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J Immunol 2006; 177:5631-5638; doi: 10.4049/jimmunol.177.8.5631
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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+25+ 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/H9253 production. This suggests that TIL-10 cells inhibit col-V-specific Th1 cells through an IL-10-dependent mechanism. Further, 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 autoimmune responses. Recently, collagen V (col-V)1 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 ratio was 8.1. Informed consent was obtained from all the patients for the study.

1 Abbreviations used in this paper col-V, collagen V; BOS, bronchiolitis obliterans syndrome; DC, dendritic cell; LT, lung transplant; MMP, metalloproteinase; gpm, spots per million cells; Treg, regulator T cell; Tpro-γ, cell, IFN-γ-producing Th1 cell; TIL-10 cell, IL-10-producing CD4+ T cell.
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 mAb (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 mAbs (100 μg/ml) were purchased from Ancell. Hybridomas for anti-HLA class II mAb ((clone MAR4), and isotype controls were also purchased from Pharmingen) and pan-specific anti-soluble TGF-β (clone RPA-T4), CD25 (clone M-A251), and isotype control Abs were purchased from BD Biosciences in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated, endotoxin-free FBS (HyClone), 10 μM HEPES, and 1% BSA for 2 h and washed three times with PBS. Subsequently, the cell lines were activated overnight using stimulating anti-CTLA4 F(ab’)2 Abs for 30 min at room temperature and then washed three times.

**Results**

**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 × 10⁵ cells were cultured in triplicate in the presence of col-V (40 μg/ml) and 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 μg/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/BST/Tween 20 was added to the wells. After 2 h, the assay was developed with 3-aminio-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 1 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 × 10⁵ 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 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.

**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 <1% 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.

**Statistical Analysis**

Statistical analysis was performed using Microsoft Excel 2002 and GraphPad Prism, version 4.02 (GraphPad Software). Paired or unpaired two-tailed Student and Fisher exact t tests were used as appropriate to compare the col-V response. Statistical significance was defined as p < 0.05.

**Tregs induce IL-10 predominant CD4⁺ T cell immuno-complex to col-V in LT patients**

The col-V-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 line and from the APCs used during stimulations. Following the last stimulation, the CD4⁺ T cells were negatively selected. 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 (291 ± 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 = 0.0001) 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.
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. This 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 T_{IL-10} cells were distinct but still required the presence of CD4^+ CD25^+ Tregs for development. 

T_{IL-10} did not express CD25 and Foxp3 constitutively

To further confirm that the T_{IL-10} 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 T_{IL-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_{IL-10} cells were CD25 and Foxp3 negative and, therefore, distinct from Tregs.

T_{IL-10} suppressed autologous T_{IFN-γ} 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_{IL-10} cells but also to the expansion of T_{IFN-γ} cells. We hypothesized that the suppression of T_{IFN-γ} cells observed by reconstituting the cultures with activated-fixed Tregs was at least partly due to the development of T_{IL-10} 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.
Foxp3). As control, the Foxp3 expression of CD25 in the IL-10 production after coculture with TIL-10 compared with estingly, the Th1 cell lines did not develop any significant increase limit Th1-autoreactivity in LT patients by IL-10 production. Inter-

per million cells.

FIGURE 2. TIL-10 cells were distinct from Tregs. Col-V-specific T cell lines were developed from the PBMCs of LT patients after stimulation with col-V. Following the last stimulation, the cell lines were rested and then CD4+ T cells were purified. The CD25+ T cells were then positively depleted from the purified CD4+ T cells. Then, the remaining CD4+ 25+ T cells were used to perform IL-10 ELISPOT in presence of autologous irradiated APCs depleted of CD25+ T cells (A) and real-time RT-PCR for Foxp3 (B). As control, the Foxp3 expression of CD25+ conventional T cells from same patients and CD4+ 25+ Tregs is shown. The data are representative of four separate experiments done in triplicate, and the SE bars are illustrated for the ELISPOT assay. The results are expressed as spots per million cells.

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 depleting CD25+ T cells (Th1 line). The initial stimulation provided the opportunity for either the TIL-10 cells or TIFN-γ 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 × 10^3 cpm vs 22.2 × 10^3 cpm, p = 0.02) and frequency of TIFN-γ cells (117 ± 16.2 spm vs 288.33 ± 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 coculture (proliferation, 44.3 × 10^3 ± 5.7 × 10^3 cpm; TIFN-γ cells, 222 ± 43.1 spm) (Fig. 4). In contrast, isotype control Abs did not reverse the inhibition (proliferation, 20.0 × 10^3 ± 10.8 × 10^3 cpm; TIFN-γ cells, 122 ± 36.0 spm), indicating that TIL-10 cells limit Th1-autoreactivity in LT patients by IL-10 production. Interestingly, the Th1 cell lines did not develop any significant increase in the IL-10 production after coculture with TIL-10 compared with baseline (46.3 ± 18.0 vs 37.7 ± 15.5 spm; p = 0.81). Therefore, although TIL-10 cells could suppress the Th1-response, the induction of suppressive properties required interactions with Tregs.

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

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 production (Fig. 5). In contrast, there was no decrease in IL-10 production with the isotype control Abs, indicating that the TIL-10 cells were HLA class II restricted.

To investigate whether TIL-10 cells could result in bystander suppression, naive 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 naive CD4+CD25- T cells was significantly reduced in the presence of TIL-10 cells as compared with coculture with CD4+CD25- T cells (45 × 10^3 ± 22 × 10^3 cpm vs 98 × 10^3 ± 14 × 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...
on Tregs following activation (Fig. 6A). We speculated that CTLA-4 may have a role in the induction of T_{IL-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 T_{IL-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 T_{IL-10} cells by Tregs.

Loss of Tregs and expansion of T_{IFN-γ} cells during BOS development

As illustrated in Table 1, 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_{IL-10} cells (Fig. 7B) and an expansion of T_{IFN-γ} cells (Fig. 7C) in these same patients.

Recently, two subsets of natural Tregs have been described, characterized by either $\alpha_{4}\beta_{7}$ or $\alpha_{5}\beta_{1}$ integrin expression (28). Therefore, we speculated whether the three patients that developed loss of T_{IL-10} cells during BOS would specifically reveal a loss of $\alpha_{4}\beta_{7}$ Tregs. To investigate this question, we analyzed the frequency of $\alpha_{4}\beta_{7}$ Tregs.
BOS was diagnosed (Table I). A significant loss of TIL-10 cells was observed during BOS development. Three (Pt1), WU07 (Pt2), and WU08 (Pt3) of the four patients (Pt1, Pt2, and Pt3) that developed BOS demonstrated a decline in the frequency of CD4+CD25+ Tregs in the peripheral blood (A). The loss of Tregs correlated with a decline in TIL-10 cells (B) and a concomitant increase in TIFN-γ cells (C). The frequency of Tregs is expressed as percentage of CD4+CD25+ T cells present in the PBMCs. TIL-10 cells and TIFN-γ cells are expressed as spots per million cells (spm). Post-Tx, posttransplantation.

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 α4β7+ Tregs was found in all the three patients (Fig. 8).

**Discussion**

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 obliterator 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.

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 might induce anergy and IL-10 production in naive 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 IL-10 cells recognizing a self-protein (Fig. 1). These IL-10 cells were distinct from Tregs because they did not express CD25 and Foxp3 constitutively (Fig. 2). Moreover, the IL-10 cells were capable of suppressing the autoreactive TIFN-γ cells through IL-10 production (Fig. 4). The suppression of TIFN-γ cells during coculture with IL-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 TIFN-γ cells.
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(data not shown). Furthermore, depletion of Tregs following development of col-V cell lines did not prevent IL-10 production (Fig. 2B). Also, it has been previously established that Tregs cannot mediate suppression of conventional T cells if separated by a physical barrier (22, 24, 45). Although TIL-10 cells suppressed T<sub>H</sub>1<sub>-</sub>10 cells, no increase in IL-10 induction was observed in Th1 cell lines after coculture (Fig. 4). This finding further suggests that direct cellular interactions with Tregs alone could induce suppressive (IL-10) activity in conventional T cells.

The mechanisms through which the Tregs induce suppressive activity in the CD25<sup>+</sup> T cells are yet unclear. In these experiments, reconstitution of activated/fixed Tregs induced T<sub>H</sub>1<sub>-</sub>10 cell expansion (Fig. 1). Moreover, blocking CTLA-4 on Tregs significantly decreased the IL-10 response (Fig. 6B). Hence, these experiments demonstrate that induction of T<sub>H</sub>1<sub>-</sub>10 cells involves direct contact-dependent interactions with Tregs and was in part mediated by CTLA-4 (Fig. 6). However, it is unclear whether the Tregs acted via CTLA-4 directly with CD25<sup>+</sup> T cells to induce IL-10 or indirectly through APCs. Tregs have recently been shown to trigger the induction of IDO in dendritic cells (DCs) through CTLA-4 signaling (47–49). IDO then catalyzes the conversion of tryptophan to kynurenine and other metabolites that have potent immunosuppressive effects in the local environment of the DCs. Furthermore, Tregs can also down-regulate expression of both CD80 and CD86 on DCs making Ag presentation inefficient (50), which might in turn anergetize the concerned T cells. However, the Tregs can also function in the absence of APCs (24). Trl-activity can also be generated by DCs in the presence of high IL-10 (51). Hence, Tregs may interact with dendritic cells through CTLA-4 to induce IL-10 production, which can induce an IL-10 predominant phenotype in conventional T cells.

Two subsets of natural Tregs have recently been described, characterized by either α<sub>4</sub>β<sub>7</sub> or α<sub>5</sub>β<sub>2</sub> integrins (28). Tregs expressing β<sub>2</sub> induce IL-10-production, whereas those with β<sub>7</sub> integrin preferentially induce TGF-β in conventional CD4<sup>+</sup> T cells upon polyclonal stimulation. In the present study, there was a loss of α<sub>4</sub>β<sub>7</sub><sup>+</sup> Tregs during BOS development with a concomitant decrease in T<sub>H</sub>1<sub>-</sub>10 cells and an increase in T<sub>H</sub>N<sub>-</sub>γ<sub>-</sub>γ<sub>-</sub> cells. Interestingly, these patients also revealed a loss of α<sub>5</sub>β<sub>2</sub><sup>+</sup> Tregs (data not shown), indicating the possibility of deranged TGF-β-mediated regulation during BOS. Taken together, our results indicate that propagation of infectious tolerance by Tregs may form an integral regulatory circuit that maintains peripheral tolerance to self-Ags in human allograft recipients.

Acknowledgments

We express our gratitude to Billie Glasscock for her secretarial assistance.

Disclosures

The authors have no financial conflict of interest.

References


