Published in final edited form as:

J Heart Lung Transplant. 2010 April ; 29(4): 410–416. doi:10.1016/j.healun.2009.10.003.

INCREASED ERYTHROCYTE C4D IS ASSOCIATED WITH KNOWN ALLOANTIBODY AND AUTOANTIBODY MARKERS OF ANTIBODY MEDIATED REJECTION IN HUMAN LUNG TRANSPLANT RECIPIENTS

Angali Golocheikine, MD^a, Dilip S. Nath, MD^d, Haseeb Ilias Basha, MD^a, Deepti Saini, PhD^a, Donna Phelan, BS^e, Aviva Aloush, RN^c, Elbert P. Trulock, MD^c, Ramsey R. Hachem, MD^c, G.Alexander Patterson, MD^d, Joseph M. Ahearn, MD^f, and Thalachallour Mohanakumar, PhD^{a,b}

^aDepartment of Surgery, Washington University School of Medicine, St. Louis, MO

^bDepartment of Pathology & Immunology, Washington University School of Medicine, St. Louis, MO

^cDepartment of Medicine, Washington University School of Medicine, St. Louis, MO

^dCardiothoracic Surgery, Washington University School of Medicine, St. Louis, MO

^eHLA Laboratory, Barnes-Jewish Hospital, St. Louis, MO

^fDepartment of Medicine, University of Pittsburgh, Pittsburgh, PA

Abstract

Background—Immune responses to mismatched donor HLA antigens play a significant role in the pathogenesis of chronic rejection. The study objective was to evaluate whether erythrocyte bound C4d (E-C4d) is associated with known alloimmune and autoimmune markers of antibody mediated rejection (AMR) following human lung transplantation (LTx).

Methods—22 LTx recipients and 15 normal subjects were analyzed for E-C4d using flow cytometry. Development of antibodies (Abs) to donor mismatched HLA (DSA) and Abs to HLA were determined using solid phase method by Luminex. Development of Abs to self-antigens, K-alpha-1-tubulin (KA1T) and collagen V (Col-V) were measured by ELISA. C3d deposition in lung biopsies was determined by immunohistochemical staining.

Results—Percent E-C4d (%E-C4d) levels in LTx patients were higher compared to normal subjects (19.9% vs. 3.7%, $p = 0.02$). DSA+ patients had higher E-C4d levels compared to DSA- patients (34.1% vs. 16.7%, $p = 0.02$). In 5 patients with preformed anti-HLA, E-C4d levels were not significantly different compared to 13 patients with no detectable anti-HLA ($p = 0.1$). Higher E-C4d levels were noted in patients who developed Abs to KA1T ($p = 0.02$) and Col-V ($p = 0.03$). Recipients with C3d tissue deposition had higher E-C4d levels compared to patients with C3d negative biopsy results ($p = 0.01$).

Conclusions—Increased % E-C4d levels are found in patients with positive DSA, high Abs titers to KA1T and Col-V, and have C3d positive lung biopsy findings. Therefore, % E-C4d can serve as a potential marker for AMR following LTx.

Corresponding Author T. Mohanakumar, Ph.D Washington University School of Medicine Department of Surgery Box 8109-3328 CSRB 660 S. Euclid Avenue St. Louis, MO 63110 Tel: 314-362-8463 Fax: 314-747-1560 kumart@wustl.edu.

There is no financial or professional conflict of interest by any of the authors of this manuscript.

Introduction

The presence of pre-existing antibodies specific to mismatched donor HLA have been strongly correlated with acute and/or chronic rejection and has generally been accepted as a contraindication for solid organ transplantation (1,2). More recent evidence suggests that post-transplant development of donor specific antibody (DSA) against mismatched donor HLA play a crucial role in acute and chronic rejection of lung allografts (3-5).

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

Our laboratory has previously demonstrated that not only alloimmune responses to donor HLA but also autoimmune response to self-antigens, namely K-alpha-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, play an important role 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 following LTx. Hence, this study was conducted to investigate whether E-C4d is associated with alloantibody and autoantibody makers of AMR in human lung transplant 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, cell-bound complement activation products such as erythrocyte C4d (E-C4d) and erythrocyte complement receptor 1 (ECR1) have been shown to be biomarkers for monitoring disease activity in patients with SLE (14). The advantage of utilizing an erythrocyte bound product is that it is more stable and has an increased half-life compared to its more transient circulating counterpart.

With recent reports that have suggested a predictive role of 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 correlate with other established markers of AMR such as DSA, Abs to self-antigens, KA1T and Col-V, and C3d deposition in lung biopsies.

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 following informed consent, patients with no active infection or primary graft dysfunction at time of study enrollment. The exclusion criteria included patient age greater than 65, patients with body mass index (BMI) greater than 40, patients with cold ischemia time greater than 6 hours at the time of LTx, patients with increased liver enzymes (elevated

transaminases or hyperbilirubinemia), patients with abnormal thyroid tests and patients with previously documented hypertension. Fifteen healthy volunteers were also recruited as control. Fresh ethylenediamine-tetraacetic acid (EDTA) anticoagulated blood was collected and E-C4d testing was performed on whole blood samples immediately after receiving the specimen. Serum and peripheral blood lymphocytes were separated and stored at -135°C . Lung biopsies were performed according to protocol. At the time of obtaining tissues samples, blood samples for E-C4d measurements were obtained simultaneously.

A diagnosis of AMR was made based on clinical, histological and serological criteria. Clinical suspicion included lung allograft dysfunction manifest by diminished FEV1, reduced PO2/FiO2 ratios in ventilated patients and lack of response to immunosuppressive therapy directed towards cellular rejection. Histological 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, serological evidence such as Ab in LTx recipients in this study comprised serological 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, CA). In brief, primary Ab coated beads and incubation buffer were placed into 96-well filter plates. Both samples and standards were incubated with the primary Ab beads at room temperature on an orbital shaker. The wells were then washed and biotinylated detector Abs were added for a further incubation period of 30 minutes. The wells were washed again and streptavidin-R-phycoerythrin solution was added and incubated for 15 minutes. Following this, the wells were washed and data read utilizing a dual-laser flow analyzer, the Luminex-100 system version 1.7. Data analysis was performed using the MasterPlex QT 1.0 system (MiraiBio) and a five-parameter regression formula was utilized to allow detection compared to 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, NY) was coated with $1\mu\text{g/ml}$ of recombinant purified KA1T or commercially available Col-V (Chemicon, CA, USA) in phosphate-buffer solution (PBS) overnight at 4°C . The antigen coated wells were blocked for non-specific binding with 1% bovine serum albumin for 2 hours. Sera from post-transplant and normal 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 IgG, IgM bound to horseradish peroxidase (Jackson ImmunoResearch Laboratory Inc, PA) and developed with tetramethylbenzidine substrate (Millipore, CA). Immunosorbance was detected at 460nm. Concentration of Ab was calculated based on a standard curve using the binding of known concentration of commercial anti-KA1T/anti-Col-V Abs (Santacruz Biotechnology, Inc. CA).

Detection of E-C4d

10 μl of fresh EDTA anticoagulated blood was washed twice in 1ml 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. 5 μl 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 mins at 4°C . Mouse IgG1 $_{\text{k}}$ was used as an isotype control. After 2 washes with FACS buffer, the pellet was treated with fluorescein isothiocyanate (FITC) conjugated goat anti mouse IgG in a dilution of 1 in 100 for 20 mins at

4°C. Following two washes with FACS buffer, the cells were resuspended in 500µl of FACS buffer and read on 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. Percent E-C4d (%E-C4d) was calculated by determining the percentage of total RBCs with a positive mean fluorescence shift ($MFI_{\text{anti-C4d}} - MFI_{\text{isotype IgG1}}$). A two 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 three times and the deviation was within 5% of each measurement.

Immunohistochemical staining for C3d

Immunohistochemical studies were conducted utilizing formalin fixed paraffin embedded lung allograft biopsies from 22 recipients. The paraffin embedded tissue was cut (4-6 microns in thickness) and placed on Fisher/Superfrost Plus slides. The slides were then placed in a 60°C oven for 1 h, 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% H₂O₂ for 30 min. Before addition of primary Ab, slides were blocked for nonspecific staining using a serum-free protein for 15 minutes at room temperature (DakoCytomation, Carpinteria, CA). Primary Ab (C3d rabbit polyclonal Abs, DakoCytomation, Carpinteria, CA) was diluted 1:500 and tissue was incubated overnight. The detection system, a labeled polymer system, Envision Plus Dual Link (DakoCytomation, Carpinteria, CA) was used and staining was visualized with Diaminobenzidine (DAB) chromogen (DakoCytomation, Carpinteria, CA). The slides were then counterstained with hematoxylin, dehydrated through graded ethanol solutions followed by xylene and cover slipped with mounting media.

Statistical Correlation

Graphpad Prism 4 software was used to analyze data. Statistical correlation with patient outcome was performed using the Mann-Whitney test. A p-value less than 0.05 was considered to be statistically significant.

Results

Patient Demographics

The study cohort consisted of 22 patients who underwent LTx from May 2006-July 2007. Mean age at transplant was 50.7 years. Eleven patients were male. 14 patients were Caucasian, 6 were African-American and 2 were classified as Other. The indication for transplant included chronic obstructive pulmonary disease (n=8; 36%), idiopathic pulmonary fibrosis (n=4, 18%), alpha-1-antitrypsin deficiency (n=3, 14%), primary pulmonary hypertension (n=2, 9%), cystic fibrosis (n=2, 9%), idiopathic interstitial lung disease (n=1, 5%), and sarcoidosis (n=1, 5%). Bilateral lung transplantation was performed in 20 patients (92%). Mean ($\pm$ standard deviation) follow-up after LTx was 20.1 ± 3.4 months and the median follow-up was 21.5 months. Of 22 patients, 5 patients experienced at least one episode of biopsy documented cellular rejection. While patients with active infection at time of study enrollment met exclusion criteria, 3 patients developed systemic infections (2 with bacterial and 1 with viral).

%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 normal volunteers and 22 post-LTx recipients on freshly collected EDTA anticoagulated blood. The test for E-C4d was performed at a mean ($\pm$ standard deviation) of 15.3 ± 4.2 months from time of LTx. Mean E-C4d ($\pm$ standard deviation) level in the normal cohort was $3.7 \pm 2.2\%$. Mean

E-C4d level in the LTx cohort was $19.9 \pm 9.7\%$ (Fig 1, $p = 0.02$). This data demonstrates that percentage E-C4d level is higher in LTx recipients compared to control. To determine whether this is due to increase in patients developing DSA which changes the %EC4d in the LTx recipients, we analyzed for development of DSA as well as Abs to HLA.

%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 ($\pm$ standard deviation) 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 three different patient groups: Group 1 consists of patients with DSA ($n=4$), Group 2 has patients who developed anti-HLA but not DSA ($n=5$) and Group 3 remained negative for both DSA and anti-HLA ($n=13$) (Fig 2). Mean E-C4d ($\pm$ standard deviation) in Group 1 was $34.1 \pm 5.9\%$. Mean C4d in Group 2 was $13.9 \pm 8.4\%$. Mean C4d in Group 3 was $17.7 \pm 6.7\%$. There was a significant difference between DSA and anti-HLA positive patients (Group 1 vs 2; $p = 0.02$), DSA and anti-HLA negative patients (Group 1 vs 3; $p = 0.03$) as well as DSA and non-DSA patients (Group 1 vs Group 2+3; $p = 0.02$). There was no significant difference between anti-HLA+ and anti-HLA- patients (Group 2 vs 3, $p = 0.1$). This data demonstrates that percentage E-C4d is higher in DSA+ LTx patients compared to those that are DSA-. Furthermore, %E-C4d is not significantly different in anti-HLA+ recipients without DSA compared to anti-HLA- recipients. These preliminary results demonstrate that patients with DSA also develop an increase in %EC4d and therefore could be used as a marker for AMR following LTx.

%E-C4d is significantly increased in LTx recipients who develop Ab to self-antigens, KA1T and/or Col-V

Post-transplant development of Abs to self-antigens has been associated with development of chronic rejection following human LTx. Mean time ($\pm$ standard deviation) 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). Therefore, we measured the E-C4d levels in patients who developed Abs to KA1T and Col-V (Fig 3). A LTx recipient was considered to have developed Abs to self antigens if titer to a self-antigen was greater than normal cohort mean + ($2 \times$ normal cohort standard deviation). Similarly, a LTx recipient had a low Ab titer to a given self-antigen if the level was less than normal mean cohort - ($2 \times$ normal cohort standard deviation). Mean KA1T ($\pm$ standard deviation) level in normal was 194 ± 52 ug/mL. Mean Col-V ($\pm$ standard deviation) level in normal was 111 ± 42 ug/mL. Of 22 lung transplant recipients, 11 patients had a high titer to KA1T (> 298 ug/mL) and 3 patients had a low titer to KA1T (< 90 ug/mL). Similarly, 14 patients had a high tier to Col-V (> 195 ug/mL) and 3 patients had a low titer to Col-V (< 27 ug/mL). Mean E-C4d ($\pm$ standard deviation) in recipients with high KA1T titers was $23 \pm 10.5\%$; in the low KA1T titer group, it was $3.4 \pm 1.4\%$ ($p = 0.02$). Similarly, mean E-C4d ($\pm$ standard deviation) in recipients with high Col-V titers was $22.9 \pm 9.7\%$; in the low Col-V titer group, it was $3.4 \pm 1.4\%$ ($p = 0.03$). This data demonstrates that %E-C4d is higher not only in patients who developed DSA following post-LTx but also in patients who developed Abs to self antigens.

%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- (Fig 4). Mean E-C4d ($\pm$ standard deviation) in C3d+ patients was 26.1 ± 10.1 . Mean E-C4d ($\pm$ standard deviation) 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

In our cohort of 22 patients, 1 patient was diagnosed with BOS and 7 had clinical, histological or serological features consistent with AMR. Of 7 patients with AMR, DSA was detected in 3 patients and Abs against KA1T and Col-V were detected in 6 patients. Only 3 patients had both DSA and Abs to KA1T and Col-V.

Discussion

Lung transplantation is accepted as an effective treatment modality for patients with end-stage lung disease (18,19). Antibody mediated rejection is typically resistant to conventional immunosuppressive strategies and has recently emerged as a potential cause of acute and chronic graft dysfunction following LTx (20-22). Both DSA to HLA and Abs to self antigens are 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 Bronchiolitis Obliterans Syndrome (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 four criteria needed to diagnose AMR include: (a) detection of DSA or preformed anti-HLA in circulation, (b) C4d deposition in the biopsy, (c) histological evidence of tissue injury, and (d) clinical evidence of graft dysfunction. But in LTx, the diagnosis of AMR is more nebulous compared to its better defined counterpart in cardiac and renal allografts (9). The detection of DSA is strongly correlated with the development of chronic rejection following lung transplantation. However, many patients do not demonstrate detectable levels of DSA in the circulation. It has been suggested the DSA may appear transiently in serum as 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 play a role in the development of AMR (23,24). In fact, induction of post-transplant 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 to serum DSA in BOS patients. Using an animal model of BOS, we have also demonstrated that alloimmunity mediated by anti-MHC 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 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 (MBL) pathway (27). Furthermore, staining for C4d in particular is not specific for the presence of AMR as it has also been noted in patients with primary graft dysfunction and infection (28). Also, C4d staining in lung allograft biopsies do not consistently identify acute or chronic humoral rejection (29,30).

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

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

There were 13 patients with no detectable anti-HLA; 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 absence of anti-HLA antibodies 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 to 6 patients with no detectable anti-HLA and no anti-KA1T and anti-Col V Abs ($24.4\% \pm 7.1$ vs. $12.8\% \pm 1.9$; $p=0.01$). 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 three patients were $31.4 \pm 2.6\%$. One patient who was DSA+, high titers of Abs to self-antigens and negative C3d staining had a high %E-C4d (29%). This patient was diagnosed with BOS. This suggests that %E-C4d can be used as a sensitive marker of humoral activation in the absence of biopsy C3d/C4d staining following LTx. Our results agree with a published report in cardiac transplant patients where measurement of E-C4d/E-CR1 ratio was used as a non-invasive marker for detecting AMR (15).

Current immunosuppressive regimen is targeted to address T-cell mediated rejection. Rejection episodes in patients who are on appropriate immunosuppressive therapy are typically secondary to humoral mediated allo and autoimmune responses. It must be noted that autoimmune responses (manifest by presence of antibodies to self-antigens, Col-V and KA1T) independent of alloimmune responses (manifest by presence of DSA or anti-HLA antibodies) 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 allo 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 warrants further investigation to evaluate the correlation between circulating E-C4d levels and histological evidence of complement deposition. The inherent selection bias of a small, single-institution patient cohort has been partially obviated by utilizing a specific inclusion/exclusion criteria. It is not clear what happens to E-C4d in terms of inflammation and infection. 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 post-Tx, we excluded patients with primary graft dysfunction from study enrollment. Hence our study is not designed to address early post-Tx 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 **demonstrates that increased %E-C4d levels 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 post-LTx patients.**

Acknowledgments

The study was supported by NIH Grant 1 R01 HL56643 (TM) and NIH Training Grant: T32 HL07776 (DSN)

References

1. Terasaki PI. Humoral theory of transplantation. *Am J Transplant* 2003;3:665–73. [PubMed: 12780557]
2. Masson E, Stern M, Chabod J, et al. Hyperacute rejection after lung transplantation caused by undetected low-titer anti-HLA antibodies. *J Heart Lung Transplant* 2007;26:642–5. [PubMed: 17543791]
3. Sundaresan S, Mohanakumar T, Smith MA, et al. HLA-A locus mismatches and development of antibodies to HLA after lung transplantation correlate with the development of bronchiolitis obliterans syndrome. *Transplantation* 1998;65:648–53. [PubMed: 9521198]
4. Giritia AL, McCurry KR, Iacono AT, et al. HLA-specific antibodies are associated with high-grade and persistent-recurrent lung allograft acute rejection. *J Heart Lung Transplant* 2004;23:1135–41. [PubMed: 15477106]
5. Giritia AL, Duquesnoy R, Yousem SA, et al. HLA-specific antibodies are risk factors for lymphocytic bronchiolitis and chronic lung allograft dysfunction. *Am J Transplant* 2005;5:131–8. [PubMed: 15636621]
6. Collins AB, Schneeberger EE, Pascual MA, et al. Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 1999;10:2208–14. [PubMed: 10505698]
7. Feucht HE, Schneeberger H, Hillebrand G, et al. Capillary deposition of C4d complement fragment and early renal graft loss. *Kidney Int* 1993;43:1333–8. [PubMed: 8315947]
8. Feucht HE. Complement C4d in graft capillaries -- the missing link in the recognition of humoral alloreactivity. *Am J Transplant* 2003;3:646–52. [PubMed: 12780555]
9. Stewart S, Fishbein MC, Snell GI, et al. Revision of the 1996 working formulation for the standardization of nomenclature in the diagnosis of lung rejection. *J Heart Lung Transplant* 2007;26:1229–42. [PubMed: 18096473]
10. Fukami N, Ramachandran S, Saini D, et al. Antibodies to MHC class I induce autoimmunity: role in the pathogenesis of chronic rejection. *J Immunol* 2009;182:309–18. [PubMed: 19109162]
11. Bharat A, Fields RC, Trulock EP, Patterson GA, Mohanakumar T. Induction of IL-10 suppressors in lung transplant patients by CD4+25+ regulatory T cells through CTLA-4 signaling. *J Immunol* 2006;177:5631–8. [PubMed: 17015751]
12. Goers TA, Ramachandran S, Aloush A, Trulock E, Patterson GA, Mohanakumar T. De novo production of K- α 1 tubulin-specific antibodies: role in chronic lung allograft rejection. *J Immunol* 2008;180:4487–94. [PubMed: 18354170]
13. Jaramillo A, Smith MA, Phelan D, et al. Development of ELISA-detected anti-HLA antibodies precedes the development of bronchiolitis obliterans syndrome and correlates with progressive decline in pulmonary function after lung transplantation. *Transplantation* 1999;67:1155–61. [PubMed: 10232567]
14. Manzi S, Navratil JS, Ruffing MJ, et al. Measurement of erythrocyte C4d and complement receptor 1 in systemic lupus erythematosus. *Arthritis Rheum* 2004;50:3596–604. [PubMed: 15529364]
15. Lee KC, Chang CY, Chuang YC, et al. Measurement of human erythrocyte C4d to erythrocyte complement receptor 1 ratio in cardiac transplant recipients with acute symptomatic allograft failure. *Transplant Proc* 2008;40:2638–42. [PubMed: 18929826]
16. Iwata T, Philipovskiy A, Fisher AJ, et al. Anti-type V collagen humoral immunity in lung transplant primary graft dysfunction. *J Immunol* 2008;181:5738–47. [PubMed: 18832733]
17. Westall GP, Snell GI, McLean C, Kotsimbos T, Williams T, Magro C. C3d and C4d deposition early after lung transplantation. *J Heart Lung Transplant* 2008;27:722–8. [PubMed: 18582800]
18. Woo MS. Overview of lung transplantation. *Clin Rev Allergy Immunol* 2008;35:154–63. [PubMed: 18821036]
19. Orens JB, Garrity ER Jr. General overview of lung transplantation and review of organ allocation. *Proc Am Thorac Soc* 2009;6:13–9. [PubMed: 19131526]

20. Ng CY, Madsen JC, Rosengard BR, Allan JS. Immunosuppression for lung transplantation. *Front Biosci* 2009;14:1627–41. [PubMed: 19273152]
21. Allan JS. Immunosuppression for lung transplantation. *Semin Thorac Cardiovasc Surg* 2004;16:333–41. [PubMed: 15635537]
22. Reinsmoen NL, Bolman RM, Savik K, Butters K, Hertz MI. Are multiple immunopathogenetic events occurring during the development of obliterative bronchiolitis and acute rejection? *Transplantation* 1993;55:1040–4. [PubMed: 7684535]
23. Schulman LL, Ho EK, Reed EF, et al. Immunologic monitoring in lung allograft recipients. *Transplantation* 1996;61:252–7. [PubMed: 8600633]
24. Scornik JC, Zander DS, Baz MA, Donnelly WH, Staples ED. Susceptibility of lung transplants to preformed donor-specific HLA antibodies as detected by flow cytometry. *Transplantation* 1999;68:1542–6. [PubMed: 10589952]
25. Takemoto SK, Zeevi A, Feng S, et al. National conference to assess antibody-mediated rejection in solid organ transplantation. *Am J Transplant* 2004;4:1033–41. [PubMed: 15196059]
26. Diuvestijn AM, Derhaag JG, van Breda Vriesman PJ. Complement activation by anti-endothelial cell antibodies in MHC-mismatched and MHC-matched heart allograft rejection: anti-MHC-, but not anti non-MHC alloantibodies are effective in complement activation. *Transpl Int* 2000;13:363–71. [PubMed: 11052273]
27. Awad MR, El-Gamel A, Hasleton P, Turner DM, Sinnott PJ, Hutchinson IV. Genotypic variation in the transforming growth factor-beta 1 gene: association with transforming growth factor beta 1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. *Transplantation* 1998;66:1014–20. [PubMed: 9808485]
28. Morrell MR, Patterson GA, Trulock EP, Hachem RR. Acute antibody-mediated rejection after lung transplantation. *J Heart Lung Transplant* 2009;28:96–100. [PubMed: 19134538]
29. Magro CM, Harman A, Pope, Klinger D, et al. Use of C4d as a diagnostic adjunct in lung allograft biopsies. *Am J Transplant* 2003;3:1143–54. [PubMed: 12919095]
30. Wallace WD, Reed EF, Ross D, Lassman CR, Fishbein MC. C4d staining of pulmonary allograft biopsies: an immunoperoxidase study. *J Heart Lung Transplant* 2005;24:1565–70. [PubMed: 16210131]

Figure 1. Increased percentage of the erythrocyte bound complement degradation product, C4d (E-C4d) fraction in the peripheral circulation of post-LTx recipients (n=22) compared to normal healthy controls (n=15)

%E-C4d levels were measured by flow cytometry in 15 normal volunteers and 22 post-LTx recipients on freshly collected EDTA anticoagulated blood. %E-C4d in the post-LTx patients was calculated by determining the percentage of circulating RBCs with a mean fluorescence shift ($MFI_{\text{anti-C4d}} - MFI_{\text{isotype IgG1}}$) of greater than two standard deviations (S.D) obtained in the control population. The percentage of circulating RBCs in the normal cohort with a positive mean fluorescence shift $\pm$ two S.D. (%E-C4d) was 3.7 ± 2.2 . %E-C4d in the post-LTx cohort was 19.9 ± 9 ($p=0.02$). Results are expressed in mean $\pm$ S.D.

Figure 2. Increased %E-C4d in post-LTx recipients with DSA compared to DSA-patients with or without detectable anti-HLA Abs

Group 1 consists of patients with DSA (n=4), Group 2 has patients who developed anti-HLA but not DSA (n=5) and Group 3 remained negative for both DSA and anti-HLA (n=13). Mean E-C4d ($\pm$ standard deviation) in Group 1 was 34.1 $\pm$ 5.9%. Mean E-C4d in Group 2 was 13.9 $\pm$ 8.4%. Mean E-C4d in Group 3 was 17.7 $\pm$ 6.7%. 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) as well as DSA and non-DSA patients (Group 1 vs Group 2+3; p=0.02). There was no significant difference between anti-HLA+ and anti-HLA- patients (Group 2 vs 3, p=0.1). This data demonstrates that %E-C4d is higher in DSA+ LTx patients compared to those that are DSA- (p=0.02).

Figure 3. Increased %E-C4d in post-LTx recipients with high Ab titers to self-antigens, KA1T and/or Col-V

Of 22 LTx recipients, 11 patients had a high titer to KA1T (> 298ug/mL) and 3 patients had a low titer to KA1T (<90ug/mL). Similarly, 14 patients had a high tier to Col-V (>195ug/mL) and 3 patients had a low titer to Col-V (<27ug/mL). Mean E-C4d (± standard deviation) 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, mean E-C4d (± standard deviation) 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).

Figure 4. Increased %E-C4d in post-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 ($\pm$ standard deviation) in C3d+ patients was 26.1 ± 10.1 . Mean E-C4d ($\pm$ standard deviation) in C3d- patients was 15.5 ± 6.8 ($p=0.01$). The higher %E-C4d noted in patients who are C3d+ on immunohistological staining further demonstrates that increased E-C4d is a biomarker for AMR in post-LTx patients.