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Pathologic findings in lung allografts with anti-HLA antibodies

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Abstract

Background—Despite data indicating a positive correlation between donor-specific anti-HLA antibodies (DSAs) and early development of bronchiolitis obliterans syndrome (BOS) in lung allografts, the role of an antibody-mediated process in acute and chronic lung allograft rejection has not been elucidated. In this study we evaluated pathologic features of transplant lung biopsies in patients with and without DSAs.

Methods—Forty-one lung transplant biopsies from 41 patients at our institution were included in our study. The biopsy H&E slides were reviewed in a blinded fashion, and scored for presence of microvascular inflammation, acute rejection, bronchiolar inflammation and acute lung injury, as well as diffuse alveolar damage (DAD). Microvascular inflammation was graded by the presence of capillary neutrophils on a scale of 0 to 4⁺. For immunohistochemical analysis, the pattern and intensity of staining for C4d and C3d deposition were evaluated in airways and alveolar capillaries.

Results—Histopathology suspicious for antibody-mediated rejection (AMR)—defined as 2⁺ neutrophilic infiltration and/or DAD—were more common in DSA-positive cases than controls (11 of 16 vs 6 of 25, $p < 0.01$). Evidence of allograft dysfunction was significantly more common among patients with both DSA and suspicious histopathology compared with controls (5 of 10 vs 3 of 25, $p = 0.03$). The combination of DSAs and histopathology suspicious for AMR was associated with both BOS ($p = 0.002$) and mortality ($p = 0.03$). Immunohistochemistry for C3d and C4d showed no correlation with each other, DSAs or histopathology.

Conclusions—Grade 2⁺ neutrophilic infiltration is the histopathologic finding most closely related to DSAs with graft dysfunction and development of BOS in lung transplant recipients and may be a marker for AMR.

Keywords

AMR; lung transplant; neutrophilic inflammation; DSA; BOS; capillaritis

Lung transplantation is well established as a viable option for patients with end-stage lung disease. The first lung transplant was performed by Dr James Hardy at the University of Mississippi in 1963, with short-term survival of only 18 days.¹ As transplantation techniques and immunosuppression therapy have improved with experience, longer term survival has become possible. Nevertheless, the current 5-year survival for all lung transplantations is only 50.6%.² The greatest limitation to long-term survival is the development of chronic rejection, manifesting as bronchiolitis obliterans syndrome (BOS). The greatest risk factor for the development of BOS is acute rejection, pathologically defined by the presence of perivascular lymphoid infiltrates of various intensities.³ This process is considered to be mediated by T lymphocytes and is termed acute cellular rejection (ACR).

In other transplanted organs, the categorization of acute rejection is divided into ACR or antibody-mediated rejection (AMR).⁴ The recognition of AMR in kidney and heart allograft biopsies followed the discovery of characteristic pathologic features and accelerated allograft deterioration in the setting of de novo donor-specific antibodies (DSAs) in the organ recipient's serum. The common pathology of AMR in the various transplant organs is microvascular (capillary) inflammation, usually with accompanying complement activation.⁴⁻¹⁰ The complement activation can be assessed by detecting deposition of C3d and C4d, subunits of C3 and C4 complement components, which are cleaved after initiation of the complement cascade by antibody interactions and covalently bond to allograft endothelial cells.^{6,7,11-14} Despite data indicating a positive correlation between development of de novo DSAs and early development of BOS in lung allografts, the role of an antibody-mediated process in lung allograft rejection has not been clarified.^{3,15,16}

Studies and case reports have postulated that the histologic manifestation of AMR in the lung is pulmonary capillaritis.^{17,18} Capillaritis is a pattern of intense capillary inflammation typically seen in autoimmune-mediated capillary inflammation, such as anti-basement membrane antibody disease. As the location of injury (capillary wall) and inflammatory mediator (antibody-mediated inflammation) are very similar, it is intuitive to speculate that this pattern of inflammation and injury could resemble AMR in lung allografts.

Acute lung injury may also manifest in a non-specific pattern of diffuse alveolar damage (DAD). The etiology of DAD is diverse and includes: post-transplant ischemia-reperfusion injury; infection-related injury; drug toxicity; or an idiopathic process. The pathophysiology of DAD may begin with epithelial or endothelial cell injury. As the endothelium is the postulated site of injury in AMR it is reasonable to suggest that AMR may be a potential etiology for DAD in the presence of DSAs and the absence of other pathologic or clinical explanations for acute lung injury.

To better optimize treatment strategies for patients and to guide clinical trials, a standardized diagnosis of AMR in lung transplantation is necessary. The purpose of this study was to evaluate the putative correlation of capillary inflammation, DAD and/or complement deposition with lung transplant biopsies that have DSAs, non-DSAs and biopsies without identifiable antibodies and to analyze the findings with outcomes data, specifically development of BOS and allograft survival.

Methods

Patients

Biopsy specimens from lung transplant recipients were retrospectively selected and grouped according to the presence or absence of a de novo DSA. All patients had been screened for the presence of anti-human leukocyte antigen (HLA) antibodies at protocol surveillance time-points. Inclusion criteria for this study required biopsy 3 weeks after lung

transplantation and within 28 days of a positive or negative test for anti-HLA DSAs. Clinical characteristics and the number of post-transplant days to biopsy were similar between groups (Table 1). The biopsy indications were: routine surveillance; follow-up surveillance (for a prior abnormal finding); or clinically indicated (prompted by symptoms or clinical findings) (Table 2). Biopsies with concurrent infection, as determined by a positive bronchoalveolar lavage (BAL), positive viral serologies or clinical suspicion of infection, and biopsies from re-transplanted organs were excluded from consideration.

Patients with a de novo DSA occurring outside of the 28-day window or concurrent with infection were excluded. No patient who ever developed DSA was included as a control case. All biopsies meeting the criteria were included in a sequential, retrospective fashion from July 2009 until a maximum of 25 had been achieved for a group. The underlying disease processes in these patients included idiopathic pulmonary fibrosis, emphysema/chronic obstructive pulmonary disease (COPD), polymyositis/dermatomyositis, scleroderma/systemic sclerosis, bronchiectasis, cystic fibrosis, sarcoidosis and Eisenmenger syndrome; no patients with primary pulmonary vasculitis were included in this study. In total, 16 patients with de novo DSAs, transplanted between August 2006 and January 2009, had an eligible biopsy and were included as DSA cases. The control group contained 25 patients transplanted between January 2006 and July 2009, who never developed DSA, including 9 with anti-HLA non-DSA antibodies and 16 without any demonstrable anti-HLA antibody.

The induction, maintenance immunosuppression and antimicrobial prophylaxis were administered in accordance with the UCLA lung transplantation protocols as previously described.¹⁹ Briefly, anti-thymocyte globulin (ATG) was given to patients <65 years of age and basiliximab to patients age ≥ 65 years of age. Standard maintenance immunosuppression included mycophenolate mofetil (MMF) plus tacrolimus and prednisone. No patients received everolimus or sirolimus. No patients received intravenous immunoglobulin (IVIg) or cytomegalovirus immunoglobulin (CMVig) prior to DSA diagnosis.

HLA typing and HLA antibody evaluation

HLA Class I and Class II typing was performed on all donors and recipients as previously described.²⁰ HLA antibodies were tested quarterly from the time of listing and at Months 1, 3, 6, 12 post-transplant and bi-annually, and at times of suspected rejection. Pre- and post-transplant sera were tested for antibodies directed against HLA Class I (A, B and C) and Class II (DR, DQ and DP) antigens using Luminex PRA and antibody specificity reagents (Gen-Probe, San Diego, CA). Single-antigen antibody identification assays (One Lambda, Canoga Park, CA) were run on positive sera to confirm the antibody specificity assignment using a normalized cut-off value >1,000 median fluorescence intensity (MFI).²⁰ A virtual crossmatch was employed for donor selection using a cut-off value of 5,000 MFI for listing unacceptable antigens.²¹ T- and B-lymphocyte cytotoxic and flow crossmatches were performed peri-operatively.²²

Histopathologic scoring and definitions

Biopsy hematoxylin-eosin (H&E)-stained slides were reviewed by two pathologists (M.M.D. and W.D.W.) in a blinded fashion, and scored for the presence of ACR and lymphocytic bronchiolitis, based on the revised ISHLT classification for pulmonary allograft rejection.³ The presence of diffuse alveolar damage or bronchiolitis obliterans was also noted. Microvascular inflammation was graded by the presence of capillary neutrophils (Figure 1A).

Capillary neutrophilia was evaluated in areas of non-atelectatic lung and scored on a scale of 0 to 4⁺ (Table 3). The overall grade is based on the field with the highest neutrophil concentration.

A priori, we defined the presence of 2⁺ capillary neutrophilia and/or unexplained acute lung injury (DAD) as suspicious for AMR.

Immunohistochemistry scoring and definitions

For immunohistochemical analysis, the paraffin blocks for all cases were re-cut and stained for C4d and C3d as per laboratory protocols. Briefly, after slide preparation for immunoperoxidase staining, the slides were incubated with 1:50 rabbit anti-human C4d and C3d antibody (C4d: American Laboratory Products Co., Windham, NH; C3d: Dako) in 3% normal goat serum (NGS) overnight at 4°C, followed by washing in Tris-buffered saline (TBS; 3 × 5 minutes). Secondary antibody (1:200 biotinylated goat anti-rabbit IgG) was applied for 40 minutes, followed by washing in TBS (3 × 5 minutes). Horseradish peroxidase-avidin (1:1,000) was applied for 30 minutes, followed by washing in TBS (3 × 5 minutes). Diaminobenzidine (DAB) was applied for 10 minutes, followed by further washing in running water. The slides were then counterstained with dilute aqueous hematoxylin, dehydrated in graded alcohols, cleared in xylene, and coverslipped.

The specific structures examined for pattern and intensity of staining for C4d and C3d were airway and alveolar capillaries. Other non-specific staining patterns were noted. Staining distribution was quantified as negative, focal, multifocal or diffuse. Staining intensity was graded from 1⁺ to 3⁺. Positive staining was defined as specific capillary endothelial-cell staining of any intensity (Figure 1B).

Positive and negative controls for all antibodies included cardiac allograft biopsies with known diagnoses of AMR or negative for AMR, respectively. Four biopsies could not be stained for immunohistochemistry due to insufficient tissue remaining in the blocks.

Statistical analyses

Data are described as medians with range or as proportions. Comparisons were made with the non-parametric Wilcoxon's test for continuous variables and with a chi-square test or Fisher's exact test for categorical variables. To explore the impact of pathologic and clinical variables at the time of biopsy on subsequent outcomes, we constructed Kaplan-Meier survival curves and compared groups using log-rank statistics. Cox proportional hazards models were also used to quantify the relative risks. Variables were entered into models as baseline (time-independent) variables with time to BOS, death or re-transplant from the date of biopsy as the dependent outcome.

Results

Biopsies from 41 patients were included in this study, including 16 DSA-positive cases and 25 DSA-negative controls. Of those in the control group, 9 developed post-transplant de novo anti-HLA antibodies that were not specific to the donor.

Histopathology findings

Thirteen of 41 biopsies were diagnosed with ACR, including 6 with minimal/A1 rejection, 5 with mild/A2 rejection and 2 with moderate/A3 rejection. Fourteen of 41 biopsies were diagnosed with bronchial/bronchiolar inflammation. There were no significant differences in the incidence of ACR or bronchial/bronchiolar inflammation between DSA-positive cases and controls (see Table 2).

Twenty-six of 41 biopsies had greater than baseline neutrophils (1^+) in the capillaries. Greater than baseline capillary neutrophilia was significantly more common in the DSA-positive cases than in controls, (14 of 16 vs 12 of 25, $p = 0.02$). In controls, the incidence of 1^+ capillary neutrophilia was similar among those with and without anti-HLA antibodies (4 of 9 vs 8 of 16, $p = 1.00$). Fourteen biopsies had 2^+ capillary neutrophilia. Capillary neutrophilia 2^+ tended to be more common in DSA-positive cases than controls, but this did not meet statistical significance (8 of 16 vs 6 of 25, $p = 0.09$). Among controls, the incidence of 2^+ capillary neutrophilia was similar between those with and without anti-HLA antibodies (3 of 9 vs 3 of 16, $p = 0.63$). Nine biopsies had acute lung injury or DAD, a finding that tended to be more common among DSA cases than controls, but this did not meet statistical significance (6 of 16 vs 3 of 25, $p = 0.12$).

Seventeen of 41 (41%) of the biopsies examined were considered suspicious for AMR by histopathology, as defined by 2^+ capillary neutrophilia and/or DAD. A biopsy suspicious for AMR was significantly more common in DSA-positive cases than controls (11 of 16 vs 6 of 25, $p < 0.01$; see Table 2).

C4d/C3d immunoperoxidase

Thirty-seven biopsies were available for C4d and C3d immunohistochemistry studies, which included 14 DSA-positive cases and 23 controls. The overall staining patterns for both C4d and C3d included extensive non-specific staining of alveolar epithelial cells, alveolar fibrin and elastic fibers of bronchiolar and vascular walls. There was relatively infrequent capillary endothelial cell staining for C4d and C3d. When positive staining was defined as any staining (focal, multifocal, diffuse) of capillary endothelium of 1^+ intensity, 10 (27%) biopsies showed C4d positivity and 15 (41%) showed C3d positivity. There was no correlation between C4d and C3d positivity ($p = 0.48$), with 5 (14%) cases being positive for both C4d and C3d. Neither C4d nor C3d positivity was more common in DSA-positive cases compared with controls (3 of 14 vs 7 of 23 [$p = 0.71$] and 3 of 14 vs 12 of 23 [$p = 0.09$], respectively). Furthermore, when we defined C4d and C3d positivity more narrowly (multifocal or diffuse staining and 2^+ intensity), C4d and C3d positivity was still not more common in DSA-positive cases compared with controls (1 of 14 vs 3 of 23 [$p = 1.00$] and 3 of 14 vs 5 of 23 [$p = 1.00$], respectively).

There was also no association of C4d positivity with histopathology findings considered suspicious for AMR. Of the 14 biopsies thought suspicious for AMR that were also available for immunohistochemistry, only 3 (18%) showed C4d positivity ($p = 0.71$). There was a significant negative correlation between C3d positivity and suspicious histopathology ($p = 0.02$). Only 2 (12%) biopsies with suspicious histopathology were positive for C3d staining, whereas 13 (56%) biopsies without suspicious histopathology were positive for C3d. When the more narrow definition of C4d and C3d positivity was used (at least multifocal staining and 2^+ intensity), there was no association with histopathology suspicious for AMR with either complement component ($p = 0.62$ and $p = 0.68$, respectively).

Allograft function and survival

Six (38%) DSA-positive cases had concurrent graft dysfunction at the time of biopsy (defined as a new hypoxia or a decline in forced expiratory volume in 1 second [FEV₁] of 10% compared with prior measurement) as compared with only 3 (12%) DSA-negative controls, but this difference did not meet statistical significance ($p = 0.12$). However, graft dysfunction was significantly more common among patients with both a de novo DSA and histopathology suspicious for AMR when compared with DSA-negative controls (5 of 10 vs 3 of 25, $p = 0.03$).

We also looked for an association between DSA and histopathologic findings and development of future morbidity and mortality. As shown in Figure 2, DSA-positive cases were significantly more likely to develop BOS ($p = 0.007$, hazard ratio [HR] 3.70, 95% confidence interval [CI] 1.43 to 10.71), but not mortality ($p = 0.11$, HR 2.48, 95% CI 0.82 to 8.25). Importantly, the combination of both de novo DSA and histopathology suspicious of AMR had an even stronger association with BOS ($p = 0.002$, HR 5.06, 95% CI 1.81 to 15.28) and was also significantly associated with mortality ($p = 0.03$, HR 3.57, 95% CI 1.13 to 12.14; Figure 2). DSA-positive cases without suspicious histopathology had no significantly greater risk of BOS ($p = 0.33$, HR 2.07, 95% CI 0.43 to 7.87) or mortality ($p = 0.84$, HR 0.80, 95% CI 0.42 to 4.99). Patients with de novo DSA, suspicious histopathology and concurrent allograft dysfunction had the highest risk for BOS ($p = 0.01$, HR 6.42, 95% CI 1.63 to 22.72) and also the highest risk of mortality compared with DSA-negative controls ($p = 0.02$, HR 5.75, 95% CI 1.41 to 21.93).

Discussion

In this study we have considered the pathology of AMR in kidney and heart transplantation in an attempt to evaluate putative features of AMR in the lung allograft. In renal transplantation, the recognized and accepted pathologic findings in AMR include deposition of C4d in peritubular capillaries, capillary inflammation and vascular thrombosis with chronic changes manifested by capillary basement membrane remodeling as a consequence of prolonged/repeated endothelial cell injury.^{5–8, 10–12} The occurrence of AMR in renal allografts is associated with shorter graft survival.²³ In cardiac allografts, the presence of DSAs has also been found to correlate with capillary inflammation and deposition of C4d and C3d complement products.^{9, 14} The features in both transplanted organs are sufficiently distinctive that the most recent Banff classification and ISHLT classification for allograft rejection in kidney and heart transplants, respectively, include criteria for AMR.^{9,24}

The inflammatory cells in the capillaries of heart and kidney AMR include monocytes, neutrophils and lymphocytes. In our study we included only neutrophils due to the large native population of monocytes and lymphocytes in the lungs that are difficult to distinguish from a reactive infiltrate.

Results from our blinded grading of capillary neutrophilic inflammation showed a positive correlation between histopathologic findings (2^+ neutrophilia [at least 2 back-to-back intracapillary neutrophils] and/or DAD) and the presence of DSA. Furthermore, pathologic findings, with concurrent DSAs, are inversely related to allograft survival. The association between pathologic findings, DSAs and allograft failure is consistent with our experience with AMR in other organ systems. The correlation found between pathologic findings and allograft failure without DSAs should be interpreted with caution. Our cohort contained only 6 patients and is not statistically robust. Furthermore, the pathologic findings of capillary neutrophilia and DAD are not specific and may be seen in other forms of lung injury that could result in shorter graft survival. It is still imperative to attempt to accurately diagnose the cause of the lung injury, because, if it is not antibody-mediated, the patients would not be expected to respond to AMR-targeted therapy.

In a prior study by our group we failed to find significant staining for C4d deposition in sequential lung allograft biopsies.²⁵ However, other groups have found varying results, so we were determined to look again at C4d deposition in the context of other assumed features of AMR and included C3d in our evaluation.^{18,26–31} We failed to find correlation between specific capillary endothelial C4d deposition and anti-HLA DSA; C3d actually had a negative correlation with DSAs. Furthermore, C4d and C3d did not correlate with each other and had no correlation with the pathologic findings we suggested to be indicative of AMR,

namely capillary inflammation and DAD. However, as noted by our group previously and by other groups, in using an immunoperoxidase technique there is extensive nonspecific staining of elastic tissue, fibrin and areas of epithelial cell injury, which makes objective evaluation of the C4d and C3d immunohistochemistry stains very difficult to obtain and unreliable. Our findings are in contrast to those in the recent study by Yousem and Zeevi, who found C4d deposits in 76% of lung transplant biopsies with anti-HLA antibodies but also in 24% of biopsies without demonstrable anti-HLA antibodies.³¹ We have not been able to reproduce these findings using our techniques. Ultimately, those investigators concluded the diagnosis of AMR is not a pathologic diagnosis alone but requires multi-disciplinary collaboration.

Our data are based on a relatively small sample size of patients and will need to be validated and duplicated. However, we detected a statistically significant pattern of pathologic change, specifically capillary neutrophilia with at least 2 back-to-back neutrophils and/or unexplained DAD, associated with DSAs and decreased survival. Unfortunately, capillary neutrophilic inflammation and DAD are not specific and may be seen in various clinical settings, including infection and other forms of acute lung injury. Also, capillary neutrophilia may be difficult to evaluate in small transbronchial biopsies that frequently have crush artifact and artifactual hemorrhage that may contain neutrophils and obscure the AMR-associated inflammation. These caveats strongly indicate that the diagnosis of AMR in the lung must be a multi-specialty diagnosis that incorporates all available clinical inflammation and is ultimately determined by the treating clinician. Nevertheless, in the appropriate clinical setting and with an adequate biopsy, the pathologist may be able to provide very practical guidance to the treating clinician.

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Figure 1.

(A) Capillary neutrophilia of 2^+ . Neutrophils (arrows) and back-to-back neutrophils (double arrows) are shown (H&E stain; original magnification 400 $\times$). (B) Specific alveolar capillary complement deposition (C4d immunoperoxidase stain; original magnification 400 $\times$).

Figure 2.

Cox hazard model of graft deterioration in the setting of histopathology suspicious for AMR and DSAs. (A–C) BOS analyses. (D–F) Survival analyses.

Table 1
Clinical Characteristics by Group

	DSA (<i>n</i> = 16)	Control (<i>n</i> = 25)	<i>p</i>-value
Male recipients, <i>n</i> (%)	9 (56)	13 (52)	0.79
Recipient age, median (range)	59 (23-71)	61 (35-71)	0.07
Transplant indication, <i>n</i> (%)			0.58
Restrictive	11 (69)	18 (72)	
Obstructive	3 (19)	5 (20)	
Cystic fibrosis	1 (6)	2 (8)	
Vascular	1 (6)	0 (0)	
Transplant type, <i>n</i> (%)			0.24
Single	6 (38)	14 (56)	
Bilateral	9 (56)	11 (44)	
Heart-lung	1 (6)	0 (0)	
Induction type, <i>n</i> (%)			0.36
Thymoglobulin	10 (62)	12 (48)	
Basiliximab	6 (38)	13 (52)	
Days to biopsy, median (range)	88 (28-617)	92 (27-627)	0.74

Table 2
Histopathologic Findings by Group

	DSA (<i>n</i> = 16)	Control (<i>n</i> = 25)	<i>p</i> -value
Indication for bronchoscopy [<i>n</i> (%)]			0.19 ^a
Surveillance—routine	8 (50)	19 (76)	
Surveillance—follow-up abnormal	1 (6)	0 (0)	
Clinically indicated	7 (44)	6 (24)	
Concurrent ACR [<i>n</i> (%)]	7 (44)	6 (24)	0.18
Concurrent LB [<i>n</i> (%)]	8 (50)	6 (24)	0.09
Any capillary neutrophilia	14 (88)	12 (48)	0.02
2 ⁺ capillary neutrophilia [<i>n</i> (%)]	8 (50)	6 (24)	0.09
ALI/DAD	6 (38)	3 (12)	0.12
Positive histology ^b [<i>n</i> (%)]	11 (69)	6 (24)	<0.01
Allograft dysfunction ^c [<i>n</i> (%)]	6 (38)	3 (12)	0.12

ACR, acute cellular rejection; ALI, acute lung injury; DAD, diffuse alveolar damage; DSA, donor-specific antibody; FEV₁, forced expiratory volume in 1 second; LB, lung biopsy.

^a Chi-square comparison of clinically indicated vs surveillance (routine and follow-up abnormal combined).

^b Defined as 2⁺ capillary neutrophilia or ALI/DAD.

^c Defined as new hypoxia or a decline in FEV₁ of ≥10% from the prior measurement.

Table 3
Neutrophilic Infiltration Score

0	Baseline level intracapillary neutrophils (rare or negative)
1+	Single/scattered intracapillary neutrophils above baseline
2+	2 back-to-back intracapillary neutrophils
3+	3 or 4 back-to-back intracapillary neutrophils
4+	Diffuse back-to-back capillary neutrophils with karyorrhectic debris and alveolar hemorrhage (capillaritis)