IL-10-producing CD4<sup>+</sup> T cells, or be required for the *in vitro* differentiation of IL-10-producing cells, CD4<sup>+</sup> T cells depleted of CD25<sup>+</sup> cells were tested in this *in vitro* differentiation system (33). As shown in Fig. 2 A, a significant increase in IL-10-producing cells was observed in cultures of CD25<sup>-</sup>CD4<sup>+</sup> T cells which had been differentiated in the presence of IL-10 and IFN- $\alpha$ , in comparison to cultures in the absence of polarizing cytokines. Importantly, as Tr1 cells also produce IFN- $\gamma$  (8, 33), a majority of the IL-10-producing cells also produced IFN- $\gamma$ . When data from five independent experiments were combined, we observed a significant increase in the percentage of cells expressing IL-10, and expressing both IL-10 and IFN- $\gamma$ , in cultures of CD25<sup>-</sup>CD4<sup>+</sup> T cells differentiated in the presence of IL-10 and IFN- $\alpha$ , in comparison to control cultures of CD25<sup>-</sup>CD4<sup>+</sup> T cells differentiated in the absence of these cytokines (Fig. 2 B). Cultures of CD25<sup>-</sup>CD4<sup>+</sup> T cells differentiated with IL-10 and IFN- $\alpha$  also displayed a significant reduction in the number of IL-4-producing cells, while the numbers of IL-2 and IFN- $\gamma$ -producing cells were equivalent to those observed in the absence of IL-10 and IFN- $\alpha$ . Thus, similarly to CD4<sup>+</sup>CD45RO<sup>-</sup> T cells (33), CD25<sup>-</sup>CD4<sup>+</sup> T cells from peripheral blood can differentiate into IL-10-producing Tr1 cells in the presence of IL-10 and IFN- $\alpha$ .

To demonstrate that CD25<sup>-</sup>CD4<sup>+</sup> T cells differentiated with IL-10 and IFN- $\alpha$  displayed suppressive function, we tested their ability to regulate the proliferation of autologous CD4<sup>+</sup> T cells in response to alloantigens. As shown in Fig. 2 C, cells from cultures performed in the absence of polarizing cytokines did not suppress proliferation in a primary MLR. In contrast, addition of cells from cultures which had been differentiated with IL-10 and IFN- $\alpha$  resulted in a dose-dependent suppression of proliferation. The average percent inhibition of primary MLRs with different ratios of cells from five independent experiments is shown in Fig. 2 D. When CD25<sup>-</sup>CD4<sup>+</sup> T cells, which had been differentiated with IL-10 and IFN- $\alpha$ , were added at a 2:1 or 1:1 ratio (polarized:naive), a consistent and highly significant suppression was observed. A less significant and more variable reduction in proliferation was observed when cells were added at a 1:2 ratio.

**Isolation and Characterization of Human CD25<sup>+</sup>CD4<sup>+</sup> T Cell Clones.** CD25<sup>bright</sup> T cells were purified by FACS<sup>®</sup> sorting from peripheral blood CD4<sup>+</sup> T cells. The resulting CD25<sup>bright</sup> and CD25<sup>-</sup> populations were 98 and 99% pure, respectively (Fig. 3 A), and were cloned by limiting dilution, as described in Materials and Methods. The CD25<sup>-</sup>CD4<sup>+</sup> T cells had a cloning efficiency of 42%, whereas the CD25<sup>+</sup>CD4<sup>+</sup> T cells had a lower cloning efficiency of 10.2%. 120 CD25<sup>+</sup>CD4<sup>+</sup> and 40 CD25<sup>-</sup>CD4<sup>+</sup> T cell clones were isolated and expanded for analysis. The TCR usage of 20 clones which were repeatedly tested in the present study was determined by TCR V $\beta$  spectratyping (36). This analysis revealed that each T cell clone tested expressed a single V $\beta$  and confirmed their clonal origin. In



**Figure 2.** Tr1 cells can be differentiated from CD25<sup>+</sup>CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells were purified, depleted of CD25<sup>+</sup> cells, and activated by anti-CD3 mAbs cross-linked on CD32<sup>+</sup>CD80<sup>+</sup>CD58<sup>+</sup> L-cells in the absence (-) or presence of IL-10 plus IFN- $\alpha$ . After two rounds of identical stimulation, T cells were restimulated with immobilized anti-CD3 (10  $\mu$ g/ml) and soluble anti-CD28 (1  $\mu$ g/ml) mAbs and cytokine production was determined by intracytoplasmic staining and cytofluorometric analysis (A and B). In B the average percent positive cells from five independent experiments is shown. \* indicates a statistically significant difference in the percentage of cytokine-producing cells in cultures with IL-10 plus IFN- $\alpha$ , in comparison to those in the absence of cytokines. Error bars represent the SEM. In parallel, polarized T cells were tested for their ability to suppress the proliferation of naive autologous CD4<sup>+</sup> T cells to alloantigens. Increasing numbers of cells which had been differentiated in the absence (-) or presence of IL-10 and IFN- $\alpha$  were added to the primary MLR, and after 4 d of culture, [<sup>3</sup>H]thymidine was added for an additional 16 h. One representative experiment is shown in C. Numbers above the bars in cultures with IL-10 and IFN- $\alpha$  represent the percent inhibition in comparison to the primary MLR alone. D represents the average reduction in proliferation of primary MLRs induced by CD25<sup>+</sup>CD4<sup>+</sup> T cells differentiated in IL-10 and IFN- $\alpha$  in five independent experiments. \* indicates a statistically significant ( $P \leq 0.05$ ) reduction in proliferation in comparison to primary MLRs in the absence of T cells differentiated in IL-10 and IFN- $\alpha$ .

addition, all suppressive clones expressed different V $\beta$  chains (data not depicted).

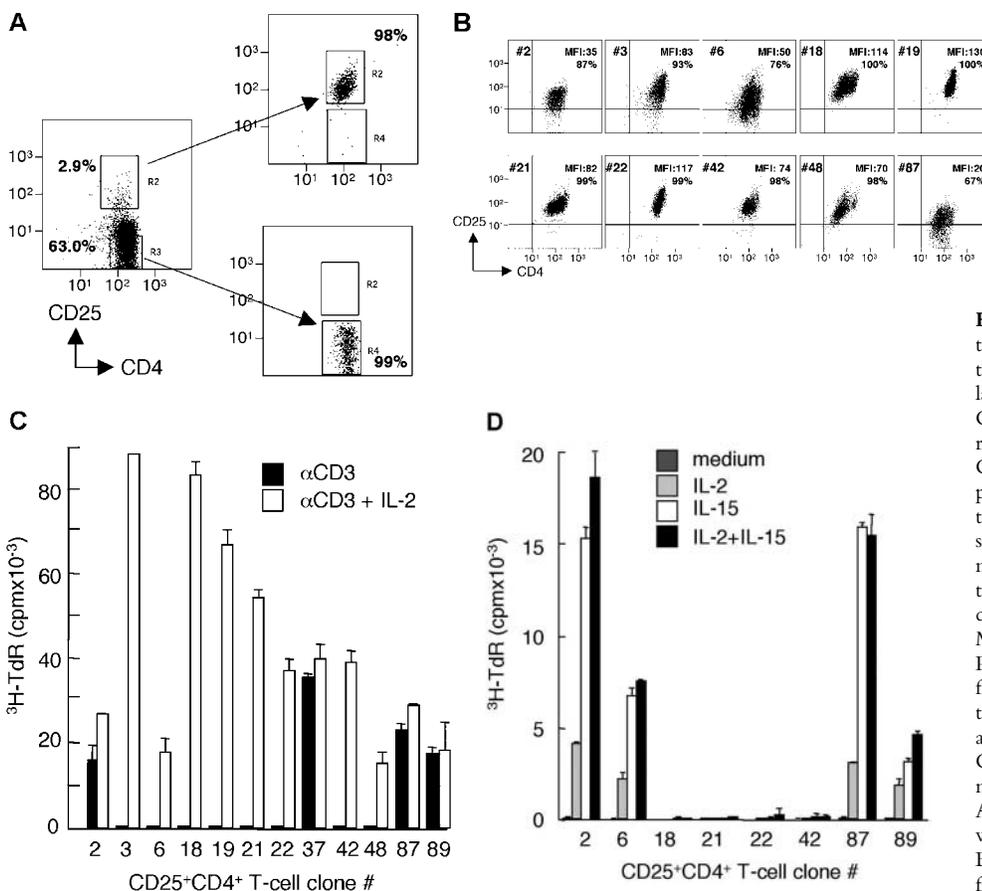
CD25<sup>+</sup>CD4<sup>+</sup> T cell clones in the resting phase displayed a heterogeneous expression of CD25 (Fig. 3 B). Approximately half of the clones remained 98–100% positive for CD25, and had a high mean fluorescence intensity (MFI). The other clones contained a significant number of CD25<sup>-</sup> cells and displayed a lower MFI. T cell clones derived from CD25<sup>-</sup>CD4<sup>+</sup> T cells consistently contained a low percentage of CD25<sup>+</sup> cells in the resting phase and consequently had a low MFI for CD25 (data not depicted).

Similar to the heterogeneity observed in the expression of CD25, the CD25<sup>+</sup>CD4<sup>+</sup> T cell clones were also heterogeneous in their proliferative capacities. The majority of the clones tested (58/72, 80%) were anergic and failed to proliferate in response to anti-CD3 mAbs, but showed good proliferative responses when activated by anti-CD3 mAbs in the presence of IL-2. The remaining clones (14/72, 20%) proliferated significantly in response to anti-CD3 mAbs even in the absence of IL-2. A representative subset of the 72 clones tested is shown in Fig. 3 C. As expected, the majority (~90%) of T cell clones which originated from CD25<sup>-</sup>CD4<sup>+</sup> T cells proliferated well in response to anti-CD3 mAbs, in the absence of IL-2 (data not depicted).

We previously reported that Tr1-cell clones are preferentially responsive to cytokines, and in particular to IL-15, in comparison to proliferation after TCR-mediated activa-

tion (12). To further address their possible relationship with Tr1 cells, we determined the ability of CD25<sup>+</sup>CD4<sup>+</sup> T cell clones to proliferate in response to cytokines. Some clones (no. 2, 6, 87, and 89) proliferated well in response to both IL-2 and/or IL-15 (Fig. 3 D). In contrast, others (no. 18, 21, 22, and 42) failed to proliferate in response to IL-2, IL-15, or the combination, in the absence of TCR-mediated activation (Fig. 3 D, and data not depicted). Thus, the CD25<sup>+</sup>CD4<sup>+</sup> T cell clones were also heterogeneous in terms of their response to IL-2 and/or IL-15.

**Suppressive Activity of CD25<sup>+</sup>CD4<sup>+</sup> T Cell Clones.** The heterogeneity of CD25<sup>+</sup>CD4<sup>+</sup> T cell clones, in terms of expression of CD25 and proliferation, suggested that some cells within the CD25<sup>bright</sup>CD4<sup>+</sup> population of PBMCs, may not be suppressor cells, and that a proportion are activated Th cells. To test this hypothesis we performed in vitro suppression assays with a total of 44 CD25<sup>+</sup>CD4<sup>+</sup> T cell clones. Indeed, as shown in Fig. 4, only a subset of the CD25<sup>+</sup>CD4<sup>+</sup> T cell clones was able to suppress the proliferative response of autologous CD4<sup>+</sup> T cells in response to anti-CD3 mAbs cross-linked on T cell-depleted PBMCs (Fig. 4 A) or immobilized on plastic (Fig. 4 B). All T cell clones which consistently inhibited the proliferation of the naive CD4<sup>+</sup> T cells by more than 20% were considered suppressive. Activation with immobilized anti-CD3 mAbs in the absence of APCs resulted in an average suppression of  $57.9 \pm 15.5\%$  (17 suppressive clones repeatedly tested).



**Figure 3.** Isolation and characterization of human CD25<sup>+</sup>CD4<sup>+</sup> T cells at the clonal level. CD4<sup>+</sup> T cells were isolated from PBMCs, stained with anti-CD4 and anti-CD25 mAbs, and separated into CD25<sup>+</sup>CD4<sup>+</sup> and CD25<sup>-</sup>CD4<sup>+</sup> T cells by FACS<sup>®</sup> sorting to a purity greater than 98 and 99%, respectively (A). Resting T cell clones were stained with anti-CD4 and -CD25 mAbs 12–14 d after the last restimulation. Numbers on the top left indicate clone number, and on the top right the MFI and percent positive cells (B). Resting T cell clones were also tested for their ability to proliferate in response to anti-CD3 mAbs (10 μg/ml) in the absence or presence of IL-2 (100 U/ml; C), or in response to IL-2, IL-15 (10 ng/ml), or IL-2 and IL-15 together. (D) After 48 h of culture, [<sup>3</sup>H]thymidine was added for an additional 16 h. For B–D results are representative of at least five independent tests.

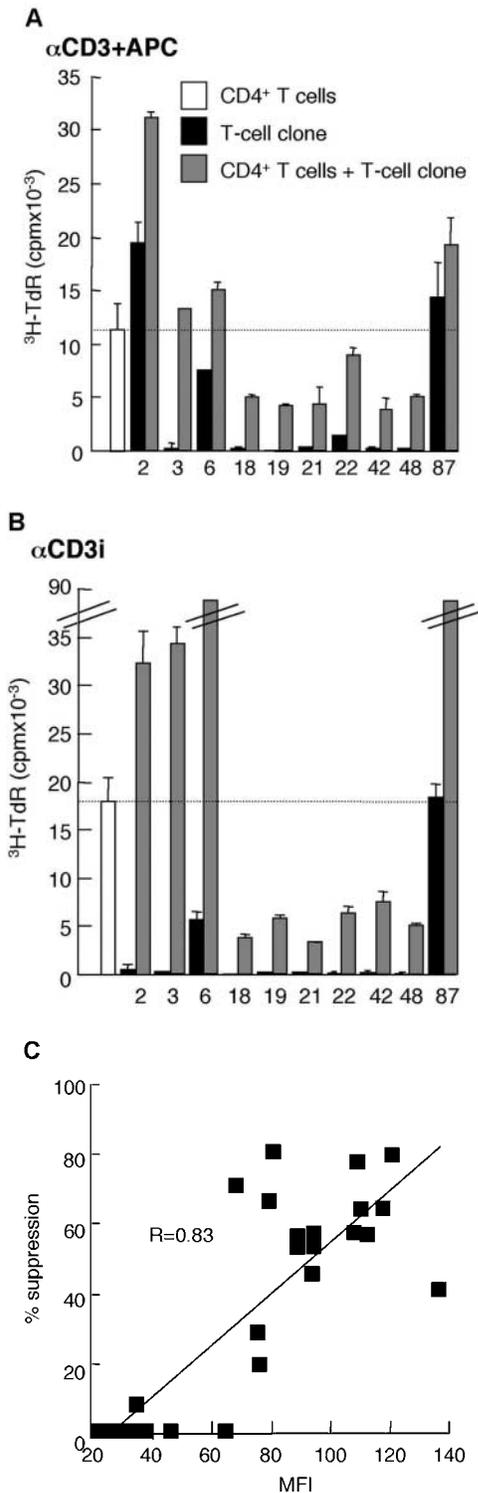
Similarly, when activated with soluble anti-CD3 mAbs and APCs, an average suppression of  $53.8 \pm 18.5\%$  (15 suppressive clones repeatedly tested) was observed.

Interestingly, only those clones which were (a) anergic, (b) did not proliferate in response to cytokines, and (c) displayed a constitutively high expression of CD25, had a suppressive phenotype. When the MFI and percent suppression of proliferation in response to immobilized anti-CD3 mAbs were plotted, there was a strong linear correlation between suppressive capacity and expression of CD25 (Fig. 4 C). Furthermore, separation of the CD25<sup>+</sup>CD4<sup>+</sup> T cell clones into suppressive and nonsuppressive groups revealed that the MFI of CD25 staining was significantly higher in the group with suppressive activity ( $P < 0.000007$ ). Lack of proliferation in response to anti-CD3 mAbs was a less reliable predictor of suppressive capacity, as several clones within the nonsuppressive category were anergic (Fig. 4, A and B). Therefore, the previously reported correlation between anergy and suppression is not absolute (37).

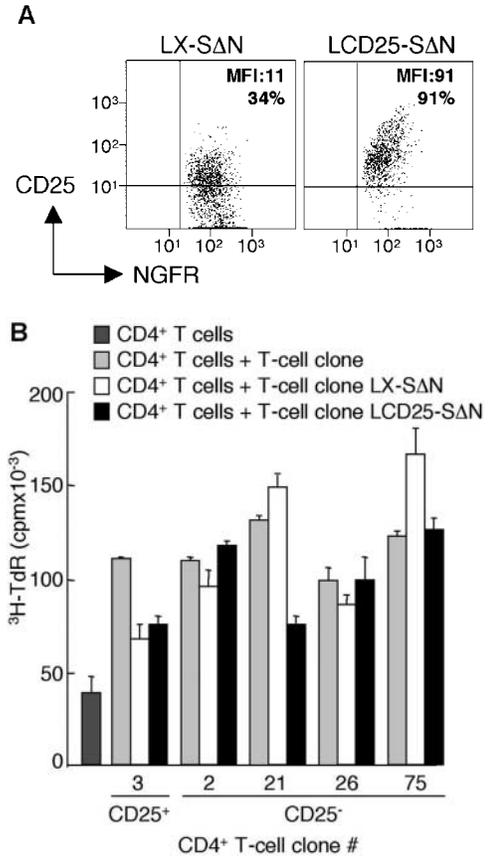
**Suppressive Activity Is Not Conferred by High Constitutive Expression of CD25.** This strong correlation between expression of CD25 and suppressive activity raised the question of whether high levels of CD25 alone may be sufficient to confer suppressive function. We have previously shown that activated effector CD4<sup>+</sup> T cells expressing high levels of CD25 do not suppress proliferation (19); however, the possibility remained that in these cells CD25<sup>+</sup> could be

down-regulated during the coculture. We therefore transduced several nonsuppressive clones derived from either CD25<sup>+</sup> or CD25<sup>-</sup>CD4<sup>+</sup> T cells with retroviral vectors encoding NGFR, as a marker gene, and with or without CD25 under control of the LTR. Transduced cells were purified by sorting for NGFR-positive cells, and subsequently analyzed for expression of CD25 in the resting phase (Fig. 5 A). T cell clones which were transduced with the LCD25-ΔN vector displayed significantly higher expression of CD25 than untransduced cells, or cells transduced with the control LX-ΔN vector. Importantly, the MFI of CD25 in the LCD25-ΔN-transduced cells was equivalent to the levels of CD25 expressed by the suppressive clones (Fig. 5 A). The transduced T cell clones were then tested for their suppressive capacity. As shown in Fig. 5 B, expression of high levels of CD25 was not sufficient to confer suppressive capacity. It is interesting to note that neither nonsuppressive, nor anergic clones, acquired suppressive capacity upon enforced expression of CD25.

**Expression of CTLA-4 and GITR on CD25<sup>+</sup>CD4<sup>+</sup> T Cell Clones.** It has been reported that polyclonal populations of CD25<sup>+</sup>CD4<sup>+</sup> T cells constitutively express high levels of CTLA-4 and GITR (14–17, 19). It was therefore of interest to determine whether expression of these two molecules correlated with suppressive activity of the T cell clones. FACS<sup>®</sup> analysis revealed that there was an absolute correlation between constitutive expression of both



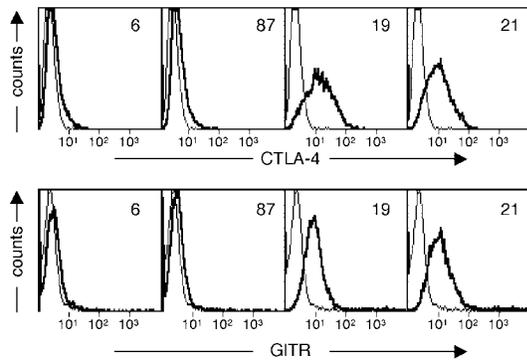
**Figure 4.** Suppression of naive T cell responses by CD25<sup>+</sup>CD4<sup>+</sup> T cell clones. Autologous CD4<sup>+</sup> T cells were purified and activated with anti-CD3 mAbs and irradiated CD3-depleted APCs (A) or anti-CD3 mAbs immobilized on plastic (B) in the absence or presence of CD25<sup>+</sup>CD4<sup>+</sup> T cell clones added at a 1:1 ratio. After 48 (A) or 72 h (B) of culture, [<sup>3</sup>H]thymidine was added for an additional 16 h. Results are representative of 2–5 independent tests for each clone. In C, the MFI of 27 individual CD25<sup>+</sup>CD4<sup>+</sup> T cell clones and the respective percent reduction in proliferation of autologous T cells activated with immobilized anti-CD3 mAbs in the presence of CD25<sup>+</sup>CD4<sup>+</sup> T cell clones were plotted.



**Figure 5.** Constitutive expression of CD25 does not confer suppressive capacity. Nonsuppressive T cell clones derived from CD25<sup>+</sup>CD4<sup>+</sup> T cells (clone #3) or CD25<sup>-</sup>CD4<sup>+</sup> T cells (clones #2, 21, 26, 75) were transduced with LCD25- $\Delta$ N or a control vector, LX- $\Delta$ N. Transduced cells were purified by FACS<sup>®</sup>-sorting for NGFR-positive cells. During the resting phase, transduced cells were tested for expression of CD25 (A). Pure populations of NGFR-positive cells transduced with LCD25- $\Delta$ N or LX- $\Delta$ N, and their untransduced counterparts were then tested for their ability to suppress the proliferation of naive CD4<sup>+</sup> T cells (1:1 ratio) in response to immobilized anti-CD3 mAbs (B). After 72 h of culture, [<sup>3</sup>H]thymidine was added for an additional 16 h. Results are representative of two independent tests for each clone.

CTLA-4 and GITR and suppressive capacity. Two representative clones from the nonsuppressive (no. 6 and 87) and suppressive groups (no. 19 and 21) are shown in Fig. 6. Of the 10 clones tested, suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones expressed four- to sixfold higher levels of both molecules. For CTLA-4, the MFI of the suppressive T cell clones was  $14.3 \pm 5.1$ , in comparison to  $3.9 \pm 0.9$  for nonsuppressive clones ( $P \leq 0.006$ ). For GITR, the MFI of the suppressive T cell clones was  $36.6 \pm 12.0$ , in comparison to  $7.4 \pm 1.9$  for nonsuppressive clones ( $P \leq 0.003$ ).

We showed previously that neutralizing anti-CTLA-4 mAbs did not affect suppression mediated by CD25<sup>+</sup>CD4<sup>+</sup> T cells (19). In the present study we investigated whether GITR may play a role in suppression mediated by human CD25<sup>+</sup>CD4<sup>+</sup> T cells. Experiments performed with polyclonal populations of CD25<sup>+</sup>CD4<sup>+</sup> T cells revealed that a commercially available monoclonal anti-GITR mAb



**Figure 6.** Expression of CTLA-4 and GITR on CD25<sup>+</sup>CD4<sup>+</sup> T cell clones. Several CD25<sup>+</sup>CD4<sup>+</sup> T cell clones were tested for expression of intracytoplasmic CTLA-4 and cell surface GITR by flow cytometric analysis (thick lines). Thin lines represent staining with the appropriate control Abs. Clones 6 and 87 are representative of nonsuppressive clones, and clones 19 and 21 of anergic and suppressive clones. Results are representative of two independent tests for each clone.

(R&D Systems) did not reverse anergy or suppression (data not depicted). Furthermore, addition of rGITR ligand at concentrations of up to 100 ng/ml also had no effect on anergy or suppression (data not depicted). Thus, similar to CD25, CTLA-4 and GITR are cell surface markers for suppressive CD4<sup>+</sup> T cells. However, it remains unclear whether any of these molecules are functionally important.

**Cytokine Production Profile of Suppressive CD25<sup>+</sup>CD4<sup>+</sup> T Cell Clones.** To further investigate whether CD25<sup>+</sup>CD4<sup>+</sup> T cells may be related to IL-10-producing Tr1 cells, we determined the cytokine production profile of the CD25<sup>+</sup>CD4<sup>+</sup> T cell clones. A number of suppressive and nonsuppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones were stimulated with anti-CD3 and anti-CD28 mAbs and supernatants were assayed for the presence of IL-2, IL-4, IL-5, IL-10, IFN- $\gamma$ , and TGF- $\beta$ . As shown in Table I, nonsuppressive clones varied in their cytokine production profile and tended to produce significant levels of most cytokines tested. This Th0-like phenotype of cytokine production was expected from CD4<sup>+</sup> T cells which had been randomly cloned in the absence of polarizing cytokines, such as IL-4 or IL-12. In striking contrast, all suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones consistently failed to produce detectable levels of IL-2 or IL-10. The majority of the suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones also did not produce significant amounts of IL-4, IL-5, or IFN- $\gamma$ . In contrast, all the suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones produced significant levels of active TGF- $\beta$ .

Cytokine production by suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones was also investigated after activation with anti-CD3 and anti-CD28 mAbs in the presence of exogenous IL-2 (i.e., under conditions in which they proliferated). Even when activated in the presence of IL-2, all suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones failed to produce detectable levels of IL-10 (data not depicted). Under these conditions, the levels of IL-4 and IL-5 produced by suppressive

**Table I.** Cytokine Production Profile of CD25<sup>+</sup>CD4<sup>+</sup> T Cell Clones

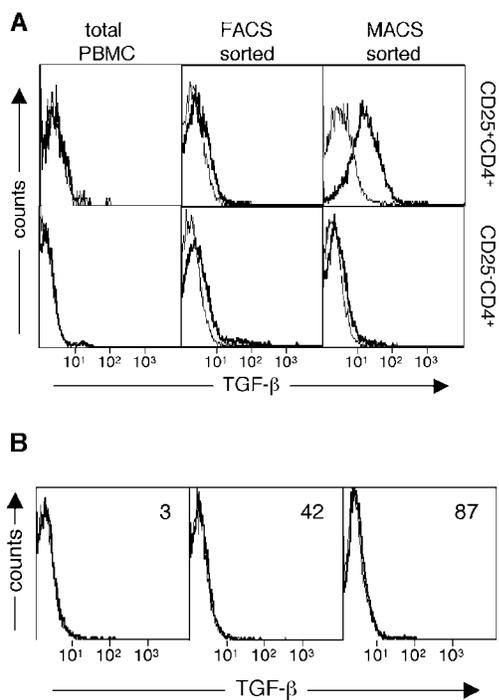
CD25 <sup>+</sup> CD4 <sup>+</sup> clone no.	IL-2	IL-4	IL-5	IL-10	IFN- $\gamma$	TGF- $\beta$
	pg/ml	pg/ml	ng/ml	pg/ml	ng/ml	pg/ml
Nonsuppressive						
2	<20	1,184	35.39	140	2.70	251
3	<20	8,002	3.45	26	0.79	242
6	<20	57	0.88	<20	0.14	113
85	<20	521	7.89	94	1.46	214
86	394	537	3.86	62	11.94	n.t.
87	<20	419	2.96	262	4.17	114
89	476	604	4.26	183	8.77	n.t.
90	1,360	618	4.59	199	12.64	n.t.
Suppressive						
4	<20	<20	<0.02	<20	<0.06	151
17	<20	67	0.05	<20	0.07	186
18	<20	<20	<0.02	<20	n.t.	274
19	<20	140	0.28	<20	0.10	351
20	<20	<20	<0.02	<20	<0.06	83
21	<20	<20	<0.02	<20	<0.06	298
22	<20	<20	<0.02	<20	<0.06	401
29	<20	<20	<0.02	<20	<0.06	291
40	<20	<20	<0.02	<20	<0.06	243
42	<20	83	0.19	<20	0.20	355
57	<20	<20	<0.02	<20	0.10	278

T cell clones were activated with  $\alpha$ CD3 and  $\alpha$ CD28 mAbs, and supernatants were collected after 24 (for IL-2), 48 (for IL-4, IL-5, IL-10, and IFN- $\gamma$ ), and 72 h (for TGF- $\beta$ ). Amounts of cytokines in the supernatants were determined by capture ELISA and/or CBA assay as described in Materials and Methods. n.t.: not tested. Numbers represent the average values obtained in 2–6 individual tests for each clone. SDs were <20%. Clones were divided into suppressive and nonsuppressive groups based on results obtained from experiments performed as described in Fig. 4.

CD25<sup>+</sup>CD4<sup>+</sup> T cells increased slightly, but always remained significantly lower than those produced by nonsuppressive clones. Production of IFN- $\gamma$  and TGF- $\beta$  by suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones remained unchanged when activated in the presence of IL-2 (data not depicted).

These data indicate that naturally occurring CD25<sup>+</sup>CD4<sup>+</sup> T cells isolated from peripheral blood are not IL-10-producing Tr1 cells. The fact that TGF- $\beta$  was the only cytokine which was consistently detected in the supernatants of all the suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones suggests that naturally occurring CD25<sup>+</sup>CD4<sup>+</sup> T cells may be more related to the TGF- $\beta$ -producing Th3 cells which were originally described in models of oral tolerance (4).

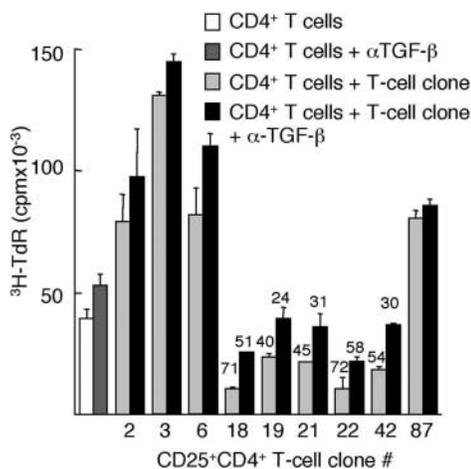
**Human CD25<sup>+</sup>CD4<sup>+</sup> T Cells Do Not Express Membrane-bound TGF- $\beta$ .** Recently, it has been reported that murine CD25<sup>+</sup>CD4<sup>+</sup> T cells and human CD25<sup>+</sup>CD4<sup>+</sup> thymocytes express high levels of TGF- $\beta$  bound to their plasma membrane by an unknown mechanism (31, 32). We therefore investigated whether human CD25<sup>+</sup>CD4<sup>+</sup> T suppressor cell clones may also express this molecule in a membrane-bound form. When expression of membrane-bound TGF- $\beta$  was analyzed on CD25<sup>+</sup>CD4<sup>+</sup> T cells present in total PBMCs, by triple-staining with anti-CD4, -CD25, and -TGF- $\beta$  mAbs, no positive signal was detected (Fig. 7 A, left panel). In addition, in CD25<sup>+</sup> and CD25<sup>-</sup>CD4<sup>+</sup> T cells freshly isolated and purified by FACS<sup>®</sup> sorting, no significant expression of membrane-bound TGF- $\beta$  was detected (Fig. 7 A, middle panel). Furthermore, none of the CD25<sup>+</sup>CD4<sup>+</sup> T cell clones tested expressed significant levels of membrane-bound TGF- $\beta$  (Fig. 7 B). However, it should be noted that in CD25<sup>+</sup>CD4<sup>+</sup> T cells purified by positive selection using magnetic beads, TGF- $\beta$  appeared to be present on the cell surface (Fig. 7 A, right panel). This false positive staining in



**Figure 7.** Human CD25<sup>+</sup>CD4<sup>+</sup> T cells or T cell clones do not express membrane-bound TGF- $\beta$ . Total PBMCs were stained with anti-CD4, -CD25, and -TGF- $\beta$  Abs. CD25<sup>+</sup>CD4<sup>+</sup> or CD25<sup>-</sup>CD4<sup>+</sup> T cells were gated, and expression of TGF- $\beta$  (thick line) on the two subsets was compared with staining by the secondary reagent alone (thin line; A, left panel). In parallel, CD25<sup>+</sup>CD4<sup>+</sup> and CD25<sup>-</sup>CD4<sup>+</sup> T cells were purified by FACS<sup>®</sup> sorting or via positive-selection with microbeads. Purified populations were subsequently stained with anti-TGF- $\beta$  Abs (thick line) or with the secondary reagent alone (thin line). Results are representative of nine independent experiments. For B, several CD25<sup>+</sup>CD4<sup>+</sup> T cell clones were tested for expression of membrane-bound TGF- $\beta$ . Clone 87 is representative of nonanergic clones, clone 3 of anergic, nonsuppressive clones, and clone 42 of anergic and suppressive clones. Results are representative of two independent tests.

MACS-sorted cells was due to the fact that the primary chicken-anti-TGF- $\beta$  Ab nonspecifically bound to microbeads attached to cells purified by positive selection (Fig. 7 A, right panel, and data not depicted).

**TGF- $\beta$  Is At Least Partially Required for Suppressive Effects of Human CD25<sup>+</sup>CD4<sup>+</sup> T Cell Clones.** The Th3-like cytokine production profile of the suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones prompted us to test whether TGF- $\beta$  was required for their suppressive effects. We therefore performed suppression experiments using immobilized anti-CD3 mAbs as a stimulus in the presence of neutralizing anti-TGF- $\beta$  mAbs. As expected, addition of anti-TGF- $\beta$  mAbs to naive CD4<sup>+</sup> T cells alone resulted in a slight increase in proliferation (Fig. 8). In contrast, addition of anti-TGF- $\beta$  mAbs to the suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones had no effect on their failure to proliferate in response to anti-CD3 mAbs (data not depicted). When the naive CD4<sup>+</sup> T cells were cocultured with suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones, a significant reversal of suppression was consistently observed in the presence of the anti-TGF- $\beta$  mAbs. Neutralizing anti-TGF- $\beta$  mAbs reduced the suppressive capacity of the six CD25<sup>+</sup>CD4<sup>+</sup> T cell clones tested by an average of  $19.5 \pm 8.4\%$  ( $P \leq 0.02$ ). As expected, anti-TGF- $\beta$  mAbs did not significantly affect the proliferation of nonsuppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones in the absence (data not depicted) or presence of naive CD4<sup>+</sup> T cells (Fig. 8). Similar experiments were performed in the presence of anti-IL-10R mAbs, which had no measurable effect on the suppressive capacity of the CD25<sup>+</sup>CD4<sup>+</sup> T cell clones. Furthermore, when both anti-IL-10R and -TGF- $\beta$  mAbs were added in the suppression experiments, results were not significantly different from those with anti-TGF- $\beta$  mAbs alone (data not depicted).



**Figure 8.** Partial requirement for TGF- $\beta$  in suppression mediated by CD25<sup>+</sup>CD4<sup>+</sup> T cell clones. Suppressive and nonsuppressive T cell clones were tested for their ability to reduce proliferation of naive CD4<sup>+</sup> T cells in response to immobilized anti-CD3 mAbs in the absence or presence of neutralizing anti-TGF- $\beta$  mAbs (1D11, 10  $\mu$ g/ml). After 72 h of culture, [<sup>3</sup>H]thymidine was added for an additional 16 h. Numbers above the bars indicate percent reduction in proliferation in comparison to naive CD4<sup>+</sup> T cells without or with the mAbs.

## Discussion

We analyzed naturally occurring human CD25<sup>+</sup>CD4<sup>+</sup> T cells at the clonal level and investigated their possible relationship with IL-10-producing Tr1 cells. Together, our data strongly support the conclusion that these two types of regulatory cells are distinct, and that CD25<sup>+</sup>CD4<sup>+</sup> T cells do not represent the anergic precursors of IL-10-producing Tr1 cells. Unlike Tr1-cell clones (12), suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones express significantly higher levels of CD25 in comparison to nonsuppressive controls, do not proliferate in response to cytokines, and most importantly do not produce IL-10. However, suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones do produce TGF- $\beta$ , and this molecule, although not bound to the cell membrane, had a small, but significant role in suppression of proliferation. Furthermore, characterization at the clonal level of CD25<sup>+</sup>CD4<sup>+</sup> T cells demonstrates that human CD25<sup>+</sup>CD4<sup>+</sup> T cells are not a homogenous population of suppressor cells.

Studies on IL-10-induced antigen-specific unresponsiveness and differentiation of IL-10-producing Tr1 cells revealed that CD25<sup>+</sup> T cells were dispensable for these immunomodulatory effects of IL-10. These data are in line with our previous observation that Tr1 cells arise from CD45RO<sup>-</sup>CD4<sup>+</sup> T cells (19), whereas CD25<sup>+</sup>CD4<sup>+</sup> T suppressor cells are CD45RO<sup>+</sup> (18). These findings support the hypothesis that a major difference between Tr1 and naturally occurring CD25<sup>+</sup>CD4<sup>+</sup> T cells is that whereas CD25<sup>+</sup>CD4<sup>+</sup> T cells exit the thymus as fully differentiated suppressor cells (6), Tr1 cells can arise from any naive peripheral CD4<sup>+</sup> T cell which encounters its antigen in a tolerogenic fashion (3).

It has been previously reported that human CD25<sup>+</sup>CD4<sup>+</sup> T cells could be split into suppressive and nonsuppressive fractions by sorting CD25<sup>bright</sup> and CD25<sup>dim</sup> cells, respectively (21). In the present study, analysis at the clonal level revealed that even the small fraction of CD25<sup>bright</sup>CD4<sup>+</sup> T cells was not a homogenous population of suppressor cells. Only 20 out of 44 (45%) CD25<sup>+</sup>CD4<sup>+</sup> T cell clones which expressed high levels of CD25 were consistently found to be suppressive. However, no conclusions about the absolute frequency of suppressive cells within the CD25<sup>bright</sup> population should be drawn, as the process of cloning and selection of proliferating cells is likely to bias against suppressive cells, which proliferate and expand very poorly in comparison to nonsuppressive cells (data not depicted).

The finding that the CD25<sup>bright</sup> subset of human CD4<sup>+</sup> T cells is not a homogeneous population indicates that the small fraction of suppressive cells must have a remarkably potent suppressor effect. One should consider that the majority of reports on human cells are based on the use of populations of CD25<sup>+</sup>CD4<sup>+</sup> T cells purified with magnetic beads, which contained mixtures of CD25<sup>dim</sup> and CD25<sup>bright</sup> cells, but nevertheless were capable of potently inhibiting proliferation and cytokine production by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (18–20, 22–24). It remains to be

determined whether or not murine CD25<sup>+</sup>CD4<sup>+</sup> T cells also represent a nonhomogenous population of suppressor cells.

Suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones retain the biological properties which were previously described in polyclonal populations. All suppressive T cell clones derived from CD25<sup>+</sup>CD4<sup>+</sup> T cells failed to proliferate in response to anti-CD3 mAbs in the absence of exogenous IL-2, as described previously in both murine and human CD25<sup>+</sup>CD4<sup>+</sup> T cells (1, 2, 18–20, 22–24). In addition, unlike Tr1-cell clones which proliferate in response to cytokines alone (12), all suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones failed to proliferate in response to IL-2, IL-15, or the combination of the two cytokines. This inability to respond to IL-2 or IL-15 was not due to a lack of expression of the IL-2R $\beta$  or  $\gamma$ c chains (data not depicted) and suggest that suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cells may have an intrinsic signaling defect. Importantly, suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones inhibited the proliferation of CD4<sup>+</sup> T cells in response to anti-CD3 mAbs both in the presence or absence of APCs, further supporting the concept that CD25<sup>+</sup>CD4<sup>+</sup> T suppressor cells have a direct effect on the target T cells (6).

Suppressive and nonsuppressive clones derived from CD25<sup>+</sup>CD4<sup>+</sup> T cells could be distinguished via analysis of CD25, CTLA-4, and GITR expression since suppressive function strongly correlated with persistent expression of all three molecules. Experiments with nonsuppressive clones transduced with retroviral vectors encoding CD25 revealed that constitutively high levels of CD25 expression alone are not sufficient to confer suppressive activity. The contribution of CTLA-4 toward the suppressive function is unclear. Some reports demonstrated a role for this molecule (16, 17, 32), whereas others failed to do so (19, 21, 26). With respect to GITR, more studies with well-characterized agonistic and antagonistic Abs, and with the natural ligand, are required to clarify its role in suppression. Although all three of these molecules are markers for suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cells, even at the clonal level, they are not specific since they are also expressed by activated CD4<sup>+</sup> T effector cells.

The finding that suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones do not produce IL-10 contrasts with several studies which reported that freshly isolated human CD25<sup>+</sup>CD4<sup>+</sup> T cells were capable of producing a number of cytokines, including IL-10, when activated by alloantigens or polyclonal stimuli (18–20, 22). The present data suggest that in previous studies performed with nonhomogeneous populations of human CD25<sup>+</sup>CD4<sup>+</sup> T cells, the IL-10 was likely produced by nonsuppressive CD25<sup>+</sup> effector T cells. The observation that IL-10 does not have a role in the suppressive capacity of CD25<sup>+</sup> clones is consistent with previous findings that neutralizing anti-IL-10 mAbs failed to reverse suppression of proliferation by polyclonal populations of human CD25<sup>+</sup>CD4<sup>+</sup> T cells (18–20, 23, 24). These data are also in accordance with the observation that murine

CD25<sup>+</sup>CD4<sup>+</sup> T cells from mice genetically deficient for IL-10 do not have an altered suppressive capacity (6). In vivo studies demonstrating a role for IL-10 in suppression mediated by naturally occurring CD25<sup>+</sup>CD4<sup>+</sup> T cells (30, 38), IL-10 may not have been produced by the suppressive fraction of CD25<sup>+</sup>CD4<sup>+</sup> T cells. Alternatively, it cannot be ruled out that CD25<sup>+</sup>CD4<sup>+</sup> T cells may acquire the capacity to produce IL-10 when stimulated under certain conditions in vivo.

The observation that all the suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones produced TGF- $\beta$ , and that this cytokine was involved in their suppressive effects was somewhat more surprising. Although it has previously been reported that polyclonal populations of human CD25<sup>+</sup>CD4<sup>+</sup> can produce TGF- $\beta$  (18, 19), reversal of suppression in the presence of anti-TGF- $\beta$  mAbs was never observed (18, 19, 23, 24). Furthermore, CD25<sup>+</sup>CD4<sup>+</sup> T cells from mice genetically deficient for TGF- $\beta$ 1 are fully suppressive, at least in vitro (28). However, at the clonal level, we found a small (~20% reversal of inhibition), but consistent and statistically significant role for TGF- $\beta$  in suppression of proliferation by all suppressive CD25<sup>+</sup>CD4<sup>+</sup> T cell clones tested. It should be noted that clonal analysis allowed us to investigate the suppressive mechanism(s) in the absence of contaminating anergic and/or effector CD25<sup>+</sup>CD4<sup>+</sup> T cells which may have confounded analysis of polyclonal populations. Although TGF- $\beta$  may partially mediate the suppressive effects of CD25<sup>+</sup>CD4<sup>+</sup> T cells, it does not appear to be sufficient, and therefore other suppressive mechanisms must exist. Thus, in vivo, CD25<sup>+</sup>CD4<sup>+</sup> T cells may be able to suppress T cell responses by a variety of mechanisms which act in concert, and whose relative contribution may depend on the microenvironment.

This partial requirement for TGF- $\beta$  in suppression mediated by CD25<sup>+</sup>CD4<sup>+</sup> T cell clones contrasts with the findings of Nakamura et al. who observed complete reversal of suppression of proliferation by murine CD25<sup>+</sup>CD4<sup>+</sup> T cells, using the same neutralizing mAb (31). In addition, it has been reported that murine CD25<sup>+</sup>CD4<sup>+</sup> T cells and human CD25<sup>+</sup>CD4<sup>+</sup> thymocytes express cell-surface bound TGF- $\beta$  (31, 32). We did not observe significant staining for membrane-bound TGF- $\beta$  on human CD25<sup>+</sup>CD4<sup>+</sup> T cells isolated from peripheral blood. Further clarification of the role of TGF- $\beta$  in human cells awaits studies with methods which allow specific and regulated abrogation of TGF- $\beta$  expression.

In conclusion, analysis of naturally occurring human CD25<sup>+</sup>CD4<sup>+</sup> T cells at the clonal level revealed that these cells are distinct from IL-10-producing Tr1 cells. This conclusion is supported by two recent reports which suggest that one of the functions of CD25<sup>+</sup>CD4<sup>+</sup> T cells may be to induce the differentiation of IL-10 and TGF- $\beta$ -producing Tr1 cells (39, 40). If true, this may clarify the confusion surrounding the role of these cytokines in suppression mediated by CD25<sup>+</sup>CD4<sup>+</sup> T cells. The finding that freshly isolated CD25<sup>+</sup>CD4<sup>+</sup> T cells from peripheral blood are not a homogeneous population of suppressor cells has important implications for their further characterization, par-

ticularly for studies in which these cells are analyzed at the molecular level. The use of human CD25<sup>+</sup>CD4<sup>+</sup> T cell clones as a homogeneous population of suppressor cells will help to reveal more specific markers, and ultimately gain a better understanding of the factors which act in concert with TGF- $\beta$  to mediate their potent suppressive effects.

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