The Journal of Immunology

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J Immunol 2004; 172:834-842; ;
doi: 10.4049/jimmunol.172.2.834
http://www.jimmunol.org/content/172/2/834

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

TGF-β1 Plays an Important Role in the Mechanism of CD4$^+$CD25$^+$ Regulatory T Cell Activity in Both Humans and Mice

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In previous studies, we have shown that murine CD4$^+$CD25$^+$ regulatory T cells produce high levels of TGF-β1 in a cell surface and/or secreted form, and blockade of such TGF-β1 by anti-TGF-β curtails the ability of these cells to suppress CD25$^-$ T cell proliferation and B cell Ig production in in vitro suppressor assays. In further support for the role of TGF-β1 in suppression by CD4$^+$CD25$^+$ T cells, we show in this study that another TGF-β1-blocking molecule, recombinant latency-associated peptide of TGF-β1 (rLAP), also reverses suppression by mouse CD4$^+$CD25$^+$ T cells as well as their human counterparts, CD4$^+$CD25^{high} T cells. In addition, we show that CD25$^-$ T cells exposed to CD4$^+$CD25$^+$ T cells in vitro manifest activation of Smad-2 and induction of CD103, the latter a TGF-β-inducible surface integrin. In further studies, we show that while CD4$^+$CD25$^+$ T cells from TGF-β1-deficient mice can suppress CD25$^-$ T cell proliferation in vitro, these cells do not protect recipient mice from colitis in the SCID transfer model in vivo, and, in addition, CD4$^+$LAP$^+$, but not CD4$^+$LAP$^-$ T cells from normal mice protect recipient mice from colitis in this model. Together, these studies demonstrate that TGF-β1 produced by CD4$^+$CD25$^+$ T cells is involved in the suppressor activity of these cells, particularly in their ability to regulate intestinal inflammation. ***The Journal of Immunology,*** **2004, 172: 834–842.**

There is accumulating evidence that the generation of regulatory T cells is necessary to maintain self-tolerance and prevent the onset of autoimmune disease. One such regulatory cell is a CD4$^+$CD25$^+$ T cell population that comprises 5–10% of the total peripheral CD4$^+$ T cells in normal adult mice and expresses surface CD25 (IL-2Rα) before activation (1). In certain strains of mice, elimination of this CD4$^+$CD25$^+$ regulatory T cell population by early thymectomy results in various autoimmune diseases, which, in turn, are preventable by its restoration by adaptive transfer (2). Similarly, transfer of CD25$^+$-depleted splenocytes into nude mice results in various autoimmune diseases that are prevented by cotransfer of CD4$^+$CD25$^+$ T cells (2). In more recent studies, CD4$^+$CD25$^+$ regulatory T cells very similar to the murine regulatory cell have also been shown to be present in humans (3–7).

In a series of in vitro studies, it has been shown that murine CD4$^+$CD25$^+$ T cells are relatively unresponsive to TCR-mediated stimulation and suppress T cell proliferation of cocultured CD4$^+$CD25$^-$ T cells (8, 9). In addition, it has been shown that such suppression acted via a cell-cell contact mechanism rather than through the secretion of suppressor cytokines such as TGF-β1 or IL-10 (8, 9). However, in a previous report, we have shown that CD4$^+$CD25$^+$ T cells are in fact capable of producing TGF-β1 either as a secreted or a cell surface protein, depending on the type and/or strength of TCR stimulation (10). Thus, when CD4$^+$CD25$^+$ T cells are stimulated with plate-bound anti-CD3 (under cross-linking conditions), they both secrete TGF-β1 and also express cell surface-bound TGF-β1; in contrast, when the same cells are stimulated by Ag plus APCs, they mainly express cell surface TGF-β1 and secrete little, if any, of this cytokine. On this basis, it seems likely that under the stimulation conditions in which CD4$^+$CD25$^+$ T cell suppressor function is generally measured in vitro CD4$^+$CD25$^+$ T cells would express cell surface TGF-β1, but not secrete TGF-β1. Thus, we postulated that in vitro CD4$^+$CD25$^+$ T cell suppressor function is mediated by contact with cell surface TGF-β1. In studies to support this concept, we showed that high concentrations of anti-TGF-β1 inhibit the suppressor function of CD4$^+$CD25$^+$ T cells in in vitro coculture assays (10). In addition, other investigators reported that suppressor function of both mouse lymphocytes and human CD4$^+$CD25$^+$ thymocytes are at least partially reversed by neutralization of TGF-β (11, 12).

Despite these findings, the involvement of TGF-β1 in CD4$^+$CD25$^+$ T cell immunoregulatory function remains controversial because in previous studies neutralization of TGF-β by anti-TGF-β failed to reverse CD4$^+$CD25$^+$ suppressor function in vitro (8, 9). In addition, it has recently been shown that CD4$^+$CD25$^+$ T cells from normal mice could suppress the proliferation of cocultured CD4$^+$CD25$^-$ T cells from TGF-βRII dominant-negative transgenic mice and Smad-3-deficient mice and, in addition, CD4$^+$CD25$^+$ T cells from TGF-β1-deficient mice could suppress cocultured CD25$^-$ T cells from normal mice, suggesting that TGF-β1 is not necessary for suppressor function in in vitro coculture system (13). These contradictory studies did not, however, lead to an alternative suppressor mechanism, and led us to again examine the role of TGF-β1 in CD4$^+$CD25$^+$ T cell regulatory function.

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Received for publication July 10, 2003. Accepted for publication November 4, 2003.

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0022-1767/04/$02.00

In the current series of studies, we showed first that in vitro suppressor function of both murine and human $CD4^+CD25^+$ regulatory T cells is inhibited by another TGF-β1-blocking agent, recombinant latency-associated peptide of TGF-β1 (rLAP),[2] in a dose-dependent fashion. We then showed that $CD4^+CD25^+$ T cells induce the expression of CD103 (α_E integrin) and activated Smad-2 in cocultured $CD25^-$ T cells, thus providing strong evidence that cell surface TGF-β1 acts as a signaling molecule in the coculture situation. Moving then to in vivo studies, we showed that while $CD4^+CD25^+$ T cells from TGF-β1-deficient mice do exhibit suppressor activity in vitro, such cells failed to prevent the development of colitis in the SCID transfer colitis model, indicating that cells from these mice do exhibit impaired suppressor function. In addition, these data show that $CD4^+CD25^+$ T cells exert suppressor activity in vivo by production of TGF-β1 rather than by inducing other cells to do so. Finally, we showed that $CD4^+LAP^+$ T cells, but not $CD4^+LAP^-$ T cells, prevent colitis in the transfer model, showing that cell surface LAP could serve as a marker for regulatory T cells.

Materials and Methods

Mice

Specific pathogen-free, 8-wk-old female BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). TGF-β1-deficient mice (14) were a kind gift from J. Letterio (National Cancer Institute, National Institutes of Health, Bethesda, MD). Splenocytes were purified from TGF-$\beta1^{+/+}$, TGF-$\beta1^{+/-}$, and TGF-$\beta1^{-/-}$ mice at the age of 1–2 wk, as TGF-$\beta1^{-/-}$ mice die before age 3 wk due to systemic autoimmune inflammation. C57BL/6 Rag-2 knockout mice and C.B-17 SCID mice were purchased from Taconic Farms (Germantown, NY). Thy-1.1^+ congenic BALB/c mice were a kind gift from J. Sprent (The Scripps Research Institute, La Jolla, CA). Animal use adhered to National Institutes of Health Animal Care Guidelines.

Reagents

Anti-mouse CD3 (145-2C11), anti-mouse CD28 (37.51), anti-mouse CTLA-4 (UC10-4F10-11), biotin-conjugated anti-mouse CD25 (7D4), FITC-conjugated anti-mouse CD4 (GK1.5 and RM4-5), PE-conjugated anti-mouse CD45RB (16A), biotin-conjugated anti-mouse IgG1, PE-conjugated streptavidin, CyChrome-conjugated streptavidin, FITC-conjugated anti-human CD4 (RPA-T4), PE-conjugated anti-human CD25 (M-A251), and 7-amino actinomycin D were purchased from BD PharMingen (San Diego, CA). Mouse anti-LAP (TGF-β1) mAb (clone 27232.11, mouse IgG1) was a kind gift from R&D Systems (Minneapolis, MN). rLAP, anti-TGF-β mAb (clone 1D11), biotin-conjugated goat anti-LAP Ab, and biotin-conjugated anti-LAP mAb (clone 27240) were purchased from R&D Systems. Streptavidin microbeads were purchased from Miltenyi Biotec (Auburn, CA). Anti-human CD3 mAb (OKT3) was purchased from Ortho Biotech (Raritan, NJ). Anti-phospho-Smad-2 (Ser^{465}, Ser^{467}) Ab was purchased from Upstate Biotechnology (Waltham, MA). Anti-Smad-2 mAb was purchased from Zymed Laboratories (South San Francisco, CA). Human rIL-2 was purchased from Life Technologies (Rockville, MD).

Cell purification

Mouse $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified, as described previously (10). Human $CD4^+CD25^+$ regulatory T cells were isolated from leukapheresis packs obtained from healthy donors after informed consent was given. In brief, PBMC isolated by Ficoll density-gradient centrifugation were passed over $CD4^+$ T cell enrichment columns (R&D Systems) to obtain $CD4^+$ T cells. The latter were then stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 and sorted by FACSVantage SE II (BD Biosciences, San Jose, CA) to obtain highly purified $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cell populations. The purity of the $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells was >85 and >95%, respectively.

Flow cytometry

Splenocytes from TGF-$\beta1^{+/+}$, TGF-$\beta1^{+/-}$, and TGF-$\beta1^{-/-}$ mice were stimulated with soluble anti-CD3 (10 μg/ml) and rIL-2 (50 U/ml) for 3 days. Cells were stained with anti-LAP mAb (clone 27232.11, mouse IgG1); washed, biotin-conjugated anti-mouse IgG1; and washed. Cells were then incubated with FITC-conjugated anti-CD4 and PE-conjugated streptavidin and washed. For exclusion of dead cells, cells were stained with 7-amino actinomycin D before analysis.

Purified human $CD4^+CD25^{high}$ and $CD25^-$ T cells were stimulated with anti-CD3 (1 μg/ml), irradiated APC, and rIL-2 (50 U/ml) for 5 days. Cells were stained with biotin-conjugated goat anti-LAP Ab, washed, and stained with FITC-conjugated anti-CD4 and PE-streptavidin. Dead cells were excluded by staining with 7-amino actinomycin D.

The analysis was performed on FACScan (BD Biosciences) flow cytometer with CellQuest II software (BD Biosciences). In some experiments, 7-amino actinomycin D staining was necessary to exclude nonspecific high background on dead cells.

Cell culture

Cell cultures of mouse $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells for in vitro suppression assays using [^{3}H]thymidine incorporation readouts were performed, as described previously (10); for this purpose, indicated cell numbers of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were placed in wells of 96-well plates. In some studies, rLAP was added to the cell cultures at various indicated concentrations.

For human $CD4^+$ T cells, 2.5×10^4 $CD4^+CD25^-$ T cells with or without the same number of $CD4^+CD25^{high}$ T cells were cultured in 96-well plates and stimulated with 1 μg/ml anti-CD3 (OKT3) and 5×10^4 irradiated syngeneic PBMC for 4 days at 37°C and pulsed with 1 μCi of [^{3}H]thymidine for the last 6 h of culture. RPMI 1640 supplemented with 3% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES (pH 7.0) was used as culture medium for human cells.

FACS analysis of CD103 expression on $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from Thy-1.1^+ congenic BALB/c mice. In parallel, $CD4^+CD25^+$ T cells were purified from normal (Thy-1.2^+) BALB/c mice. Thy-1.1^+ $CD25^-$ T cells were stimulated in the presence or absence of Thy-1.2^+ $CD25^+$ T cells with soluble anti-CD3 (10 μg/ml), irradiated APC, and rIL-2 (50 U/ml) for 5 days. For positive control, Thy-1.1^+ $CD25^-$ T cells were stimulated in the presence of rTGF-β1 (5 ng/ml). In parallel, Thy-1.1^+ $CD25^+$ T cells were stimulated for comparison. After cultivation, cells were stained with FITC-conjugated anti-Thy-1.1, PE-conjugated anti-CD103, or isotype-matched control Ab, and CyChrome-conjugated anti-CD4. Expression of CD103 on Thy-1.1^+ $CD4^+$ cells was analyzed by FACScan (BD Biosciences) flow cytometer.

Detection of Smad-2 phosphorylation

To detect induction of Smad-2 phosphorylation in $CD4^+CD25^-$ T cells cocultured with $CD4^+CD25^+$ T cells, murine $CD4^+$ T cells were purified by CD4 purification column (R&D Systems), and then separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells using PE-conjugated anti-CD25 and anti-PE microbeads ($CD4^+CD25^+$ regulatory T cell isolation kit; Miltenyi Biotec). $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were then stimulated with soluble anti-CD3 (10 μg/ml) plus irradiated adherent APCs (non-CD4 fraction) for 3 h. The nonadherent $CD4^+$ T cells were then collected, washed three times, and mixed with freshly isolated resting $CD4^+CD25^-$ T cells. The cell mixtures thus obtained were added back to the washed original wells, briefly spun down to ensure cell-cell contact, and incubated at 37°C for 90 min. Negative controls consisted of $CD4^+CD25^-$ T cells incubated with irradiated APCs in the absence of $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells. After incubation, $CD4^+CD25^-$ T cells were negatively repurified with biotin non-CD4 T cell Ab mixture, followed by the treatment of PE anti-mouse CD25, and solubilized in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.5, 2 mM EDTA, 10 mM NaF, and 1 mM Na_3VO_4 with protease inhibitors). Finally, a Western blot was performed, as described previously (10), using anti-phospho-Smad-2 Ab to detect phosphorylated Smad-2 and anti-Smad-2 mAb to detect total Smad-2 protein (on the stripped membrane).

Transfer of wild-type and TGF-β1-deficient $CD4^+CD25^+$ T cells with $CD45RB^{high}$ T cells into Rag-2 knockout recipients

Splenocytes from C57BL/6 mice were stained with FITC-conjugated anti-mouse CD4 (RM4-5) and PE-conjugated anti-mouse CD45RB, after which $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells were purified by FACS sorting. Wild-type and TGF-β1-deficient $CD4^+CD25^+$ T cells were purified, as described previously (10). $CD4^+CD45RB^{high}$ T cells (3×10^5)

[2] Abbreviation used in this paper: LAP, latency-associated peptide of TGF-β.

were injected i.p. into Rag-2-deficient mice, accompanied, in some recipients, by CD4$^+$CD45RB^{low} T cells (1.5×10^5) from normal C57BL/6 or CD4$^+$CD25$^+$ T cells (1.2×10^5) from wild-type or TGF-β1-deficient mice. After transfer, recipient mice were weighted weekly until they were euthanized for histopathologic analysis.

Transfer of LAP$^+$ and LAP$^-$CD4$^+$ T cells with CD45RB^{high} T cells into C.B-17 SCID recipients

Splenocytes from BALB/c mice were stained with FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD45RB, after which CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} T cells were purified by FACS sorting. In parallel, CD4$^+$ T cells stimulated with plate-bound anti-CD3 (10 μg/ml), soluble anti-CD28 (2 μg/ml), and rIL-2 (50 U/ml) for 3 days were collected and stained with FITC-conjugated anti-mouse CD4 (RM4-5) and anti-LAP mAb (27232.11), biotin-conjugated anti-mouse IgG1, and PE-conjugated streptavidin. CD4$^+$LAP$^+$ and CD4$^+$LAP$^-$ T cells were purified by FACS sorting. Dead cells were excluded by 7-amino actinomycin D staining. CD4$^+$CD45RB^{high} T cells (3×10^5) were injected i.p. in C.B-17 SCID mice, accompanied in some recipients by CD4$^+$CD45RB^{low} (1.5×10^5), CD4$^+$LAP$^+$ (1×10^5), or CD4$^+$LAP$^-$ (1×10^5) T cells. After transfer, recipient mice were weighted weekly until they were euthanized for histopathologic analysis.

Results

rLAP inhibits suppressor function of murine CD4$^+$CD25$^+$ regulatory T cells

To explore further the role of TGF-β in the suppressor function of murine CD4$^+$CD25$^+$ T cells in vitro, we conducted suppressor arrays in the presence and absence of rLAP, a molecule that could block TGF-β activity by capturing active TGF-β or by blocking the association of latent TGF-β with an activating molecule, such as thrombospondin-1 and $\alpha_v\beta_6$ integrin (15–17). Accordingly, we set up cultures in which CD4$^+$CD25$^-$ T cells were stimulated with soluble anti-CD3 and APC in the presence or absence of CD4$^+$CD25$^+$ T cells, as well as with various amounts of rLAP. As shown in Fig. 1, in the absence of rLAP, CD4$^+$CD25$^-$ T cells proliferated well in response to stimulation, while CD4$^+$CD25$^+$ T cells proliferated poorly. In addition, coculture of CD4$^+$CD25$^-$ T cells with CD4$^+$CD25$^+$ T cells resulted in profound suppression of CD4$^+$CD25$^-$ T cell proliferation. However, in the presence of rLAP, proliferation of CD4$^+$CD25$^-$ T cells was further increased and, more importantly, suppression by CD4$^+$CD25$^+$ T cells was significantly reversed in a dose-dependent fashion. Of note, reversal of suppression was obtained with much lower concentrations of rLAP than reversal by anti-TGF-β (10). These results thus lend additional credence to the idea that such reversal involves a TGF-β1-mediated mechanism. The fact that CD4$^+$ CD25$^-$ T cell proliferation increases in the presence of rLAP may be due to the fact that, as shown previously, CD4$^+$CD25$^-$ T cells do produce some TGF-β when stimulated with a variety of conditions (10) or are being suppressed by TGF-β known to be present in the FCS in the culture medium. Such augmentation of proliferation was previously seen when anti-TGF-β was added to the cultures (10). Finally, it should be noted that while rLAP caused substantial inhibition of suppression by CD4$^+$CD25$^+$ T cells, such inhibition was not complete. Several possible explanations for this can be put forward, but we think the most cogent is that CD4$^+$CD25$^+$ T cells inhibit CD25$^-$ T cells via a second mechanism as well under certain stimulation conditions (see discussion below).

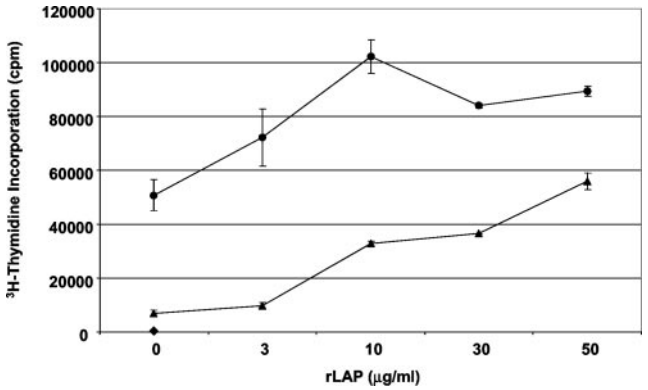

FIGURE 1. rLAP reverses suppressor function of murine CD4$^+$CD25$^+$ T cells. Mouse CD4$^+$CD25$^-$ T cells (4×10^4/well) were stimulated with 10 μg/ml soluble anti-CD3 and irradiated APC in the presence or absence of CD4$^+$CD25$^+$ T cells (3×10^4/well). Various concentrations of rLAP were added to the cell culture. ●, CD4$^+$CD25$^-$ T cell; ▲, CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cell; ◆, CD4$^+$CD25$^+$ T cell. Representative results of three independent experiments are shown.

Expression of cell surface-bound LAP on human CD4$^+$CD25$^+$ T cells and inhibition of human CD4$^+$CD25$^+$ suppressor function by rLAP

Recently, it was reported that human CD4$^+$CD25$^+$ T cells, comprising ~5–15% of CD4$^+$ peripheral blood T cells, contain a subpopulation with suppressor function similar to that exhibited by murine CD4$^+$CD25$^+$ T cells (3–6). However, further work reported previously and corroborated in this study indicated that only CD4$^+$CD25^{high} T cells, comprising 1–2% of circulating CD4$^+$ T cells, possess suppressor capability (7). In view of these findings, we investigated expression of cell surface TGF-β1 on CD4$^+$CD25^{high} and CD4$^+$CD25$^-$ T cells. First, we isolated CD4$^+$CD25^{high} and CD4$^+$CD25$^-$ T cells from human peripheral blood by flow cytometric sorting (Fig. 2*A*). We then stimulated the cells with soluble anti-CD3, APC, and rIL-2, and after 5 days stained the cells thus activated with polyclonal goat anti-LAP Ab. As shown in Fig. 2*B*, the expression level of cell surface LAP was high on CD25^{high} T cells after stimulation, whereas only a small amount of LAP was found on CD25$^-$ T cells. Similar results were obtained with another biotin-conjugated mAb against LAP (clone 27240) (data not shown). However, using an Ab against active TGF-β1, we could detect only marginal amount of TGF-β1 on the cell surface; this contrasts with results obtained in mouse cells.

Next, we conducted studies to determine whether the suppressor function of human CD4$^+$CD25^{high} T cells can also be blocked by rLAP. We therefore set up assays of human cells in the presence or absence of rLAP, similar to those in the previously described studies of mouse cells. As shown in Fig. 3, human CD4$^+$CD25$^-$ T cells proliferated well in response to stimulation with soluble anti-CD3 and APC and human CD4$^+$CD25^{high} T cells profoundly suppressed proliferation of these cells, as reported previously (7). Also in concert with the mouse data, addition of rLAP to the cell cultures caused significant reversal of suppression in a dose-dependent fashion. These results indicate that suppressor function of human CD4$^+$CD25^{high} regulatory T cells also involves a TGF-β-dependent mechanism.

CD4$^+$CD25$^+$ regulatory T cells induce TGF-βR signaling in the target cells

To provide further evidence that cell surface TGF-β on the surface of CD4$^+$CD25$^+$ T cells is involved in the suppression mediated by these cells, we sought evidence that such TGF-β signals the CD25$^-$ target cells. In an initial series of experiments along these lines, we took advantage of the fact that CD103 expression can be induced by TGF-β1, and thus, expression of this integrin on CD4$^+$CD25$^-$ target cells should increase during coculture with CD4$^+$CD25$^+$ T cells in the presence of cell surface-bound TGF-β1. Accordingly, we cocultured Thy-1.2$^+$ CD4$^+$CD25$^+$ T cells with Thy-1.1$^+$ CD4$^+$CD25$^-$ target cells and stimulated with anti-CD3, APC, and

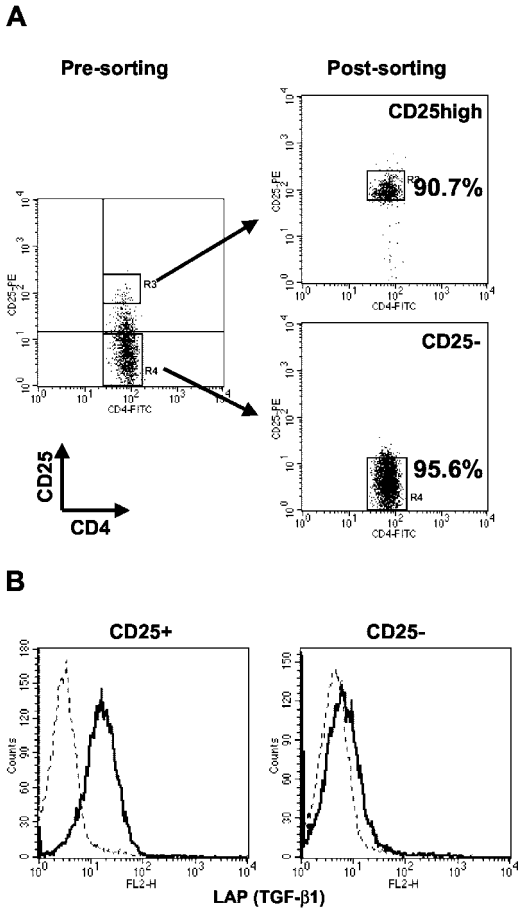

FIGURE 2. Purification of human $CD4^+CD25^{high}$ T cells and expression of LAP on activated $CD4^+CD25^{high}$ T cells. *A*, Purified human $CD4^+$ T cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25. $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were then purified by FACS sorting. *B*, Purified $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were stimulated with soluble anti-CD3 (1 μg/ml), irradiated APC, and rIL-2 (50 U/ml) for 5 days. Cells were then stained with FITC-conjugated anti-CD4, biotin-conjugated anti-LAP, and PE-conjugated streptavidin. Expression of LAP on $CD4^+$ gate is shown. Results are representative of six independent experiments.

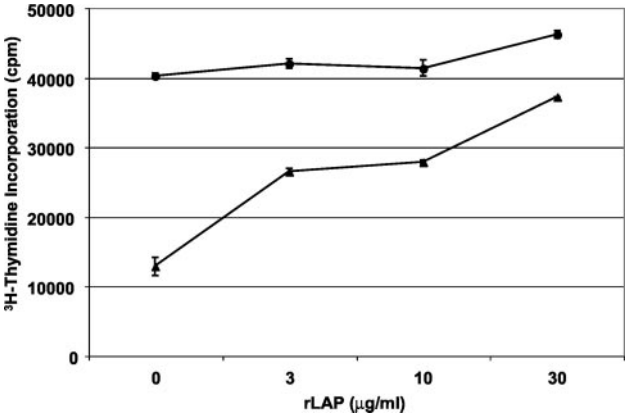

FIGURE 3. rLAP reverses suppressor function of human $CD4^+CD25^{high}$ T cells. Human $CD4^+CD25^-$ T cells were stimulated with 1 μg/ml soluble anti-CD3 and irradiated APC in the presence or absence of $CD4^+CD25^{high}$ T cells. Various concentrations of rLAP were added to the cell culture. ●, $CD4^+CD25^-$ T cell; ▲, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cell. Results are representative of three independent experiments.

rIL-2. We then stained the cells and discriminated suppressors and targets on the basis of Thy-1.1 expression. As shown in Fig. 4, cultured Thy-1.1$^+$ $CD4^+CD25^-$ T cells contained very few $CD103^+$ cells (~1.1%), whereas the same cells cultured in the presence of $CD4^+CD25^+$ T cells contain as many $CD103^+$ cells as the same cells cultured with rTGF-β1. As reported previously (18), $CD4^+CD25^+$ T cells contained a substantial subpopulation of $CD103^+$ cells, possibly due to autoactivation.

In a second series of experiments, we determined TGF-β1 signaling in $CD25^-$ target T cells, by measuring the appearance of activated Smad-2 in these cells following admixture with $CD4^+CD25^+$ suppressor T cells. In these studies, we mixed activated $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells with resting $CD4^+CD25^-$ T cells (target cells) and then briefly centrifuged the cell mixture to promote cell-cell contact. We then reisolated the $CD4^+CD25^-$ target cells and subjected lysates of these cells to Western blot analysis using anti-phospho-Smad-2 Ab. As shown in Fig. 5, $CD4^+CD25^-$ target cells in contact with $CD4^+CD25^+$ T cells gave rise to a distinctly stronger phosphorylated Smad-2 signal than $CD4^+CD25^-$ T cells in contact with $CD4^+CD25^-$ T cells or $CD4^+CD25^-$ T cells incubated alone. In contrast, total Smad-2 protein was similar in all cultures. Together, these studies show that TGF-β1 on the surface of $CD4^+CD25^+$ T cells signals $CD4^+CD25^-$ T cells: in one case, to induce expression of a TGF-β-dependent integrin, and, in another, to directly stimulate Smad-2.

TGF-β1 produced from $CD4^+CD25^+$ T cells is important in suppression of intestinal inflammation in vivo

The above results strongly support the idea that TGF-β1 is involved in $CD4^+CD25^+$ T cell-mediated suppression of T cell proliferation in vitro. However, the opposite conclusion was drawn from studies in which $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ mice were shown to mediate suppression in vitro (13). To further explore these results, we assessed the suppressor function of $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ mice both in vitro or in vivo. In initial in vitro studies shown in Fig. 6, we found that TGF-β1-deficient $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ could indeed suppress T cell proliferation in vitro to the same changes as $CD4^+CD25^+$ T cells from wild-type mice.

However, a very different result was obtained in in vivo studies of $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ mice. In this case, as shown in Fig. 7*A*, Rag-2-deficient recipients of $CD4^+CD45RB^{high}$ T cells derived from normal mice showed progressive weight loss that was inhibited by cotransfer of $CD4^+CD45RB^{low}$ T cells or $CD4^+CD25^+$ T cells from wild-type mice, whereas, in contrast, such weight loss was not inhibited by cotransfer of $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ mice. In addition, as shown in Figs. 7*B* and 8*A*, this pattern of weight loss corresponded to the macroscopic and microscopic changes present in the colons of the various groups. Finally, perhaps most importantly, these weight curves also corresponded with the pooled histological scores of mice in the various groups, which shows again that $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ did not protect Rag-2 knockout recipients of $CD45RB^{high}$ T cells from the development of colitis (Fig. 8*B*). It should be noted that these results cannot be explained by postulating that $CD4^+CD25^+$ T cells from TGF-$\beta1^{-/-}$ have an intrinsic proinflammatory effect because the development of weight loss in recipients of these cells was limited to the colon and did not occur until after a long latent period, presumably due to the long time necessary for the development of colitis in the transfer model. It should also be noted that these results correlate with previous

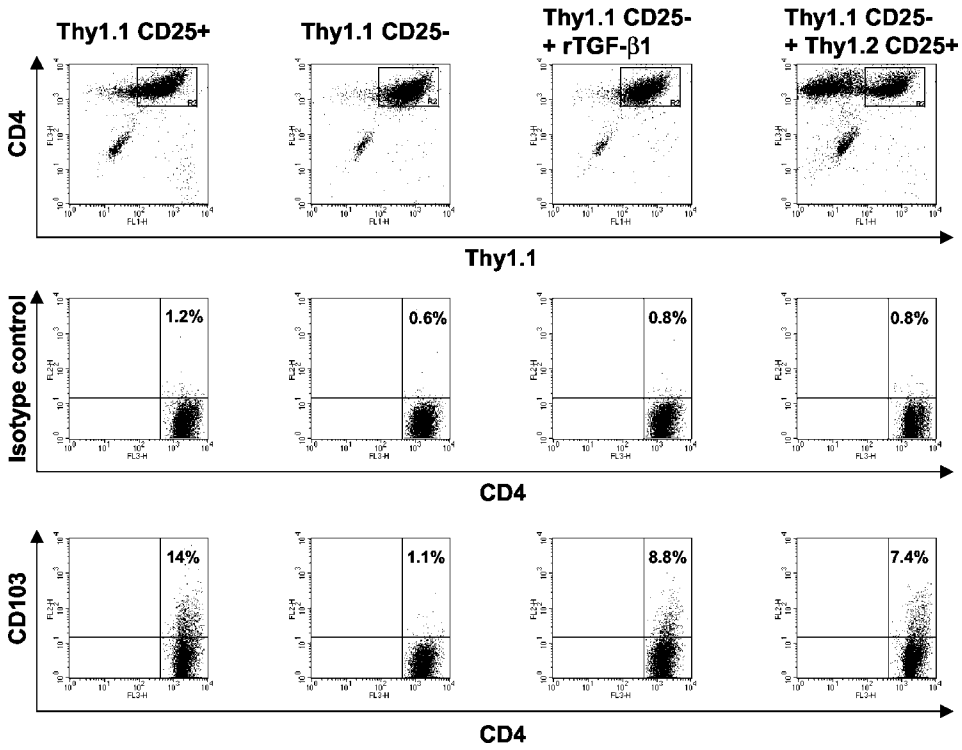

FIGURE 4. CD4$^+$CD25$^+$ T cells induce CD103 expression on CD4$^+$CD25$^-$ T cells. Thy-1.1$^+$CD4$^+$CD25$^+$ T cells (*left panels*), Thy-1.1$^+$CD4$^+$CD25$^-$ T cells in the absence (*second left panels*) and the presence (*second right panels*) of rTGF-β1 (5 ng/ml), and Thy-1.1$^+$CD4$^+$CD25$^-$ in the presence of Thy-1.2$^+$CD4$^+$CD25$^+$ T cells (*right panels*) were stimulated with soluble anti-CD3 (10 μg/ml), irradiated APC, and IL-2 (50 U/ml) for 5 days. Cells were stained with FITC-conjugated anti-Thy-1.1, PE-conjugated anti-CD103 or isotype control, and CyChrome-conjugated anti-CD4. *Top panels*, The expression of Thy-1.1 and CD4. Staining with anti-CD103 (*bottom panels*) and isotype control Ab (*middle panels*) on Thy-1.1$^+$CD4$^+$ gate is shown. These data are representative of three independent experiments.

studies showing that anti-TGF-β administration abrogates the protective effect of CD4$^+$CD25$^+$ T cells (19).

In vivo regulatory function of CD4$^+$LAP$^+$ and CD4$^+$LAP$^-$ T cells

In a final series of studies, we examined the capacity of TGF-β1-bearing T cells from normal mice to mediate regulatory function in vivo, using a SCID transfer model of colitis. In initial studies, we determined the percentage of spleen CD4$^+$ T cells expressing LAP on the cell surface after activation. As shown in Fig. 9*A*, 7.5% of activated CD4$^+$ T cells obtained from wild-type mice were cell surface LAP positive after staining with anti-LAP mAb. To confirm the specificity of the staining, we stained CD4$^+$ T cells obtained from TGF-$\beta1^{-/-}$ mice, and, as expected, they were negative for LAP expression. In addition, 3.5% of CD4$^+$ T cells from TGF-$\beta1^{+/-}$ heterozygous mice expressed cell surface LAP, approximately half as many cells as in wild-type mice.

In further studies, we determined the in vivo regulatory potential of LAP$^+$ and LAP$^-$ CD4$^+$ T cells from normal mice. As shown in Fig. 9*B*, we purified LAP$^+$ and LAP$^-$ cells from activated CD4$^+$ T cells by FACS sorting, and then transferred the sorted cells into SCID recipients along with CD4$^+$CD45RB^{high} T cells. As shown in Fig. 10*A*, SCID recipients of CD45RB^{high} T cells derived from normal mice revealed progressive weight loss that was inhibited by cotransfer of CD4$^+$CD45RB^{low} T cells or CD4$^+$LAP$^+$ T cells; in contrast, it was not inhibited by cotransfer of CD4$^+$LAP$^-$ T cells. As shown in Fig. 10*B*, macroscopic study of colons of the recipients of CD45RB^{high} T cells revealed marked swelling and shortening, which was almost completely suppressed in mice cotransferred CD45RB^{low} cells; cotransfer of LAP$^+$ T cells also suppressed inflammation, although in this case, slight signs of edema remained. Finally, as shown in Fig. 10*B*, colons of recipients of CD45RB^{high} and LAP$^-$ T cells showed severe swelling and shortening similar to that observed in mice transferred CD45RB^{high} T cell alone. Finally, as shown in Fig. 11, histologic examination and colitis scoring confirmed these results. These findings provide a clear indication that surface LAP is reflecting a

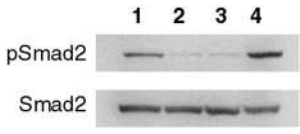

FIGURE 5. CD4$^+$CD25$^+$ T cells induce Smad-2 phosphorylation in CD4$^+$CD25$^-$ T cells. Purified CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were stimulated with soluble anti-CD3 (10 μg/ml) in the presence of irradiated APC. Then freshly isolated CD4$^+$CD25$^-$ T cells were mixed with or without activated CD4$^+$CD25$^+$ T cells, CD4$^+$CD25$^-$ T cells, or rTGF-β1, and briefly spun down and incubated for 90 min. Then CD4$^+$CD25$^-$ T cells were repurified by magnetic beads and lysed. Phosphorylated Smad-2 (*top panel*) and total Smad-2 protein (*bottom panel*) were detected by Western blot. CD4$^+$CD25$^-$ T cells were mixed with CD4$^+$CD25$^+$ T cells and APC (*lane 1*); mixed with CD4$^+$CD25$^-$ T cells and APC (*lane 2*); incubated with APC (*lane 3*); and incubated with rTGF-β1 (5 ng/ml) and APC (*lane 4*).

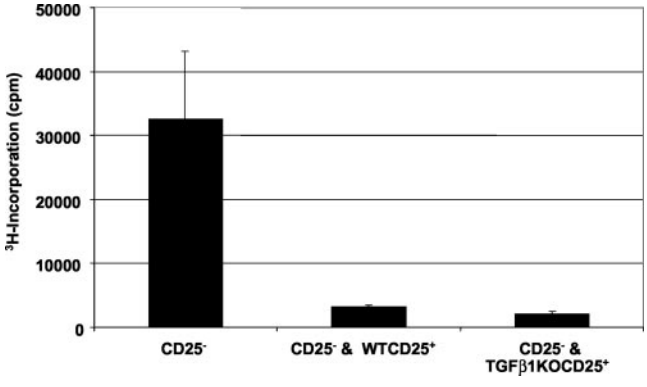

FIGURE 6. TGF-β1-deficient CD4$^+$CD25$^+$ T cells mediate suppression in vitro. CD4$^+$CD25$^-$ T cells (4×10^4/well) from wild-type mice were stimulated with 10 μg/ml of soluble anti-CD3 and irradiated APC in the presence or absence of CD4$^+$CD25$^+$ T cells (4×10^4/well) from wild-type or TGF-β1-null mice.

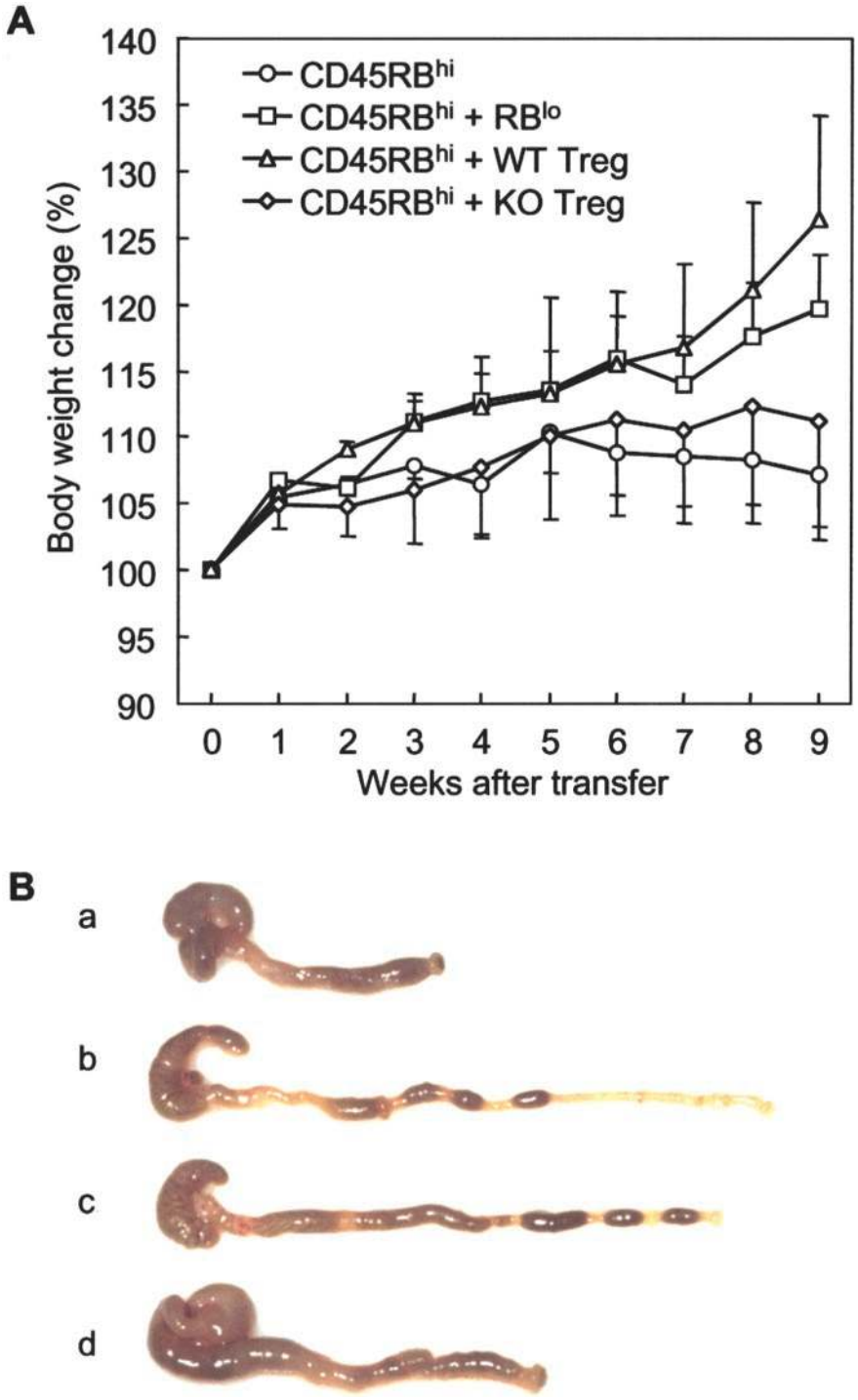

FIGURE 7. In vivo suppressor function of $CD4^+CD25^+$ T cells from TGF-β1-deficient mice in the SCID transfer model of colitis. Weight curves and gross appearance of colons. *A*, Rag-2-deficient mice were reconstituted with $CD4^+CD45RB^{high}$ T cells (○), $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells (□), $CD4^+CD45RB^{high}$ and wild-type $CD4^+CD25^+$ T cells (△), or $CD4^+CD45RB^{high}$ and TGF-β1-deficient $CD4^+CD25^+$ T cells (◇). The change in weight was monitored weekly after the transfer. *B*, The gross appearance of colons in each group. Recipients of *a*, $CD4^+CD45RB^{high}$ T cells; *b*, $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells; *c*, $CD4^+CD45RB^{high}$ and wild-type $CD4^+CD25^+$ T cells; and *d*, $CD4^+CD45RB^{high}$ and TGF-β1-deficient $CD4^+CD25^+$ T cells.

capability of cells with protective regulatory function in intestinal inflammation.

Discussion

In recent years, several molecules have been implicated in the suppressor mechanism underlying the immunoregulatory function of $CD4^+CD25^+$ T cells, including cell surface molecules such as CTLA-4 and glucocorticoid-induced TNFR, cell surface or secreted molecules such as TGF-β1, and intracellular molecules such as FoxP3 (10, 18–22). However, the interrelation of these molecules or their final common pathway has yet to be defined. In a previous study, we showed that $CD4^+CD25^+$ T cells after stimulation express high levels of TGF-β1 in a surface-bound and/or a secreted form, and addition of anti-TGF-β to in vitro cocultures of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells, or $CD4^+CD25^+$, $CD4^+CD25^-$ T cells, and B cells results in reversal of suppression of T cell proliferation and B cell Ig production, respectively (10). These findings have been subsequently corroborated by several other groups that have also found that addition of anti-TGF-β1 or soluble TGF-βR to in vitro cocultures reversed suppression of $CD4^+CD25^+$ T cells, at least in part (11, 12, 23). Nevertheless, the involvement of TGF-β1 in the suppressor function of $CD4^+CD25^+$ T cells remains controversial, mainly because, as already mentioned, $CD4^+CD25^+$ T cells can still suppress $CD25^-$ T cells from mice in whom TGF-β1 signaling is impaired and $CD4^+CD25^+$ T cells from TGF-β1-deficient mice can still suppress $CD4^+CD25^-$ T cells from normal mice in vitro (13). Therefore, in

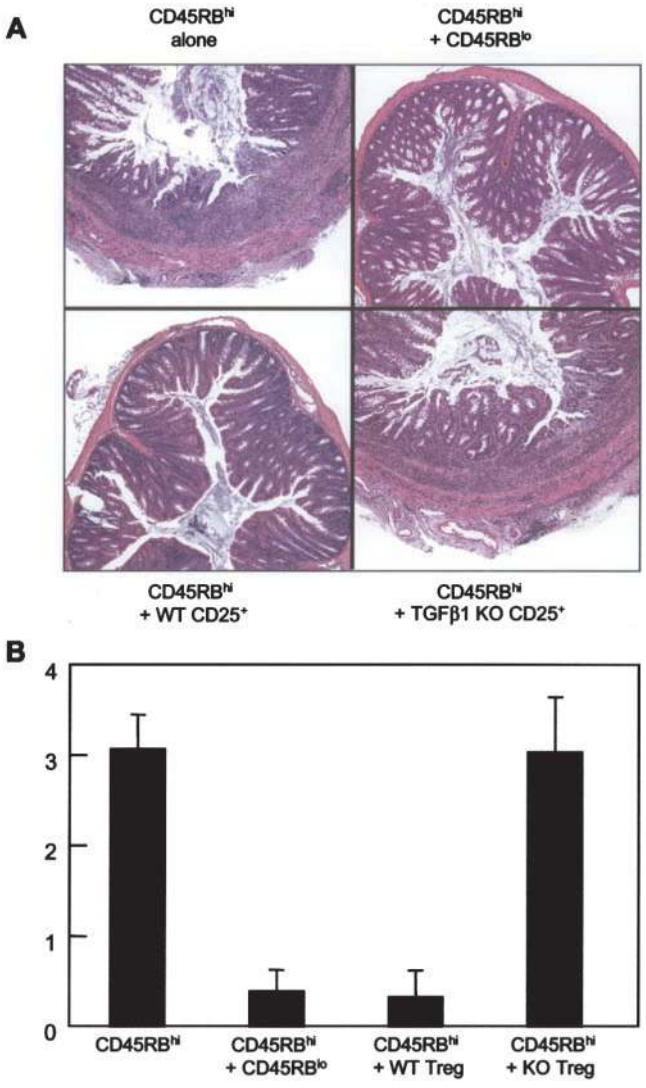

FIGURE 8. In vivo suppressor function of $CD4^+CD25^+$ T cells from TGF-β1-deficient mice in SCID transfer model of colitis. Microscopic appearance and colonic score. *A*, Representative microscopic appearance of colon of the Rag-2-deficient recipients reconstituted with $CD4^+$ $CD45RB^{high}$ T cells, $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells, $CD4^+CD45RB^{high}$ and wild-type $CD4^+CD25^+$ T cells, or $CD4^+$ $CD45RB^{high}$ and TGF-β1-deficient $CD4^+CD25^+$ T cells. *B*, The score of colonic inflammation in each group is shown. Similar results were obtained in two independent experiments.

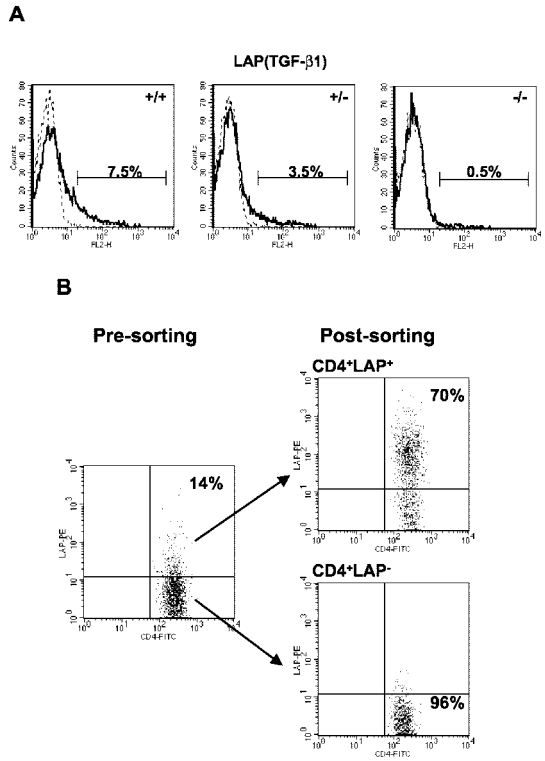

FIGURE 9. Purification of LAP^+ and LAP^- cells from activated mouse $CD4^+$ T cells. *A*, Expression of cell surface LAP on wild-type, but not on TGF-β1-deficient $CD4^+$ T cells. Total splenocytes purified from TGF-$\beta1^{+/+}$, TGF-$\beta1^{+/-}$, and TGF-$\beta1^{-/-}$ mice were stimulated with soluble anti-CD3 (10 μg/ml) and rIL-2 (50 U/ml) for 3 days. Cells were stained with FITC-conjugated anti-CD4 and anti-LAP mAb (clone 27232.11, mouse IgG1), biotin-conjugated anti-mouse IgG1, and PE-conjugated streptavidin. Cells were stained with 7-amino actinomycin D before analysis for dead cell exclusion. $CD4^+$ cells were gated, and the expression of LAP was shown. Results shown are representative of three independent experiments. *B*, LAP^+ and LAP^- $CD4^+$ T cells were purified by FACS sorting.

this study, we re-examined the involvement of TGF-β1 in $CD4^+CD25^+$ T cell suppressor function and present new evidence buttressing the role of this molecule in the suppressor process.

Recognizing that the results of studies with anti-TGF-β have been variable in their ability to demonstrate inhibition of $CD4^+CD25^+$ T cell-mediated suppression, we sought another inhibiting agent. We ultimately chose to evaluate rLAP as a possible inhibitor because this molecule is as TGF-β1 specific as anti-TGF-β1 and binds to TGF-β1 with at least as high an affinity as anti-TGF-β1 (24). This substance could conceivably block TGF-β1 suppressor activity by either of two mechanisms: 1) by combining with newly released active TGF-β1 and converting it back into a latent form; and 2) by competitive inhibition of latent TGF-β1 (LAP) for potential binding sites at which conversion of latent TGF-β1 to an active form normally occurs. Indeed, we found that rLAP inhibited $CD4^+CD25^+$ T cell-mediated suppression in the in vitro assay in a dose-dependent fashion and was able to do so in assays of either murine or human cells. These results provide independent confirmation that TGF-β1 is at least partially involved in the in vitro suppressor activity of $CD4^+CD25^+$ T cells. In retrospect, we would suggest that the failure of some investigators to obtain inhibition of suppression with anti-TGF-β1 can be attributed to either the use of too low a concentration of Ab or an Ab that does not block the ability of latent TGF-β1 to bind to sites where conversion to an active form must occur or alternatively to block the ability of TGF-β1 to bind to the TGF-βR after conversion to an active form. In addition, we have noted that blockade by anti-TGF-β varies with the degree of suppression and that at higher levels of suppression one observes less blockade. This may relate to the fact that at higher levels of suppression there is sufficient redundancy in the system to preclude effective blockade at reasonable Ab doses. Thus, the study of inhibition by anti-TGF-β should be done at a variety of E:T cell ratios. Finally, because TGF-β1-mediated suppression would necessarily involve both an activation step (resulting in dissociation from LAP) and a receptor interaction step and because these steps may be interrelated, it may be difficult to inhibit with Ab, unless one uses a great excess of Ab. LAP would be less subject to this difficulty because it can act before activation of TGF-β1.

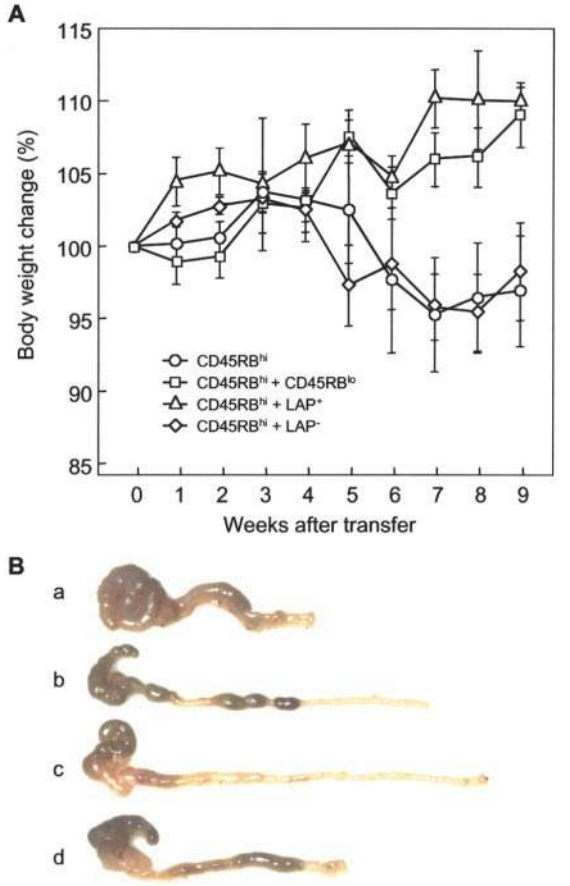

FIGURE 10. In vivo suppressor function of LAP^+ and LAP^- T cells in the SCID transfer colitis. Weight curves and gross appearance of colons. *A*, C.B-17 SCID mice were reconstituted with $CD4^+CD45RB^{high}$ T cells (○), $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells (□), $CD4^+CD45RB^{high}$ and $CD4^+LAP^+$ T cells (△), or $CD4^+CD45RB^{high}$ and $CD4^+LAP^-$ T cells (◇). The change in weight was monitored weekly after the transfer. *B*, The gross appearance of colons in each group. Recipients of *a*, $CD4^+CD45RB^{high}$ T cells; *b*, $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells; *c*, $CD4^+CD45RB^{high}$ and $CD4^+LAP^+$ T cells; and *d*, $CD4^+CD45RB^{high}$ and $CD4^+LAP^-$ T cells.

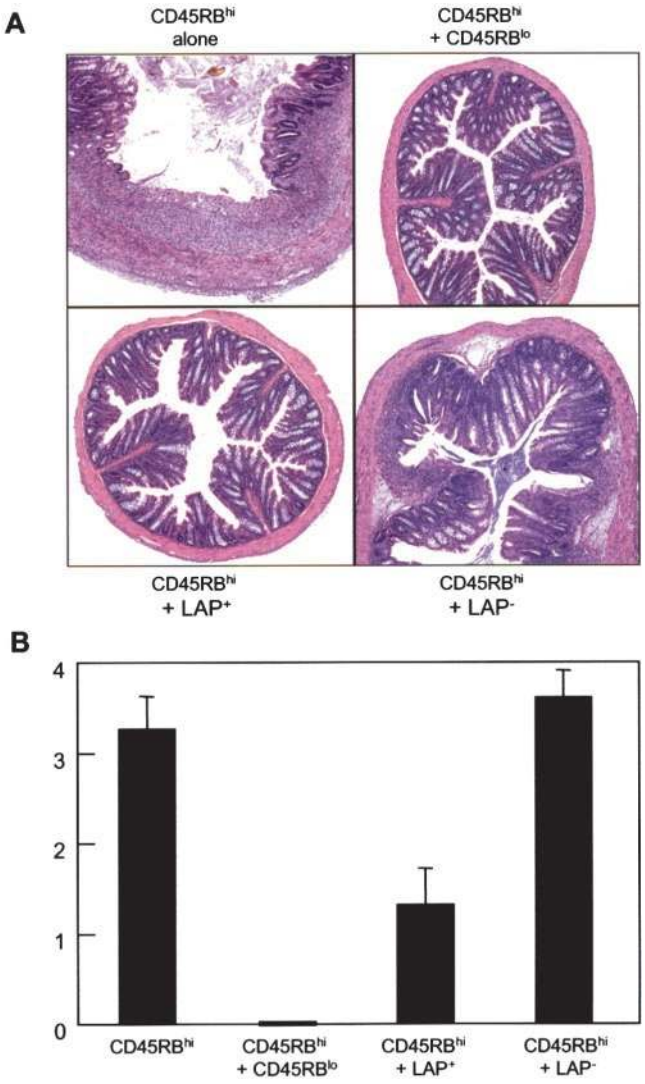

FIGURE 11. In vivo suppressor function of LAP^+ and LAP^- T cells in SCID transfer colitis. Microscopic appearance and colonic score. *A*, Representative microscopic appearance of colon of the C.B-17 SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells, $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ T cells, $CD4^+CD45RB^{high}$ and$CD4^+LAP^+$ T cells, or $CD4^+CD45RB^{high}$ and $CD4^+LAP^-$ T cells. *B*, The score of colonic inflammation in each group is shown. Similar results were obtained in another independent experiment.

In a second approach to investigating whether TGF-β1 is acting as a suppressor factor in $CD4^+CD25^+$ T cell-mediated suppression, we sought evidence that TGF-β1 could signal $CD25^-$ T cells via TGF-βR. We showed that this was indeed true in that $CD25^-$ target cells in the in vitro assay when mixed with $CD4^+CD25^+$ T cells (but not with $CD4^+CD25^-$ T cells) undergo Smad-2 phosphorylation. Such activation indicates that the target cells have been signaled via TGF-βR. TGF-β1 signaling of target cells was also demonstrated somewhat more indirectly by the fact that $CD25^-$ target cells cocultured with $CD4^+CD25^+$ T cells were induced to express CD103 (α_E integrin), an integrin previously shown to be regulated by TGF-β1 (25). This finding has added weight in the light of the recent finding that stimulated $CD4^+$ T cells in which TGF-β1 signaling is blocked by overexpression of Smad-7 do not express $\alpha_E\beta_7$, indicating that TGF-β1 is necessary for $\alpha_E\beta_7$ expression, at least in vitro (26). Finally, it is interesting that a significant fraction of $CD4^+CD25^+$ T cells expresses CD103, presumably because they are autostimulated by TGF-β1.

A finding that is seemingly at odds with the TGF-β1 blocking and signaling studies reported in this work concerns the fact that, as reported previously, $CD4^+CD25^+$ T cells from TGF-β1-deficient mice were able to mediate suppression of $CD4^+CD25^-$ T cells in the in vitro assay system. There are a number of possible explanations of these discordant results, but the one that appears most likely to us is that in the absence of TGF-β1, $CD4^+CD25^+$ T cells express and/or up-regulate alternative suppressor mechanisms that ordinarily play a secondary role in the mechanism of suppression. This possibility would accord with the fact mentioned above that anti-TGF-β blockade is more effective at lower levels of suppression (when secondary mechanisms may be less evident) and the finding of Chen and Wahl (23) that $CD4^+CD25^+$ T cells from TGF-β1-deficient mice suppressed less well at the usual ratio of regulatory cells to target cells, but could exert suppression at high ratios. This explanation notwithstanding, further work will be necessary to resolve this discrepancy. Finally, the finding that $CD4^+CD25^+$ T cells are capable of suppressing $CD25^-$ T cells from mice with TGF-β1 signaling defects (13) is not persuasive to us because these cells had leaky defects. Thus, in our hands, cells from either Smad3-deficient mice or mice bearing a dominant-negative TGF-βR were still subject to inhibition of proliferation by

the addition of rTGF-β to the cultures (data not shown). Thus, these cells do not allow a rigorous test of TGF-β-mediated suppressor function.

Additional support for the involvement of TGF-β1 in CD4$^+$CD25$^+$ T cell regulatory function came from in vivo studies in which we showed that while CD4$^+$CD25$^+$ T cells from TGF-β1-deficient mice manifest suppressor function in the in vitro suppressor assay, they cannot prevent colitis mediated by CD45RB^{high} T cells transferred to Rag-2-deficient recipients, whereas as shown previously and again in this study, such cells from wild-type mice can prevent colitis. We believe this is a more robust test of the role of TGF-β1 in the CD4$^+$CD25$^+$ T cell regulatory cell function of cells from TGF-β1-deficient mice than the in vitro study, because in this case the regulatory cells are presumably acting via surface expression or secretion of TGF-β1, which cannot be compensated by another suppressor mechanism.

Previous studies have shown that anti-TGF-β administration to SCID or Rag-2-deficient mice transferred CD45RB^{high} T cells and protective CD45RB^{low} abrogates the protective effect and that CD4$^+$CD25$^+$ T cells are the subpopulation that mediate protection (19, 27). These studies left unanswered the question of whether the CD4$^+$CD25$^+$ T cells were producing the TGF-β1 themselves or inducing other cells to produce this cytokine. This question is answered by the present findings showing that the TGF-β1 must in fact come from the CD4$^+$CD25$^+$ T cells themselves.

In related studies, we showed that CD4$^+$LAP$^+$ T cells, but not CD4$^+$LAP$^-$ T cells, from normal mice prevent colitis in the transfer model. This again provides yet additional evidence for the link between cell surface TGF-β1 expression and regulatory function. Recently, Oida et al. (28) reported that CD25$^-$LAP^{high} cells also prevent colitis in the transfer model and such prevention is also TGF-β dependent. This cell population may represent a population in a different state of activation or differentiation than the cells investigated in this study, but nevertheless, the results are consistent with our finding that LAP$^+$ cells (cells bearing cell surface TGF-β1) are suppressor cells whether or not they express CD25$^+$.

Taken together, these studies show that TGF-β1 produced by CD4$^+$CD25$^+$ regulatory T cells are an important mechanism by which these cells exert suppression in vitro and more, particularly, in vivo. However, they leave open the possibility that other suppressor mechanisms may also exist and may come to the fore under some circumstances. Finally, studies presented in this work and previously strongly suggest that the presence of LAP on the cell surface is an excellent marker of regulatory cells, perhaps better than CD25 itself.

Acknowledgments

We thank Calvin Eigsti for technical assistance with FACS sorting. We also thank Dr. Mizuko Mamura for valuable help in providing TGF-β1 knockout mice. In addition, we acknowledge Akiyo Kondo for her technical assistance and Sara Kaul for editorial assistance.

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