Brief Definitive Report

Infectious Tolerance: Human CD25$^+$ Regulatory T Cells Convey Suppressor Activity to Conventional CD4$^+$ T Helper Cells

Helmut Jonuleit,[1] Edgar Schmitt,[2] Hacer Kakirman,[1] Michael Stassen,[2] Jürgen Knop,[1] and Alexander H. Enk[1]

[1]*Department of Dermatology and the* [2]*Institute of Immunology, University of Mainz, 55101 Mainz, Germany*

Abstract

Regulatory CD4$^+$CD25$^+$ T cells (Treg) are mandatory for maintaining immunologic self-tolerance. We demonstrate that the cell-cell contact–mediated suppression of conventional CD4$^+$ T cells by human CD25$^+$ Treg cells is fixation resistant, independent from membrane-bound TGF-β but requires activation and protein synthesis of CD25$^+$ Treg cells. Coactivation of CD25$^+$ Treg cells with Treg cell–depleted CD4$^+$ T cells results in anergized CD4$^+$ T cells that in turn inhibit the activation of conventional, freshly isolated CD4$^+$ T helper (Th) cells. This infectious suppressive activity, transferred from CD25$^+$ Treg cells via cell contact, is cell contact–independent and partially mediated by soluble transforming growth factor (TGF)-β. The induction of suppressive properties in conventional CD4$^+$ Th cells represents a mechanism underlying the phenomenon of infectious tolerance. This explains previously published conflicting data on the role of TGF-β in CD25$^+$ Treg cell–induced immunosuppression.

Key words: human regulatory T cells • CD4$^+$CD25$^+$ T cells • infectious tolerance • T cell inhibition • TGF-β

Introduction

The process of TCR generation, that is based on random rearrangements, as well as the promiscuity of the resulting receptor expressed by mature T cells requires central and peripheral mechanisms of tolerance induction (1). CD25$^+$ T regulatory (Treg) cells play a central role in the maintenance of peripheral tolerance as the depletion of these cells in a murine adoptive transfer model leads to various autoimmune diseases (2, 3). These CD4$^+$ T cells express CD25 but do not or only marginally proliferate after polyclonal or antigen-specific activation. However, although they seem to be immunologically inert, activated CD25$^+$ Treg cells strongly suppress the proliferation of coactivated conventional CD4$^+$CD25$^-$ T cells in vitro (4, 5). We and others characterized a human equivalent of murine CD25$^+$ Treg cells that has comparable properties and can be isolated from human peripheral blood (6–8). The suppressive capacity of these human CD25$^+$ Treg cells depends on direct cell–cell contact but the mechanism of suppression is largely unknown. Several studies implied that signaling through CTLA-4 might be responsible for the inhibitory potency of CD25$^+$ Treg cells, whereas others could not confirm this finding (2). Studies using a murine IBD model suggest that TGF-β and IL-10 are potent mediators of suppression (9, 10). However, neither anti–IL-10 nor anti–TGF-β antibodies could abrogate the suppressive capacity of human CD25$^+$ Treg cells in vitro (6). On the other hand, it was shown that blocking anti–TGF-β antibodies abrogated suppressor activity in vivo (9, 11, 12). Hence, there are a lot of conflicting and contradictory findings regarding the suppressive mechanisms of CD25$^+$ Treg cells. Herein we demonstrate that coculture of human CD25$^+$ Treg cells with CD25$^-$CD4$^+$ T cells results in the development of an additional CD4$^+$ T suppressor cell population. These induced CD4$^+$ Treg cells emerge from the CD25$^-$CD4$^+$ T cell population and suppress the proliferation of freshly isolated conventional CD4$^+$ T cells. This process is partially mediated by soluble TGF-β.

Materials and Methods

Culture Medium. X-VIVO-15 supplemented with 1% autologous plasma was used for culture of dendritic cells (DCs) and without plasma for culture of T cells (BioWhittaker).

Address correspondence to H. Jonuleit, Dept. of Dermatology, University of Mainz, D-55101 Mainz, Germany. Phone: 49-6131-173541; Fax: 49-6131-175505 or 17-473541; E-mail: jonuleit@hautklinik.klinik.uni-mainz.de

Cytokines. All cytokines used in this study were recombinant human proteins. Final concentrations: 800 U/ml GM-CSF (Leukomax), IL-4, 1,000 U/ml IL-6 (Strathmann Biotech GmbH), IL-1β, 10 ng/ml TNF-α (Strathmann), and PGE_2 (Minprostin; Pharmacia-Upjohn) 1 μg/ml. The DCs were generated from buffy coats of healthy volunteers as described previously (13). For preactivation of T cells: IL-2 (Proleukin; Chiron) 10 U/ml.

Antibodies. These mAbs were used for the staining of MACS®-sorted T cells: FITC- or PE-conjugated CD3, CD4, CD25, CTLA-4, anti–HLA-A,B,C, and FITC- and PE-conjugated mouse IgG (Beckman Coulter and Immunotech). These mAbs were used for the staining of membrane-bound TGF-β: LAP (biotinylated mAb, R&D Systems, used according to the manufacturer's instructions) detected by streptavidin-Cy5 (Dianova). The anti–TGF-β (R&D Systems, used according to the manufacturer's instructions) and anti–IL-10 mAb (JES-19F1.1.1; American Type Culture Collection (ATCC), blocking capacity tested in proliferation assays using IL-10 receptor transfected Baf3 cells) were used for blocking experiments (14). For sorting of preactivated HLA-A2–positive conventional $CD4^+$ T cells, purified anti–HLA-A2–positive-specific mAb (BB7.2; ATCC) and anti–mouse IgG microbeads (Miltenyi Biotec) were used. Labeled T cells were analyzed by flow cytometry (FACScalibur™ and CELLQuest™; Becton Dickinson). Necrosis versus apoptosis were determined by propidium iodide and annexin-V staining according to the manufacturer's instructions (BD PharMingen).

Isolation and Stimulation of T Cell Populations. Conventional $CD4^+$ Th cells and $CD4^+CD25^+$ regulatory T cells were isolated from buffy coats of healthy volunteers as described previously (6). Briefly, CD4-MACS®-Multisort-Beads (Miltenyi Biotec) were used for isolation of $CD4^+$ T cells. After detaching, cells were washed and $CD4^+CD25^+$ T cells were positively selected according to the instructions of the manufacturer using CD25 microbeads. For some experiments, $CD4^+CD25^+$ T cells and conventional $CD4^+$ T cells were preactivated with 0.5 μg/ml anti-CD3 mAb at 37°C for 20 h in the presence of 10 U/ml IL-2. Aliquots of the cultures were used for proliferation assays performed in X-VIVO-15 and in the presence of different numbers of allogeneic DCs or anti-CD3/CD28 stimulation in 96-well plates. T cell proliferation was measured after 3–4 d of incubation and an additional 16-h pulse with ^{3}[H]Tdr (37 kBq/well) using a liquid scintillation counter.

Isolation of Precultured $CD4^+$ T Cells. 10^6 freshly isolated HLA-A2-negative $CD4^+$ T cells and 10^6 HLA-A2-positive $CD25^+$ Treg from a different donor were coactivated with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) mAb. After 6 d of coculture the $CD25^+$ Treg cells were stained with a HLA-A2-specific mAb and depleted from the anergized $CD4^+$ T cells using anti–mouse IgG microbeads (purity >95%). The anergized $CD4^+$ T cells were restimulated with anti-CD3/CD28 mAb as described previously. 24–72 h after restimulation aliquots were used for detection of TGF-β by ELISA (DRG-Instruments, detection limit of biologically active TGF-β: 4.7 pg/ml).

Transwell Experiments. Transwell experiments were done in 24-well plates as described previously (14). Briefly, 10^6 $CD4^+$ conventional T cells or $CD4^+CD25^+$ T cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) in 1.5 ml. Additionally, 10^6 $CD4^+CD25^+$ T cells or precultured $CD4^+$ T cells (Thsup) were either directly added to cultures of activated conventional $CD4^+$ T cells or were placed in transwell chambers (Millicell, 0.4 μm; Millipore) in the same well. After 3 d of culture, activated T cells (200 μl per well) were transferred to 96-well plates in triplicates. Proliferation was measured after an additional 16-h pulse with ^{3}[H]Tdr using a liquid scintillation counter.

Fixation of Freshly Isolated and Preactivated T Cells. For some experiments, conventional $CD4^+$ T helper cells and $CD25^+$ Treg were fixed with 1% paraformaldehyde for 10 min in PBS, either directly after isolation or 20 h after preactivation with 0.5 μg/ml anti-CD3 mAb and 10 U/ml IL-2. Additionally, $CD25^+$ Treg were preactivated in the presence of 10 μg/ml cycloheximide or 1 μg/ml monensin. These concentrations of cycloheximide and monensin showed no toxic effects but blocked the upregulation of CTLA-4 on $CD25^+$ Treg and CD25 on conventional $CD4^+$ T cells during preactivation (unpublished data). Fixed cells were washed intensively with RPMI 1640 plus 10% FCS and used for coculture with freshly isolated $CD4^+$ T cells in proliferation assays.

Results and Discussion

$CD25^-$ conventional $CD4^+$ T cells and $CD25^+$ Treg cells were isolated from buffy coats of healthy volunteers as described previously (6). Freshly isolated, $CD25^+$ Treg cells showed a fourfold increased expression of membrane-bound TGF-β as compared with conventional $CD4^+$ Th cells (Fig. 1 A). However, polyclonal activation using anti-CD3 in combination with anti-CD28 antibodies resulted in an upregulated surface expression of TGF-β on conventional $CD4^+$ T cells, whereas TGF-β on $CD25^+$ Treg cells was downregulated. Both populations, $CD25^+$ Treg cells and conventional $CD4^+$ Th cells, either resting or activated, showed no significant production of biologically active soluble TGF-β (see Fig. 4 C).

To analyze the potential inhibitory effect of membrane-bound TGF-β on the surface of freshly isolated human $CD25^+$ Treg cells, as postulated for murine $CD25^+$ Treg cells (15), we stimulated freshly isolated $CD4^+$ Th cells in coculture with human $CD25^+$ Treg cells and in the presence or absence of blocking antibodies against TGF-β. As shown in Fig. 1 B, the presence of anti–TGF-β antibodies could not reverse the cell contact–dependent inhibitory effect of the $CD25^+$ Treg population on the proliferation of $CD4^+$ Th cells, neither upon allogeneic nor polyclonal stimulation of Th cells. Thus, these data indicate that membrane-bound TGF-β is not responsible for the suppressive effects of freshly isolated human $CD25^+$ Treg cells. In addition, the fact that highly proliferative $CD4^+$ Th cells express the same amounts of TGF-β as highly suppressive $CD25^+$ Treg cells (Fig. 1 A) also strongly argues against the assumption that membrane-bound TGF-β is responsible for the suppressive capacity of human $CD25^+$ Treg cells.

To analyze the suppressive mechanism in more detail, we stimulated $CD4^+$ T cells in the presence of fixed $CD25^+$ Treg cells. In coculture experiments, freshly isolated human $CD25^+$ Treg cells inhibited the proliferation and cytokine production of coactivated conventional $CD4^+$ T cells in a dose-dependent manner. If $CD25^+$ Treg cells were fixed directly after isolation, no suppressive activity could be detected (Fig. 2 A). However, if

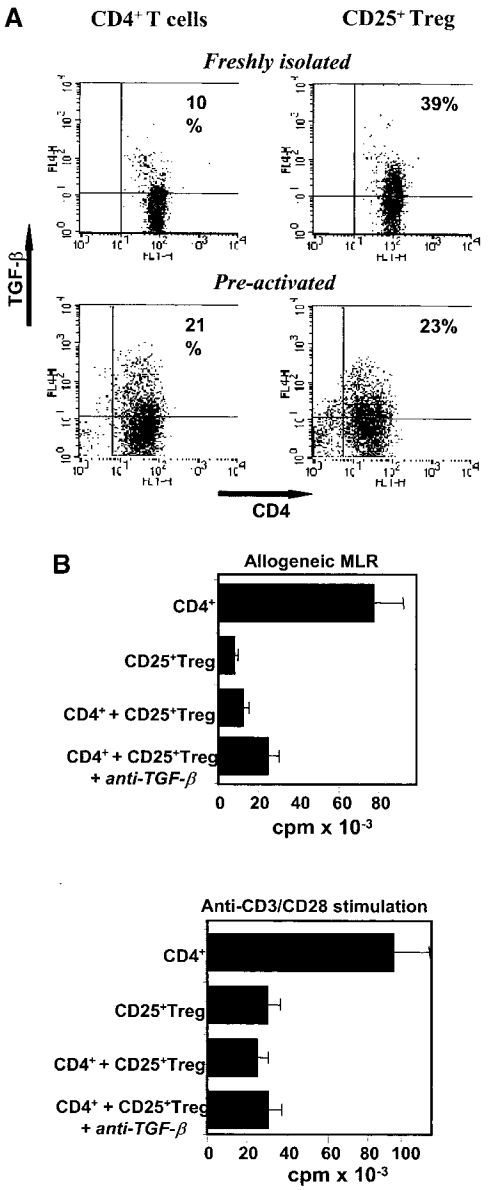

Figure 1. The suppressive activity of human CD25$^+$ Treg cells is independent from membrane-bound TGF-β. CD4$^+$ and CD4$^+$CD25$^+$ T cells were isolated from buffy coats of healthy volunteers by positive selection using paramagnetic beads. (A) Surface expression of TGF-β by freshly isolated CD4$^+$ T cells and CD25$^+$ Treg cells in comparison to the same T cell populations preactivated for 48 h with anti-CD3 (OKT3, 1 μg/ml) and anti-CD28 mAb (CD28.2, 2 μg/ml). The figure shows the expression of TGF-β (LAP-biotinylated) detected by streptavidin-Cy5 and CD4 (RPAT4-FITC). (B) CD4$^+$ T cells (10^5 cells per well) or CD25$^+$ Treg cells (10^5 cells per well), alone or in coculture (1:1), were stimulated with allogeneic mature DC (10^4 cells per well) or by anti-CD3 (1 μg/ml) plus anti-CD28 mAb (2 μg/ml). Neutralizing anti-TGF-β mAb (10 μg/ml) was added to the cocultured cells as indicated. ^{3}[H]Tdr was added after 3 (polyclonal stimulation) or 4 d (allogeneic MLR) of culture for the final 16 h.

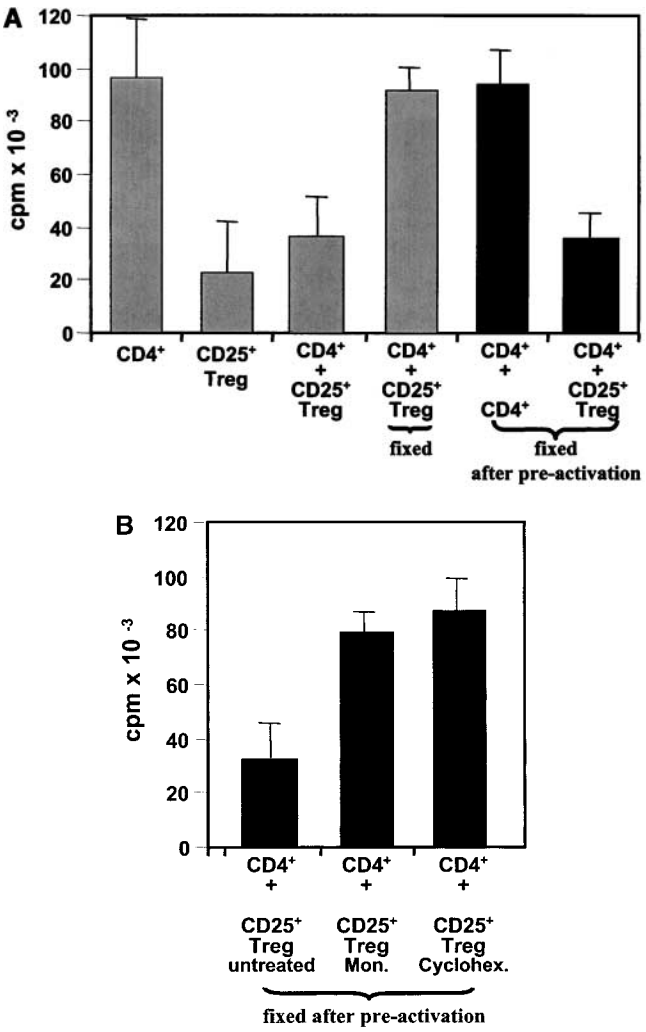

Figure 2. The suppressive activity of activated CD25$^+$ Treg cells is fixation-resistant but the activation is sensitive to treatment with monensin or cycloheximide. (A) Freshly isolated CD4$^+$ T cells (10^5 cells per well) and CD25$^+$ Treg cells (10^5 cells per well) or a combination of both (1:1) were activated with anti-CD3 (1 μg/ml) and anti-CD28 mAb (2 μg/ml). In addition, CD4$^+$ T cells were coactivated with CD25$^+$ Treg cells or conventional CD4$^+$ T cells that were immediately fixed after isolation (1% paraformaldehyde, 10 min) or were fixed after preactivation with 0.5 μg anti-CD3 mAb plus 10 U/ml IL-2 for 20 h (black bars). (B) CD4$^+$ T cells were coactivated either with preactivated and fixed CD25$^+$ Treg cells or with CD25$^+$ Treg cells treated with monensin (1 μg/ml) or cycloheximide (10 μg/ml) during preactivation before fixation. ^{3}[H]Tdr was added after 3 d of culture for the final 16 h.

CD25$^+$ Treg cells were preactivated overnight with anti-CD3 antibodies (0.5 μg/ml) and 10 U/ml IL-2 before fixation, they showed a comparable suppressive capacity for CD4$^+$ T cells as unfixed CD25$^+$ Treg cells. In contrast, Treg-depleted and activated conventional CD4$^+$ T helper cells did not exert any suppressive activity, although such cells express comparable amounts of TGF-β (Fig. 1 A). These data suggest that the inhibitory function of human CD25$^+$ Treg cells is activation dependent. Additional experiments revealed that the induction of suppressor activity requires protein synthesis as it can be inhibited by the presence of cycloheximide or monensin (Fig. 2 B). However, once activated, the suppressive activity of human CD25$^+$ Treg cells is fixation-resistant (Fig. 2 A). These data also strongly corroborate our findings that the inhibi-

tory function of human CD25+ Treg cells is independent of soluble mediators (6). Furthermore, the activation of suppressor function of human CD25+ Treg is also independent of costimulation, since the addition of mature DCs or soluble anti-CD28 antibodies during overnight preactivation with anti-CD3 antibodies did not alter/enhance the functional activities of human CD25+ Treg cells (unpublished data).

We and others could not detect any influence of TGF-β on the cell contact–mediated inhibitory function of CD25+ Treg cells in vitro (2, 6), whereas several groups demonstrated that neutralizing antibodies against TGF-β abrogated the suppressive function of murine CD25+ Treg cells in vivo (9, 15). One possible explanation for this discrepancy could be that CD25+ Treg cells exert an additional suppressive mechanism that is presumably also involved in the phenomena of bystander suppression or infectious tolerance (10, 16) but is not directly mediated by the CD25+ Treg cells themselves. Consequently, we analyzed the functional properties of the anergized conventional CD4+ Th cells after coculture with human CD25+ Treg cells. To distinguish both T cell populations, HLA-mismatched CD25+ Treg cells were used and depleted of the anergized CD4+ T cells after coculture with the aid of HLA-specific mAbs as illustrated in Fig. 3 A. Coculture of HLA-mismatched conventional CD4+ T cells served as a control. As shown in Fig. 3 B, the purified CD4+ T cells, anergized by CD25+ Treg cells, revealed a strong suppressive activity for conventional CD4+ Th cells that was comparable to the inhibitory capacity of CD25+ Treg cells. Thus, human CD25+ Treg cells, in a cell contact–dependent fashion, suppress the proliferation of conventional CD4+ T cells and simultaneously induce suppressive activity in these CD4+ Th cells (Fig. 3 B). In contrast, conventional CD4+ T cells preactivated in the presence of HLA-mismatched conventional CD4+ T cells showed no suppressive activity for freshly isolated Th cells (Fig. 3 B, lowest bar).

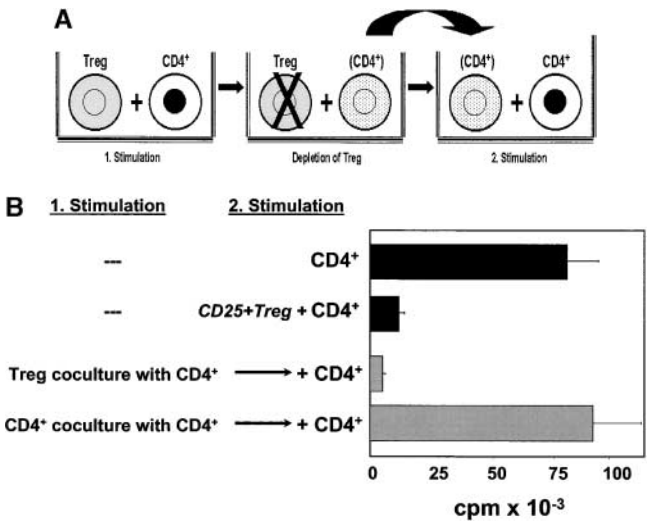

Figure 3. CD25+ Treg cells convey a suppressive activity to conventional CD4+ Th cells. (A) Cocultured HLA-A2-positive CD25+ Treg cells (10^6/ml) and HLA-mismatched CD4+ T cells (10^6/ml, HLA-A2-negative) were primarily activated by anti-CD3 (1 μg/ml) and anti-CD28 mAb (2 μg/ml). As a control HLA-A2-negative CD4+ T cells were also coactivated with HLA-A2-positive CD4+ T cells. After 6 d of culture the HLA-A2-positive CD25+ Treg cells and HLA-A2-positive CD4+ T cells were depleted from HLA-A2-negative CD4+ T cells using a HLA-specific mAb (BB7.2) and paramagnetic microbeads (purity of precultured CD4+ T cells >95%). (B) The remaining precultured CD4+ T cells (10^5 cells per well) were γ-irradiated (3,000 rad) and secondarily stimulated with freshly isolated CD4+ responder T cells (10^5 cells per well, 1:1) using anti-CD3 and anti-CD28 mAb. In addition, freshly isolated CD25+ Treg cells were used as a control population. ^{3}[H]Tdr was added after 3 d of culture for the final 16 h.

To elucidate the mechanism of this conveyed suppressive activity, we analyzed the properties of the anergized CD4+ T cells (subsequently termed Th_{sup}) in comparison to the original CD25+ Treg population in transwell stimulations. The semipermeable transwell membrane prevents direct cell–cell contact between the responsive Th cell population and the suppressor T cells. As shown recently, the physical separation by the transwell membrane abrogated the cell contact–dependent suppression of CD4+ Th cells by CD25+ Treg cells (6). In contrast, the conveyed suppressor activity of Th_{sup} cells could not be abrogated by the semipermeable membrane (Fig. 4 A). However, fixation of such Th_{sup} cells completely abrogated their suppressor function while it could not inhibit the suppressive properties of activated CD25+ Treg cells (Fig. 4 B). These data strongly support our primary finding that the induced secondary suppressor activity of Th_{sup} cells is cell contact independent. In addition, these results were further confirmed by the fact, that the suppressive activity of Th_{sup} cells could be partially abrogated upon the addition of neutralizing anti–TGF-β antibodies (Fig. 4 A), whereas neutralizing anti–IL-10 antibodies showed no effect (data not shown). These data strongly suggest that the suppressive activity of Th_{sup} cells is mediated partially by biologically active TGF-β. Therefore, the production of TGF-β was assessed by using an ELISA that specifically detects biologically active material. As shown in Fig. 4 C, Th_{sup} cells produce biologically active TGF-β immediately after polyclonal stimulation. In contrast, neither conventional CD4+ Th cells nor CD25+ Treg cells, alone or in coculture, produced detectable amounts of TGF-β. This finding again strongly supports our thesis that resident human CD25+ Treg cells convey a suppressor function to conventional CD4+ Th cells that is completely cell contact independent and mediated mainly by TGF-β.

In summary, our findings help to explain several conflicting and contradictory results published previously. In accordance with published data on the properties of resident murine and human CD25+ Treg in vitro (2, 6, 8), we show that the primary functional activity of CD25+ Treg is strictly activation- and cell contact–dependent and independent of soluble mediators. Moreover, our data clearly indicate that classical human CD25+ Treg cells can, in a cell contact–dependent manner, direct the differentiation of conventional CD4+ Th cells toward an additional population of regulatory T cells (Th_{sup}). Both

regulatory T cell populations can inhibit Th cell proliferation through distinct suppressor mechanisms (cell contact–dependent versus soluble mediators). Thus, these results imply that human CD25$^+$ Treg cells can amplify their suppressive capacity through the recruitment of an additional population of suppressor T cells that exert a distinct but complementary suppressor mechanism. Data from murine in vivo studies suggest that the suppressive properties of CD25$^+$ Treg cells are mediated via soluble mediators such as TGF-β (2). In contrast, in vitro studies clearly revealed that murine and human CD25$^+$ Treg cells inhibit T cell proliferation via cell contact and independent from soluble factors (6, 8). These apparently discordant results could be reconciled by the assumption that in vitro a local cell contact–dependent mechanism is sufficient to inhibit the proliferation of the responding T cell population. However, in vivo, e.g., in the murine model of IBD (9), additional soluble suppressive mediators such as TGF-β, derived from anergized CD4$^+$ Th cells, are important for the systemic control of autoreactive T effector cells (Fig. 5). Finally, the regulation of immune responses that includes bystander suppression and infectious tolerance (10, 16) obviously requires a transfer of tolerizing potencies from resident or induced CD25$^+$ Treg cells to another T cell population with a different antigen specificity. Hence, we would like to speculate that the spreading of suppression from human CD25$^+$ Treg cells to conventional CD4$^+$ Th cells is one of the fundamental mechanisms that is involved in the induction or maintenance of peripheral tolerance.

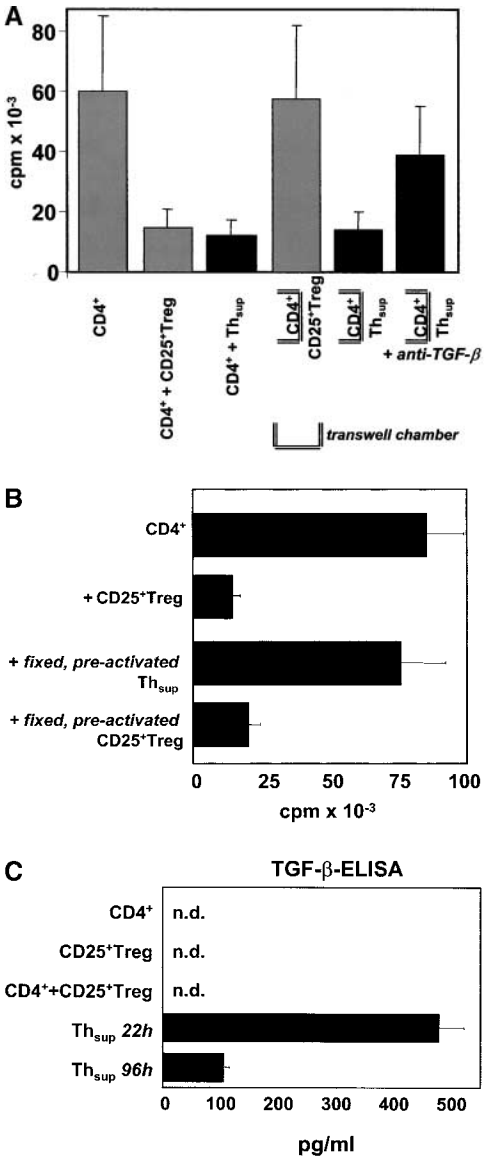

Figure 4. The suppressive activity of CD251 Treg-induced CD4$^+$ Th cells is partially mediated by soluble TGF-β. (A) 10^6 Freshly isolated CD4$^+$ T cells were activated alone or in coculture with 10^6 CD25$^+$ Treg cells or 10^6 Th_{sup} cells by anti-CD3 (1 μg/ml) and anti-CD28 mAb (2 μg/ml). Transwell chambers were used to prevent the direct cell contact between the T cell populations, neutralizing mAb against TGF-β were used at 10 μg/ml. After 3 d, aliquots from all approaches were transferred to 96-well microplates and ^{3}[H]Tdr was added for the final 16 h. (B) Freshly isolated CD4$^+$ T cells were activated alone or in coculture with freshly isolated CD25$^+$ Treg cells. In addition, such CD4$^+$ T cells were coactivated with CD25$^+$ Treg cells or Th_{sup} cells that had been fixed after preactivation with 0.5 μg/ml anti-CD3 mAb plus 10 U/ml IL-2 for 20 h (1:1). ^{3}[H]Tdr (1 μCi per well) was added after 3 d of culture for the final 16 h. (C) Freshly isolated CD4$^+$ T cells and CD25$^+$ Treg cells or a mixture of both were activated with anti-CD3 (1 μg/ml) and anti-CD28 mAb (2 μg/ml) for 3 d. Th_{sup} cells were also activated under the same conditions for 20 and 96 h, respectively. The content of biologically active TGF-β of the resulting supernatants was assessed by a TGF-β–specific ELISA.

The authors are grateful to Dr. E. Rüde and Dr. K. Mahnke for critical reading of this manuscript and helpful discussions. We also thank L. Paragnik, A. Kandemir, S. Fondel, and P. Hoelter for expert technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft, grant A6SFB548 (to E. Schmitt) and grant A3SFB548 (to A.H. Enk).

Submitted: 11 March 2002
Revised: 23 April 2002
Accepted: 5 June 2002

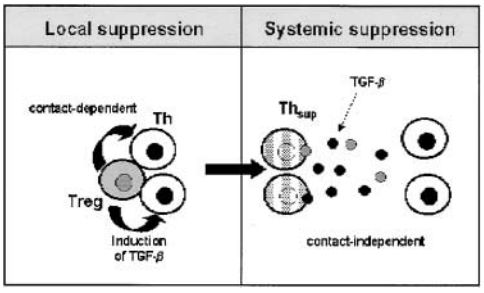

Figure 5. Model of T effector cell regulation. We propose the following model of immunoregulation by CD25$^+$ Treg in vivo: resident or induced CD25$^+$ Treg suppress the activation of conventional Th cells. This is a cell contact–dependent local inhibitory effect. The induced secondary T suppressor cells (Th_{sup}) produce inhibitory mediators such as biologically active TGF-β which itself inhibits the activation of T effector cells. This secondary systemic suppressive effect is cell contact independent.

References

1. Mason, D. 1998. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol. Today*. 19: 395–404.
2. Shevach, E.M., R.S. McHugh, C.A. Piccirillo, and A.M. Thornton. 2001. Control of T-cell activation by $CD4^+$ $CD25^+$ suppressor T cells. *Immunol. Rev*. 182:58–67.
3. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev*. 182:18–32.
4. Thornton, A.M., and E.M. Shevach. 2000. Suppressor effector function of $CD4^+CD25^+$ immunoregulatory T cells is antigen nonspecific. *J. Immunol*. 164:183–190.
5. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell*. 101:455–458.
6. Jonuleit, H., E. Schmitt, M. Stassen, A. Tuettenberg, J. Knop, and A.H. Enk. 2001. Identification and functional characterization of human $CD4^+CD25^+$ T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med*. 193:1285–1294.
7. Dieckmann, D., H. Plottner, S. Berchtold, T. Berger, and G. Schuler. 2001. Ex vivo isolation and characterization of $CD4^+CD25^+$ T cells with regulatory properties from human blood. *J. Exp. Med*. 193:1303–1310.
8. Ng, W.F., P.J. Duggan, F. Ponchel, G. Matarese, G. Lombardi, A.D. Edwards, J.D. Isaacs, and R.I. Lechler. 2001. Human $CD4^+CD25^+$ cells: a naturally occurring population of regulatory T cells. *Blood*. 98:2736–2744.
9. Powrie, F. 1995. T cells in inflammatory bowl disease: protective and pathogenic roles. *Immunity*. 3:171–174.
10. Weiner, H.L., A. Friedman, A. Miller, S.J. Khoury, A. al Sabbagh, L. Santos, M. Sayegh, R.B. Nussenblatt, D.E. Trentham, and D.A. Hafler. 1994. Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu. Rev. Immunol*. 12:809–837.
11. Mason, D., and F. Powrie. 1998. Control of immune pathology by regulatory T cells. *Curr. Opin. Immunol*. 10:649–655.
12. Fuss, I.J., M. Boirivant, B. Lacy, and W. Strober. 2002. The interrelated roles of TGF-β and IL-10 in the regulation of experimental colitis. *J. Immunol*. 168:900–908.
13. Jonuleit, H., U. Kuhn, G. Muller, K. Steinbrink, L. Paragnik, E. Schmitt, J. Knop, and A.H. Enk. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol*. 27:3135–3142.
14. Jonuleit, H., E. Schmitt, G. Schuler, J. Knop, and A.H. Enk. 2000. Induction of interleukin 10-producing, nonproliferating $CD4^+$ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med*. 192:1213–1222.
15. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by $CD4^+CD25^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor β. *J. Exp. Med*. 194:629–644.
16. Waldmann, H., and S. Cobbold. 2001. Regulating the immune response to transplants. a role for $CD4^+$ regulatory cells? *Immunity*. 14:399–406.