ORIGINAL ARTICLE

Eosinophils in transbronchial biopsies: a predictor of chronic lung allograft dysfunction and reduced survival after lung transplantation – a retrospective single-center cohort study

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ABSTRACT

Long-term outcomes after lung transplantation remain inferior to those of other solid organ groups. The significance of eosinophils detected on transbronchial biopsies (TBBx) after lung transplantation and their relationship to long-term outcomes remain unknown. A retrospective single-center cohort study was performed of patients transplanted between January 01, 2001, and July 31, 2018, who had at least 1 TBBx with evaluable parenchymal tissue. Multivariable Cox proportional hazard models were used to assess the associations between eosinophil detection and: all-cause mortality and Chronic Lung Allograft Dysfunction (CLAD). 8887 TBBx reports from 1440 patients were reviewed for the mention of eosinophils in the pathology report. 112 (7.8%) patients were identified with eosinophils on at least one TBBx. The median (95% CI) survival time for all patients was 8.28 (7.32–9.31) years. Multivariable analysis, adjusted for clinical variables known to affect post-transplant outcomes, showed that the detection of eosinophils was independently associated with an increased risk of death (HR 1.51, 95% CI 1.24–1.85, $p < 0.01$) and CLAD (HR 1.35, 95% CI 1.07–1.70, $P = 0.01$). Eosinophils detected in TBBx are associated with an increased risk of CLAD and death. There may be benefit in specifically reporting the presence of eosinophils in TBBx reports and incorporating their presence in clinical decision-making.

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Key words
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Introduction

Lung transplantation is an established therapeutic option to improve the lung function and quality of life for patients with advanced lung disease. However, the survival after lung transplantation, at a median of 6.5 years worldwide, remains inferior to that observed in other solid organ transplant groups [1]. Chronic lung allograft dysfunction (CLAD) is regarded as a leading determinant of poor patient and graft survival [2,3]. A restrictive phenotype of CLAD, with decreased lung volumes and parenchymal fibrosis, termed restrictive

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allograft syndrome (RAS), has been shown to have a particularly grim prognosis [2,4–5]. The identification of predictive factors associated with survival and CLAD is imperative to improve outcomes for lung transplant recipients.

Since the commencement of lung transplantation, eosinophils have been detected in transbronchial biopsy (TBBx) specimens of recipients [6–11]. In nontransplant pulmonary medicine, eosinophilic inflammation is associated with a wide spectrum of conditions, which include allergy, auto-immunity, helminthic infections, and drug reactions. In small lung-transplant patient cohorts, the presence of perivascular eosinophils in TBBx has been correlated with acute cellular rejection (ACR) [6–9]. The international consensus guidelines on the histologic grading of pulmonary ACR include tissue eosinophils in acute rejection grades 2 and higher [12]. In other solid organ transplant groups including kidney, liver, and heart, allograft eosinophils seen on biopsy have been similarly shown to correlate with ACR [13–15].

Eosinophils detected in bronchoalveolar lavage (BAL) fluid have been shown to independently predict both overall recipient survival and the development of CLAD [16,17]. Importantly, BAL eosinophils have also been associated with RAS [17,18].

While tissue eosinophils have been recognized as components of histologic high-grade ACR, the relationship between their presence and histologic location on transbronchial biopsies (TBBx), and long-term outcomes after lung transplantation have not been assessed. We hypothesized that the presence of TBBx eosinophils, as reported by experienced lung-transplant pathologists, predicts a shorter time to CLAD and death. The aim of this study was to determine whether the presence and pattern of eosinophilia in lung-transplant recipient TBBx is associated with survival and/or CLAD outcomes in a large single-center cohort.

Methods

Subjects

A retrospective cohort analysis was performed using a database of all patients transplanted at Toronto General Hospital between January 01, 2001, and July 31, 2018. The study was approved by the University Health Network Research Ethics Board (protocol number 15-9531-AE). The 'eosinophils' group was defined as the first presence of eosinophils reported in at least one of their TBBx. All other patients transplanted during the study time period, without eosinophils detected on TBBx, were considered as the comparison 'without eosinophils' group. All TBBx with evaluable parenchymal tissue, defined as at least 1 evaluable parenchymal fragment (EPF > 1), for study subjects and controls, were included to achieve the highest sensitivity for detection of TBBx eosinophils in the context of real-world reporting. Recipient and donor baseline characteristics and cause of death were included in the database. The maximum recipient blood eosinophil count in the 14 days prior to TBBx was recorded. Active patient medications at the time of TBBx eosinophil detection, with an established association with pulmonary eosinophilia based on the Pneumotox database, were recorded for the 'eosinophils' group [19].

Bronchoscopic surveillance

After transplantation, patients underwent surveillance bronchoscopies with BAL and TBBx as per our hospital protocol (at 0.5, 1.5, 3, 6, 9, 12, 18, and 24 months). Additional diagnostic bronchoscopies were performed as clinically indicated. All TBBx were reviewed by pulmonary pathologists with expertise in lung transplantation and were classified according to the International Society for Heart and Lung Transplantation nomenclature [12]. ACR was defined as those biopsies with ≥ A1 grade (A1-A4) or ≥ B1R (B1R-B2R) grade components. A standardized cumulative A rejection score was defined for each biopsy as the sum of all A grades divided by the number of available evaluable biopsies up until this timepoint [4]. TBBx with evaluable parenchymal tissue (EPF > 1), deemed ungradeable (AX) by ACR criteria, were included and analyzed as equal to A0 to generate the cumulative A rejection score. Additional pathologic findings, including the histologic location and pattern of eosinophils, were noted. The concurrent matched FEV_1 immediately preceding the TBBx occasion was compared with the average baseline (average of the two best FEV_1 measured ≥ 3 weeks apart) and the recent baseline (the highest of the 2 previous FEV_1) to determine a %-change [20].

Concurrent BAL microbiology and cytology data were recorded. BAL cytology was categorized into "Positive = 1" for eosinophils (Eosinophils> 2%) and "Negative = 0". The cumulative cytology score was defined at the time of each bronchoscopy as the sum of all eosinophil-positive cytology specimens divided by the number of total BAL specimens until this timepoint. BAL microbiology specimens were categorized into "Positive = 1" for significant pathogenic organisms and "Negative = 0" samples. The cumulative infection score was generated at the time of each bronchoscopy as the number of

positive BAL specimens, divided by the number of total BAL specimens obtained until this timepoint. The presence of de novo donor-specific antibodies (dn DSA) at the time of biopsy was recorded.

Immunosuppression and CLAD treatment

Each patient received maintenance immunosuppression including cyclosporine with therapeutic drug monitoring and azathioprine 1.5–2 mg/kg/day. Maintenance corticosteroid dosing was administered as follows; methylprednisone 0.5 mg/kg for 3 days followed by prednisolone 0.5 mg/kg/day tapering to 0.25 mg/kg/day at 3 months, 0.15 mg/kg/day at 6 months, and 0.075 mg/kg/day at 12 months post-transplant. Basiliximab (20 mg IV for two doses) was given to patients with early acute kidney impairment with temporary cessation of calcineurin inhibitor. Induction immunosuppression was not administered. Since 2008, patients with a positive virtual crossmatch, defined as a pretransplant donor-specific antibody, were treated with a postoperative desensitization protocol comprised of plasma exchange, intravenous immunoglobulin (IVIG: 1 g/kg), and rabbit anti-thymocyte globulin (3–5 mg/kg) [21]. Patients with pretransplant or de novo donor-specific HLA antibodies were treated with mycophenolate as a substitute for azathioprine. Symptomatic or spirometrically significant A1, or any>= A2, acute cellular rejection was treated with augmented immunosuppression, most commonly with pulse corticosteroids, and a switch to tacrolimus. Additional adjustments were made as needed in case of side effects.

Treatment for chronic lung allograft dysfunction (CLAD)

At the onset of CLAD, patients were typically changed from cyclosporine to tacrolimus, initiated on a trial of azithromycin, and aggressively treated for gastroesophageal reflux. Retransplantation was considered for appropriate candidates. No patients received Montelukast.

Clinical outcomes

The primary outcome was all-cause death or retransplantation. The cause of death was recorded where available.

The secondary outcome was the time from transplant to CLAD onset. CLAD was calculated in an automated fashion according to published guidelines, and each case was subsequently confirmed by physician review [2]. Lung allograft dysfunction from causes other than CLAD was noted. Patients were excluded from CLAD analysis if they had < 4 PFT measurements post-transplant, <90 days survival, or if they developed CLAD prior to detection of eosinophils in TBBx. CLAD phenotype was defined by the 2019 Consensus criteria [2,22,23].

Statistical analysis

Statistical analyses were performed using R version 3.4.3. Statistical significance was set at a 2-sided level of 0.05. Descriptive statistics were summarized by the mean and standard deviation (SD) and median with interquartile range (IQR) and compared using the two-sample t-test for continuous variables and the chi-square test for categorical variables. Time-dependent multivariable Cox proportional hazards models were used to determine the association between the first presence of TBBx eosinophils and survival and CLAD. Data were right censored at the time of the last pulmonary function test or July 31, 2018, whichever came first. Explanatory variables were each tested for the proportional hazards assumption and for correlation. Variables of interest potentially associated with survival and CLAD outcomes after transplantation were established a priori and recorded including recipient age, donor age, donor-recipient sex matching, type of transplant, native lung disease, CMV sero-status matching, and transplantation era [1]. Univariate analysis was performed to identify predictor variables with a significant hazard ratio for time-to-death and time-to-CLAD, and then incorporated for multivariable assessment. The cumulative A score, cumulative eosinophil-positive cytology score, and cumulative infection score were analyzed as time-dependent co-variates. The landmark Kaplan–Meier approach was adopted to visualize time-to-event probabilities in each group, conditional on the group membership of individual patients at 1 and 2 years follow-up [24]. For CLAD analysis, death and lung allograft dysfunction without CLAD were analyzed as censoring events. Kaplan-Meier curves were generated by plotting overall survival (or CLAD-free survival) in the groups with and without TBBx eosinophils and compared using the Log rank test. Additionally, a time-dependent univariate Cox proportional hazards model was used to measure the association between TBBx eosinophils and the RAS/Mixed CLAD phenotype. The 'Other' CLAD phenotypes included BOS, undefined and unclassified.

Results

Descriptive statistics

8887 TBBx reports from 1440 patients in the Toronto Lung Transplant Program database, between January 01, 2001, and July 31, 2018, were available containing at least one evaluable parenchymal fragment (Fig. 1). The reports were searched for any mention of the words: eosinophil, eosinophilic, and eosinophilia. 112 patients had eosinophils on at least one TBBx. Only a small proportion of patients, 9/112 (8.0%), had recurrent eosinophils in subsequent TBBx. There was a significant difference in the median (IQR) number of biopsies per patient for the TBBx eosinophils groups was 8 (3), and for those without TBBx, eosinophils was 6 (4), $P < 0.01$. The majority of positive TBBx eosinophils occurred in the 1st (38.4%) and 2nd (21.4%) sampling occasion (Table S1).

Baseline patient demographics are summarized in Table 1 and show significant differences in recipient age and native lung disease. TBBx eosinophils were detected more frequently in the early era 75/112 (67%) compared with the latter era 37/112 (33%). There was a significant difference in the median (IQR) number of TBBx per patient performed in 2001–2009 at 7 (2) compared with 2010–2018 at 6 (4) ($P < 0.01$). Recipient post-transplant characteristics are summarized in Table 2, showing a significantly higher A score and proportion of deaths because of CLAD in the eosinophil group. Complete HLA antibody data were available for 307 patients between January 01, 2008, and December 29, 2011. There were no significant differences in the proportions of dn DSA positive between the TBBx eosinophils (21/38, 55.3%) compared with those without (134/269, 49.8%), $P = 0.60$.

Clinical characteristics at the time of TBBx demonstrating eosinophils versus all those samples without eosinophils are summarized in Table 3. Where a biopsy indication was recorded, there was a significantly greater proportion of diagnostic biopsies in the TBBx eosinophils group at 17/79 (21.5%) compared to those without eosinophils at 587/6644 (8.8%), $P < 0.01$. The median (range) time to first presence of eosinophils was 48.5 (279.8) days. There was a higher proportion of concurrent BAL eosinophilia at the time of TBBx eosinophils (27.7% vs. 1.6%), $P < 0.01$. There was a significant association between TBBx eosinophils and BAL eosinophils with OR 33.6 (95% CI 12.8–88.5), $P < 0.01$. The mean peak blood eosinophil count preceding TBBx was significantly higher in those with eosinophils compared to those without (0.51 vs. 0.17), $P < 0.01$. Concurrent BAL infections are shown in the table. The median (IQR) concurrent A-grade ACR value was 2 (2) in patients with TBBx eosinophils compared with 0 (1) in patients without. There was a significantly greater relative decline from the average and recent baseline in the matched FEV_1 for patients with TBBx eosinophils compared to those without. The proportion of patients prescribed medications associated with pulmonary eosinophilia at the time of TBBx is summarized in Table 3. After adjustment for transplant era, there was a significantly higher proportion of patients administered acetaminophen, antibiotics (penicillins, fluoroquinolone,

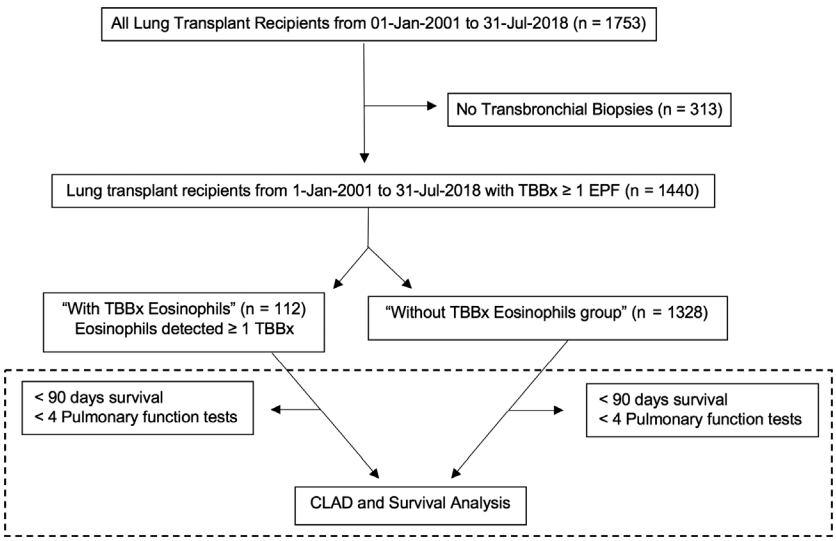

Figure 1 Consort diagram.

Table 1. Baseline patient demographics at the time of transplantation.

Patient Characteristics	With TBBx Eosinophils (n = 112)	Without TBBx Eosinophils (n = 1328)	P value
Mean Recipient Age in years (SD)	46.7 (14.5)	52.3 (14.6)	<0.01
Native lung disease (n, % total)			0.02
COPD	29 (25.9%)	338 (25.5%)	
Cystic fibrosis	30 (26.8%)	212 (16.0%)	
Interstitial lung disease	34 (30.4%)	544 (41.0%)	
Other	19 (17.0%)	234 (17.6%)	
Sex-matching (Recipient/Donor) (n, % total)			0.69
F/F	38 (33.9%)	401 (30.2%)	
F/M	14 (12.5%)	164 (12.3%)	
M/M	13 (11.6%)	206 (15.5%)	
M/F	47 (42.0%)	557 (41.9%)	
CMV match-status (n, % total)			0.40
D−/R−	32 (28.6%)	314 (23.6%)	
R+	53 (47.3%)	738 (55.6%)	
D+/R−	27 (24.1%)	275 (20.7%)	
Mean Donor Age (SD)	43.47 (17.5)	45.14 (16.8)	0.33
Transplant (n, % total)			0.93
First	109 (97.3%)	1284 (96.7%)	
Second	3 (2.7%)	44 (3.3%)	
Type of transplant (n, % total)			0.71
Bilateral	96 (85.7%)	1101 (82.9%)	
Single Left	6 (5.4%)	106 (8.0%)	
Single Right	9 (8.0%)	100 (7.5%)	
Heart/Lung	1 (0.9%)	21 (1.6%)	
Transplantation Era (n, % total)			<0.01
2001–2009	75 (67%)	486 (36.6%)	
2010–2018	37 (33%)	842 (63.4%)	

The chi-square test was used for nominal variables, the two-sample t-test for continuous variables and (n) denotes the number of patients.

Table 2. Recipient characteristics after lung transplantation.

Patient characteristic	With TBBx Eosinophils (n = 112)	Without TBBx Eosinophils (n = 1328)	P value
Cause of death (n/total death, %total death)			<0.01
CLAD	34/60 (54.0%)	153/545 (28.1%)	
Infection	11/60 (17.5%)	127/545 (23.3%)	
Malignancy	6/60 (9.5%)	67/545 (12.3%)	
Cardiovascular	4/60 (6.3%)	13/545 (2.4%)	
Other	8/60 (13.3%)	90/545 (16.5%)	
CLAD Status (n, %total)			<0.01
CLAD	63 (56.3%)	523 (39.4%)	
No CLAD	41 (36.6%)	720 (54.2%)	
Insufficient PFTs	1 (0.9%)	13 (1%)	
Non-CLAD cause of allograft dysfunction	7 (6.3%)	57 (4.3%)	
Not available	0 (0%)	15 (1.1%)	
Patients with recurrent eosinophils on subsequent TBBx, n (% total)	9 (7.8%)	N/A	N/A
Mean A Score (SD)	0.54 (0.37)	0.33 (0.33)	<0.01
Proportion with positive de novo donor-specific antibodies	21/38 (55.3%)	134/269 (49.8%)	0.60

The chi-square test was used for nominal variables, the two sample t-test for continuous variables and (n) denotes the number of patients. A score refers to the sum of all TBBx A-grade components divided by the total number of biopsies.

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Table 3. Patient characteristics based on TBBx sampling occasion for biopsies demonstrating first detection of eosinophils versus all those without.

Recipient characteristics	Biopsies with Eosinophils (n = 112)	Biopsies without Eosinophils (n = 8764)	P value
Median days (Range) to detection of TBBx eosinophils post-transplant	48.5 (24–302.75)	**N/A**	
Proportion of diagnostic biopsies where indication was reported	17/79 (21.5%)	587/6644 (8.8%)	<0.01
Concurrent BAL eosinophilia ≥ 2%	31 (27.7%)	140 (1.6%)	<0.01*
Mean peak peripheral eosinophilia (</=14 days TBBx) ×10e9/L [SD]	0.5 (0.9)	0.2 (0.2)	<0.01*
Significant pathogens identified on BAL microbiology			
Bacteria	20 (17.9%)	1407 (16.1%)	
Fungi	3 (2.7%)	1089 (12.4%)	
Mycobacteria	0 (0%)	189 (2.2%)	
Virus	2 (1.8%)	317 (3.6%)	
Active medications associated with pulmonary eosinophilia	Total n = 111	Total n = 8735	*
Bactrim	94 (84.7%)	7900 (90.4%)	0.65
Azathioprine	67 (60.4%)	3205 (36.7%)	0.27
SSRI Antidepressant	20 (20.7%)	1806 (18.0%)	0.52
Acetaminophen	58 (52.3%)	3180 (36.4%)	<0.01
Macrolide Antibiotic	15 (13.5%)	1476 (16.9%)	0.30
Penicillin Antibiotic	13 (11.7%)	377 (4.3%)	0.03
Fluoroquinolone Antibiotic	12 (10.8%)	778 (8.9%)	0.05
Dapsone	12 (10.8%)	616 (7.1%)	0.36
ACE Inhibitor	10 (9.0%)	894 (10.2%)	0.40
Colistin	8 (7.2%)	210 (2.4%)	0.03
Aspirin	7 (6.3%)	1607 (18.4%)	0.01
SNRI Antidepressant	6 (5.4%)	408 (4.7%)	<0.01
Carbapenem Antibiotic	6 (5.4%)	161 (1.8%)	0.02
Ceftazidime	6 (5.4%)	107 (1.2%)	<0.01
Ranitidine	5 (4.5%)	860 (9.8%)	0.64
Isoniazid	4 (3.6%)	92 (1.1%)	0.54
Mirtazapine	4 (3.6%)	302 (3.5%)	0.57
Vancomycin	3 (2.7%)	114 (1.3%)	0.09
Concurrent A-grade rejection (n, %)			
AX	12 (10.7%)	1543 (17.6%)	
A0	26 (23.2%)	5226 (59.6%)	
A1	21 (18.8%)	1573 (17.9%)	
A2	43 (38.4%)	393 (4.5%)	
A3	7 (6.3%)	27 (0.3%)	
A4	3 (2.7%)	2 (0.0%)	
Concurrent B-grade rejection (n, %)			
Bx	85 (75.9%)	5978 (68.2%)	
B0	20 (17.9%)	2591 (29.6%)	
B1R	7 (6.3%)	195 (2.2%)	
B2R	0 (0.0%)	1 (0.0%)	
Mean FEV_1 %-Change from average post-transplant baseline† (SD)	−14.4 (18.0)	−8.2 (14.4)	0.01
Mean FEV_1 %-Change recent baseline (highest of 2 previous FEV_1) (SD)	−7.0 (13.9)	−2.2 (10.4)	<0.01

The two groups in this table represent dependent, repeated measurement data. For A- and B-grade rejection, percentages are based on the number of evaluable biopsies.

*Mixed-effects models were used to assess significance differences in the two groups for these variables. For active medications analysis, transplant era (2001–2008 vs. 2009–2018) was adjusted for in the final model.

†Defined as the average of the 2 best FEV1 measurements ≥ 3 weeks apart.

Bold values are statistically significant (p<0.05).

ceftazidime, colistin, and carbapenems) and SNRI antidepressants at the time of TBBx eosinophils compared to the TBBx without eosinophils. A significantly greater proportion of patients were administered aspirin in TBBx without eosinophils.

The pattern of eosinophils on TBBx histology was determined for each biopsy and presented in Table 4. Representative histologic images of each pattern are shown in Fig. 2. The eosinophils were airway-predominant in 24/112 (21.4%), parenchymal-predominant in 29/112 (25.9%), perivascular in 56/112 (50%), and mixed pattern in 3/112 (2.7%) case. There were no significant differences in the median (IQR) time to first presence of TBBx eosinophils between these different patterns. The concurrent median A-grade and concurrent administration of an eosinophil-associated medication were both most common in the case of perivascular eosinophils. There were no significant differences in the frequency of concurrent BAL infection frequency based on the pattern of TBBx eosinophils.

Survival analysis

The total number of patients eligible for time-to-death/retransplant analysis was 1440. The overall median (95% CI) survival time was 8.28 (7.32–9.31) years. 42% (605/1440) patients were deceased before the completion of the study follow-up including 60/112 (53.6%) in the TBBx eosinophil group and 545/1328 (41.0%) in the group without. Univariate Cox proportional hazards analysis found an increased risk of death (HR 1.38, 95% CI 1.06–1.79, $P = 0.02$) once eosinophils were detected (for the first time) during follow-up. Multivariable analysis showed that, after adjustment for clinical variables known to affect survival, eosinophil detection was independently associated with reduced survival (HR 1.51,

Table 4. Concurrent clinic-pathologic features for different patterns of eosinophil distribution on TBBx ($n = 109$) including perivascular, parenchymal-predominant, and airway-predominant.

Concurrent Clinicopathologic feature	Perivascular ($n = 56$)	Parenchymal-predominant ($n = 29$)	Airway-predominant ($n = 24$)	*P* value
Median A grade (IQR)	2 (2–2)	1 (0–2)	0 (0–1.5)	<0.01
Clinically significant infection	9/56 (16.1%)	6/29 (20.7%)	6/23 (26.1%)	0.58
Eosinophil-associated drug	42/56 (75.0%)	15/29 (51.7%)	13/24 (54.2%)	0.05
Median days post-transplant (IQR)	45 (172)	176 (515)	51 (295)	0.17
Mean FEV_1 %-Change from Baseline (SD)	−10 (16)	−21 (20)	−17 (18)	0.15
Mean FEV_1 %-Change Recent Baseline (Highest of 2 previous FEV_1) (SD)	−5 (13)	−9 (14)	−7 (13)	0.81

The Kruskal–Wallis nonparametric test was used for continuous variables and the chi-square test for categorical variables. In 3 patients, no defined pattern could be determined.

Bold values are statistically significant (p<0.05).

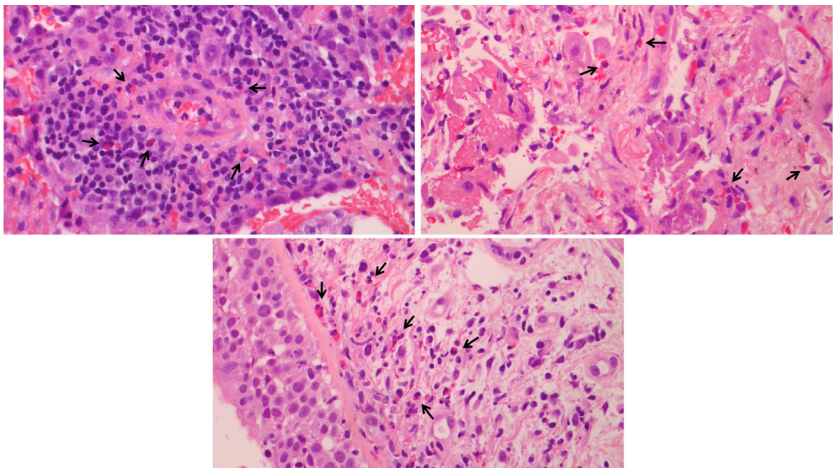

Figure 2 Representative histologic slides for each TBBx Eosinophil biopsy pattern. All images taken at 40X magnification. From left to right: Perivascular, Interstitial, Airway (below) patterns. Black arrows represent eosinophils.

95% CI 1.24–1.85, $P < 0.01$). The relative hazards for all-cause death/retransplant are summarized in Table 5. The total number of patients who died after 2 years was 380 (26.4%), including 43 (38.4%) with TBBx Eosinophils and 337 (25.4%) in the group without. Landmark Kaplan–Meier survival curves were generated for patients with eosinophils detected within 1 year or 2 years versus those without in Fig. 3 and Supplementary Fig. 1. There was a significant difference in survival when comparing patients with and without eosinophils in the first two years ($P = 0.02$).

Chronic lung allograft dysfunction (CLAD)

The total number of patients eligible for CLAD analysis was 1192. In these patients, the median (95%CI) time to CLAD was 6.00 (5.38–6.96) years. In univariate analysis, if eosinophils were detected for the first time during follow-up, the risk of CLAD was significantly increased (HR 1.41, 95% CI 1.04–1.90, p = 0.03). There was no significant interaction between TBBx eosinophils and cumulative A rejection score ($P = 0.36$). In multivariable analysis, after adjustment for clinical variables associated with CLAD, the presence of eosinophils was independently associated with an increased risk of CLAD (HR 1.35, 95% CI 1.07–1.70, $P = 0.01$). Table 6 is a summary of the multivariable Cox proportional hazards for time-to-CLAD. Upon model fitting, we detected a significant correlation between TBBx and BAL eosinophils and, therefore, BAL eosinophils could not be included as a co-variate for multivariable analysis because of collinearity. Landmark Kaplan–Meier CLAD-free survival curves in patients with eosinophils detected within 1 year and 2 years versus those without are shown in Fig. 4 and Supplementary Fig. 2.

Restrictive allograft syndrome (RAS)

Since eosinophils have been associated with RAS in prior publications, we sought to evaluate this association in our dataset. The largest cohort of patients who have been fully phenotyped using the 2019 consensus criteria included recipients transplanted between January 01, 2009, and January 01, 2015. This included 217 patients with CLAD. 29 (13.4%) patients developed RAS/Mixed phenotype. In univariate analysis, the presence of TBBx eosinophils was not significantly associated with an increased risk of RAS/Mixed phenotype (HR 2.45, 95% CI 0.74–8.14), $P = 0.14$, although there was a trend toward increased risk. When assessing patients at 1 and 2 years post-transplant, recipients with TBBx eosinophils had a lower probability of RAS/Mixed CLAD-free survival compared to the recipients without TBBx eosinophils ($P = 0.03$) (Fig. 5 and Supplementary Fig. 3).

Discussion

Our results from a large retrospective cohort of lung transplant recipients show that the detection of eosinophils in TBBx is independently associated with an increased risk of all-cause mortality and CLAD.

Since the earliest era of histopathologic assessment after lung transplantation, TBBx eosinophils have been associated with potentially injurious allograft processes [6–9]. Clelland *et al.* noted bronchiolar and perivascular eosinophilic infiltrates in the TBBx of 21 heart-lung recipients with ACR. A reduction in the size of the infiltrates was observed after administration of pulse corticosteroids [6]. Yousem reviewed 112 TBBx and noted that eosinophils were present in 22%, 78%, and 100% cases with mild, moderate, and severe ACR respectively. In TBBx where> 50% of the infiltrating cells were eosinophils, 5 cases were detected early after transplantation and thought concurrent with ACR. The other 4 cases had established CLAD and concurrent active infection without ACR [7]. In a study of 16 patients with untreated, clinically silent grade A2 ACR, TBBx eosinophils were found more commonly in patients who demonstrated progressive rejection on subsequent biopsies than those without [8]. In a review of 780 TBBx from 91 lung-transplant recipients, the presence of TBBx eosinophils in the parenchyma was associated with later development of histopathologic fibrosis regardless of the rejection grade [11]. In a more recent study, immunohistochemical analysis of 18 explanted lungs with RAS showed a significantly higher number of eosinophils per area in RAS lungs compared with 19 explants with bronchiolitis obliterans syndrome (BOS) and 22 controls [18]. To our knowledge, our study is the largest cohort report of eosinophils in TBBx detected in recipients after lung transplantation and their relation to patient characteristics and long-term outcomes. In our study, patients in the TBBx eosinophils group had a higher average A-grade rejection score but similar detection of donor-specific antibodies, compared to the no eosinophils group.

Eosinophils detected in bronchoalveolar lavage (BAL) from lung transplant patients have been the focus of previous analyses in the literature: Unlike TBBx eosinophils, BAL eosinophilia has not been shown to be specific to ACR [25–27]. One study demonstrated that BAL

Table 5. Relative hazards for all-cause death or retransplant in a time-dependent Cox regression model.

Variable	Univariate hazard ratio (95% CI)	Univariate *P* value	Multivariate hazard ratio (95% CI)	Multivariate *P* value
Eosinophils on TBBx	1.38 (1.06–1.79)	0.02	1.51 (1.24–1.85)	<0.01
Recipient age (per 5-unit change)	1.05 (1.02–1.08)	<0.01	1.02 (0.99–1.05)	0.22
Donor age (per 5-unit change)	1.02 (0.99–1.04)	0.15	1.01 (0.99–1.03)	0.17
Recipient sex (Male)	1.18 (1.00–1.39)	0.05		
Single and heart/lung transplant	1.33 (1.07–1.66)	<0.01	1.29 (1.08–1.53)	<0.01
Native lung disease				
Cystic fibrosis	0.87 (0.66–1.15)	0.34	0.85(0.67–1.07)	0.16
Interstitial lung disease	1.23 (0.97–1.56)	0.08	1.03 (0.84–1.26)	0.77
COPD	1.20 (0.94–1.54)	0.15	1.03 (0.84–1.26)	0.76
Other	Reference level			
Sex Matching (Recipient/Donor)				
F/M	1.14 (0.87–1.49)	0.35	1.18 (0.96–1.45)	0.11
M/F	1.20 (0.94–1.53)	0.14	1.09 (0.90–1.32)	0.36
M/M	1.24 (1.02–1.50)	0.03	1.19 (1.02–1.38)	0.03
F/F	Reference level			
CMV Serostatus				
D+/R− (Mismatch)	1.90 (1.50–2.41)	<0.01	1.93 (1.62–2.31)	<0.01
R+	1.25 (1.01–1.54)	0.04	1.12 (0.95–1.31)	0.17
D−/R−	Reference level			
Era of Transplantation				
After 2009	0.82 (0.68–0.97)	0.02	0.78 (0.68–0.89)	<0.01
Before 2009	Reference level			
Cumulative A rejection score (per 1-unit change)	1.08 (0.86–1.35)	0.53	0.93 (0.77–1.12)	0.44
Cumulative BAL Eosinophil-positive score (per 0.1-unit change)	1.06 (0.96–1.16)	0.25		
Cumulative Infection Score (per 0.1-unit change)	1.15 (1.06–1.25)	<0.01	1.07 (0.98–1.16)	0.15

Relative hazards for death or retransplant for lung transplant recipients 2001–2018 expressed as hazard ratios (95% CI). Univariate analysis is presented on the left representing the relative hazard contribution of unadjusted variables. Multivariate analysis is presented on the right demonstrating the hazard contributions for adjusted variables. The standardized cumulative scores for infection, rejection, and cytology were calculated at the time of each measurement as the sum of all positive events (or A grades for rejection) up to this time point, divided by the total number of sampling occasions. Recipient sex was not included for multivariate assessment because of the correlation with Sex Matching. BAL Eosinophil Cytology score was not added for multivariate analysis given the correlation with TBBx Eosinophils.

eosinophilia, with a quantitative cut-off ≥ 2%, independently predicted both overall survival and the development of CLAD [16]. Importantly, BAL eosinophilia and peripheral blood eosinophils have both been associated with the restrictive phenotypes of CLAD and post-restrictive-CLAD survival [16,17]. In our study, concurrent BAL eosinophilia was detected in only 31/112 (27.7%) of patients with TBBx eosinophils. In our cohort, BAL eosinophilia ≥ 2%, when measured as a cumulative score, was not associated with death or CLAD in univariate analyses. In a moderate-sized cohort of CLAD phenotyped patients by 2019 consensus criteria, we observed an association between the presence of TBBx and the RAS and Mixed CLAD phenotypes which was not statistically significant in Cox regression analysis. This association should be further verified in more definitive cohorts but may suggest a potential role for TBBx eosinophils in the pathogenic pathways leading to parenchymal allograft fibrosis.

There are several potential etiologies for TBBx eosinophils in lung transplant recipients, in addition to rejection processes. In biopsies with TBBx eosinophils, there was concurrent positive BAL bacterial infection in 20/112 (17.9%) cases. It has been demonstrated that the expression of bactericidal, oxygen-dependent, cytoplasmic granules are important anti-bacterial effector functions of eosinophils [28–31]. Eosinophils appear to be involved in host responses to fungi and respiratory viral

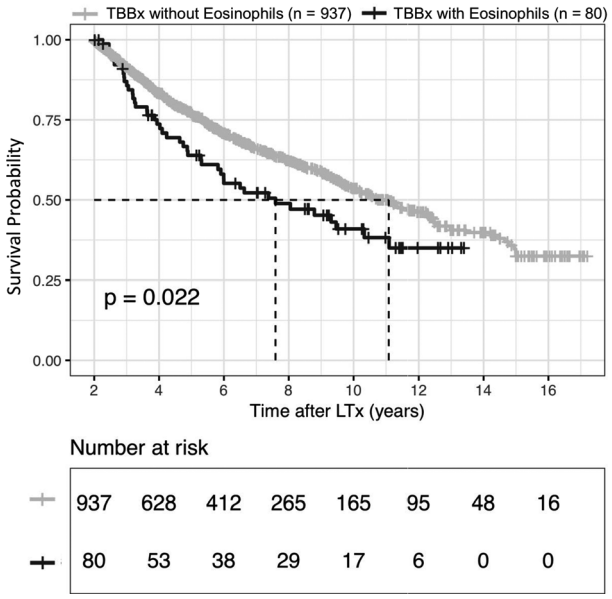

Figure 3 Kaplan–Meier survival curves for patients with TBBx eosinophils detected in the first two years versus those without. Curves compared using the Log rank test. Dashed lines indicate median survival in each group.

infections; however, we did not observe high frequencies of these pathogens concurrent with TBBx eosinophils [32–34]. We found that the association between TBBx eosinophils and graft loss was independent of the cumulative BAL infection score. Clarification of the interaction between eosinophilic inflammation in pulmonary allografts and infection should be a focus of future research. Differences were observed in the proportion of patients prescribed medications associated with pulmonary eosinophilia at the time of TBBx. Acetaminophen and antibiotics are commonly prescribed for patients with acute lung allograft dysfunction; however, drug-induced eosinophilic graft inflammation may be an inciting event for further injurious allograft processes. We do not expect SNRI anti-depressants to be more commonly prescribed during acute lung allograft dysfunction and clinicians should be alert to this drug association. The mainstay of treatment for eosinophilic drug reactions is modification of the regimen, with cessation or substitution of the culprit medication undertaken, when it is considered clinically safe to do so.

We observed that the frequency of TBBx eosinophil detection was higher in the earlier era of transplantation. There was a significant difference in the median (IQR) number of TBBx per patient performed in 2001–2009 at 7 (2) compared with 2010–2018 at 6 (4) ($P < 0.01$). We believe that the difference between 7 and 6 biopsies per patient in each transplant era is not clinically significant for our analysis. Importantly, however, transplant era was included in the final multivariable models as a potential confounder and the association between TBBx eosinophils and long-term outcomes remained independent despite this co-variate. Without re-reviewing all 8887 biopsies acquired over the 20 years included in this study, we cannot rule out a variation in reporting. Changes in immunosuppression may explain differences in TBBx eosinophil frequency over time. A peri-operative desensitization protocol for highly sensitized lung transplant recipients, which includes anti-thymocyte globulin, was introduced in the latter era. A higher proportion of patients with TBBx eosinophils (60.4%) were administered azathioprine compared to those without (36.7%); however, this difference was dependent on the transplant era. In the latter transplant era, there has also been a shift from azathioprine to mycophenolate as the predominant cell-cycle inhibitor. A switch from cyclosporine to tacrolimus has been increasingly used in our program for early CLAD onset. Greater immunosuppression may relate to a decreased presence of TBBx eosinophils.

Three major patterns of TBBx eosinophilia were identified in this study including airway-associated, parenchymal-predominant, and perivascular. The perivascular location of TBBx eosinophils suggested that they were likely part of the ACR lesions in these biopsies. A significantly higher concurrent median A grade was noted for TBBx eosinophils with a perivascular location compared with airway- and parenchymal-predominant patterns. We initially hypothesized that the effect of overall presence of eosinophils on graft survival would be dependent on ACR. Multivariable analysis, however, confirmed that this association is independent and, as such, the presence of TBBx eosinophils appears to provide additional information above that of ACR alone. No specific pattern was associated with significantly higher frequency of concurrent BAL infection. The numbers of patients in each pattern group were small which precluded further analysis with long-term outcomes. The findings of this study provide evidence for a potential value in the systematic reporting of the gradation and pattern of TBBx eosinophils, and future studies should assess the effect of such reporting on outcomes.

Regardless of the cause of the eosinophilic inflammation, the downstream effects may be similar. We showed that there was greater associated functional impairment of the allograft in TBBx with eosinophil, as measured by FEV1 %-change, as compared to TBBx without eosinophils. These cells may represent surrogate markers of

Table 6. Relative hazards for time-to-CLAD in a time-dependent Cox regression model.

Variable	Univariate hazard ratio (95% CI)	Univariate *P* value	Multivariate hazard ratio (95% CI)	Multivariate *P* value
Eosinophils detected on TBBx	1.41 (1.04–1.90)	0.03	1.35 (1.07–1.70)	0.01
Recipient Age (per 5-unit change)	0.97 (0.95–1.00)	0.08	0.94 (0.91–0.95)	<0.01
Donor age (per 5-unit change)	1.02 (1.00–1.05)	0.07	1.04 (1.01–1.06)	<0.01
Recipient sex (Male)	1.12 (0.94–1.33)	0.22		
Native lung disease				
Cystic fibrosis	1.19 (0.94–1.58)	0.13	0.99 (0.79–1.24)	0.92
Interstitial lung disease	1.01 (0.78–1.31)	0.95	1.11 (0.90–1.38)	0.34
COPD	1.22 (0.94–1.58)	0.13	1.33 (1.07–1.65)	0.01
Other	Reference level			
Sex matching				
F/M	1.15 (0.86–1.53)	0.35	1.08 (0.86–1.35)	0.51
M/F	0.98 (0.74–1.29)	0.88	1.00 (0.80–1.23)	0.97
M/M	1.24 (1.01–1.52)	0.04	1.25 (1.07–1.46)	0.01
F/F	Reference level			
CMV Sero-status				
R+	1.37 (1.10–1.71)	<0.01	1.43 (1.21–1.70)	<0.01
D+/R−	1.46 (1.12–1.91)	0.01	1.49 (1.22–1.82)	<0.01
D−/R−	Reference level			
Era of transplantation				
After 2009	0.78 (0.65–0.94)	<0.01	0.80 (0.70–0.92)	<0.01
Before 2009	Reference level			
Cumulative A rejection score	1.20 (0.93–1.54)	0.17	1.09 (0.89–1.33)	0.40
Cumulative BAL Eosinophil-Positive Cytology Score (per 0.1-unit change)	1.11 (0.99–1.25)	0.07		
Cumulative Infection Score (per 0.1-unit change)	1.03 (0.91–1.16)	0.68	1.03 (0.94–1.13)	0.48

Relative hazards for time-to-CLAD for lung transplant recipients 2001–2018 expressed as hazard ratios (95% CI). Univariate analysis is presented on the left representing the relative hazard contribution of unadjusted variables. Multivariate analysis is presented on the right demonstrating the hazard contributions for time-to-CLAD for adjusted variables. Recipient sex was not included for multivariate assessment because of the correlation with sex matching. BAL Eosinophil Cytology score was not added for multivariate analysis given the correlation with TBBx Eosinophils.

injurious inflammatory processes, immunomodulatory actors in tolerance and repair, or end-stage effectors that trigger pathogenic pathways toward CLAD [35,36]. Specifically, eosinophil-induced airway epithelial cell injury may represent an inciting trigger for CLAD [37]. Eosinophil cationic protein stimulates the release of the profibrotic cytokine, TGF-β1, and attracts fibroblasts in vivo [38,39]. Eosinophil granules, including major basic protein are capable of causing tissue damage and dysfunction via increased membrane permeability and ciliary damage [40–42]. We noted a significantly higher proportion of patients with TBBx eosinophils were transplanted for cystic fibrosis. We hypothesize that stronger age-related innate immune responses, including degranulation response to IL-5 stimulation, may account for a higher prevalence of TBBx in this younger cohort [43]. Future studies should be designed to establish the potential mechanistic role of eosinophils in the development of CLAD.

There are several limitations to this study. TBBx eosinophils were reported at the discretion of seven pulmonary pathologists over the 17-year study period. They were mentioned if the cells were present in large quantities (>2–3 per high power field) or in a pattern which assisted with clinical interpretation of the inflammatory process. Given the absence of a standardized pathologist reporting scheme for TBBx eosinophilia, selection bias may have distorted the accurate characterization of our patient and biopsy groups. It was not feasible to re-review all TBBx to determine the gradation and pattern of TBBx eosinophils and we cannot rule out variation in reporting. Our results do however reflect real-world reporting. Based on the results of the large number of biopsies in this retrospective study,

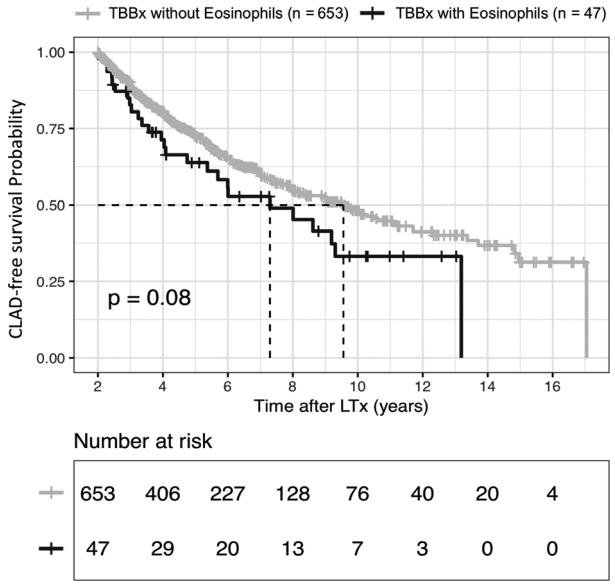

Figure 4 Kaplan–Meier CLAD-free survival for patients with eosinophils in the first two years versus those without. Curves compared using the Log rank p test. Dashed lines indicate median CLAD-free survival. Death and lung allograft dysfunction without CLAD were analyzed as censoring events.

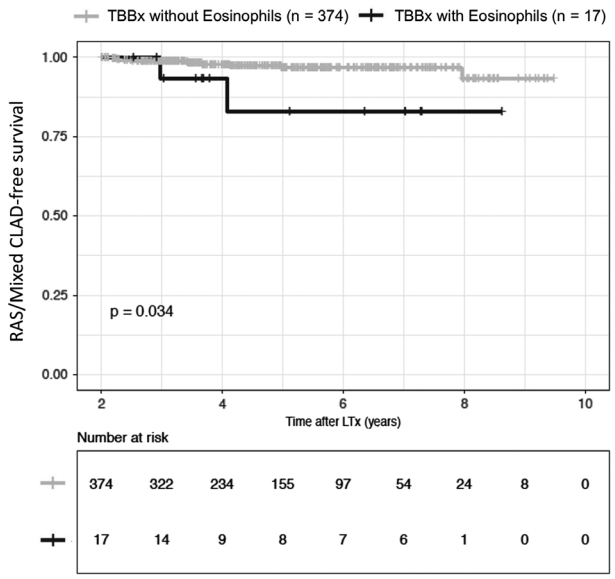

Figure 5 Kaplan–Meier RAS/Mixed CLAD-free survival for patients with eosinophils in the first two years versus those without. Curves compared using the Log rank p test. Death, lung allograft dysfunction without CLAD and 'Other' CLAD phenotype were analyzed as censoring events.

there is a need to implement and prospectively validate a systematic TBBx eosinophil reporting scheme. It was not possible to retrospectively determine the concurrent steroid dose in 8887 biopsies. The nature of concurrent corticosteroid administration on the presence, gradation, and pattern of TBBx eosinophils remains unknown. Our study design allows only for identification of predictive factors associated with poor patient outcomes. The focus of future, prospective studies should be designed to establish the causative mechanisms for reduced survival in patients with TBBx eosinophilia to identify treatment targets for these individuals.

Conclusion

The detection of eosinophils on transbronchial biopsies is independently associated with an increased risk of patient or graft death and CLAD after lung transplantation. There may be additional benefit in the systematic reporting of these. The exact causative mechanism and pathogenic roles of eosinophils remain unknown.

Authorship

DRD: contributed to the study design, data acquisition, analysis, and writing of the manuscript. JM, EH, and GT: participated in data analysis. PF, LL, DMH, WK, RZ, and SK: made substantial contributions to acquisition, analysis, and interpretation of data. LGS and JMT: participated in research design, supervision, and manuscript writing. TM supervised the project regarding research design, data collection, data analysis, manuscript writing, and final approval of the manuscript. All authors agree to be accountable for all aspects of the work relating to accuracy and integrity.

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Conflict of interest

Dr Pierre Fiset reports personal fees from Roche Canada, Pfizer Canada, Astra Zeneca Canada, and Merck Canada, all of which are outside the submitted work. There are no planned, pending or issued patents relevant to the work. There are no other conflicts to report.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Kaplan-Meier survival curves for patients with TBBx eosinophils detected in the first year versus those without.

Figure S2 Kaplan-Meier CLAD-free survival for patients with eosinophils in the first year versus those without.

Figure S3 Kaplan-Meier RAS/Mixed CLAD-free survival for patients with eosinophils in the first year versus those without.

Table S1 Eosinophils were detected in the following order of biopsies, counting from the time of transplant.

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