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Induction of IL-10 Suppressors in Lung Transplant Patients by CD4+25+ Regulatory T Cells through CTLA-4 Signaling

Ankit Bharat,* Ryan C. Fields,* Elbert P. Trulock,§ G. Alexander Patterson,*‡ and Thalachallour Mohanakumar.2*†

T cell-mediated autoimmunity to collagen V (col-V), a sequestered yet immunogenic self-protein, can induce chronic lung allograft rejection in rodent models. In this study we characterized the role of CD4+CD25+ regulatory T cells (Tregs) in regulating col-V autoimmunity in human lung transplant (LT) recipients. LT recipients revealed a high frequency of col-V-reactive, IL-10-producing CD4+ T cells (TIL-10 cells) with low IL-2-, IFN-γ-, IL-5-, and no IL-4-producing T cells. These TIL-10 cells were distinct from Tregs because they lacked constitutive expression of both CD25 and Foxp3. Expansion of TIL-10 cells during col-V stimulation in vitro involved CTLA-4 on Tregs, because both depleting and blocking Tregs with anti-CTLA4 F(ab)2 mAbs resulted in loss of TIL-10 cells with a concomitant increase in IFN-γ-producing Th1 cells (TIFN-γ cells). A Transwell culture of col-V-specific TIL-10 cells with Th1 cells (those generated in absence of Tregs) from the same patient resulted in marked inhibition of IFN-γ and proliferation of TIFN-γ cells, which was reversed by neutralizing IL-10. Furthermore, the TIL-10 cells were HLA class II restricted because blocking HLA class II on APCs resulted in loss of IL-10 production. Chronic lung allograft rejection was associated with the loss of Tregs with a concomitant decrease in TIL-10 cells and an increase in TIFN-γ cells. We conclude that LT patients have col-V-specific T cells that can be detected in the peripheral blood. The predominant col-V-specific T cells produce IL-10 that suppresses autoreactive Th1 cells independently of direct cellular contact. Tregs are pivotal for the induction of these “suppressor” TIL-10 cells. The Journal of Immunology, 2006, 177: 5631–5638.

Lung allografts sustain multiple injuries due to ischemia-reperfusion, alloimmunity, external pathogens, and gastroesophageal reflux (10–13). Such an inflammatory milieu is conducive for the development of autoimmunity. Recently, collagen V (col-V) has been demonstrated to represent a sequestered self-protein localized in the lung tissue that can induce autoimmunity and contribute to lung allograft and isograft rejection in both animal models and human subjects (14–16). However, the pathogenesis of autoimmunity to col-V following lung transplantation remains unclear. CD4+25+ T cells have emerged as potent modulators of both alloimmunity and autoimmunity (17). Nevertheless, the mechanisms of CD4+25+ T cell-mediated suppression of conventional T cells are unclear. In addition, the role of CD4+25+ T cells in maintaining peripheral tolerance to self-proteins after human allograft transplantation has not been investigated. In this study, we demonstrate that CD4+25+ T cells prevent autoimmunity to col-V following human lung transplantation by inducing the development of IL-10-producing T cells.

Materials and Methods

Human subjects

Ten patients that underwent lung transplantation at the Washington University Medical Center/Barnes-Jewish Hospital were randomly selected for the study after obtaining informed consent. The PBMCs were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia) and stored at −135°C until evaluation. The cell yield ranged between 0.1 and 4.0 × 10⁶ PBMCs per milliliter of whole blood. The samples selected for analysis were obtained at least 1 year posttransplantation and when the patients were free of any acute or chronic rejection. The mean age of transplantation was 52.0 ± 8.1, and the male to female

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*Department of Surgery, †Department of Pathology, ‡Division of Cardiothoracic Surgery, and §Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110

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*Address correspondence and reprint requests to Dr. Thalachallour Mohanakumar, Washington University School of Medicine, Department of Surgery, Box 8109-3328 Clinical Sciences Research Building, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail address: kumarw@wustl.edu

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ratio was 4:6. The end stage pulmonary pathologies were chronic obstructive pulmonary disease (n = 7), α-1-antitrypsin deficiency (n = 1), and cystic fibrosis (n = 2). Most of the transplants were bilateral (n = 9). Chronic lung allograft rejection (bronchiolitis obliterans syndrome (BOS)) was diagnosed according to standard International Society for Heart and Lung Transplantation guidelines (18). The standard immunotherapy protocol for all patients consisted of cyclosporine A, azathioprine, and prednisone.

**Antibodies**

The unconjugated and fluorochrome-conjugated mouse anti-human CD4 (clone RPA-T4), CD25 (clone M-A251), and isotype control Abs were purchased from BD Biosciences. Anti-human APC-CD49d (integrin α4 chain; clone 9F10), PE/Cy5-integrin β2 (clone FIB504), PE/Cy5/CD29 (integrin β1; clone MAR4), and isotype controls were also purchased from BD Biosciences. Anti-human IL-10-antibody (50 μg/ml; clone JES-19F1; BD Pharmingen) and pan-specific anti-soluble TGF-β Abs (20 μg/ml; AB-100-NA; R&D Systems) were used for neutralization experiments. Anti-human CTLA-4 F(ab')2 mAb (10 μg/ml) were purchased from Ancell. Hybridomas for anti-HLA class II mAb KuIA2 (framework anti-HLA-DR) were produced in our laboratory (19).

**Development of cell lines**

PBMCs from lung transplant (LT) patients were stimulated with irradiated (3000 rad) autologous PBMCs (1:1 ratio) with 20 μg/ml human col-V (BD Biosciences) in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated, endotoxin-free FBS (Hy Clone), l-glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), penicillin (100 U/ml), streptomycin (100 μg/ml); 1% BSA for 2 h and washed three times with PBS. Subsequently, 3 × 10^5 cells were cultured in triplicate cultures in flat-bottomed 96-well microtiter plates (NUNC) with 0.4-μm membrane supports. For all experiments the ratio of Treg cells on either side of membrane was 1:1. At the end of coculture, CD4^- T cells were negatively selected and col-V-specific response was measured using ELISPOT assays.

**ELISPOT assay**

ELISPOT assays were performed as described previously (20). Briefly, MultiScreen 96-well filtration plates (Millipore) were coated overnight at 4°C with 5.0 μg/ml capture human cytokine-specific mAb (BD Biosciences) in 0.05 M carbonate-bicarbonate buffer (pH 9.6). The plates were blocked with 1% BSA for 2 h and washed three times with PBS. Subsequently, 3 × 10^5 cells were cultured in triplicate in the presence of col-V (40 ng/ml) irradiated feeder autologous PBMCs (APCs) (1:1 ratio). After 48 h, the plates were washed three times with PBS and three times with 0.05% PBS/Tween 20. Then, 2.0 ng/ml biotinylated human cytokine-specific mAb (BD Biosciences) in PBS/BSA/Tween 20 was added to the wells. After an overnight incubation at 4°C, the plates were washed three times and HRP-labeled streptavidin (BD Biosciences) diluted 1/2000 in PBS/BSTA/Tween 20 was added to the wells. After 2 h, the assay was developed with 3-amin-9-ethylcarbazole substrate reagent (BD Biosciences) for 5–10 min. The plates were washed with tap water to stop the reaction and air dried. The spots were analyzed in an Immunospot Series I analyzer (Cellular Technology), and the results were expressed as spots per million cells (spm). Any spots obtained by culturing T cell lines with APCs alone were subtracted from the number of spots in the experimental cultures.

**Proliferation assay**

Cells from col-V-specific cell lines were seeded in triplicate cultures in flat-96 well plates (Falcon; BD Labware) at a concentration of 2 × 10^5 cells/well in the presence of col-V (40 μg/ml) and APCs (1:1). After 4 days, the cultures were pulsed with [3H]thymidine (1 μCi/well) for 18 h, after which [3H]thymidine incorporation into DNA was determined by means of liquid scintillation counting. The results were expressed as counts per minute after subtracting the counts obtained from the culturing cells with autologous APCs.

**Coculture**

Coculture experiments were performed in 24-well Transwell plates (Costar) with 0.4-μm membrane supports. For all experiments the ratio of T cells obtained was 1:1. At the end of coculture, CD4^- T cells were negatively selected and col-V-specific response was measured using ELISPOT assays.

**Activation-fixation of regulatory T cells (Tregs)**

Activation-fixation of natural Tregs was performed as described earlier (21). Briefly, CD4^-CD25^- T cells were separated from the PBMCs using immunomagnetic separation (Miltenyi Biotec). The yield of Tregs varied between 0.3 and 8%. First, CD4^- T cells were negatively purified using the non-CD4 depletion Ab mixture. Then, CD25^- T cells were positively selected from the enriched CD4^- T cells. The purity of CD4^-CD25^- T cells obtained by this protocol was >98% (not shown). The CD4^-CD25^- T cells obtained were then treated with anti-CD3/CD28 Abs (Dynal Biotech). Following this treatment, the CD4^-CD25^- T cells were washed thoroughly and fixed in 10% paraformaldehyde for 15 min. In parallel, PBMCs were depleted of the CD25^- T cells using anti-CD25 microbeads. The feeder PBMCs depleted of CD25^- T cells, which had >95% CD25^- T cells, were kept in overnight culture and then irradiated (3000 rad). The irradiated PBMCs and the isolated CD4^-CD25^- T cells were then added with col-V to the developing cell lines (1:1 ratio). In separate experiments, non-stimulating anti-CTLA4 F(ab')2 Abs were used to block CTLA-4 on CD4^-CD25^- T cells. CD4^-CD25^- T cells were activated overnight using anti-CD3/CD28 treatment. Following this step, the activated CD4^-CD25^- T cells were incubated with the anti-CTLA4 F(ab')2 (10 μg/ml) Abs for 30 min at room temperature and then washed three times.

**Results**

Tregs induce IL-10 predominant CD4 T cell immunity to col-V in LT patients

The CD4^- T cell-specific response was first analyzed in either the presence or absence of Tregs. The PBMCs from LT patients were stimulated with col-V in the presence of irradiated autologous PBMCs acting as APCs (n = 10) to develop cell lines. The clinical and demographic profile of these patients is presented in Table I. In parallel, the PBMCs from five of these patients were stimulated in the absence of CD25^- T cells. CD25^- T cells were depleted from the original PBMC sample used to generate the cell lines and from the APCs used during stimulations. Following the last stimulation, the CD4^- T cells were negatively purified. The cells obtained were >90% CD4^- (data not shown). Col-V-specific response was measured by performing ELISPOT assays in the presence of irradiated autologous APCs and col-V. For cell lines generated in the absence of Tregs, the autologous APCs used for the ELISPOT assay were also depleted of CD25^+ T cells. Of the 10 LT patients tested, cell lines could be established in eight. These cell lines generated in the presence of CD4^-CD25^- T cells revealed a high frequency of IL-10-producing CD4^+ T cells (TIL-10 cells) reactive to col-V (29.1 ± 24.17 spm) and a low frequency of IFN-γ-producing T cells (TIFN-γ cells, 57.33 ± 24.84 spm). The TIL-10 cells did not constitutively produce any IL-10 in the absence of APCs and col-V (0 spm). In addition, there was low cross-reactivity with an unrelated protein OVA (12.4 ± 9.5 spm) and collagen type II (32.2 ± 10.5 spm). However, depletion of CD25^- T cells led to IFN-γ predominance (250.67 ± 52.67 spm) with low IL-10 (39.84 ± 13.84 spm; p = 0.001) in all of the five patients tested (Fig. 1). This result indicated that the amplification of col-V-specific TIL-10 cells was dependent on Tregs. Depletion of Tregs led to the preferential expansion of TIFN-γ cells that represent Th1 cells.

TIL-10 cells were distinct from Tregs

The possibility of differentiation of Tregs into TIL-10 cells was investigated next. The PBMCs were stimulated with col-V in the
Table I. Demographic and clinical profile of lung transplant recipients

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<th>Type (R/B)</th>
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<th>Ischemia Time (min)</th>
<th>Time of Analysis Posttransplant (mos)</th>
<th>No. of Acute Rejections</th>
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<td>N</td>
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</table>

* Patient identifier.

† M, male; F, female.

‡ C, Caucasian.

§ A1A, α-1-antitrypsin deficiency; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease.

¶ R, right lung; B, bilateral.

∥ UA, unavailable.

# RT, right lung; LT, left lung.

* Y, yes; N, no.

presence of activated but fixed Tregs. The original PBMCs were depleted of the CD25+ T cells as described in Materials and Methods. The autologous APCs were also depleted of CD25+ T cells. CD4+CD25+ Tregs were isolated using immunomagnetic separation and cultured in the presence of anti-CD3/CD28 beads overnight, after which they were fixed in paraformaldehyde and washed thoroughly. Then, the CD25+ T cell-depleted APCs were irradiated and mixed with the activated-fixed Tregs and used as feeders for the CD25+ T cell-depleted PBMCs at a 1:1 ratio in the presence of col-V. The procedure prevents any cytokine production from CD4+CD25+ Tregs yet maintains their cell contact-dependent suppressive functions (21, 22). The cell lines generated in this fashion had high IL-10 (221 ± 25.10 spm) and low IFN-γ (85 ± 25.12 spm) in response to col-V (Fig. 1), suggesting that the predominant TIL-10 cells were distinct but still required the presence of CD4+CD25+ Tregs for development.

T<sub>IL-10</sub> did not express CD25 and Foxp3 constitutively

To further confirm that the T<sub>IL-10</sub> cells were distinct from Tregs, we analyzed the expression of CD25 and Foxp3. Following in vitro stimulation, the cells were rested for 10 days and the CD4 T cells were purified by negative selection as described in Materials and Methods. Following this step, CD25+ T cells were positively depleted from the purified CD4 T cells. The remaining CD4+CD25- T cells were used to perform a col-V-specific IL-10 ELISPOT assay in the presence of CD25+ T cell-depleted autologous APCs and in a real-time RT PCR to quantify Foxp3 expression. There was no significant decrease in the IL-10 response after depleting the CD25+ T cells from the cell cultures (291.0 ± 24.17 spm vs 212.0 ± 24.25 spm; p = 0.08), indicating that the predominant TIL-10 cells did not express CD25 constitutively (Fig. 2A). Further, the real-time PCR assay indicated that these cells did not express Foxp3 (Fig. 2B). These results indicated that the T<sub>IL-10</sub> cells were CD25 and Foxp3 negative and, therefore, distinct from Tregs.

T<sub>IL-10</sub> suppressed autologous T<sub>HIFN-γ</sub> cells (Th1 cells) without direct contact

Previous studies have clearly demonstrated that in vitro suppression mediated by CD4+CD25+ Tregs is cytokine independent and cell contact-dependent (23–27). We first confirmed these findings. Neutralization with high concentrations of anti-IL-10 or anti-TGF-β did not reverse the suppression of conventional CD25+ T cells during coculture with CD4+CD25+ Tregs (Fig. 3A). Moreover, Transwell separation of CD4+CD25+ Tregs from the CD4+CD25- T cells completely abrogated the suppression of CD4+CD25- T cells (Fig. 3B).

It was interesting to note that depletion of Tregs in the previous experiments led not only to the loss of T<sub>IL-10</sub> cells but also to the expansion of T<sub>HIFN-γ</sub> cells. We hypothesized that the suppression of T<sub>HIFN-γ</sub> cells observed by reconstituting the cultures with activated-fixed Tregs was at least partly due to the development of T<sub>IL-10</sub> cells and mediated by IL-10 produced by these cells. To investigate this question, cell lines generated in presence and absence of CD4+CD25+ Tregs from the same patient were cocultured. Cell
As control, the Foxp3 expression of CD25

Interestingly, the Th1 cell lines did not develop any significant increase

limit Th1-autoreactivity in LT patients by IL-10 production. Inter-

cells from same patients and CD4
lines. Following the last stimulation, the col-V-specific response

Abs were supplemented in the cocultures of IL-10 and Th1 cell

were cocultured in a Transwell plate at the ratio of 1:1. In parallel

original PBMCs and APCs used at each stimulation. The cells

were irradiated APCs depleted of CD25

col-V. Following the last stimulation, the cell lines were rested and then

Th1 (Th1) predominance. Hence, PBMCs from the same patient

were stimulated either in the presence of CD25+

line) of or after deleting CD25+

line (Th1 line). The initial stimulation

provided the opportunity for either the TIL-10 cells or

T cells to preferentially expand in the respective cell lines.

The IL-10 and Th1 cell lines were then cocultured for another three rounds of stimulation in the presence of autologous APCs

and col-V. For the Th1 line, CD25+ T cells were depleted from the original PBMCs and APCs used at each stimulation. The cells

were cocultured in a Transwell plate at the ratio of 1:1. In parallel

experiments, neutralizing anti-IL-10 or isotype (rat IgG2a) control

Abs were supplemented in the cocultures of IL-10 and Th1 cell

lines. Following the last stimulation, the col-V-specific response

was analyzed. There was a significant inhibition in both the cellular

proliferation ($54.5 \times 10^3$ cpm vs $22.2 \times 10^3$ cpm, $p = 0.02$)

and frequency of TIL-10 cells ($117 \pm 16.2$ spm vs $288.33 \pm 45.6$

spm) in the Th1 cell lines after coculture with the IL-10 cell line.

Moreover, this inhibition was reversed by neutralizing IL-10 in the

Suppression of Th1 responses was further investigated by stimulating

naive CD4+CD25+ control T cells with CD3/CD28 beads and comparing

different concentrations of Tregs. The TIL-10 cells were activated with

autologous APCs and col-V, whereas those generated after depleting

Tregs revealed IL-10 immunity to col-V, although TIL-10 cells could suppress the Th1-response, the induc-

and frequency of TIFN-

Although TIL-10 cells could suppress the Th1-response, the induc-

action of suppressive properties required interactions with Tregs.

TIL-10 cells were self-HLA class II restricted and resulted in bystander

We investigated whether TIL-10 cells were self-HLA class II restricted. Following stimulation, TIL-10 cells were activated with

autologous APCs and col-V in the presence of increasing doses of anti-HLA class II (KuIA2) or isotype control (mouse IgG1) Abs.

KuIA2 mAbs demonstrated a dose-dependent suppression of IL-10 produ-

To investigate whether TIL-10 cells could result in bystander suppression, naïve CD4+CD25+ T cells were stimulated with anti-

CD3/CD28 beads in the presence of either TIL-10 cells or control

CD4+CD25+ T cells in a Transwell coculture setting. The TIL-10

cells were activated using autologous APCs and col-V, whereas

the control CD4+CD25+ T cells were activated using anti-CD3/

CD28 beads. The proliferation of naïve CD4+CD25+ T cells was significantly reduced in the presence of TIL-10 cells as compared

with coculture with CD4+CD25+ T cells (45 $\pm 10^3$ vs 22 $\times 10^3$

cpm vs 98 $\times 10^3$ ± 14 $\times 10^3$ cpm; $p = 0.01$), indicating that

activation of TIL-10 cells can cause bystander suppression of T

cells, possibly including suppression of alloimmunity.

Induction of TIL-10 by CTLA-4 on Tregs

The induction of TIL-10 cells by Tregs was next investigated. As

previously described (26), CTLA-4 was found to be up-regulated

FIGURE 2. TIL-10 cells were distinct from Tregs. Col-V-specific T cell

lines generated in presence of Tregs had shown IL-10 immunity to

col-V, whereas those generated after depleting Tregs revealed

IFN-γ (Th1) predominance. Hence, PBMCs from the same patient

were stimulated either in the presence of CD25+ T cells (IL-10

line) or after deleting CD25+ T cells (Th1 line). The initial stimula-
tion provided the opportunity for either the TIL-10 cells or

T cells from LT patients at a 1:1 ratio were cocultured and

stimulated with CD3/CD28 beads for 6 days. In parallel, neutralizing IL-10

or TGF-β mAbs were added to the cultures. Total cellular proliferation

was measured using a [3H]thymidine incorporation assay and has been

illuminated as counts per minute.

FIGURE 3. CD4+CD25+ T cell-mediated suppression of conventional

T cells is contact dependent. A, Freshly isolated CD4+CD25+ or

CD4+CD25+ T cells from LT patients at a 1:1 ratio were cocultured and

stimulated with CD3/CD28 beads for 6 days. In parallel, neutralizing IL-10

or TGF-β mAbs were added to the cultures. Total cellular proliferation

was measured using a [3H]thymidine incorporation assay and has been

illuminated as counts per minute.

TIL-10 were HLA class II restricted and resulted in bystander

suppression
These results indicate that CTLA-4 was in part responsible for B served by using isotype control Abs (251.0 ± 30.23 spm; \( p < 0.039 \)) Abs. These activated but CTLA-4-blocked CD4 cells were depleted from the original PBMC sample and the APCs were used for another three rounds of col-V stimulation either in the presence or absence of anti-IL-10 mAbs or isotype (rat IgG2a) control Abs. After six stimulations, the col-V-specific response was analyzed by IFN-γ ELISPOT assays (solid line). Isotype control (mouse IgG1) Abs were used as control (broken line). The data are representative of three separate experiments done in triplicate, and the SE bars are illustrated. The results are expressed as spots per million cells.

Recently, two subsets of natural Tregs have been described, characterized by either \( \alpha_\gamma \beta_7 \) or \( \alpha_\beta_2 \) integrin expression (28). Whereas \( \alpha_\gamma \beta_7 \) Tregs induce IL-10, \( \alpha_\beta_2 \) Tregs induce TGF-β in conventional CD4+ T cells (28). Therefore, we speculated whether the three patients that developed loss of TIL-10 cells during BOS would specifically reveal a loss of \( \alpha_\gamma \beta_7 \) Tregs. To investigate this question, we analyzed the frequency of \( \alpha_\gamma \beta_7 \) on Tregs following activation (Fig. 6A). We speculated that CTLA-4 may have a role in the induction of TIL-10, cells by Tregs. To investigate this possibility, the CD25+ T cells were positively depleted from the original PBMC sample and the APCs were used to generate the cell line. The isolated CD4+CD25+ Tregs were activated using the CD3/CD28 ligation. Then, CTLA-4 on CD4+CD25+ T cells was blocked by nonstimulating anti-CTLA-4 F(ab′)2 Abs. These activated but CTLA-4-blocked CD4+CD25+ Tregs were reconstituted back into the cell cultures along with irradiated (CD25+ T cell-depleted) autologous APCs and col-V. The cells were treated similarly at each round of stimulation. We found a significant decline in the frequency of TIL-10 cells by blocking the CTLA-4 molecule of Tregs (136.2 ± 45.1 vs 280.0 ± 30.23 spm; \( p = 0.039 \)). However, the IL-10 response was preserved by using isotype control Abs (251.0 ± 45.2; \( p = 0.67 \)) (Fig. 6B). These results indicate that CTLA-4 was in part responsible for the induction of TIL-10 by Tregs.

**FIGURE 4.** T<sub>IL-10</sub> cells suppressed col-V reactive T<sub>IFN-γ</sub> cells. PBMCs from the same patients were stimulated with col-V either in the presence (IL-10 cell line) or absence (IFN-γ cell line) of CD25+ T cells three times. Following this step, the IL-10 and IFN-γ cell lines were transferred to a Transwell plate separating the IL-10 (above) and IFN-γ cells (below) at 1:1 ratio. Under these conditions, the IL-10 and IFN-γ cell lines were cocultured for another three rounds of col-V stimulation either in the presence or absence of anti-IL-10 mAbs or isotype (rat IgG2a) control Abs. After six stimulations, the col-V-specific response was analyzed by IFN-γ (open bars) and IL-10 (filled bars) ELISPOT assays (A) and [3H]thymidine incorporation (B). For the IFN-γ cell lines all APCs used were depleted of CD25+ T cells. The data represent the effects of coculture in IFN-γ cell lines from three separate experiments, and the SE bars are represented (1–3; \( p < 0.01 \)). The results for ELISPOT assays are expressed as spots/million cells (spm) and for [3H]thymidine incorporation as counts per minute.

**FIGURE 5.** T<sub>IL-10</sub> cells were HLA class II restricted. Col-V cell lines were developed from the PBMCs of LT patients after stimulation with col-V and autologous APCs. Following stimulation, the HLA class II on the APCs was first blocked by incubating with increasing concentrations of KuA2 mAbs, and then the IL-10 response was analyzed using ELISPOT assays (solid line). Isotype control (mouse IgG1) Abs were used as control (broken line). The data are representative of three separate experiments done in triplicate, and the SE bars are illustrated. The results are expressed as spots per million cells.

**FIGURE 6.** Expansion of T<sub>IL-10</sub> cells involves CTLA-4 on Tregs. A. CD4+CD25+ Tregs up-regulate CTLA-4 within 24 h of activation. B, Col-V specific cell lines were developed from the PBMCs of LT patients after stimulation with col-V. In parallel cultures Tregs were depleted from the cell cultures. The isolated Tregs were activated using CD3/CD28 co-stimulation and then incubated with anti-CTLA-4 F(ab′)2, Abs. Then, the activated and CTLA-4-blocked Tregs were reconstituted back into the cell cultures. Isotype mouse IgG1 Abs were used as control. The frequency of T<sub>IL-10</sub> cells was analyzed using IL-10 ELISPOT assay. The data are representative of four separate experiments done in triplicate, and the SE bars are illustrated. The results are expressed as spots per million cells.

**Loss of Tregs and expansion of T<sub>IFN-γ</sub> cells during BOS development**

As illustrated in Table I, four of the patients included in the study developed BOS. Three of these BOS patients, and none of the BOS-negative patients, revealed a significant decline in the frequency of CD4+CD25+ Tregs at the time of BOS development (\( p = 0.04 \), Fig. 7A). Concomitantly, there was a loss of T<sub>IL-10</sub> cells (Fig. 7B) and an expansion of T<sub>IFN-γ</sub> cells (Fig. 7C) in these same patients.
Tregs in the CD4+/CD25+ T cells population within the PBMCs. Samples were obtained from a pre-BOS visit and at the time when BOS was diagnosed (Table I). A significant loss of Tregs during BOS development. Three (Pt1), WU07 (Pt2), and WU08 (Pt3) of the four patients (Pt1), WU07 (Pt2), and WU08 (Pt3), and whose cells did not expand had cystic fibrosis (WU06 and WU10). Further, one of them (WU06) had single lung transplantation, no donor HLA class I or class II mismatch, and short ischemia time (Table I). It is possible that the level of injury sustained by these patients was below a certain threshold required to induce col-V-specific T cells. Similarly, the inability to readily elicit col-V immune response in healthy subjects can be attributed to lack of significant injuries and tissue remodeling in the native lungs, thus preventing col-V sensitization.

Naturally occurring Tregs have emerged as potent mediators of peripheral tolerance. Depletion of Tregs has been shown to result in the development of autoimmunity (17). Tregs have also been shown to modulate transplantation tolerance (43, 44). Several previous reports have established that the suppressive properties of Tregs in vitro are dependent on direct cellular interactions (24, 45, 46). However, if the suppressive properties of Tregs are dependent on direct cellular contact, it is difficult to explain how a subset of T cells that comprises only ~5–6% of total PBMCs can effectively mediate suppressive functions. Using in vitro model of mitogenic stimulation, Dieckmann et al. (21) have recently shown that Tregs may mediate suppressive effects on CD4+ T cells, converting them into suppressor cells, a phenomenon termed "infectious tolerance". This may serve to complement the peripheral suppression of Tregs. We demonstrated that the Tregs were crucial for the development of TIL-10 cells recognizing a self-protein (Fig. 1). These TIL-10 cells were distinct from Tregs because they did not express CD25 and Foxp3 constitutively (Fig. 2). Moreover, the col-V-specific T cell lines derived from rat lung allograft recipients but not in normal hosts (14), and col-V-reactive T cells are present in rat lung allografts undergoing rejection. Moreover, col-V-specific T cell lines derived from rat lung allografts with obliterative bronchiolitis, the histological correlate of BOS, induced rejection of isografts when adoptively transferred into isograft recipients without significantly affecting the native lungs. Interestingly, col-V is deposited in the alveolar interstitium and pulmonary capillary basement membranes of smaller airways (33, 37, 41). These are also the important sites where mononuclear cell infiltration is seen during human lung allograft rejection (42). In this study we were able to develop col-V-reactive T cells from eight of 10 patients tested. Interestingly, both patients whose cells did not expand had cystic fibrosis (WU06 and WU10). Further, one of them (WU06) had single lung transplantation, no donor HLA class I or class II mismatch, and short ischemia time (Table I). It is possible that the level of injury sustained by these patients was below a certain threshold required to induce col-V-specific T cells. Similarly, the inability to readily elicit col-V immune response in healthy subjects can be attributed to lack of significant injuries and tissue remodeling in the native lungs, thus preventing col-V sensitization.

Human allografts undergo significant inflammation and tissue remodeling after transplantation due to ischemia-reperfusion and alloimmunity. Lung allografts are also susceptible to injuries by exogenous pathogens and gastroesophageal reflux (10–12, 29–31), creating an inflammatory milieu conducive for the development of autoimmunity. We found that immune responses could be elicited in LT patients against col-V, a minor component of lung collagen (32). During inflammation and lung tissue repair, the col-V levels in the lungs increase significantly (33, 34). The col-V molecule has several unique properties (35). Being incorporated into collagen types I and III (33, 36, 37), it is considered to be “sequestered” under normal conditions, yet it maintains immunogenic potential. Matrix metalloproteinases (MMP) 2 and 9 are capable of cleaving collagen molecules (38). The activity of MMP-2 and MMP-9 has been shown to be up-regulated after lung transplantation (39, 40). Also, col-V can be detected in the bronchoalveolar lavage fluid from rat lung allograft recipients but not in normal hosts (14), and col-V-reactive T cells are present in rat lung allografts undergoing rejection. Moreover, col-V-specific T cell lines derived from rat lung allografts with obliterative bronchiolitis, the histological correlate of BOS, induced rejection of isografts when adoptively transferred into isograft recipients without significantly affecting the native lungs. Interestingly, col-V is deposited in the alveolar interstitium and pulmonary capillary basement membranes of smaller airways (33, 37, 41). These are also the important sites where mononuclear cell infiltration is seen during human lung allograft rejection (42). In this study we were able to develop col-V-reactive T cells from eight of 10 patients tested. Interestingly, both patients whose cells did not expand had cystic fibrosis (WU06 and WU10). Further, one of them (WU06) had single lung transplantation, no donor HLA class I or class II mismatch, and short ischemia time (Table I). It is possible that the level of injury sustained by these patients was below a certain threshold required to induce col-V-specific T cells. Similarly, the inability to readily elicit col-V immune response in healthy subjects can be attributed to lack of significant injuries and tissue remodeling in the native lungs, thus preventing col-V sensitization.

**Discussion**

Naturally occurring Tregs have emerged as potent mediators of peripheral tolerance. Depletion of Tregs has been shown to result in the development of autoimmunity (17). Tregs have also been shown to modulate transplantation tolerance (43, 44). Several previous reports have established that the suppressive properties of Tregs in vitro are dependent on direct cellular interactions (24, 45, 46). However, if the suppressive properties of Tregs are dependent on direct cellular contact, it is difficult to explain how a subset of T cells that comprises only ~5–6% of total PBMCs can effectively mediate suppressive functions. Using in vitro model of mitogenic stimulation, Dieckmann et al. (21) have recently shown that Tregs may mediate suppressive effects on CD4+ T cells, converting them into suppressor cells, a phenomenon termed "infectious tolerance". This may serve to complement the peripheral suppression of Tregs. We demonstrated that the Tregs were crucial for the development of TIL-10 cells recognizing a self-protein (Fig. 1). These TIL-10 cells were distinct from Tregs because they did not express CD25 and Foxp3 constitutively (Fig. 2). Moreover, the TIL-10 cells were capable of suppressing the autoreactive TIL-10 cells through IL-10 production (Fig. 4). The suppression of TIL-10 cells during coculture with TIL-10 cells in the Transwell plates was not mediated by Tregs (present in IL-10 cell lines), because activated Tregs alone in a similar setup did not inhibit TIL-10 cells
crease in TIL-10 cells and an increase in TIFN- 
reconstitution of activated/fixed Tregs induced TIL-10 cell expan-
TIFN- 
propagation of infectious tolerance by Tregs may form an integral
regulation during BOS. Taken together, our results indicate that
sive (IL-10) activity in conventional T cells.

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Disclosures

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