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Research Article **Transplantation**

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IL-17–dependent cellular immunity to collagen type V predisposes to obliterative bronchiolitis in human lung transplants

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Bronchiolitis obliterans syndrome (BOS), a process of fibro-obliterative occlusion of the small airways in the transplanted lung, is the most common cause of lung transplant failure. We tested the role of cell-mediated immunity to collagen type V [col(V)] in this process. PBMC responses to col(II) and col(V) were monitored prospectively over a 7-year period. PBMCs from lung transplant recipients, but not from healthy controls or col(IV)-reactive Goodpasture's syndrome patients after renal transplant, were frequently col(V) reactive. Col(V)-specific responses were dependent on both $CD4^+$ T cells and monocytes and required both IL-17 and the monokines TNF-α and IL-1β. Strong col(V)-specific responses were associated with substantially increased incidence and severity of BOS. Incidences of acute rejection, HLA-DR mismatched transplants, and induction of HLA-specific antibodies in the transplant recipient were not as strongly associated with a risk of BOS. These data suggest that while alloimmunity initiates lung transplant rejection, de novo autoimmunity mediated by col(V)-specific Th17 cells and monocyte/macrophage accessory cells ultimately causes progressive airway obliteration.

Introduction

Organ transplantation is the only definitive therapy for many forms of end-stage organ failure. Current immunosuppressive regimens are effective in reversing acute cellular rejection, yet are ineffective against the fibroproliferative process of chronic rejection that causes failure of most organ transplants (1). In lung transplantation, chronic rejection takes the form of obliterative bronchiolitis (OB). OB was first described in heart-lung transplant recipients as fibrous lesions occluding the terminal bronchioles, rapidly progressing between 2 and 3 years after transplant (2). Because of the patchy nature of OB, its diagnosis via transbronchial biopsy is difficult. Thus, bronchiolitis obliterans syndrome (BOS), defined as a sustained decline of 20%–50% in forced expiratory volume in 1 second (FEV1) relative to the maximum post-transplant value, has become the standard clinical marker of OB. Once initiated, the obliterative process has no effective remedy and causes failure of more than 50% of lung allografts worldwide by 5 years after transplant (3).

OB histopathology suggests that both inflammation and injury responses precede small airway obliteration. Acute rejection and alloantibody formation, primarily triggered by ubiquitous donor HLA proteins, are classically thought of as the basis for acute allograft rejection. Both are known to be associated with BOS onset (4, 5). Yet despite newer therapeutic agents that have reduced the incidence of lung transplant acute rejection, the incidence and severity of BOS remains unchanged. While deposition of complement cleavage products and alloantibodies to HLA class I and class II has been strongly associated with chronic rejection of kidney transplants (6), their association with BOS has been less consistent (5, 7–9).

An alternate hypothesis is that chronic rejection is the end result of transplant-induced autoimmunity. Ischemically injured organs express exposed or modified normal protein constituents. These changes may be inconsequential in an isograft setting because of the immune system's capacity to buffer autoreactivity with regulatory T cells and dendritic cells. Yet in an allograft setting, alloreactive T and B cell responses to polymorphic HLA antigens may undermine immunoregulatory mechanisms, allowing de novo host T and B cell responses against nonpolymorphic graft neoantigens to develop. While both Ab-mediated (10–12) and cell-mediated (13, 14) autoimmune responses may have pathogenic consequences, to our knowledge, it has yet to be shown that they can account for the fibro-obliterative occlusion of vascular and epithelial spaces seen in chronic rejection of human organ transplants.

Collagen type V [col(V)], a minor fibrillar collagen abundant in lung, skin, and placenta, is essential for tissue elasticity and compliance (15). Normally cryptic components of extracellular matrix, overlaid by major collagens I and III within mature collagen fibrils (16), col(V) fragments are released into the extracellular milieu after lung transplantation and can trigger T cell–dependent immunity (17). Col(V)-specific $CD4^+$ T cell clones, derived from rejected rat lung allografts, induce acute rejection-

Nonstandard abbreviations used: BOS, bronchiolitis obliterans syndrome; col(V), collagen type V; DTH, delayed-type hypersensitivity; FEV1, forced expiratory volume in 1 second; GPS, Goodpasture's syndrome; HEL, hen egg lysozyme; OB, obliterative bronchiolitis; TT, tetanus toxoid; TT/DT, tetanus and diphtheria toxoid; TV-DTH, trans-vivo DTH; WKY, Wistar-Kyoto (rat).

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Table 1
Description of study subjects

	Lung transplant	Renal transplant	Controls
Total study subjects	54	5	6
Male recipients (*n*)	28 (52%)	4 (80%)	3 (50%)
Mean age (yr)			
Recipient	48 ± 12.5	50.2 ± 15.7	29.9 ± 9.3
Donor	31 ± 13.1	48.5 ± 10.5	–
Immunosuppression[A]	Yes	Yes	No
HLA match ≥1 (*n*)			
HLA-A	24 (44%)	5 (100%)	–
HLA-B	17 (31.5%)	5 (100%)	–
HLA-DR	22 (41%)	5 (100%)	–
Primary disease (*n*)			
AAD	11 (20%)	–	–
CF	12 (22%)	–	–
COPD	17 (32%)	–	–
IPF	7 (13%)	–	–
GPS	–	5 (100%)	–
Other[B]	7 (13%)	–	–

Lung transplants were 57% single lung, 43% bilateral. All renal transplants were single organs. AAD, α-1 antitrypsin deficiency; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis. [A]Standard triple therapy includes prednisolone, mycophenolate mofitil, and cyclosporine or tacrolimus. Rapamycin was used in place of calcineurin inhibitors when nephrotoxicity developed. [B]Includes bronchiectasis, ciliary dyskinesia, lymphangioleiomyomatosis, idiopathic bronchiolitis obliterans, pulmonary fibrosis, primary pulmonary hypertension, and sarcoidosis.

like pathology in rat lung isografts upon adoptive transfer (13). Similarly, LN cells transferred from col(V)-immunized syngeneic rats cause acute rejection pathology in isografted lungs (18). In the latter model, vasculitis and bronchiolitis correlated with the local expression of IL-17 transcripts and acquisition of systemic autoimmunity to col(V) in the adoptive host, measured by delayed-type hypersensitivity (DTH) response to ear challenge (18). Here we tested the hypothesis that cell-mediated autoimmunity specific to col(V) is a critical step in BOS progression in human lung transplants.

Results

$CD4^+$ T cell– and monocyte-dependent cellular immunity to col(V) after lung transplant. The clinical characteristics of 65 study subjects are detailed in Table 1. Patients with primary lung transplants from deceased donors ($n = 54$) were enrolled in a prospective monitoring trial; their demographics and lung disease at the time of transplant are shown. Five patients with Goodpasture's syndrome (GPS), all of whom had renal transplants from 1 HLA-haplotype matched living donors, and 6 healthy nonsmoking subjects were included as controls.

Collagen-specific cell-mediated immunity was assessed using the trans-vivo DTH (TV-DTH) assay. Figure 1A shows responses to recall antigen and to 3 different collagens for PBMCs of healthy normal subjects, renal transplant recipients, and a random sample of 15 of the 54 lung transplant recipients. The latter were donating blood at their regular clinic visits to monitor responses to col(II) and (V); col(IV), a ubiquitous basement membrane collagen (19), was added as a further specificity control. Renal transplant patients were selected because of the known autoimmunity to col(IV) in GPS (19) and because their immunosuppression regimen was similar to that of lung transplant patients. As shown in Figure 1A, all 3 groups had positive responses ($\geq 25 \times 10^{-4}$ in. net swelling) to EBV or tetanus and diphtheria toxoid (TT/DT) recall antigens, indicating that memory T cell–dependent immunity remained intact despite immunosuppression. Col(II), a fibrillar collagen specific to articular cartilage, did not elicit a TV-DTH response in patients or controls. Renal transplant recipients with GPS had significantly ($P < 0.001$) elevated mean swelling responses to col(IV). Lung transplant recipients did not respond to col(IV), but did respond to col(V) ($P < 0.001$ versus controls or renal transplant recipients). The level of response to col(V) was quite variable, with 5 of 15 patients having a response in the positive range ($\geq 25 \times 10^{-4}$ in.), including 2 with responses of 50×10^{-4} in. or more at a single sampling time point. Using recombinant α1(V) and α2(V) chains prepared from gene-transferrent fibroblast cell lines, the TV-DTH reactivity in 2 of these high-responder patients mapped to the α1(V) chain (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI28031DS1).

To characterize the cells and cytokines mediating the swelling response to col(V), we obtained leukaphereses from 4 lung transplant recipients with col(V)-specific TV-DTH responses of 30×10^{-4} in. or greater. PBMCs and antigens were coinjected into the mouse footpad along with cytokine-neutralizing Abs. A strong swelling response to both TT/DT and col(V) was observed in the presence of isotype control IgG (Figure 1B). However, the responses differed markedly in cytokine requirements. The response to TT/DT was strongly inhibited by antibodies to human IFN-γ, but not by antibodies to TNF-α or IL-1β, nor by Ab to human IL-17. In contrast, col(V)-specific TV-DTH responses were blocked by Abs neutralizing IL-17, TNF-α, or IL-1β, but not by anti–IFN-γ.

The differences in cytokine dependence of TV-DTH responses to col(V) and TT/DT were mirrored by their different cellular requirements. The response to TT/DT was abrogated by removal of $CD4^+$ T cells, but was unaffected by $CD14^+$ cell depletion (Figure 1C). In contrast, removal of either $CD4^+$ or $CD14^+$ cells markedly reduced col(V)-specific swelling. Depletion of $CD8^+$ T cells had no effect on either response (Figure 1C and data not shown).

PBMCs from the col(V)-sensitized lung transplant recipients were then compared with PBMCs from 6 healthy controls for the in vitro release of cytokines IFN-γ, TNF-α, and IL-1β in response to antigen stimulation. IFN-γ was released into supernatants of tetanus toxoid–stimulated (TT-stimulated) PBMCs in both controls and patients (Figure 2A), along with a relatively low amount of TNF-α and no IL-1β. Control PBMCs did not respond to col(V). In contrast, the supernatants of col(V)–stimulated PBMCs from transplant recipients were enriched in TNF-α and IL-1β relative to control PBMC cultures ($P < 0.05$) and relatively low in IFN-γ (P = NS). IL-17 release from col(V)-stimulated whole PBMCs was below the limits of detection by ELISA (data not shown).

Cellular requirements for the TNF-α response to col(V) were similar to those for the TV-DTH response, being unaffected by CD8 depletion, reduced by CD4 depletion, and eliminated by CD14 depletion (Figure 2B). Importantly, TNF-α release was inhibited in a dose-dependent manner by addition of anti–IL-17 Ab, while anti–IFN-γ Ab enhanced the TNF-α response to col(V) (Figure 2B). A nonspecific toxic effect of anti–IL-17 Ab could be ruled out because IFN-γ responses in the same cultures were either unaffected or increased (data not shown).

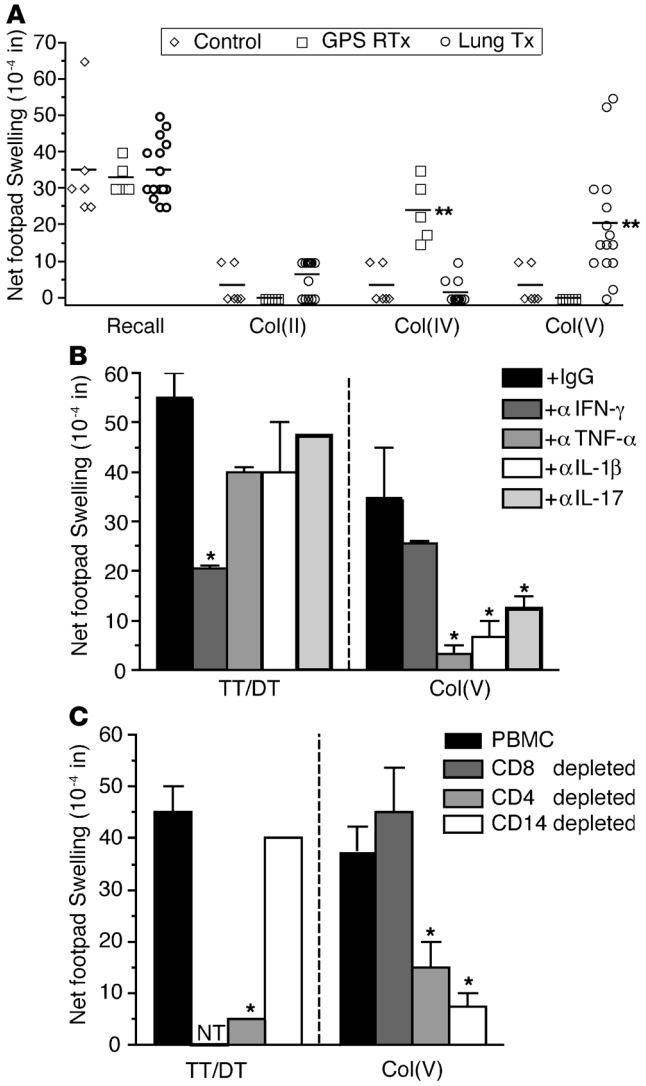

Figure 1

CD4⁺ T cell–dependent, col(V)-specific cell-mediated immunity in lung transplant (Tx) recipients. (**A**) TV-DTH responses by PBMCs obtained from normal healthy controls (n = 6), renal transplant recipients with GPS at 2–10 yr after transplant (n = 5), or lung transplant recipients at 0.5–3.5 yr after transplant (n = 15). Lung primary disease types represented are chronic obstructive pulmonary disease (n = 7), cystic fibrosis (n = 3), idiopathic pulmonary fibrosis (n = 2), α-1 antitrypsin deficiency (n = 2), and other (n = 1). All had stable graft function on standard immunosuppression at time of testing. TV-DTH responses to EBV and TT/DT were determined separately and averaged to yield a positive control swelling response (Recall) for each subject. Responses to 5 μg col(II), col(IV), or col(V) were averaged from duplicate tests and are shown as individual data points. Horizontal bars denote group means. $^{**}P < 0.001$ among treatment groups in response to a specific collagen, Wilcoxon rank-sum test. (**B**) TV-DTH responses (mean ± SEM) to TT/DT and col(V) in col(V)-reactive patients L52 and L84 in the presence of isotype control, anti–IFN-γ, anti–TNF-α, anti–IL-1β, or anti–IL-17 Abs. $^{*}P < 0.05$ versus IgG, Student's t test. (**C**) TV-DTH responses (mean ± SEM) to TT/DT and col(V) by sham-depleted whole PBMCs (10×10^6) or subset-depleted PBMCs (8×10^6) of patients L84 (α-1 antitrypsin deficiency) and L52 and L16 (chronic obstructive pulmonary disease). NT, not tested. $^{*}P < 0.05$ versus whole PBMC, Student's t test.

It has previously been shown that recombinant human IL-17 triggers rapid TNF-α and IL-1β release from monocytes of healthy controls (20). If the col(V)-induced cellular immune response of lung transplant patients relies on this same pathway, then the monocyte, and not the CD4⁺ T cell, would be the predicted source of TNF-α. To assess this possibility, we performed intracellular cytokine staining on short-term (5 h), brefeldin-treated cultures of whole PBMCs from patient L84 stimulated with col(II) or col(V). As shown in Figure 2C, the intracellular staining for TNF-α was largely confined to CD14⁺ monocytes and was specifically induced by col(V) and not by col(II) (3.23% and 0.82% positive cells, respectively). Few CD3⁺ T cells made TNF-α specifically in response to col(V), and this was largely confined to CD8⁺ rather than the CD4⁺ (CD8⁻) T cell subset (Figure 2C).

Cell-mediated immunity to col(V) is strongly associated with onset of BOS. To test the hypothesis that development of col(V)-specific cell-mediated immunity is involved in the process of OB, we conducted a prospective monitoring trial in 54 lung transplant recipients. The incidence of BOS was 26% (14 of 54) with a mean follow-up of 3.5 ± 1.9 yr. From time-course analysis of pulmonary function, 6 of 14 patients were classified as BOS-1 only (Supplemental Figure 2A) while 8 of 14 progressed to the most severe forms of respiratory failure, BOS-2 or BOS-3 (Supplemental Figure 2B).

Post-transplant cell-mediated immunity to col(V) was evaluated by the TV-DTH assay at multiple sampling time points, shown as dots on individual patient timelines (Figure 3). Col(II) served as a control antigen in all monitoring tests, which were performed prospectively using fresh blood samples by an individual blinded to the patient's clinical status. Overall, the mean response to col(V) in 54 recipients was significantly elevated relative to col(II) ($P < 0.0001$; Supplemental Figure 3), confirming the results of the random sampling of 15 patients shown in Figure 1A, and there were no significant differences in mean levels of post-transplant col(V)-specific TV-DTH reactivity among primary lung disease categories (Supplemental Figure 3).

Figure 3 depicts the time dependence of cell-mediated immune response to col(V) in peripheral blood, along with outcome data, in patients with maximum TV-DTH responses classified as low ($<25 \times 10^{-4}$ in., n = 22; Figure 3A), intermediate (25×10^{-4} in., n = 8; Figure 3B) or high ($>25 \times 10^{-4}$ in., n = 24; Figure 3C). None of the 22 patients whose responses to col(V) remained low developed severe BOS: 19 (81%) maintained excellent allograft function, 2 (patients L44 and L51) developed late-onset (>4 yr) BOS-1 that has not progressed further, and patient L18 expired with a functioning graft after 1 yr (Figure 3A). In the intermediate col(V) response group (Figure 3B), there was 1 case of BOS (patient L57) that occurred following a change from a negative to a positive anti-col(V) response. This patient progressed rapidly to BOS-2 and died 2.3 yr after transplant.

Patients with at least 1 time point of strongly positive TV-DTH response to col(V) had the highest incidence of severe BOS (7 of 24, 29%; Figure 3C). There were 4 cases of BOS-1 only, including 1 patient (L15) whose FEV1 values were declining precipitously but died before reaching BOS-2, and 2 patients (L52 and L65) whose pulmonary function declined to a BOS-2 level but partially recovered to stabilize at BOS-1 (Figure 3C and Supplemental Figure 2).

To estimate the relative risk for BOS over time after transplant we used a Cox proportional hazards model. We analyzed the data in 2 different ways: (a) by treating response at any time after transplant as a risk factor, and (b) with response treated as a time-varying, bina-

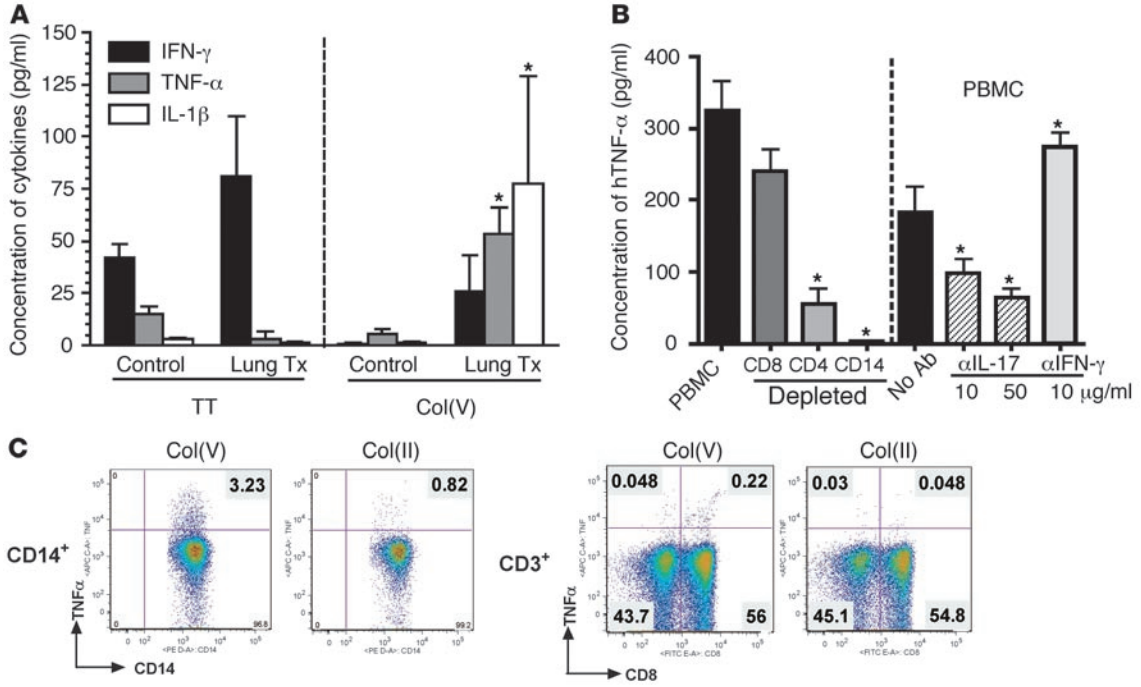

Figure 2

In vitro cytokine responses to col(V) indicate a key role for monocytes. (**A**) PBMCs from controls (n = 6) and lung transplant recipients (n = 4; patients L84, L52, L16, and L143) were stimulated with TT or col(V). Release of IFN-γ, TNF-α, or IL-1β was measured in culture supernatants after 20 h. Release of TNF-α and IL-1β was significantly higher for lung transplant recipients compared with controls. *P = 0.03 versus respective control, Wilcoxon rank-sum test. (**B**) TNF-α responses of patient L84 to col(V) in sham-depleted PBMCs or PBMCs depleted of the indicated subpopulations after 20 h culture, or in the presence of media alone, anti–IL-17, or anti–IFN-γ at the indicated concentrations. *P < 0.05 versus PBMCs or media alone as appropriate, Student's t test. (**C**) Most intracellular TNF-α is derived from monocytes. PBMCs from patient L84 were cultured with col(V) or col(II) for 5 h. TNF-α$^+$ cells are shown for CD14$^+$ monocytes and CD3$^+$ T cells.

ry covariate. Using the former criteria, the hazard ratios for having a strongly positive post-transplant TV-DTH response to col(V) were very high: 5.4 for BOS-1 and 9.8 for BOS-2 (both P = 0.03; Table 2). At 3.5–5.7, hazards ratios were somewhat lower, but still statistically significant, when col(V) response was considered as time-varying, reflecting a tendency toward a positive response occurring at the last test before the BOS event (Figure 3, B and C). Response to col(II), tested concurrently, was not a significant BOS risk factor.

Antibodies to HLA were tested in 170 serum/plasma samples from 52 patients; 14 of 52 (27%) were positive for anti-HLA Ab at 1 or more time points after transplant. The hazards ratio associated with a single time point of post-transplant anti-HLA (class I and/or class II) Ab positivity, regardless of specificity, was estimated at 1.5 and 1.1 for BOS-1 and BOS-2, respectively, but neither reached statistical significance (Table 2). The risks of BOS associated with the number of acute rejection episodes also were not significant in the 54-patient study group. However, in a larger cohort of 281 primary lung transplants, the acute rejection-associated risk ratios for BOS-1 and BOS-2 were estimated at 1.4 and 1.5, respectively, both of which were highly significant (P < 0.0001). HLA-DR mismatch in the large cohort was a significant risk factor for severe BOS (relative risk, 1.9; P < 0.01), but not for BOS-1. Of the other risk factors tested, including post-transplant CMV infection, HLA-A or -B mismatch, recipient age or gender, bilateral versus single lung transplant, donor age, CMV-positive donor to CMV-negative recipient, or primary disease, none were significant for BOS either in the prospective study group or in the larger retrospective analysis (data not shown).

The striking association of IL-17 and monokine-dependent TV-DTH responses to col(V) with onset of severe BOS in lung transplant patients suggests that allograft-induced, col(V)-specific Th17 cells and monocytes are causally linked to OB itself. To determine whether cell-mediated immunity to col(V) can generate an OB lesion in the absence of confounding factors such as alloreactivity and immunosuppression present in the setting of a clinical lung transplant, we used an orthotopic rat left lung isograft and adoptive transfer model. This model system has 3 clinically relevant features: (a) col(V) immunization of Wistar-Kyoto (WKY) rats upregulates IL-17 and IL-23 gene transcripts in LN cells; (b) adoptive transfer of such primed LN cells to a naive WKY rat results in systemic immunity to col(V) detected by direct DTH (ear challenge) assay; and (c) adoptive hosts that receive a WKY lung isograft develop acute lung injury with bronchiolitis and vasculitis confined to the isograft 1 day later (21). We analyzed lung immunopathology in these rats at 30 days after transplant. As shown in Figure 4, control LN cells from hen egg lysozyme–immunized (HEL-immunized) rats failed to cause any long-term airway pathology either in the isograft or in the native lung. In contrast, LN cells from col(V)-immunized rats caused classical OB lesions in the lung isograft by day 30 (Figure 4). Extensive collagen deposition typical of clinical OB was evident by trichrome staining. By immunostaining, col(V) was detected within the bronchiolar mucosal lamina propria, within the heavily fibrosed bronchial smooth muscle layer, and at especially high levels within the dense connective tissue constituting the outer-

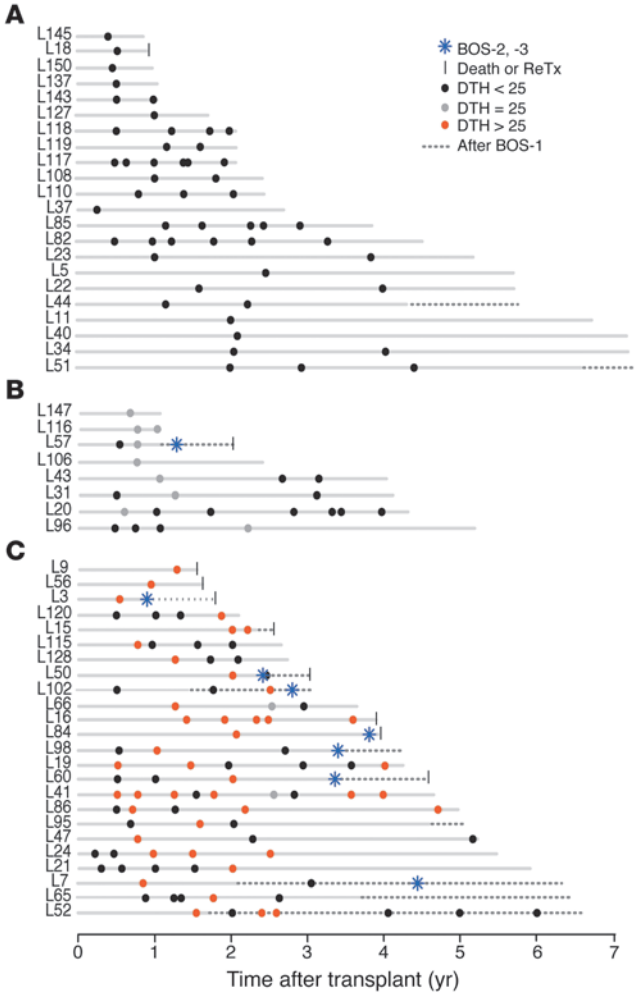

Figure 3
Lung transplant recipients develop anti-col(V) TV-DTH responsiveness prior to onset of severe BOS. Patient timelines (n = 54) were grouped by maximum post-transplant TV-DTH response to col(V) as assessed by net footpad swelling, either low (**A**, DTH <25 × 10^{-4} in.; n = 22), intermediate (**B**, DTH 25 × 10^{-4} in.; n = 8) or strongly positive (**C**, DTH >25 × 10^{-4} in.; n = 24). Each dot represents a separate blood sample/test result. Patient timelines were sorted by follow-up time. The outcomes as of December 31, 2005, include time points of BOS-1, BOS-2, and death or retransplant.

most layer of the bronchial wall and continuous with the alveolar septae, which were thickened in the OB lung (Figure 4). The same pattern of deposition of immunoreactive col(V) was seen in an adjacent obliterated vessel. Immunohistochemical staining for col(V) was mostly negative in the native lung of the same animal, except for faint immunostaining around the subepithelial basement membrane, a normal finding.

Discussion

Chronic rejection of human lung transplants remains an enigmatic and clinically intractable problem more than 2 decades after its first description (2). Early studies of human lung allografts showed an elevated IgG2/IgG1 ratio in bronchoalveolar lavage fluid of patients with acute rejection (22, 23). However, instead of Abs to polymorphic HLA proteins, such as those commonly found in serum during lung transplant acute rejection (5), analysis of the IgG2 Abs in bronchoalveolar lavage revealed a dominant specificity for an ECM protein, the minor col(V) (D.S. Wilkes, unpublished observations). Under normal circumstances, col(V) is largely confined to the interior of mature col(I):col(V) heterotypic fibrils; acid treatment or limited protease digestion is required to expose col(V) epitopes (16). The ability of col(V) to become antigenic for a Th-dependent B cell response in lung transplants suggested both disruption of normal ECM structure and neoantigen creation. After extensive studies in rat indicated a critical role for col(V) autoimmunity in lung allograft tolerance and rejection (reviewed in ref. 24), human translational studies were initiated. Our present finding of a high incidence of CD4-dependent TV-DTH responses to col(V), but not to col(IV) or col(II), in PBMCs from lung transplant recipients and the previous finding of outgrowth of col(V)-specific $CD4^+$ T cell lines from long-term cultures of patient PBMCs (25) confirm that col(V) becomes immunogenic after human lung transplantation. However, to our knowledge, evidence for a direct association of col(V)-specific cell-mediated immunity with lung transplant chronic rejection has been lacking until now.

The introduction of the TV-DTH assay (26, 27) when our prospective clinical study of BOS began was fortuitous, because it detected cell-mediated immune responses dependent on either Th1 (IFN-γ) or Th17 (IL-17) and monocyte (TNF-α, IL-1β) products (Figure 1). It was also quite sensitive. The Th17 subset of $CD4^+$ T memory cells is in low frequency in human peripheral blood relative to the Th1 (IFN-γ–producing) subset and typically produces 10- to 100-fold lower levels of cytokine after polyclonal stimulation (28). We have thus far been unsuccessful in detecting col(V)-induced IL-17 protein or mRNA in cultures of lung transplant patient PBMCs and have relied on the much stronger monokine signal as an in vitro correlate of the col(V)-specific TV-DTH response (Figure 2). Taking into account the low frequency of Th17 memory cells, and the fact that all patients were taking multiple immunosuppressive drugs, the detection of col(V)-specific cell-mediated immune responses in the majority of lung transplant recipients is quite remarkable. The critical role of both $CD14^+$ monocytes and $CD4^+$ T cells suggests an explanation: that immunosuppressive regimens currently used in lung transplantation may fail to prevent de novo Th17-dependent immune responses (29). An interesting exception may be the macrolide antibiotic azithromycin, currently being used to treat established BOS; its unique ability to block downstream effects of IL-17 on lung tissue may account for its efficacy (30). The phenomenon of monocyte–$CD4^+$ T cell interactions leading to monokine production closely parallels findings in rheumatoid arthritis (31), a disease that is resistant to calcineurin inhibitors but often responsive to treatment with TNF-α antagonists (32). The correlation of col(V)-specific autoimmunity with BOS in our prospective study is also consistent with the ability of anti–TNF-α and anti–IL-1β Abs to block airway obliteration in the mouse tracheal allograft model (33) and with the prominent role of monokines and monocyte recruitment in the alloantigen-independent phase of classical chronic rejection (34).

Antibodies to HLA class I or class II antigens were detected in 14 of 52 (27%) patients tested, but did not prove to be a significant risk factor for BOS (Table 2). In contrast, other published studies using ELISA and flow cytometry techniques have found a significant correlation between anti-HLA Abs in general, or donor-specific HLA Abs

Table 2
Risk factors for development of BOS

			Risk ratio (95% confidence interval)	
Parameter	**Cutoff**	**Analysis**	**BOS-1 (*n* = 14)**	**BOS-2 (*n* = 8)**
Col(V) DTH$^+$ (*n* = 54)	>25 × 10^{-4} in.	Any time[A]	5.4 (1.5–19.7)[E]	9.8 (1.2–81.2)[E]
Col(V) DTH$^+$ (*n* = 54)	>25 × 10^{-4} in.	Time-varying[B]	3.5 (1.2–10.6)[E]	5.3 (1.2–23.1)[E]
Col(II) DTH$^+$ (*n* = 54)	>25 × 10^{-4} in.	Any time	1.9 (0.6–6.0)	1.4 (0.3–7.1)
HLA Ab (*n* = 52)	120 MFU[C]	Any time	1.5 (0.5–4.7)	1.1 (0.25–5.1)
Acute rejection (*n* = 54)	≥A2 or R_x[D]	No. rejections	1.12 (0.8–1.8)	1.5 (0.2–12.7)
			BOS-1 (*n* = 106)	**BOS-2 (*n* = 74)**
Acute rejection (*n* = 281)	≥A2 or R_x[D]	No. rejections	1.4 (1.2–1.6)[F]	1.5 (1.3–1.8)[F]
HLA-DR match (*n* = 279)	0	Categorical	1.4 (0.9–2.1)	1.9 (1.2–3.1)[G]

[A]Risk was assumed positive if the subject had at least 1 test at cutoff value. [B]Risk was assumed to be time-varying and could change at each measurement. [C]MFU, mean fluorescence units; arbitrary cutoff based on positive and negative control values. [D]Biopsy score greater than or equal to that of A2 or antirejection drug treatment. [E]$P < 0.05$. [F]$P < 0.001$. [G]$P < 0.01$.

in particular, and subsequent BOS development (5, 9). Sample size may be one limiting factor in our study: for example, neither acute rejection nor HLA-DR mismatch were significant risk factors for BOS in the study subgroup (*n* = 54), but both became significant in analysis of the cohort of 281 lung transplant recipients (Table 2).

The magnitude of estimated risk of BOS associated with col(V)-specific cell-mediated immunity was 3- to 5-fold for BOS-1; this risk increased to 5- to 10-fold for BOS-2, the severe form of lung chronic rejection. Besides col(V) TV-DTH response, certain other risk factors such as HLA-DR mismatch (Table 2 and ref. 35) and the appearance of lymphocytic bronchitis/bronchiolitis on surveillance biopsy (5) have also been associated with a higher risk for BOS-2 versus BOS-1. HLA-DR mismatch tends to prevent establishment of immune regulation to allografts (36); thus it may also predispose to an imbalance between self-reactive T regulatory and T effector cells that could promote a breakdown of col(V)-specific tolerance (25). Lymphocytic bronchitis itself could be an indicator of an immune attack on areas of col(V) exposure (18).

This possibility is supported by the ability, demonstrated here, (a) to generate an OB-like lesion in a rat lung isograft by adoptive transfer of LN cells from col(V)-immunized donors and (b) to detect col(V) antigen in the rat OB lung under nondenaturing conditions (Figure 4). The latter finding is significant because col(V) epitopes cannot be readily detected by immunohistochemistry in normal tissues unless these tissues are first denatured to disrupt col(I) and/or col(V) fibrils, thereby exposing col(V) epitopes (16). Col(V) transcripts are among the most highly upregulated of all matrix proteins in recently transplanted allografts (37). Once the initial graft injury has healed, there may be lingering deposits of exposed col(V) within airway parenchyma and perivascular interstitium (21). These deposits can serve as an antigen source for uptake and presentation of col(V) peptides, for example by monocytes and macrophages, to col(V)-specific Th17 effector cells that reach the graft long after the initial repair process has subsided. Furthermore, gastroesophageal reflux disorder (38) and community-acquired respiratory viruses (39) may promote BOS by causing acid- or collagenase-mediated exposure of col(V) from a normally sequestered position in the interior of mature heterotypic col(I) and/or col(V) fibrils (16). In particular, during ECM repair, normally rare col(V) homotrimers, composed of 3 α1(V) chains, can occur. These would be excluded from heterotypic col(I)/col(V) fibrils and thus expose α1(V) epitopes (40), which include the immunodominant antigens for CD4$^+$ T cells detected by TV-DTH (Supplemental Figure 1B).

Persistent col(V) reactivity in the PBMCs without BOS development (for example, patients L16, L41, and L24; Figure 3C) may indicate a failure of monocytes and col(V)-specific CD4$^+$ T cells to mobilize from the blood to the graft. Chemokine receptors specifically expressed by human Th17 cells have recently been identified (28). Their ligands, when expressed by the target organ, may be particularly important in the mobilization process. In addition, collagen-specific activating receptors such as discoidin domain receptor 1 on monocytes and fibroblasts (41) may synergize with signaling via IL-17 receptors, propagating lung fibroproliferative lesions.

We acknowledge limitations to our study. First, we relied upon a single measure of cell-mediated immunity, the TV-DTH assay, to prospectively monitor lung transplant recipients over a 7-yr period. This test requires crosstalk between human cytokines and cells in the SCID mouse footpad leading to chemokine-driven recruitment of a granulocyte-rich mouse leukocyte infiltrate (ref. 27 and L.D. Haynes, E. Jankowska-Gan, W. Burlingham, and J. Torrealba, unpublished observations). While in vitro analysis of cytokine release from antigen-stimulated PBMCs tends to validate the TV-DTH assay for type 1 and type 17 responses, we have not evaluated other types of human T cell–dependent responses, such as type 2 reactions, that may also be important in OB and/or BOS (42). Second, there were gaps in the follow-up of some patients, while others were sampled more regularly; importantly, the average frequency of sampling was not different among low, intermediate, and high col(V) responders. Third, PBMCs from 1 of 4 col(V) TV-DTH$^+$ patients produced IFN-γ in response to col(V) challenge in vitro; this finding suggests heterogeneity in the types of CD4$^+$ T cells responding to this autoantigen and merits caution in the conclusion that all such cells are Th17 type.

In summary, prospective monitoring of human lung transplant patients revealed a critical role of col(V)-specific cellular immunity in the progression of OB, while confirming previous studies that indicate a role for HLA-specific immunity and acute rejection in the initiation of this process. Rat studies confirmed that col(V)-immune lymphoid cells caused OB lesions in a lung isograft but not in native lung of a syngeneic recipient. Analysis of patient responses suggests that an interplay between Th17 cells and monocytes is critically important in this allograft-induced autoimmune response. Recent data implicating IL-1β as the critical monokine driving differentiation of human Th17 cells (43) is entirely in line with our findings and suggests an amplification loop whereby col(V) immunoreactivity begets more autoimmune effector T cells. The immunobiology of col(V)-reactive CD4$^+$ effector and regulatory T cells, the biochemistry of exposure of the α1 chain of col(V) in the human lung transplant, and the mechanism of human Th17 cell–dependent monocyte activation and pulmonary fibrosis all warrant further investigation. Current clinical practice with respect to prophylaxis of BOS is clearly inadequate and may benefit substantially from consideration of the autoimmune component of this disease.

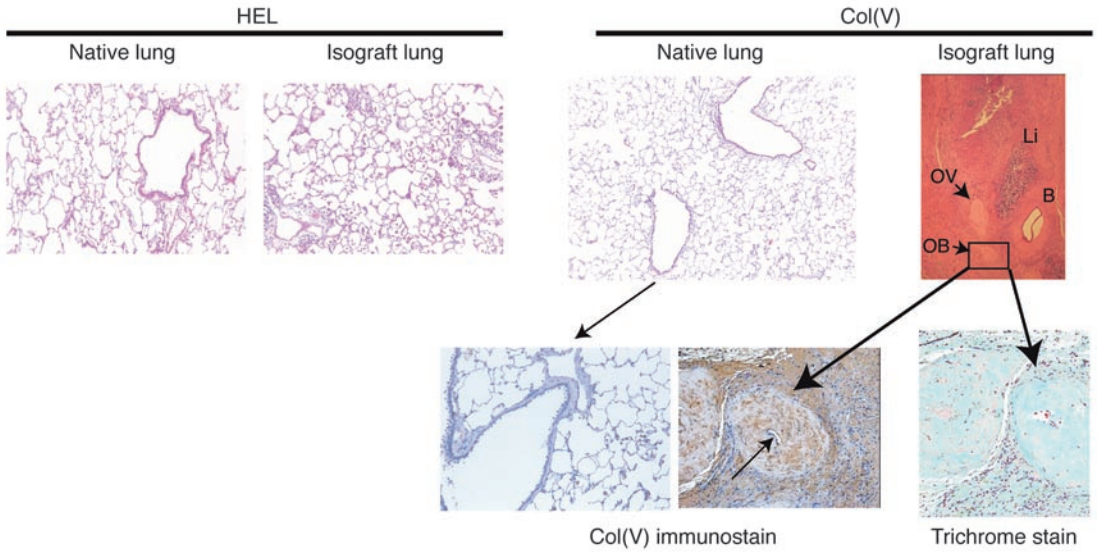

Figure 4
An alloantigen-independent form of OB is induced by transfer of col(V)-immune rat LN cells. LN cells from WKY rats immunized with HEL or col(V) were adoptively transferred to naive WKY rats 1 d prior to transplantation of a WKY lung isografts. Top: H&E staining of both native lungs and isografted lungs (30 d after transplant) from animals transferred with LN cells from HEL-immunized animals and col(V)-immunized animals. Bottom: Trichrome staining and col(V) immunostaining of native and isograft lungs from animals that received LN cells from col(V)-immunized animals was performed to determine tissue exposure of col(V). H&E staining revealed lymphocytic infiltrates (Li), an open bronchus (B), an occluded vessel (OV), and an occluded bronchiole (OB). Arrow on occluded bronchiole shows residual airway. Original magnification, ×200 (top); ×400 (bottom).

Methods

Subjects. Immunologic monitoring was performed prospectively on blood samples obtained at 3- to 6-month intervals, and annually after year 2, from 54 recipients of primary lung transplants at the University of Wisconsin–Madison between May 1998 and June 2005. Subject consent was obtained using human subjects committee–approved, written, informed consent procedures at the University of Wisconsin Hospital and Clinics. Blood samples were collected and processed as described previously (36), and the PBMC fraction was tested within 24 hours. PBMC samples from 6 healthy nontransplanted volunteers and 5 renal transplant recipients with GPS served as controls. Detailed analyses of col(V)-specific T cell response in selected patients and controls were performed using cryopreserved leukapheresis samples. Plasma samples obtained at the time of blood collection were frozen for retrospective analysis of anti-HLA Ab. Immunosuppression therapy and acute rejection diagnosis and treatment were described previously (44).

Pulmonary function tests were performed at regular clinic visits; additional visits were scheduled when home spirometry measurements indicated a decline in FEV1. Maximum FEV1 was established by the average of the 2 highest measurements taken at least 3 wk apart in the first year. BOS was diagnosed by a sustained drop in FEV1 below 80% of maximum, designated BOS-1, or below 65% of maximum, designated BOS-2 (3). BOS-2 or higher represented a relatively severe impairment of airflow, most likely indicative of OB.

TV-DTH assay. CB-17 SCID mice were purchased (Harlan Sprague-Dawley Inc.) or were bred locally. All animals were housed and treated in accordance with NIH guidelines, using protocols approved by the Research Animal Resources Centers of University of Wisconsin and University of Indiana Medical School. TV-DTH was performed by cotransfer of human PBMCs and antigens into the footpads of SCID mice as described previously (26, 27, 45). Inactivated EBV (Viral Antigens Inc.) and TT/DT (Aventis-Pasteur Inc.) were used as positive control recall antigens. Two sources of col(V) from human placenta were used: (a) purified col(V) (obtained from D. Brand, University of Tennessee, Memphis, Tennessee, USA) as described elsewhere (17), and (b) tissue-culture grade col(V) purchased from BD Biosciences. The former was used for TV-DTH monitoring tests, the latter for in vitro assays. Human col(IV) (Fluka Inc.) and col(II) (Southern Biotech) were purchased. Collagens were tested at 5 μg per injection for TV-DTH monitoring tests. Background swelling due to PBMCs with buffer alone was subtracted to determine antigen-specific response. In some experiments, immunomagnetic beads (MACs Beads; Miltenyi Biotec) were used to deplete $CD4^+$, $CD8^+$, or $CD14^+$ cells from PBMCs prior to TV-DTH or in vitro cytokine assays. In some assays, cytokines were neutralized with 5 μg coinjection of antibodies to human IFN-γ, TNF-α (both from BD Biosciences), IL-1β (eBiosciences), or IL-17 (R&D Systems). The antibodies did not crossreact with mouse cytokines.

In vitro cytokine analysis. Whole or subset-depleted PBMCs (5×10^5 cells/well) were cultured with human col(V) (20 μg/ml; BD Biosciences) or TT (0.5 μg/ml, a gift from J.E. Gumperz, University of Wisconsin–Madison) in RPMI1640 with 10% FCS for 20 h. Cells were spun down, and supernatant was harvested for ELISA. The Ab pairs and standard for TNF-α, IL-Iβ, and IFN-γ were purchased from BD Biosciences, eBiosciences, and Endogen, respectively. Horseradish peroxidase (Poly-HRP-20; Research Diagnostics) was used as a third reagent. The ELISA was developed with SureBlue TMB Microwell Substrate and Stop Solution (KPL).

For intracellular staining of TNF-α, PBMCs were cultured with either col(V) or col(II) (20 μg/ml) in the presence of brefeldin A for 5 h. Harvested cells were fixed and permeabilized using BD Cytofix/Cytoperm and stained with CD8-FITC, CD14-PE, anti-human TNF-α–APC, and CD3–Alexa Fluor 700 (BD Biosciences). Flow cytometry was performed on SLR II (BD Biosciences) and analyzed with FlowJo (Tree Star) software.

Anti-HLA Ab detection. Samples of plasma were thawed, encoded, and tested in blinded fashion at a 1:3 dilution for anti-HLA Ab by indirect immunofluorescence using the PRA class I and class II mixed antigen screening system (LabScreen mixed antigen beads; One Lambda Inc.). Single HLA-coated beads were used as a secondary screen to be sure that weak

positive reactions were not overlooked. Positive and negative cutoff values were determined based on specific immunofluorescence values for a given batch of HLA-coated beads.

Rat lung transplantation model. The orthotopic transplantation of left lung isografts (WKY→WKY) and adoptive transfer of LN cells from col(V) or HEL-immunized WKY rats (1 d prior to transplant) was performed as previously reported (21). No immunosuppressive therapy was given at any time during the experimental period. Thirty days after transplantation, native and transplanted lungs were harvested, fixed, sectioned, and stained. Grading for rejection pathology was performed in a blinded fashion by a pulmonary pathologist using ISHLT standard criteria.

Statistics. The criterion used for a positive human TV-DTH response was a footpad swelling of $\geq 25 \times 10^{-4}$ in. over PBMC-plus-PBS background swelling. Analyses of BOS risk were done using TV-DTH response of 25×10^{-4} in. and greater. A Cox proportional hazards model was used to evaluate the association between suspected risk factors, some of which were time-varying, and BOS-free survival. In the time-varying analysis, the covariate was considered on when a response is positive and off when it becomes negative, etc.

For time-varying covariates in the Cox model, we carried the most recently measured values forward to each event time. A Wilcoxon rank-sum test was used for all other comparisons unless otherwise specified. *P* values less than 0.05 were considered significant. All analyses were performed using SAS statistical software release 6.12 (SAS Institute Inc.).

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