

# Phenotype, Localization, and Mechanism of Suppression of CD4<sup>+</sup>CD25<sup>+</sup> Human Thymocytes

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## Abstract

Phenotypic markers, localization, functional activities, and mechanisms of action in vitro of CD4<sup>+</sup>CD25<sup>+</sup> T cells, purified from postnatal human thymuses, were investigated. These cells showed poor or no proliferation in mixed lymphocyte culture (MLC), and suppressed in a dose-dependent fashion the proliferative response to allogeneic stimulation of CD4<sup>+</sup>CD25<sup>-</sup> thymocytes. Virtually all CD4<sup>+</sup>CD25<sup>+</sup> thymocytes constitutively expressed cytoplasmic T lymphocyte antigen (CTLA)-4, surface tumor necrosis factor type 2 receptor (TNFR2), and CCR8. They prevalently localized to perivascular areas of fibrous septa and responded to the chemoattractant activity of CCL1/I-309, which was found to be produced by either thymic medullary macrophages or fibrous septa epithelial cells. After polyclonal activation, CD4<sup>+</sup>CD25<sup>+</sup> thymocytes did not produce the cytokines interleukin (IL)-2, IL-4, IL-5, IL-13, interferon  $\gamma$ , and only a very few produced IL-10, but all they expressed on their surface CTLA-4 and the majority of them also transforming growth factor (TGF)- $\beta$ 1. The suppressive activity of these cells was contact dependent and associated with the lack of IL-2 receptor (IL-2R)  $\alpha$ -chain (CD25) expression in target cells. Such a suppressive activity was partially inhibited by either anti-CTLA-4 or anti-TGF- $\beta$ 1, and was completely blocked by a mixture of these monoclonal antibodies, which were also able to restore in target T cells the expression of IL-2R  $\alpha$ -chain and, therefore, their responsiveness to IL-2. These data demonstrate that CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes represent a population of regulatory cells that migrate in response to the chemokine CCL1/I-309 and exert their suppressive function via the inhibition of IL-2R  $\alpha$ -chain in target T cells, induced by the combined activity of CTLA-4 and membrane TGF- $\beta$ 1.

Key words: MLC • CCR8 • I-309 • CTLA-4 • TGF- $\beta$ 1

## Introduction

Spontaneously occurring populations of T cells that regulate autoimmune reactions have been detected in animals (1, 2). One such population is the CD4<sup>+</sup>CD25<sup>+</sup> subset found in normal mice (3). Neonatally thymectomized mice, which are deficient in this population of T cells develop multiorgan autoimmune disease, and the adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> cells from normal mice can protect these animals from autoimmune diseases (4–6). The existence of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg)\* cells has

recently been reported also in human peripheral blood (PB) (7–14) and thymus (13). However, the phenotypic features of CD4<sup>+</sup>CD25<sup>+</sup> human Treg cells so far described are controversial. In addition, the mechanism by which these cells exert their suppressive activity is unclear. This is at least in part due to the fact that human PB CD4<sup>+</sup>CD25<sup>+</sup> T cells represent a heterogeneous population containing not only regulatory, but also activated IL-2 receptor (IL-2R) $\alpha$ -chain (CD25)-expressing effector, T cells (14).

In this study, we have assessed phenotypic markers, localization, and functional activity of CD4<sup>+</sup>CD25<sup>+</sup> T cells purified from postnatal human thymuses. The results provide evidence that CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes exhibit well recognizable phenotypic and functional features. These cells showed indeed poor or no proliferation in

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\*Abbreviations used in this paper: MLC, mixed lymphocyte culture; PB, peripheral blood; SP, single-positive; Treg, regulatory cell.

mixed lymphocyte culture (MLC) and suppressed in a dose-dependent fashion the proliferative response to allogeneic stimulation of CD4<sup>+</sup>CD25<sup>-</sup> T cells. CD4<sup>+</sup>CD25<sup>+</sup> thymocytes constitutively expressed CTLA-4 in their cytoplasm, as well as TNF type 2 receptor (TNFR2), and CCR8 on their surface membrane. These cells prevalently localized to fibrous septa and the medulla of human thymus, and responded to the chemoattractant activity of CCL1/I-309, which was found to be produced by either macrophages or epithelial cells present in the same areas. After polyclonal activation, CD4<sup>+</sup>CD25<sup>+</sup> thymocytes did not produce IL-2, IL-4, IL-5, IL-13, IFN- $\gamma$ , and only a few produced IL-10, but virtually all they expressed on their surface CTLA-4 and the majority of them also expressed membrane TGF (mTGF)- $\beta$ 1. Finally, the suppressive activity of these cells was contact dependent, and appeared to be related to their ability to inhibit the expression of IL-2R  $\alpha$  chain in target T cells. Such a suppressive activity was partially inhibited by neutralizing mAbs against either CTLA-4 or TGF- $\beta$ 1, and was completely blocked by a mixture of these mAbs, which were also able to restore the expression of IL-2R  $\alpha$ -chain in target T cells and, therefore, their responsiveness to IL-2. These data strongly suggest that the regulatory activity of CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes is mediated by the combined action of CTLA-4 and TGF- $\beta$ 1 which are expressed on their surface membrane after activation, via the inhibition of IL-2R  $\alpha$ -chain expression in target T lymphocytes.

## Materials and Methods

**Reagents and Abs.** The medium used throughout was RPMI 1640 (Seromed), supplemented with 2 mM L-glutamine, 1% nonessential aminoacids, 1% pyruvate,  $2 \times 10^{-5}$  M 2-ME (all from GIBCO BRL). Unconjugated and FITC-, PE-, allophycocyanin-, or peridin chlorophyll protein-conjugated anti-CD3 (SK7, mouse IgG1), anti-CD3 (UCHT1, mouse IgG1 used for cell stimulation and immunohistochemistry stainings), anti-CD4 (SK3, mouse IgG1), anti-CD8 (SK1, mouse IgG1), anti-CD11c (S-HCL-3, mouse IgG2b), anti-CD14 (M $\Phi$ 9, mouse IgG2b), anti-CD16 (3G8, mouse IgG1), anti-CD19 (4G7, mouse IgG1), anti-CD25 (2A3, mouse IgG1), anti-CD28 (CD28.2, mouse IgG1 used for cell stimulation), anti-CD34 (My10, mouse IgG1), anti-CD56 (My31, mouse IgG1), anti-CD69 (L78, mouse IgG1), anti-CD83 (HB15e, mouse IgG1), anti-TCR- $\gamma\delta$  (11F2, mouse IgG1), anti-CTLA-4 (BNI3, mouse IgG2a used for flow cytometry and cell cultures), anti-CCR4 (1G1, mouse IgG1), anti-IL-2 (5344.111, mouse IgG1), anti-IL-4 (3010.211, mouse IgG1), anti-IL-5 (TRFK5, rat IgG1), anti-IL-10 (JES3-9D7, rat IgG1), anti-IL-13 (JES10-5A2, rat IgG1), and anti-IFN- $\gamma$  (4S.B3, mouse IgG1) mAbs were purchased from Becton Dickinson. The anti-glycophorin A and B (E3, mouse IgG1), and the anti-cytokeratin (C-11, mouse IgG1) mAbs were from Sigma-Aldrich.

The anti-CD68 (KP1, mouse IgG1) mAb used for immunohistochemistry was from Dako. The PE-conjugated anti-TNFR2 (22235.311, mouse IgG2a), the purified anti-TGF- $\beta$ 1 (9016.2, mouse IgG1) neutralizing mAb, TGF- $\beta$ R2 (goat IgG) neutralizing Ab, and biotin-conjugated anti-TGF- $\beta$ 1 (chicken IgY) and anti-LAP (TGF- $\beta$ 1) (goat IgG) Abs were purchased from R&D Systems. All conjugated and unconjugated isotype-matched con-

trol Abs were purchased from Southern Biotechnology Associates, Inc., (mouse IgG1: clone 15H6; mouse IgG2a: clone HOPC-1; mouse IgG2b: clone A-1, rat IgG, goat IgG, and rabbit IgG). Human recombinant IL-2 was gifted by Eurocetus (Milan, Italy). IL-15, CCL1/I-309, and CCL22/MDC human recombinant cytokine and chemokines were from R&D Systems. The anti-CCR8 rabbit polyclonal Ab used for flow cytometry was from AMS Biotechnology. The anti-CCR8 (second extracellular domain) goat polyclonal Ab used for immunohistochemistry was from Alexis Biotechnology. The anti-I-309 rabbit polyclonal Ab used for immunohistochemistry was from PeproTech. The goat anti-mouse IgG and anti-CD25 Abs conjugated with magnetic beads were obtained from Miltenyi Biotec. The CFSE was from Molecular Probes.

**Human Thymuses.** Normal postnatal thymus specimens were obtained from 15 children, aged between 5 d and 3 yr, who underwent corrective cardiac surgery at the Apuano Pediatric Hospital of Massa Carrara, Italy. The procedures followed in the study were in accordance with the ethical standards of the Regional Committee on Human Experimentation.

**Flow Cytometry Analysis.** Flow cytometry analysis on thymic cell suspensions was performed as described previously (15, 16). To assess the expression of cytoplasmic CTLA-4, cells were washed twice with PBS, pH 7.2, fixed 15 min with formaldehyde (2% in PBS, pH 7.2), washed twice with 0.5% BSA in PBS, pH 7.2, permeabilized with PBS, pH 7.2, containing 0.5% BSA and 0.5% saponin, and then incubated for 15 min at room temperature with the specific mAbs. Cells were then washed and analyzed on a BD-LSR cytofluorimeter using the CELLQuest<sup>TM</sup> software (Becton Dickinson). The area of positivity was determined using an isotype-matched mAb, a total of  $10^4$  events for each sample were acquired.

**Isolation of CD4<sup>+</sup>CD25<sup>+</sup> Thymocytes.** Negative selection of CD4<sup>+</sup> single-positive (SP) thymocytes was first performed by high-gradient magnetic cell sorting, as described previously (15). In brief, thymic MNC suspensions were incubated for 20 min with anti-CD8, anti-CD14, anti-CD16, anti-CD19, anti-CD34, anti-CD56, anti-TCR- $\gamma\delta$ , anti-glycophorin A and B mAbs, extensively washed, and then incubated for additional 20 min with goat anti-mouse polyclonal Ab conjugated to colloidal superparamagnetic microbeads, according to the MACS<sup>®</sup> system (Miltenyi Biotec). After washing, cells were separated on a CS<sup>+</sup> column. The remaining SP CD4<sup>+</sup> T cells were then separated into CD25<sup>+</sup> or CD25<sup>-</sup> by positive selection by the use of anti-CD25 MACS<sup>®</sup> microbeads. The positive selections were performed on LS<sup>+</sup> columns. According to manufacturer's instructions, positively selected cells were used in all the experiments without removing the microbeads conjugated Ab. To exclude possible artifacts, related to the presence in culture of microbeads used for positive selection of CD4<sup>+</sup>CD25<sup>+</sup> thymocytes, these cells were also isolated by FACS<sup>®</sup> sorting (FACSVantage<sup>TM</sup>) by using a PE-coupled anti-CD25 mAb and the responsiveness of the two populations was compared.

**Immunohistochemistry.** Immunohistochemical staining of thymic sections with 5  $\mu$ g/ml anti-CD3, 10  $\mu$ g/ml anti-CD4, 10  $\mu$ g/ml anti-CD11c, 10  $\mu$ g/ml anti-CD25, 5  $\mu$ g/ml anti-CD68, 6  $\mu$ g/ml anti-CD83, anti-cytokeratin (1:1,000 dilution) mAb, 2  $\mu$ g/ml anti-I-309 or 5  $\mu$ g/ml anti-CCR8 rabbit polyclonal Ab, was performed as detailed in previous papers (15, 16). Double immunostaining was performed by using the avidin-biotin-peroxidase system with two different substrates, as described previously (15, 16). To identify on the same specimen two different proteins, the 3-amino-9-ethyl-carbazole (AEC) (red color) and

the Vector SG (bluish gray) substrates were used, respectively (15, 16).

**Chemotactic Assay.** The chemotactic assay was performed according to the technique described previously (15, 16). In brief, CCL1/1-309 or CCL22/MDC were added at optimal concentrations (100 nM) in RPMI 1640 containing 0.5% BSA to the bottom well of a transwell chamber (3- $\mu$ m pore size, 12 wells; Costar).  $2 \times 10^6$  freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> thymocytes were resuspended in the same buffer and loaded into the top well. Cell migration was allowed to occur for 4 h at 37°C and cells migrating to the lower chamber were harvested and counted by BD-LSR cytofluorimeter. Cells that migrated in the presence of medium alone served as a negative control.

**Labeling of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> Thymocytes with CFSE.** Labeling of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> thymocytes with CFSE was performed as described previously (17). In brief, cells were extensively washed and resuspended at final concentration of  $10^7$  cells per milliliter in PBS. CFSE was added at a final concentration of 5  $\mu$ M and incubated for 4 min at room temperature. The reaction was stopped by cell washing with RPMI 1640, containing 10% heat inactivated FCS.

**Polyclonal Stimulation of CFSE-labeled CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> Thymocytes.** CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> purified thymocytes, after labeling with CFSE, were stimulated in RPMI 1640 medium containing 10% FCS serum on plates coated with anti-CD3 (5  $\mu$ g/ml) plus soluble anti-CD28 (10  $\mu$ g/ml) mAbs. The cells were monitored every 2 d for CTLA-4, TGF- $\beta$ 1 expression, and CFSE content and stimulated at day 6 for cytokine production.

**Cytometric Analysis of Intracellular Cytokines Versus Proliferation.** Intracytofluorimetric analysis of IL-2, IL-4, IL-5, IL-10, IL-13, and IFN- $\gamma$  synthesis at single cell level was performed as described previously (18), the only difference being stimulation of cells already labeled with CFSE. In brief,  $10^6$  cells were stimulated with 10 ng/ml PMA plus 1  $\mu$ M ionomycin for 6 h, the last two of which in the presence of 5  $\mu$ g/ml brefeldin A. After stimulation, cells were washed twice with PBS, pH 7.2, fixed 15 min with formaldehyde (2% in PBS, pH 7.2), washed twice with 0.5% BSA in PBS, pH 7.2, permeabilized with PBS, pH 7.2, containing 0.5% BSA and 0.5% saponin, and then incubated for 15 min at room temperature with the specific mAb. Cells were then washed and analyzed on a BD-LSR cytofluorimeter using the CELLQuest™ software (Becton Dickinson). The area of positivity was determined using an isotype-matched mAb, a total of  $10^4$  events for each sample were acquired.

**Proliferation Assays.** To assess the proliferative response of human CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> thymocytes, allogeneic PBMNCs, depleted of T cells by the use of anti-CD3 MACS® microbeads were used.  $10^5$  irradiated (6,000 rad) T cell-depleted PBMNCs were cultured with  $5 \times 10^4$  purified CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> thymocytes. On day 5, after 8-h pulsing with 0.5  $\mu$ Ci <sup>3</sup>[H]TdR/well (Amersham Pharmacia Biotech), cultures were harvested and radionuclide uptake measured by scintillation counting.

For the assessment of suppressive properties,  $5 \times 10^4$  CD4<sup>+</sup>CD25<sup>-</sup> thymocytes were cultured with  $10^5$  irradiated T cell-depleted allogeneic PBMNCs in the presence of different numbers of CD4<sup>+</sup>CD25<sup>+</sup> autologous thymocytes. In some experiments, 10  $\mu$ g/ml anti-TGF- $\beta$ 1, 10  $\mu$ g/ml anti-CTLA-4, anti-TGF- $\beta$ 1 plus anti-CTLA-4, or isotype control (IgG1+IgG2a 10  $\mu$ g/ml each) mAbs were added to the cultures. On day 5, after 8-h pulsing with 0.5  $\mu$ Ci <sup>3</sup>[H]TdR/well (Amersham Pharmacia Biotech), cultures were harvested and radionuclide

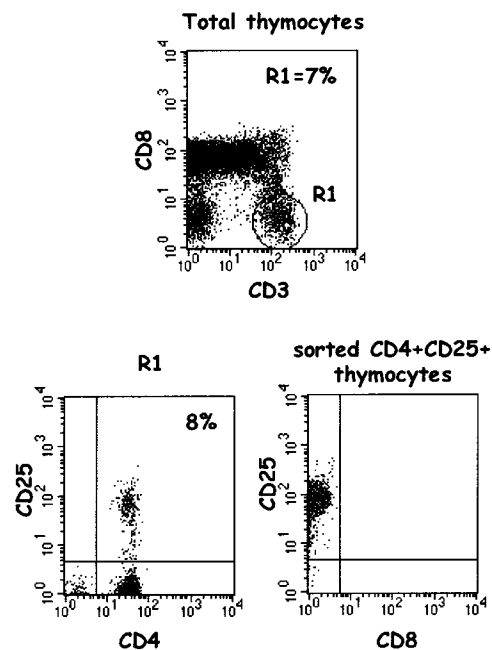
uptake measured by scintillation counting. In other experiments, the ability of CD4<sup>+</sup>CD25<sup>+</sup> cells to inhibit the proliferation of CFSE-labeled CD4<sup>+</sup>CD25<sup>-</sup> thymocytes in MLC to irradiated allogeneic human adult PB non-T cells was evaluated at different time intervals by flow cytometry. In the same experiments, the expression of CD25 and CD69 in both the CFSE<sup>-</sup>CD3<sup>+</sup> regulatory population and CFSE<sup>+</sup>CD3<sup>+</sup> target population was also evaluated. In parallel, MLCs were performed in presence or absence of 10 IU/ml IL-2 or 10 ng/ml IL-15 and thymidine incorporation was evaluated on day 5.

**Transwell Experiments.** Transwell experiments were performed in 24-well transwell plates (0.22  $\mu$ m pore size; Costar).  $5 \times 10^5$  CD4<sup>+</sup>CD25<sup>-</sup> thymocytes were stimulated with  $10^6$  irradiated T cell-depleted allogeneic PBMNCs in the bottom chamber, in the absence or presence of  $5 \times 10^5$  CD4<sup>+</sup>CD25<sup>+</sup> thymocytes placed in the same or in the top chamber. On day 5, after 8-h pulsing with 0.5  $\mu$ Ci <sup>3</sup>[H]TdR/well (Amersham Pharmacia Biotech), the cells of the lower chambers were harvested and radionuclide uptake was measured by scintillation counting.

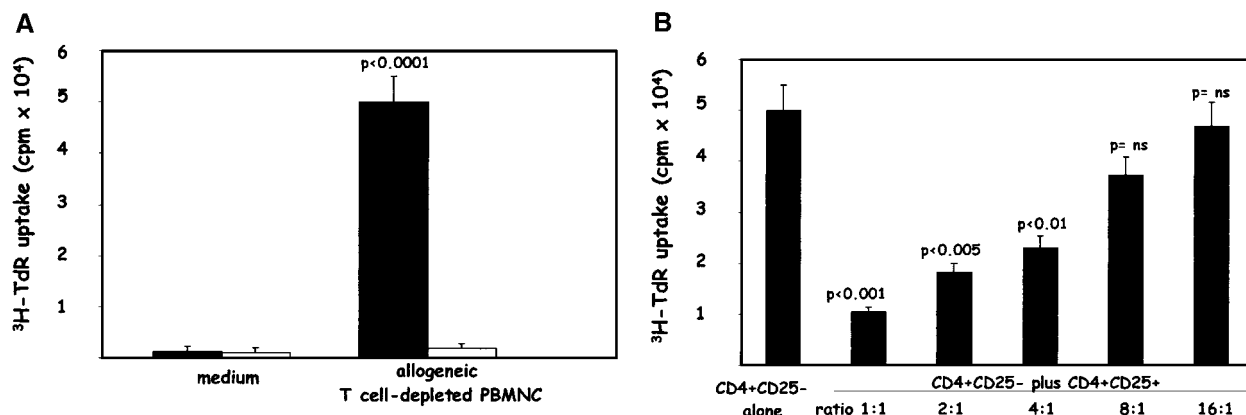
**Statistical Analysis.** Statistical analysis was performed using the Student's *t* test.

## Results

**Functional Properties of CD4<sup>+</sup>CD25<sup>+</sup> Human Thymocytes.** CD4<sup>+</sup>CD25<sup>+</sup> T cells were purified from six postnatal human thymuses by negative selection of CD4<sup>+</sup>, followed by positive selection of CD25<sup>+</sup> cells. The purified population consistently contained >98% CD4<sup>+</sup>CD25<sup>+</sup> thymocytes, meanly representing 7% ( $\pm$ 2) of the entire SP CD4<sup>+</sup>CD3<sup>+</sup> thymocyte population (Fig. 1). The ability of purified CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> thymocytes to



**Figure 1.** Identification and purification of CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes. Freshly isolated human thymocytes were assessed for the expression of CD3, CD4, CD8, and CD25 by flow cytometry. After MACS® sorting, purified CD4<sup>+</sup>CD25<sup>+</sup> thymocytes were assessed for CD8 and CD25 expression. One representative experiment is shown.

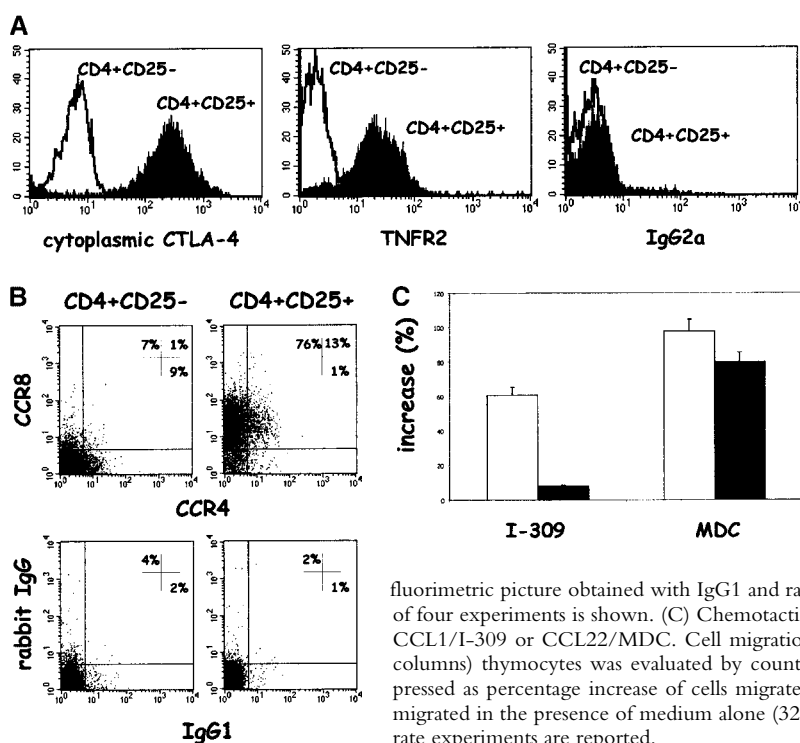


**Figure 2.** CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes do not proliferate in MLC and suppress the proliferation in MLC of CD4<sup>+</sup>CD25<sup>-</sup> thymocytes. (A) Proliferative response of purified CD4<sup>+</sup>CD25<sup>+</sup> (white columns) and CD4<sup>+</sup>CD25<sup>-</sup> (black columns) human thymocytes to allogeneic stimulation. Mean values ( $\pm$ SD) obtained in six separate experiments are reported. (B) Suppression by CD4<sup>+</sup>CD25<sup>+</sup> thymocytes of the proliferative response of autologous CD4<sup>+</sup>CD25<sup>-</sup> thymocytes to allogeneic T cell-depleted PBMCs. Mean values ( $\pm$ SD) obtained in six separate experiments are reported.

proliferate in MLC to irradiated allogeneic human adult PB non-T cells was then assessed. As shown in Fig. 2 A, CD4<sup>+</sup>CD25<sup>+</sup> thymocytes virtually did not proliferate in response to allogeneic stimulation, whereas under the same experimental conditions, the CD4<sup>+</sup>CD25<sup>-</sup> thymocyte fraction showed strong proliferation. The suppressive activity of the CD4<sup>+</sup>CD25<sup>+</sup> thymocyte population was then evaluated by adding increasing numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells to the autologous CD4<sup>+</sup>CD25<sup>-</sup> counterpart, stimulated with irradiated allogeneic non-T cells. As shown in Fig. 2 B, CD4<sup>+</sup>CD25<sup>+</sup> thymocytes inhibited in a dose-dependent fashion the MLC response obtained with the CD4<sup>+</sup>CD25<sup>-</sup> thymocyte population. In additional experi-

ments, in which CD4<sup>+</sup>CD25<sup>+</sup> thymocytes were isolated paralleled by either the MACS<sup>®</sup> system or FACS<sup>®</sup> sorting, quite comparable results were obtained (unpublished data).

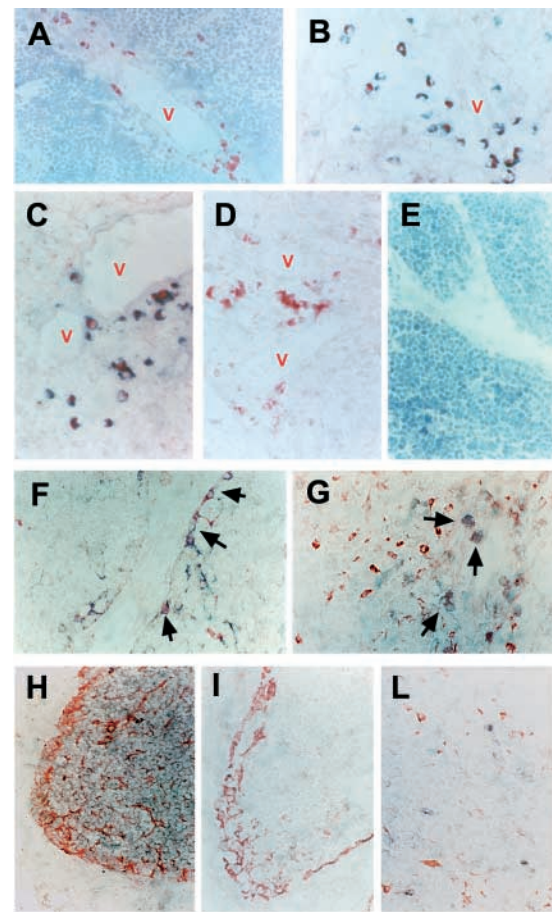
**Markers and Localization of CD4<sup>+</sup>CD25<sup>+</sup> Regulatory Thymocytes.** Having established that purified CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes showed the classic in vitro functional activities described for murine Treg cells, i.e., poor or no proliferation, as well as suppressive activity, the expression on these cells of some markers was analyzed. Virtually all CD4<sup>+</sup>CD25<sup>+</sup> thymocytes constitutively expressed cytoplasmic CTLA-4, as well as surface TNFR2 (Fig. 3 A). Moreover, they expressed CCR8, whereas CCR4 was detectable on a small proportion of these cells and was also



**Figure 3.** All CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes constitutively express cytoplasmic CTLA-4 and surface TNFR2, CCR8 and exhibit chemotactic response to CCL1/I-309. (A) Freshly purified CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> thymocytes were assessed for the expression of cytoplasmic CTLA-4 and surface TNFR2 by flow cytometry. The cytofluorimetric picture obtained with IgG2a isotype control is also depicted. One representative of four experiments is shown. (B) Expression of CCR4 and CCR8 on the surface of CD4<sup>+</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD25<sup>+</sup> thymocytes. The cytofluorimetric picture obtained with IgG1 and rabbit IgG isotype controls is also depicted. One representative of four experiments is shown. (C) Chemotactic response of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> thymocytes to CCL1/I-309 or CCL22/MDC. Cell migration of CD4<sup>+</sup>CD25<sup>+</sup> (white columns) or CD4<sup>+</sup>CD25<sup>-</sup> (black columns) thymocytes was evaluated by counting migrated cells with BD-LSR cytometer. Values are expressed as percentage increase of cells migrated in response to CCL1/I-309 or CCL22/MDC versus cells migrated in the presence of medium alone ( $32.3 \pm 1.7 \times 10^3$ ). Mean values ( $\pm$ SD) obtained in three separate experiments are reported.

present on a number of CD4<sup>+</sup>CD25<sup>-</sup> thymocytes (Fig. 3 B). CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> thymocyte populations were then assessed for their ability to respond to the chemoattractant activity of CCR8 and CCR4 ligands, CCL1/I-309 and CCL22/MDC, respectively. CCL22/MDC induced migration of both CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> thymocyte populations, whereas CCL1/I-309 induced migration of the latter alone (Fig. 3 C). By using immunohistochemical staining with anti-CD25 mAb, CD4<sup>+</sup>CD25<sup>+</sup> thymocytes were mainly detected in the fibrous septa with prevalent perivascular localization (Fig. 4, A and B), lower numbers of these cells also being present in the medullary areas (unpublished data). Immunostaining with anti-CCR8 Ab confirmed such a localization (Fig. 4, C–E). The nature and the localization of thymic cells producing the CCR8 ligand, CCL1/I-309, was also analyzed. Most CCL1/I-309-producing cells were detected in the fibrous septa, and some of them also in the medulla (Fig. 4, F–L). Part of CCL1/I-309-producing cells costained for cytokeratin (Fig. 4 F), revealing their nature of epithelial cells, whereas a proportion of them costained for CD68 (Fig. 4 G), but never for CD3 (Fig. 4 H), CD83 (Fig. 4 I) or CD11c (unpublished data), thus suggesting they were macrophages. Of note, virtually all CCL1/I-309-producing cells in the septa were epithelial cells, whereas CCL1/I-309-producing cells present in the medullary areas were either epithelial cells or macrophages.

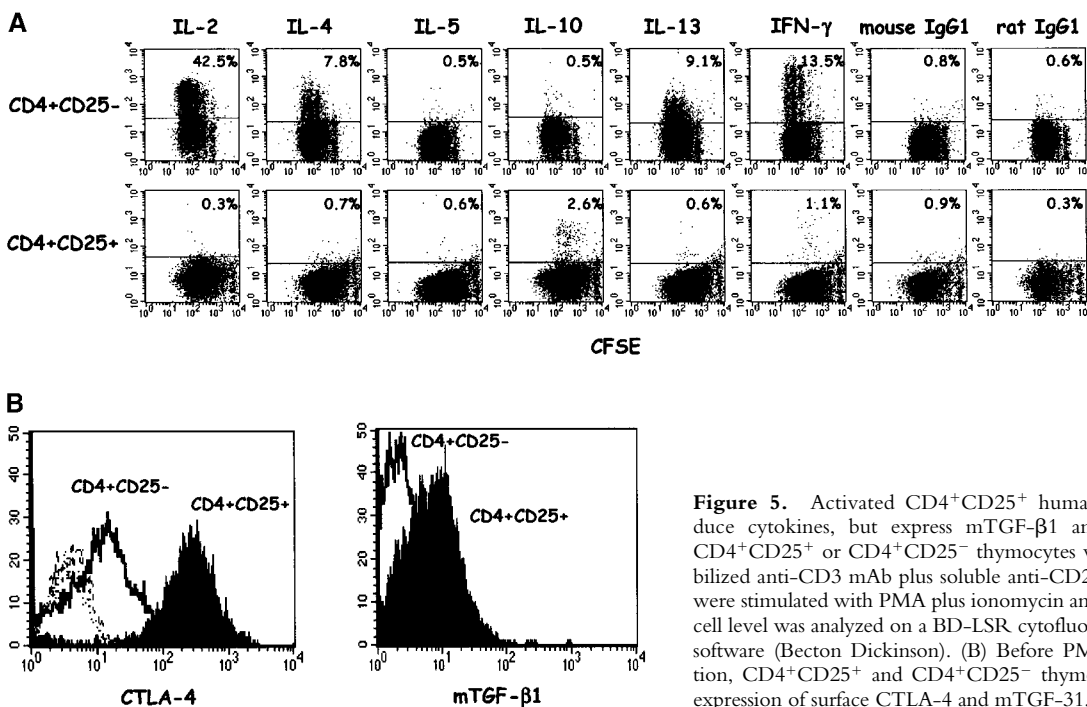
*The Suppressive Activity of CD4<sup>+</sup>CD25<sup>+</sup> Regulatory Thymocytes Is Contact-dependent and Mediated by CTLA-4 and mTGF- $\beta$ 1.* After 6 d of stimulation with insolubilized anti-CD3 plus soluble anti-CD28 mAbs of CFSE-labeled CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> thymocytes, cells were further stimulated with PMA plus ionomycin. In agreement with the results obtained by measuring <sup>3</sup>[H]TdR incorporation, the CD4<sup>+</sup>CD25<sup>+</sup> cells showed strongly reduced proliferation, as assessed by CFSE content (Fig. 5 A). Moreover, the same cells did not produce IL-2, IL-4, IL-5, IL-13, or IFN- $\gamma$ , and only a very few of them produced IL-10, as assessed by flow cytometry at single cell level (Fig. 5 A). However, stimulation with insolubilized anti-CD3 plus soluble anti-CD28 mAbs, induced virtually all CD4<sup>+</sup>CD25<sup>+</sup> thymocytes to express CTLA-4 on their surface and about a half of them also mTGF- $\beta$ 1 (Fig. 5 B). The latter finding is of interest, since mTGF- $\beta$ 1 has recently been suggested to play a role in the suppressive activity of murine CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (19). To provide direct evidence that mTGF- $\beta$ 1 was also involved in the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes, we first assessed the effect of purified CD4<sup>+</sup>CD25<sup>+</sup> thymocytes on the response of CD4<sup>+</sup>CD25<sup>-</sup> T cells in MLC by using transwell chambers, in which the proliferative response of CD4<sup>+</sup>CD25<sup>-</sup> T cells to irradiated allogeneic non-T cells placed in the lower chamber was evaluated in absence (top chamber) or in presence (bottom chamber) of CD4<sup>+</sup>CD25<sup>+</sup> T cells. As shown in Fig. 6 A, the proliferative response of CD4<sup>+</sup>CD25<sup>-</sup> T cells was strongly inhibited by CD4<sup>+</sup>CD25<sup>+</sup> T cells, but only when they were present in the same chamber, indicating that the suppressive activity



**Figure 4.** Localization of CD4<sup>+</sup>CD25<sup>+</sup> and of CCL1/I-309-expressing cells in human thymus. (A) Thymic sections stained with anti-CD25 mAb (red color) and counterstained with Gill's Hematoxylin (original magnification:  $\times 250$ ). (B) Double staining of the same thymus with anti-CD4 (bluish gray) and anti-CD25 (red color) mAb; no counterstain was applied (original magnification:  $\times 400$ ). (C) Double staining with anti-CD25 mAb (bluish gray) and anti-CCR8 polyclonal Ab (red color); no counterstain was applied (original magnification:  $\times 400$ ). (D) Double staining with anti-CCR8 polyclonal Ab (red color) and an isotype-matched mAb with irrelevant specificity (bluish gray); no counterstain was applied (original magnification:  $\times 400$ ). (E) Absence of staining in the same thymus using a rabbit polyclonal Ab with irrelevant specificity (original magnification:  $\times 400$ ). (F) Double staining with anti-CCL1/I-309 (red) and anti-CK (bluish gray) Abs. Arrows indicate cells showing double staining for CCL1/I-309 and CK; no counterstain was applied (original magnification:  $\times 400$ ). (G) Double staining with anti-CCL1/I-309 (red) and anti-CD68 (bluish gray). Arrows indicate cells showing double staining for CCL1/I-309 and CD68; no counterstain was applied (original magnification:  $\times 400$ ). (H) Double staining with anti-CCL1/I-309 (red) and anti-CD3 (bluish gray); no counterstain was applied (original magnification:  $\times 400$ ). (I) Double staining with anti-CCL1/I-309 polyclonal Ab (red color) and an isotype-matched mAb with irrelevant specificity (bluish gray); no counterstain was applied (original magnification:  $\times 400$ ). (L) Double staining with anti-CCL1/I-309 (red) and anti-CD83 (bluish gray); no counterstain was applied (original magnification:  $\times 400$ ). V, vessel.

of these cells was mediated by cell-to-cell contact and not via the release of soluble factors.

The nature of molecule(s) present on the surface of CD4<sup>+</sup>CD25<sup>+</sup> thymocytes that were responsible for their suppressive activity was then investigated. To do this, dif-

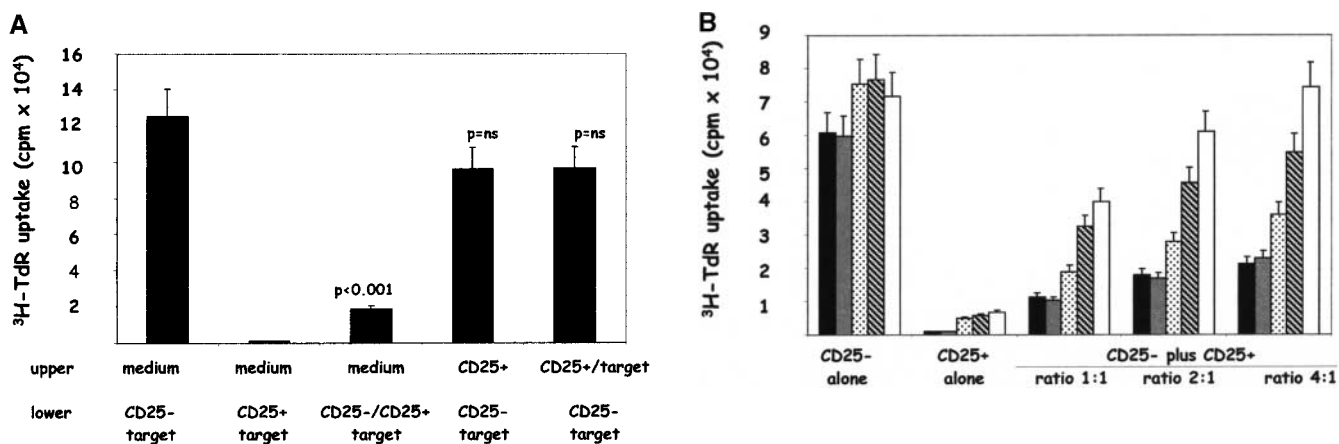


**Figure 5.** Activated CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes do not produce cytokines, but express mTGF- $\beta$ 1 and CTLA-4. CFSE-labeled CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>-</sup> thymocytes were incubated with insolubilized anti-CD3 mAb plus soluble anti-CD28 mAb. (A) On day 6, cells were stimulated with PMA plus ionomycin and cytokine synthesis at single cell level was analyzed on a BD-LSR cytofluorimeter using CELLQuest™ software (Becton Dickinson). (B) Before PMA plus ionomycin stimulation, CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> thymocytes were assessed for the expression of surface CTLA-4 and mTGF- $\beta$ 1, by flow cytometry.

ferent numbers of purified CD4<sup>+</sup>CD25<sup>+</sup> thymocytes were added to cultures of CD4<sup>+</sup>CD25<sup>-</sup> T cells stimulated in MLC in absence or presence of neutralizing anti-CTLA-4, anti-TGF- $\beta$ 1, anti-CTLA-4 plus anti-TGF- $\beta$ 1, or isotype control, mAbs. The results of these experiments are summarized in Fig. 6 B. The addition of isotype control mAbs did not exert any effect on the inhibitory activity of CD4<sup>+</sup>CD25<sup>+</sup> thymocytes on the proliferative response of CD4<sup>+</sup>CD25<sup>-</sup> T cells. By contrast, the addition of either anti-CTLA-4 or anti-TGF- $\beta$ 1 mAb partially, but consis-

tently, reduced the inhibitory activity of CD4<sup>+</sup>CD25<sup>+</sup> thymocytes, and such a suppressive activity was completely abolished by the contemporaneous addition of the two mAbs. Of note, similar effects as those induced by the anti-TGF- $\beta$ 1 mAb were observed by adding in culture an anti-TGF- $\beta$ 1R mAb (unpublished data).

*CTLA-4 and TGF- $\beta$ 1 Exert Their Suppressive Activity by Inhibiting the Expression of IL-2R  $\alpha$ -Chain and the Responsiveness of Target T Cells to IL-2.* The mechanism by which CTLA-4 and/or mTGF- $\beta$ 1 exerted their inhibitory



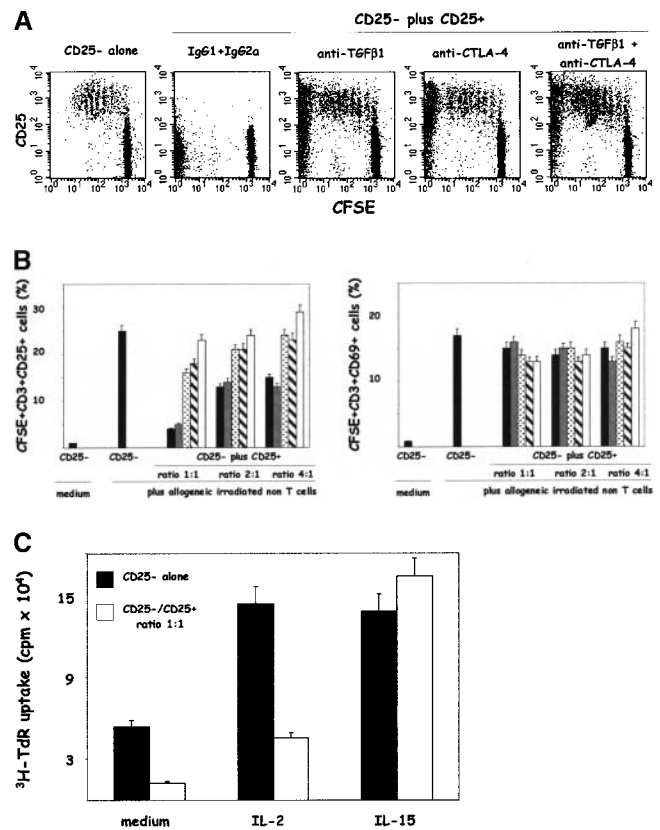
**Figure 6.** Cell contact-dependent suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes is abrogated by the combined action of anti-CTLA-4 and anti-TGF $\beta$ 1 mAbs. (A) CD4<sup>+</sup>CD25<sup>-</sup> thymocytes were stimulated with irradiated T cell-depleted allogeneic PBMCs in the lower chamber of a transwell plate in the absence or presence of CD4<sup>+</sup>CD25<sup>+</sup> thymocytes, placed in the same or in the top chamber. On day 5, cells present in the bottom chamber were harvested and their proliferation assessed by measuring <sup>3</sup>H]TdR uptake. (B) CD4<sup>+</sup>CD25<sup>-</sup> thymocytes were stimulated with allogeneic irradiated T cell-depleted PBMCs in the presence of different numbers of autologous purified CD4<sup>+</sup>CD25<sup>+</sup> thymocytes without (black columns) or with isotype control (IgG1+IgG2a) (gray columns), anti-CTLA-4 (hatched columns), or anti-TGF- $\beta$ 1 mAb (dotted columns), or mixtures of them (white columns). Cell proliferation was measured by assessment of <sup>3</sup>H]TdR uptake. Mean values ( $\pm$ SD) obtained in four separate experiments are reported.

activity on the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> thymocytes was finally investigated. To this end, CD4<sup>+</sup>CD25<sup>-</sup> thymocytes stimulated in MLC were cultured alone or together with autologous CD4<sup>+</sup>CD25<sup>+</sup> thymocytes, in presence of neutralizing anti-CTLA-4, anti-TGF-β1, anti-CTLA-4 plus anti-TGF-β1, or isotype control, mAbs. The proliferative response of CD4<sup>+</sup>CD25<sup>-</sup> thymocytes, as well as their ability to express CD25 under this different experimental conditions, was then assessed by flow cytometry. As expected, CD4<sup>+</sup>CD25<sup>-</sup> thymocytes stimulated in absence of CD4<sup>+</sup>CD25<sup>+</sup> cells showed strong proliferation, and proliferating cells expressed high CD25 levels, whereas those stimulated in presence of CD4<sup>+</sup>CD25<sup>+</sup> cells did neither proliferate, nor expressed CD25. However, CD25 expression by these cells was restored by the addition of anti-CTLA-4, anti-TGF-β1, and even more by anti-CTLA-4 plus anti-TGF-β1, mAbs, whereas the addition of isotype control mAbs had no effect. By contrast, the expression of another T cell activation marker, such as CD69, was not altered (Fig. 7, A and B). These data suggest that both CTLA-4 and mTGF-β1 contribute to suppress the proliferation of stimulated CD4<sup>+</sup>CD25<sup>-</sup> thymocytes by inhibiting the expression of IL-2R α-chain, thus rendering these cells unresponsive to IL-2.

To support this possibility, IL-2 or IL-15 were added to cultures of CD4<sup>+</sup>CD25<sup>-</sup> thymocytes, stimulated in MLC in absence or presence of autologous CD4<sup>+</sup>CD25<sup>+</sup> cells, and the degree of radionuclide incorporation was assessed. As shown in Fig. 7 C, the suppressive activity exerted by CD4<sup>+</sup>CD25<sup>+</sup> thymocytes on the proliferation of the CD4<sup>+</sup>CD25<sup>-</sup> counterpart, was not significantly affected by the addition in culture of IL-2, whereas IL-15 completely restored the proliferative response of these cells.

## Discussion

CD4<sup>+</sup>CD25<sup>+</sup> Treg cells are essential for the maintenance of self-tolerance in mice (1–5). Similar cells have also been described in humans (6–13), but the phenotypic and functional features of human CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, as well as their mechanism of action, are controversial. This is due, at least in part, to the fact that most studies were performed by using purified PB CD4<sup>+</sup>CD25<sup>+</sup> T cells, which represent a heterogeneous population, containing activated effector, in addition to regulatory, T cells (14). For example, it was reported that PB CD4<sup>+</sup>CD25<sup>+</sup> cells do not produce any cytokine (7, 14), or secrete IL-2 or IL-4, but low levels of IFN-γ and higher levels of IL-10 and TGF-β (9), or IL-10 alone (11), or higher levels of IL-4, and similar amounts of IL-10, as CD4<sup>+</sup>CD25<sup>-</sup> cells (13). The possible phenotypic markers of human CD4<sup>+</sup>CD25<sup>+</sup> human PB Treg cells are also partially known. These cells express CD45RO and HLA-DR (8, 9, 11, 14), but these molecules are also present on the surface of effector T cells. Most studies reported CTLA-4 upregulation in these cells (7–11). The CD62L has also been found to be expressed (8), whereas CD40L is downregulated (8) or is expressed at



**Figure 7.** CTLA-4 and TGF-β1-mediated suppression is due to the inhibition of IL-2R α-chain expression and is overcome by IL-15. (A) CFSE-labeled CD4<sup>+</sup>CD25<sup>-</sup> thymocytes were stimulated with irradiated allogeneic PB non-T cells in absence or presence of autologous CD4<sup>+</sup>CD25<sup>+</sup> thymocytes with anti-CTLA-4, anti-TGF-β1, anti-CTLA-4 plus anti-TGF-β1 or isotype-matched (IgG1 plus IgG2a) mAbs, and assessed by flow cytometry for proliferation and surface CD25 expression. One representative of three separate experiments is shown. (B) CD25 (left) and CD69 (right) expression by CD4<sup>+</sup>CD25<sup>-</sup> thymocytes cultured under the experimental conditions mentioned in (A). CD4<sup>+</sup>CD25<sup>-</sup> thymocytes without mAb added (black columns), or containing isotype control (gray columns), anti-TGF-β1 (dotted columns), anti-CTLA-4 (hatched columns), or anti-CTLA-4 plus anti-TGF-β1 (white columns) mAbs. Columns represent mean values (±SD) found in three separate experiments. (C) Effect of the addition of exogenous IL-2 or IL-15 on the proliferative response of CD4<sup>+</sup>CD25<sup>-</sup> thymocytes stimulated with irradiated allogeneic non-T cells in absence or presence of CD4<sup>+</sup>CD25<sup>+</sup> autologous thymocytes. Columns represent mean values (±SD) obtained in three separate experiments.

the same levels than in CD4<sup>+</sup>CD25<sup>-</sup> T cells (11). In a recent report, it has been found that CD4<sup>+</sup>CD25<sup>+</sup> PB Treg cells express the chemokine receptors CCR4 and CCR8 and are chemoattracted by the respective ligands, CCL17/TARC, CCL22/MDC, and CCL1/I-309 (12). The mechanism of action of CD4<sup>+</sup>CD25<sup>+</sup> PB Treg cells also remains to be determined. Several studies agree that the suppressive activity of these cells requires cell-to-cell contact and is cytokine independent (7, 8, 11). However, although a possible role for CTLA-4 and PDL1 in T cell–T cell regulation has recently been suggested (14), the surface molecules involved in the interactions responsible for suppression have not yet been clearly defined.

In this study, we attempted to better characterize the phenotype of human CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, as well the mechanisms involved in their suppressive activity by assessing the CD4<sup>+</sup>CD25<sup>+</sup> population present in postnatal human thymuses. Indeed, we hypothesized that CD4<sup>+</sup>CD25<sup>+</sup> thymocytes may represent a less heterogeneous population than PB CD4<sup>+</sup>CD25<sup>+</sup> T cells, thus avoiding possible interferences due to contaminating CD4<sup>+</sup>CD25<sup>+</sup> T cells with different or even opposite functions. So far, only one study dealing with CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes has been performed (13), but no information other than the suppressive activity of these cells and their inability to produce cytokines was reported (13). The results of the present study allow to draw a number of conclusions on the phenotype, localization, and some functional features of CD4<sup>+</sup>CD25<sup>+</sup> cells, as well as on the mechanisms involved in their suppressive activity. First, in agreement with the results already reported by Stephens et al. (13), CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes were unable to proliferate and suppressed in a dose-dependent fashion the proliferation of other CD4<sup>+</sup> T cells *in vitro*. Second, they were characterized by the selective constitutive expression of CD25<sup>hi</sup>, cytoplasmic CTLA-4, surface TNFR2, and CCR8, whereas their surface CCR4 expression was limited and not selective. Third, these cells were mainly observed in the context of the fibrous septa of the thymus with prevalent perivascular localization, and responded to the chemoattractant activity of CCL1/I-309. Characterization of CCL1/I-309-producing cells by double immunostaining revealed that epithelial cells were the source of this chemokine in the fibrous septa, whereas small numbers of epithelial cells and macrophages producing CCL1/I-309 were found in the medullary areas. Taken together, these data support the view that CCL1/I-309-CCR8 interaction is probably essential for the migration of this subset within the thymus or from the thymus to the periphery.

In addition, after polyclonal stimulation with PMA plus ionomycin, CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes did not produce IL-2, IL-4, IL-5, IL-13, IFN- $\gamma$ , and only a very few of them produced IL-10. However, stimulation with anti-CD3 plus anti-CD28 mAbs induced virtually all CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes to express on their surface membrane CTLA-4, and ~50% of them also TGF- $\beta$ 1. Since the regulatory activity of CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes was found to be clearly dependent by cell-to-cell contact, we asked whether the neutralization of molecules selectively expressed by these cells could also inhibit their suppressive activity. While the addition in culture of isotype control mAbs had no effect, blocking of either CTLA-4, TGF- $\beta$ 1, or TGF- $\beta$ 1R, partially, but consistently, inhibited the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes. More importantly, such a suppressive activity was completely abolished by the contemporaneous addition of anti-CTLA-4 and anti-TGF- $\beta$ 1 mAbs. These findings suggest that both CTLA-4 and mTGF- $\beta$ 1 are involved in the suppressive activity of these cells.

The results reported in this study are not fully consistent with those of studies performed so far by using PB

CD4<sup>+</sup>CD25<sup>+</sup> T cells, which failed to demonstrate reversal of suppression with anti-CTLA-4 or anti-TGF- $\beta$  Abs (8, 9, 11, 13, 14). This discrepancy may be due to either the heterogeneity of PB in comparison with thymus-derived CD4<sup>+</sup>CD25<sup>+</sup> T cells, or to the different assays used to assess the suppressive activity. However, the role of CTLA-4 in the regulatory activity of murine CD4<sup>+</sup>CD25<sup>+</sup> T cells has been clearly demonstrated *in vivo* (5, 6). More importantly, in a recent study it was shown that the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> murine T cells was abolished by the neutralization of TGF- $\beta$  and that stimulated CD4<sup>+</sup>CD25<sup>+</sup> T cells expressed high and persistent levels of TGF- $\beta$ 1 on their cell surface (19). Since CTLA-4 engagement has been found to enhance the secretion of TGF- $\beta$  (20), it is reasonable to explain why in this study a complete inhibition of the suppressive activity exerted by activated CD4<sup>+</sup>CD25<sup>+</sup> human thymocytes could be achieved only when both CTLA-4 and TGF- $\beta$ 1 were neutralized. Considering that complete anti-CTLA-4 mAb instead of its Fab fragment was used, the possibility of signaling instead of blocking effects cannot be excluded. However, based on the fact that similar effects were obtained by using either anti-CTLA-4, anti-TGF- $\beta$ 1, or anti-TGF- $\beta$ 1R mAbs, and even more, when anti-CTLA-4 and anti-TGF- $\beta$ 1 mAbs were combined, this possibility appears to be unlikely.

The results of this study also provide insights into the mechanisms by which TGF- $\beta$ 1 expressed on the surface of CD4<sup>+</sup>CD25<sup>+</sup> thymocytes is able to suppress the proliferative response of stimulated CD4<sup>+</sup>CD25<sup>-</sup> cells. Indeed, we found that under these conditions, the latter cells could express the T cell activation marker CD69, but not the IL-2R  $\alpha$ -chain and, therefore, became unresponsive to IL-2. This possibility was supported by the observation that the inability of target T cells to proliferate was not affected by the addition of exogenous IL-2, but it was overcome by the addition of IL-15, a T cell stimulatory cytokine that does not use the high affinity IL-2R (21). These findings are consistent with the results of a recent study, showing suppression of IL-2-induced human T cell proliferation and phosphorylation of signal transducer and activator of transcription (STAT)-3 and STAT-5 by TGF- $\beta$  (22). The suppression of intracellular signaling and proliferation mediated by TGF- $\beta$  could be completely overcome by treating T cells with IL-15 (22). Thus, the results of the experiments here reported not only allow to better characterize some phenotypic and functional aspects of human CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, but also provide new insights into their mechanism of action.

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