Increased erythrocyte C4D is associated with known alloantibody and autoantibody markers of antibody-mediated rejection in human lung transplant recipients

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BACKGROUND: Immune responses to mismatched donor human leukocyte antigens (HLA) are important in the pathogenesis of chronic rejection. This study evaluated whether erythrocyte-bound C4d (E-C4d) is associated with known alloimmune and autoimmune markers of antibody-mediated rejection after human lung transplantation (LTx).

METHODS: Flow cytometry was used to analyze 22 LTx recipients and 15 healthy individuals for E-C4d. Development of antibodies to donor-mismatched HLA (donor-specific antibody [DSA]) and antibodies to HLA were determined using the solid-phase method by Luminex. Development of antibodies to self-antigens, K-α-1-tubulin (KA1T) and collagen V (Col-V), were measured by enzyme-linked immunosorbent assay. C3d deposition in lung biopsy specimens was determined by immunohistochemical staining.

RESULTS: Percent E-C4d (%E-C4d) levels were 19.9% in LTx patients vs 3.7% in healthy individuals ($p = 0.02$). DSA+ patients had higher E-C4d levels than DSA− patients (34.1% vs 16.7%, $p = 0.02$). In 5 patients with preformed anti-HLA, E-C4d levels were not significantly different vs 13 patients without detectable anti-HLA ($p = 0.1$). E-C4d levels were higher in patients who developed antibodies to KA1T ($p = 0.02$) and Col-V ($p = 0.03$). Recipients with C3d-positive tissue deposition had higher E-C4d levels than patients with C3d-negative biopsy results ($p = 0.01$).

CONCLUSIONS: Increased %E-C4d levels are found in patients with positive DSA, high antibody titers to KA1T and Col-V, and have C3d+ lung biopsy findings. Therefore, %E-C4d can serve as a potential marker for antibody-mediated rejection after LTx.
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Pre-existing antibodies (Abs) specific to mismatched donor human leukocyte antigen (HLA) have been strongly correlated with acute and/or chronic rejection and their presence has generally been accepted as a contraindication for solid organ transplantation.[1,2] More recent evidence suggests that post-transplant development of donor-specific Ab

(DSA) against mismatched donor HLA has a crucial role in acute and chronic rejection of lung allografts.[3–5]

Ab-mediated rejection (AMR) in renal allografts has been well characterized by DSA in circulation and C4d deposition in the allograft at the time of rejection.[6–8] In contrast, AMR is not a well-defined entity in lung transplantation (LTx).[9] Although a diagnosis for cellular rejection can be made from the biopsy specimen, the often-nebulous diagnostic criteria for AMR include clinical, histologic, and immunologic evidence. Specifically, histologic evidence of capillary injury and neutrophilic infiltration, staining for C4d in endothelial cells, and the presence of DSA in serum in conjunction with clinical evidence of allograft dysfunction are used to reach a diagnosis.[9] The diagnosis of AMR in the setting of superimposed cellular rejection is challenging.

Our laboratory has previously demonstrated that not only alloimmune responses to donor HLA but also the autoimmune response to self-antigens, namely K-α-1-tubulin (KA1T) and collagen V (Col-V), is a risk factor for the development of chronic rejection (bronchiolitis obliterans) in LTx.[10–12] There is considerable evidence that de novo development of Abs to donor HLA, as well as to self-antigens, are important in the pathogenesis of human lung allograft rejection.[3,13] However, it is less clear if the detection of DSA and tissue deposition of complement degradation products such as C4d can be used as markers of humoral activation after LTx. Hence, this study was conducted to investigate whether E-C4d is associated with allo-Ab and auto-Ab makers of AMR in human LTx recipients.

In systemic lupus erythematosus (SLE), a circulating breakdown product in the complement activation process bound to erythrocytes has been proven to be an important adjunct to patient management.[14] Specifically, studies have shown cell-bound complement activation products such as erythrocyte C4d (E-C4d) and erythrocyte complement receptor 1 (E-CR1) are biomarkers for monitoring disease activity in patients with SLE.[14] The advantage of using an erythrocyte-bound product is that it is more stable and has an increased half-life than its more transient circulating counterpart.

With recent reports that have suggested a predictive role of the E-C4d/E-CR1 ratio in cardiac transplantation,[15] we investigated whether an increased percentage E-C4d+ red blood cells is an indicator of AMR in post-LTx patients[16] and correlates with other established markers of AMR, such as DSA, Abs to the self-antigens KA1T and Col-V, and C3d deposition in lung biopsy specimens.

Materials and Methods

Study population

From May 2006 to July 2007, 22 consecutive LTx patients at Barnes-Jewish Hospital–Washington University were prospectively enrolled in the study in accordance with a protocol approved by the Institutional Review Board. The inclusion criteria were patients who agreed to enroll after providing informed consent and patients with no active infection or primary graft dysfunction at time of study enrollment. The exclusion criteria included age older than 65 years, a body mass index (BMI) greater than 40 kg/m^2, a cold ischemia time greater than 6 hours at the time of LTx, increased levels of liver enzymes (elevated transaminases or hyperbilirubinemia), abnormal thyroid test results, and previously documented hypertension. Also recruited as controls were 15 healthy volunteers.

Fresh ethylenediamine-tetraacetic acid (EDTA) anticoagulated blood was collected, and E-C4d testing was performed on whole blood samples immediately after the specimen was received. Serum and peripheral blood lymphocytes were separated and stored at $-135°C$. Lung biopsies were performed according to protocol. Blood samples for E-C4d measurements were obtained simultaneously with the tissue samples.

A diagnosis of AMR was made based on clinical, histologic, and serologic criteria. Clinical symptoms suggestive of AMR included lung allograft dysfunction manifest by diminished forced expiratory volume in 1 second (FEV$_1$), reduced partial pressure of oxygen (Po$_2$)/fraction of inspired oxygen (Fio$_2$) ratios in ventilated patients, and lack of response to immunosuppressive therapy directed towards cellular rejection. Histologic criteria, including intra-alveolar and septal wall fibrin, thrombi, and presence of neutrophils, was noted with absence of conclusive features of cellular rejection. Given that not only the presence of donor-specific HLA Ab but also the development of Ab to self-antigen can lead to humoral activation, serologic evidence such Ab in LTx recipients in this study comprised the serologic criteria for AMR.

Detection of Abs to HLA

The presence of Abs to mismatched donor HLA (DSA) and other HLA (anti-HLA) in post-transplant sera was identified using a solid-phase assay by Luminex technology (BioSource International Inc, Camarillo, CA). In brief, primary Ab-coated beads and incubation buffer were placed into 96-well filter plates. Samples and standards were incubated with the primary Ab beads at room temperature on an orbital shaker. The wells were washed, and biotinylated-detector Abs were added for a further 30-minute incubation. The wells were washed again and streptavidin-R-phycoerythrin solution was added and incubated for 15 minutes. The wells were washed and data read using a dual-laser flow analyzer, the Luminex-100 v1.7 system. Data analysis was performed using the MasterPlex QT 1.0 system (MiraiBio, South San Francisco, CA), and a 5-parameter regression formula was used to allow detection compared with standard curves.

Detection of Abs to self-antigens KA1T and Col-V

The sera were tested for the presence of Abs to KA1T and Col-V by enzyme linked immunosorbent assay (ELISA). A

96-well plate (Nunc, Rochester, NY) was coated with 1 μg/ml recombinant purified KA1T or commercially available Col-V (Chemicon, Temecula, CA) in phosphate-buffered saline (PBS) overnight at 4°C. The antigen-coated wells were blocked for non-specific binding with 1% bovine serum albumin (BSA) for 2 hours. Sera from post-HTx and healthy volunteers were tested at 2 specific titers (1:500 and 1:1000) for the presence of Abs against KA1T and Col-V. Commercially available anti-KA1T and anti-Col-V Abs were used as positive controls. Specific binding was detected with anti-human immunoglobulin (Ig) G, IgM bound to horseradish peroxidase (Jackson ImmunoResearch Laboratory Inc, West Grove, PA), and developed with tetramethylbenzidine substrate (Millipore Corp, Temecula, CA). Immunosorbance was detected at 460 nm. The concentration of Ab was calculated based on a standard curve using the binding of known concentration of commercial anti-KA1T/anti-Col-V Abs (Santa Cruz Biotechnology Inc, Santa Cruz, CA).

Detection of E-C4d

Fresh EDTA anti-coagulated blood (10 μl) was washed twice in 1 ml of fluorescent-activator cell sorter (FACS) wash buffer. The buffer was prepared by using 0.1% BSA and 0.02% sodium azide in PBS. The washed pellet was resuspended in 200 μl of FACS buffer. Five microliters of the resuspension was mixed with 20 μl of FACS buffer and 1 μl of murine monoclonal anti-human C4d (Quidel, San Diego, CA) and incubated for 20 minutes at 4°C. Mouse IgG1$_k$ was used as an isotype control. The pellet was washed twice with FACS buffer and treated with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG in a dilution of 1:100 for 20 minutes at 4°C.

After 2 washes with FACS buffer, the cells were resuspended in 500 μl of FACS buffer and read on a FACS Caliber machine with instrumental settings for erythrocytes. The red blood cells were electronically gated based on forward and side scatter properties to include only single cells. Percentage binding was read in comparison with isotype control. Percentage of E-C4d (%E-C4d) was calculated by determining the percentage of total red blood cells with a positive mean fluorescence shift (MFI$_{anti-C4d}$ − MFI$_{isotype\ IgG1}$). A 2 standard deviation from the mean fluorescence shift obtained in the control population was used as a positive cutoff value to calculate %E-C4d in the post-LTx patients. To ensure reproducibility, each sample was run through the FACS machine 3 times and the deviation was within 5% of each measurement.

Immunohistochemical staining for C3d

Immunohistochemical studies were conducted using formalin-fixed paraffin-embedded lung allograft biopsy specimens from 22 HTx recipients. The paraffin-embedded tissue was cut (4- to 6-μm thick) and placed on Superfrost Plus slides (Fisher Scientific, Hampton NH). The slides were placed in a 60°C oven for 1 hour, cooled, deparaffinized in xylene, and then rehydrated using sequential immersion in graded ethanol solutions, and finally in water. The endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 30 minutes. Before addition of the primary Ab, slides were blocked for non-specific staining using a serum-free protein for 15 minutes at room temperature (DakoCytomation, Carpinteria, CA). Primary Ab (C3d rabbit polyclonal Abs, DakoCytomation) was diluted 1:500 and tissue was incubated overnight. The detection system, a labeled polymer system, Envision Plus Dual Link (DakoCytomation) was used, and staining was visualized with diaminobenzidine (DAB) chromogen (DakoCytomation). The slides were then counterstained with hematoxylin, dehydrated through graded ethanol solutions, followed by xylene, and coverslipped with mounting media.

Statistical correlation

Prism 4 software (GraphPad, San Diego, CA) was used to analyze data. Statistical correlation with patient outcome was performed using the Mann-Whitney test. Mean data are presented with the standard deviation. A value of $p < 0.05$ was considered to be statistically significant.

Results

Patient demographics

The study cohort consisted of 22 patients (11 men) who underwent LTx from May 2006 to July 2007. Mean age at LTx was 50.7 years. Fourteen patients were Caucasian, 6 were African American, and 2 were classified as other. The indication for LTx included chronic obstructive pulmonary disease in 8 (36%), idiopathic pulmonary fibrosis in 4 (18%), α-1-antitrypsin deficiency in 3 (14%), primary pulmonary hypertension in 2 (9%), cystic fibrosis in 2 (9%), idiopathic interstitial lung disease in 1 (5%), and sarcoidosis in 1 (5%). Bilateral LTx was performed in 20 patients (92%). Mean follow-up after LTx was 20.1 ± 3.4 months (median, 21.5 months). Of the 22 patients, 5 patients experienced at least 1 episode of biopsy-documented cellular rejection. Although patients with active infection at time of study enrollment were excluded, systemic infections developed in 3 patients, 2 bacterial and 1 viral.

The %E-C4d is significantly increased in the circulation of LTx recipients

E-C4d levels were measured on the circulating erythrocytes by flow cytometry in 15 healthy volunteers and 22 post-LTx recipients on freshly collected EDTA anti-coagulated blood. The test for E-C4d was performed at a mean of 15.3 ± 4.2 months from the time of LTx. The mean E-C4d levels were $3.7\% \pm 2.2\%$ in the healthy cohort and $19.9\% \pm 9.7\%$ in the LTx cohort ($p = 0.02$; Figure 1). This demonstrates that the %E-C4d level is higher in LTx recipients than in con-
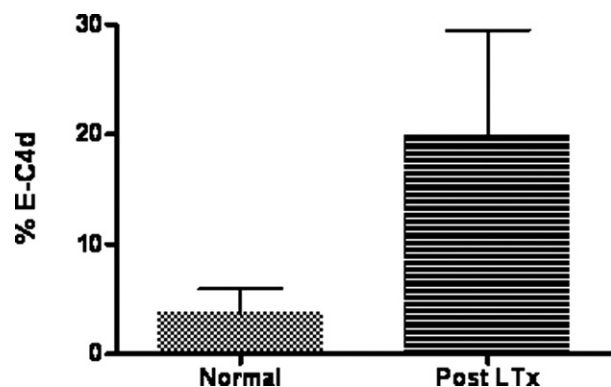
Figure 1 An increased percentage of the erythrocyte-bound complement degradation product C4d (E-C4d) fraction was found in the peripheral circulation of 22 post-lung transplant (LTx) recipients compared with 15 healthy controls. Results are expressed in mean ± SD.

trols. To determine whether this is due to an increase in DSA developing in patients, which changes the %EC4d in the LTx recipients, we analyzed for development of DSA as well as Abs to HLA.

The %E-C4d is significantly increased in LTx recipients with DSA

The presence of DSA is associated with humoral immune activation in LTx recipients. Mean time to DSA detection from time of LTx was 13.4 ± 2.9 months for the study cohort. To determine whether the development of DSA will correlate with an increase in EC4d, we determined E-C4d levels in 3 patient groups: 4 patients with DSA (Group 1), 6 patients who developed anti-HLA but not DSA (Group 2), and 13 patients who remained negative for both DSA and anti-HLA (Group 3; Figure 2). Mean E-C4d was $34.1\% \pm 5.9\%$ in Group 1, $13.9\% \pm 8.4\%$ in Group 2, and $17.7\% \pm 6.7\%$ in Group 3. There was a significant difference between DSA and anti-HLA+ patients (Group 1 vs 2; $p = 0.02$), DSA and anti-HLA− patients (Group 1 vs 3; $p = 0.03$), and DSA and non-DSA patients (Group 1 vs Group 2 and 3; $p = 0.02$). There was no significant difference between anti-HLA+ and anti-HLA− patients (Group 2 vs 3, $p = 0.1$). These data demonstrate that %E-C4d is higher in DSA+ LTx patients compared with those who are DSA−. Furthermore, %E-C4d is not significantly different in anti-HLA+ recipients without DSA compared with anti-HLA− recipients. These preliminary results demonstrate that patients with DSA also develop an increase in %EC4d, and therefore, this could be used as a marker for AMR after LTx.

The %E-C4d is significantly increased in LTx recipients who develop Abs to self-antigens KA1T or Col-V

Post-LTx development of Abs to self-antigens has been associated with development of chronic rejection after hu-

man LTx. Mean time to Ab to self-antigen detection from time of LTx was 17.8 ± 2.5 months for the study cohort. Further, it has been suggested that alloimmune responses can induce an immune response to self antigens.[10,12] We therefore measured the E-C4d levels in patients who developed Abs to KA1T and Col-V (Figure 3).

A LTx recipient was considered to have developed Abs to self-antigens if the titer to a self-antigen was greater than in the healthy cohort mean + (2 × healthy cohort standard deviation). Similarly, a LTx recipient had a low Ab titer to a given self-antigen if the level was less than in the normal mean cohort − (2 × healthy cohort standard deviation). The mean levels in healthy individuals were $194 \pm 52\ \mu g/ml$ for KA1T and $111 \pm 42\ \mu g/ml$ for Col-V. In 22 LTx recipients, the titer to KA1T was high ($>298\ \mu g/ml$) in 11 patients and low ($<90\ \mu g/ml$) in 3. Similarly, the titer to Col-V was high ($>195\ \mu g/ml$) in 14 patients and low ($<27\ \mu g/ml$) in 3. The mean E-C4d was $23\% \pm 10.5\%$ in recipients with high KA1T titers and $3.4\% \pm 1.4\%$ in the group with low titers ($p = 0.02$). Similarly, the mean E-C4d was $22.9\% \pm 9.7\%$ in recipients with high Col-V titers and $3.4\% \pm 1.4\%$ in those with low titers ($p = 0.03$). These data demonstrate that %E-C4d is higher not only in patients who developed DSA after LTx but also in patients who developed Abs to self-antigens.
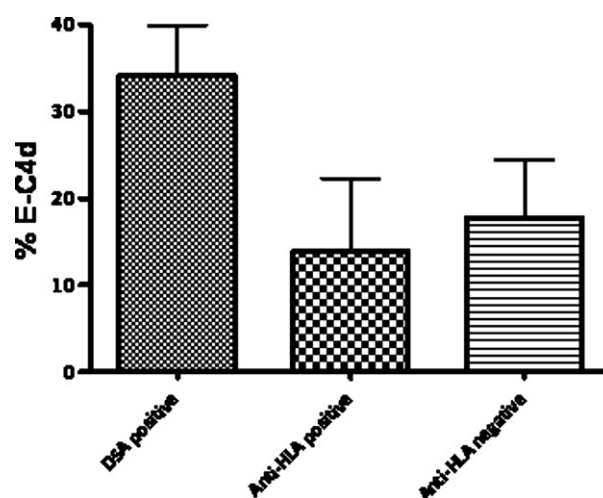

Figure 2 Increased %E-C4d in post-LTx recipients with donor-specific antibody (DSA) compared with DSA− patients with or without detectable anti-human leukocyte antigen (HLA) antibodies (Abs). Group 1 comprised 4 patients with DSA, Group 2 comprised 5 patients who developed anti-HLA but not DSA, and the 13 patients in Group 3 remained negative for both DSA and anti-HLA. There was a significant difference between DSA and anti-HLA+ patients (Group 1 vs 2; $p = 0.02$), DSA and anti-HLA− patients (Group 1 vs 3; $p = 0.03$), and DSA and non-DSA patients (Group 1 vs Group 2 + 3; $p = 0.02$). The difference between anti-HLA+ (Group2) and anti-HLA- patients (Group 3) was not significant ($p = 0.1$). %E-C4d is higher in DSA+ LTx patients compared with those who are DSA− ($p = 0.02$).
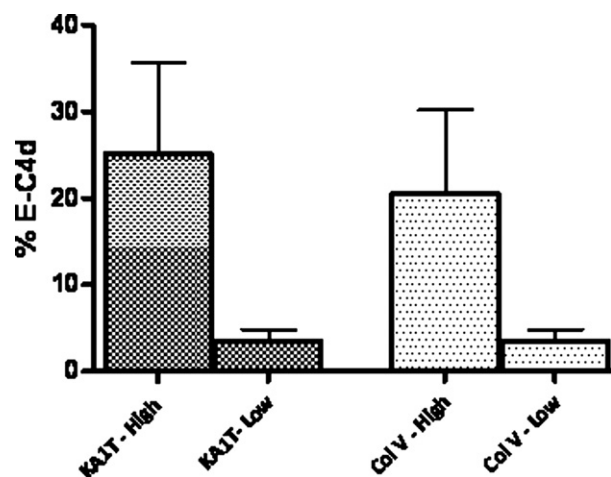
Figure 3 Increased %E-C4d in post-lung transplant (LTx) recipients with high antibody titers to self-antigens, K-α-1-tubulin (KA1T) and collagen V (Col-V). Of 22 LTx recipients, 11 patients had a high titer to KA1T (>298 μg/ml) and 3 patients had a low titer to KA1T (<90 μg/ml). Similarly, 14 patients had a high tier to Col-V (>195 μg/ml) and 3 patients had a low titer to Col-V (<27 μg/ml). Mean E-C4d in recipients with high KA1T titers was 23%+10.5%; in the low KA1T titer group, it was 3.4% ± 1.4% ($p = 0.02$). Similarly, E-C4d in recipients with high Col-V titers was 22.9% ± 9.7%; in the low Col-V titer group, it was 3.4% ± 1.4% ($p = 0.03$). Mean data are shown with the standard deviation.

The %E-C4d is significantly increased in LTx recipients who demonstrated deposition of C3d on lung biopsy tissue

Positive C3d staining in lung biopsy tissue of recipients is associated with humoral immune response.[17] Of 22 LTx recipients, 9 patients were C3d+ and 13 patients were C3d− (Figure 4). Mean E-C4d in C3d+ patients was 26.1 ± 10.1. Mean E-C4d in C3d− patients was 15.5 ± 6.8 ($p = 0.01$). Therefore, higher %E-C4d is noted in patients who are C3d+ on immunohistological staining and provides further evidence that E-C4d is a biomarker for AMR in LTx patients.

Clinical correlation

One patient in our cohort of 22 was diagnosed with as bronchiolitis obliterans syndrome (BOS), and 7 had clinical, histologic, or serologic features consistent with AMR. DSA was detected in 3 of 7 patients with AMR, and Abs against KA1T and Col-V were detected in 6. Only 3 patients had both DSA and Abs to KA1T and Col-V.

Discussion

LTx is accepted as an effective treatment modality for patients with end-stage lung disease.[18,19] AMR is typically resistant to conventional immunosuppressive strategies and has recently emerged as a potential cause of acute and chronic graft dysfunction after LTx.[20–22] DSA to HLA and

Abs to self-antigens are both thought to be involved in the pathogenesis of rejection and graft failure. Several laboratories, including ours, have confirmed the role of DSA, pre-formed anti-HLA, and Abs to self-antigens (KA1T and Col-V) in the pathogenesis of chronic rejection, clinically diagnosed as BOS in post-LTx patients.[3,23,24]

A recent national conference was convened to propose a classification to assess AMR in solid organ transplantation.[25] There was a consensus that the 4 criteria needed to diagnose AMR include: (1) detection of DSA or pre-formed anti-HLA in circulation, (2) C4d deposition in the biopsy, (3) histologic evidence of tissue injury, and (4) clinical evidence of graft dysfunction. But the diagnosis of AMR in LTx is more nebulous compared with its better-defined counterpart in cardiac and renal allografts.[9] The detection of DSA is strongly correlated with the development of chronic rejection after LTx.

Many patients, however, do not demonstrate detectable levels of DSA in the circulation. It has been suggested the DSA may appear transiently in serum because it is likely bound to the allograft at the time of rejection.[12,23] Several studies have revealed that even in the absence of circulating DSA, there is evidence of complement deposition in the allograft, suggesting that Abs to non-HLA may be involved in the development of AMR.[23,24] In fact, induction of post-LTx de novo Abs to self-antigens KA1T and Col-V has been correlated with development of BOS.[12,16] Recent studies in BOS patients in our laboratory (unpublished data) have validated the more resilient nature of Abs to self-antigens compared with serum DSA in BOS patients. Using an animal model of BOS, we have also demonstrated that alloimmunity mediated by anti-major histocompatibility complex class I can lead to autoimmunity against KA1T and Col-V, which results in chronic rejection.[10]

C3d and C4d deposition in the allograft tissues is a sign of humoral immune activation leading to tissue damage and
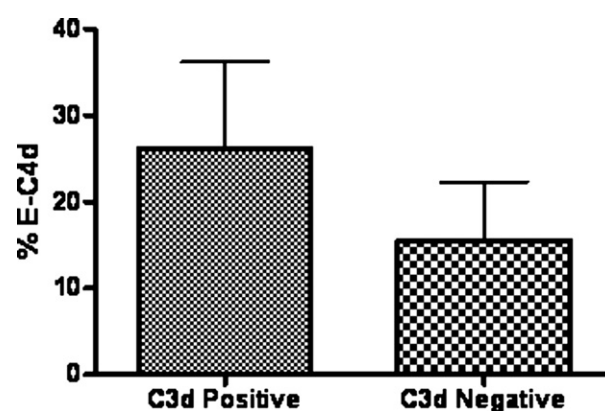

Figure 4 Increased %E-C4d in post-lung transplant (LTx) recipients who demonstrated deposition of C3d on lung biopsy tissue. Of 22 lung transplant recipients, 9 patients were C3d+ and 13 patients were C3d−. Mean E-C4d in C3d+ patients was 26.1+10.1. Mean E-C4d in C3d− patients was 15.5 ± 6.8 ($p = 0.01$). The higher %E-C4d noted in patients who were C3d+ on immunohistological staining further demonstrated that increased E-C4d is a biomarker for antibody-mediated rejection in post-LTx patients. Mean data are presented with the standard deviation.

allograft dysfunction.[17] In conjunction with serum markers, the complement activation products C3d and C4d have been used as markers of Ab-mediated injury in LTx recipients. However, C3d and C4d deposition has been documented to be mediated by Ab-independent mechanisms of complement activation, such as the mannose-binding lectin pathway.[26,27] Furthermore, staining for C4d, in particular, is not specific for the presence of AMR because it has also been noted in patients with primary graft dysfunction and infection [28] C4d staining in lung allograft biopsy specimens also does not consistently identify acute or chronic humoral rejection.[29,30]

E-C4d has been used as a novel marker for monitoring the activation of humoral immune responses in autoimmune diseases such SLE.[14] Similarly, the measurement of the EC4d/E-CR1 ratio has been correlated with AMR in cardiac transplantation and can serve as a marker for AMR in these recipients.[15]

In our study, there was an increased % E-C4d level in post-LTx recipients compared with healthy volunteers (19.9% ± 9.7% vs 3.7% ± 2.2%; $p = 0.02$; Figure 1). We categorized the LTx recipients based on presence of DSA and pre-formed anti-HLA Abs. Among the 22 post-LTx patients, 4 (18%) were DSA+, 5 (23%) had pre-formed anti-HLA Abs, and the remaining 13 (59%) had no detectable anti-HLA Abs. The %E-C4d was higher in the DSA+ group vs the DSA− group (34.1% ± 5.9% vs 16.7% ± 7.2%, $p = 0.02$; Figure 2). It is of significance that only DSA+ LTx recipients—but not those who developed anti-HLA—had significant increase in the EC4d (Figure 2), demonstrating the potential value of monitoring for EC4d in the diagnosis of AMR.

Anti-HLA was not detectable in 13 patients; of these, 4 patients had high Abs titers to KA1T and 7 patients to Col-V. Further, Abs to KA1T and Col-V are detectable in circulation in the absence of anti-HLA Abs in these patients. The 4 patients in our study with no detectable anti-HLA and high titers to both KA1T and Col-V had a high %E-C4d level compared with the 6 patients with no detectable anti-HLA and no anti-KA1T and anti-Col V Abs (24.4% ± 7.1% vs 12.8% + 1.9%; $p = 0.01$). Three patients who were DSA+ had high titers of Abs to KA1T and Col-V and demonstrated C3d deposition in lung biopsy tissue. The % E-C4d levels for these 3 patients were 31.4% ± 2.6%. One DSA+ patient had high titers of Abs to self-antigens, negative C3d staining, and a high %E-C4d (29%), and was diagnosed with BOS. This suggests that %E-C4d can be used as a sensitive marker of humoral activation in the absence of C3d/C4d staining after LTx on the biopsy specimen. Our results agree with a published report in cardiac transplant recipients where the E-C4d/E-CR1 ratio was used as a non-invasive marker for detecting AMR.[15]

Current immunosuppressive regimens are targeted to address T-cell–mediated rejection. Rejection episodes in patients who are receiving appropriate immunosuppressive therapy are typically secondary to humoral mediated alloimmune and autoimmune responses. We note that autoimmune responses (manifest by presence of Abs to the self-

antigens Col-V and KA1T) independent of alloimmune responses (manifest by presence of DSA or anti-HLA Abs) can cause chronic rejection. Therefore, %E-C4d can be used a non-invasive marker to monitor humoral activation in post-LTx patients. However, it is important to consider that complement independent pathways of humoral alloimmune and autoimmune responses can induce graft injury.

A major limitation of our cross-sectional study is that we have a small cohort of patients from a single institution and our preliminary results warrant further investigation to evaluate the correlation between circulating E-C4d levels and histologic evidence of complement deposition. The inherent selection bias of a small, single-institution patient cohort has been partially obviated by our use of specific inclusion and exclusion criteria. It is not clear what happens to E-C4d in terms of inflammation and infection. In any process in which complement is activated—be it infection or inflammation—we would expect that the E-C4d would be increased. Our exclusion criteria for the study included patients with active infections at the time of enrollment. Furthermore, of the 3 patients who later developed bacterial or viral infection, we did not have any blood samples within 6 months of developing the infection. Hence we do not have the data to comment definitively on the effect of infection on E-C4d.

In terms of inflammation early after LTx, we excluded patients with primary graft dysfunction from study enrollment. Hence, our study was not designed to address early post-LTx inflammation or ischemia injury parameters. The reliability of E-C4d in the context of sensitivity and specificity can be only explored with the enrollment of patients in a large, prospective multi-center study.

In summary, our study findings demonstrate that increased %E-C4d is strongly associated with known markers of alloimmune and autoimmune humoral responses noted in AMR and that the test has the potential to be used as a simple and reliable method of monitoring the activation of complement-dependent Ab-mediated responses in patients after LTx.

Disclosure statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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