ORIGINAL ARTICLE

Immunological diversity in phenotypes of chronic lung allograft dysfunction: a comprehensive immunohistochemical analysis

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SUMMARY

Chronic rejection after organ transplantation is defined as a humoral- and cell-mediated immune response directed against the allograft. In lung transplantation, chronic rejection is nowadays clinically defined as a cause of chronic lung allograft dysfunction (CLAD), consisting of different clinical phenotypes including restrictive allograft syndrome (RAS) and bronchiolitis obliterans syndrome (BOS). However, the differential role of humoral and cellular immunity is not investigated up to now. Explant lungs of patients with end-stage BOS ($n = 19$) and RAS ($n = 18$) were assessed for the presence of lymphoid (B and T cells) and myeloid cells (dendritic cells, eosinophils, mast cells, neutrophils, and macrophages) and compared to nontransplant control lung biopsies ($n = 21$). All myeloid cells, with exception of dendritic cells, were increased in RAS versus control (neutrophils, eosinophils, and mast cells: all $P < 0.05$, macrophages: $P < 0.001$). Regarding lymphoid cells, B cells and cytotoxic T cells were increased remarkably in RAS versus control ($P < 0.001$) and in BOS versus control ($P < 0.01$). Interestingly, lymphoid follicles were restricted to RAS ($P < 0.001$ versus control and $P < 0.05$ versus BOS). Our data suggest an immunological diversity between BOS and RAS, with a more pronounced involvement of the B-cell response in RAS characterized by a structural organization of lymphoid follicles. This may impact future therapeutic approaches.

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Introduction

Chronic rejection has been the Achilles' heel of transplantation medicine and a major focus of transplant immunologist's research across different organs [1]. The definition of chronic rejection, set by pioneers including Sir Peter Medawar and Sir Edward Donnall Thomas, was a chronic humoral- and cell-mediated response of the recipient leading to irreversible tissue fibrosis, failure of the organ and eventually graft loss

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[2]. Clinical transplant physicians, however, have been in search for a practical and accurate clinical correlate of chronic rejection. In lung transplantation (LTx), chronic rejection is clinically described by the term chronic lung allograft dysfunction (CLAD), defined by an irreversible decline in forced expiratory volume in 1 s (FEV_1) of at least 20% compared to the mean of the two best postoperative values and for which no other cause can be identified. Within CLAD, two major clinical phenotypes of chronic rejection were recently defined, being the classical bronchiolitis obliterans syndrome (BOS) and a newly identified restrictive allograft syndrome (RAS) [3]. BOS is believed to be the clinical correlate of obliterative bronchiolitis (OB), which is the typical pathological finding in such lungs. Current belief is that the origin of OB is a trigger causing damage to the epithelial bronchial wall, causing infiltration of mononuclear cells in the airway wall and proliferation of mesenchymal cells. This ultimately results in intimal thickening, epithelial damage, and fibrotic occlusion of the airway lumen leading to progressive airway obstruction, explaining the irreversible decline in pulmonary function [4,5]. Conversely, RAS is histologically characterized by pleural/septal thickening and pleuroparenchymal fibrosis in combination with a restrictive lung function defect, although OB is also found in the majority of cases. Compared to BOS (3-5 years), RAS patients experience a worse prognosis limited to about 1-1.5 years after diagnosis [3,6] because efficient therapeutic treatment is lacking. Further mechanistic insight might lead to new targets which are needed to improve long-term outcome after CLAD diagnosis.

Both innate and adaptive immunity are believed to be involved in chronic rejection. Different risk factors such as smoking, air pollution, bacteria, viruses, gastroesophageal reflux, and ischemia injury induce an innate immune response, leading to an influx of neutrophils causing damage to the airway epithelium [7,8]. A repetitive (chronic) injury can induce an adaptive immune response leading to excessive airway remodeling and eventually airway fibrosis [9]. Historically, lung transplant rejection has been regarded as a predominantly T-cell-mediated process where immunosuppressive therapy is used to target T-cell proliferation and function [10]. Nowadays, emerging data suggest that humoral immunity plays an important role in the development of chronic rejection [11–13]. Our aim was to investigate the immunological differences in lungs of patients diagnosed with end-stage BOS and RAS.

Materials and methods

Study design

Patients' inclusion was based on a retrospective diagnosis of BOS and RAS evidenced by histopathology in combination with spirometry and radiology (CT scan) data at the moment of retransplantation, death or rarely in case of autopsy or video-assisted thoracic surgery (VAT)-biopsy. End-stage lung biopsies, retrieved by the lung pathology department, were analyzed for BOS ($n = 19$) and RAS patients ($n = 18$). All included patients were transplanted between 2000 and 2011, and the last retrieved explant biopsy was at the end of 2014. Nontransplant control biopsies ($n = 22$) were obtained during autopsies of patients without any underlying lung disease ($n = 10$) or nondiseased lung tissue obtained from resections in lung carcinoma patients with a normal spirometry ($n = 12$) (Fig. 1, Tables S1–S3). No infections were present at the time of sampling, as excluded per standard diagnostic evaluation/culture for bacterial, viral or fungal infection. All patients gave informed consent for biobanking of tissue for scientific research. This study and the use of the human material were approved by the Ethics Committee of the Leuven University Hospitals (S57742) and the local Biobank Board (S51577).

Patient characteristics

Patients' characteristics included gender, age, type of LTx (single or bilateral), underlying disease and immunosuppressive therapy. Airway colonization was defined as repeated detection of a micro-organism in BAL fluid during follow-up, without clinical symptoms such as fever and persistent infiltrates [14] Respiratory infection was defined as a pulmonary infection (viral, bacterial or fungal), with clinical complaints such as dyspnea or fever requiring treatment. CLAD was diagnosed as a persistent decline in FEV_1 of at least 20% compared to the two best postoperative values and was attributed to chronic rejection when no other cause could be identified. A further subdivision was made between BOS and RAS: if total lung capacity (TLC) was available, RAS was diagnosed by a decrease in TLC $\geq$10% in combination with a 20% decrease in FEV_1. If TLC was unavailable, a FEV_1/FVC ratio >0.70 in combination with persistent infiltrates on CT and a persistent decrease in FEV_1 and forced vital capacity (FVC) was considered as RAS. The last available CT scans before reLTx or

death were scored for air-trapping, bronchial dilatation, centrilobular nodules, airway wall thickening, consolidation, ground glass opacities, pleural thickening, reticular pattern, and volume loss by a radiologist with expertise in lung transplantation (JV) using the Fleischner Society guidelines, as previously described [15]. Pathological findings were assessed in collaboration with an experienced lung pathologist (EKV). Both CT and pathology readers were blinded to study results and patients' diagnosis. Anti-human leukocyte antigen antibodies (HLA) were detected by Luminex according to the institutions' protocol and were classified as either positive or negative. In general, an anti-HLA screening result was considered negative if the median fluorescence intensity (MFI) was <500 or positive when MFI $\geq$ 500. A positive screening was retested to identify the HLA sensitivity and the presence or absence of donor-specific antibodies (DSA). Additional supporting information may be found in the online supplement.

Immunohistochemistry

From each biopsy, 10-μm-thick paraffin sections were prepared and stained for T helper cells (CD4), cytotoxic T cells (CD8), B cells (CD20), dendritic cells (CD1a and CD207), eosinophils (EG-2), mast cells (tryptase), neutrophils (MPO), and macrophages (CD68). Extra details are provided in Table S4.

Myeloid cells (eosinophils, dendritic cells, neutrophils, macrophages, and mast cells) were quantified in 30 random high-power fields equally divided into three compartments: around airways, in parenchyma and around blood vessels (peripheral pulmonary arterioles). Cells were expressed as number of positive cells per high-power fields, using a 200× magnification (or 400× for the eosinophils), within the three compartments or as an average of the different compartments. Quantification was different for lymphoid B and T cells, all scattered and aggregated cells (grouped as a follicle) were summarized and results were normalized over the total area of the section. The localization of follicles (airway, parenchyma, and blood vessels) was taken into account for analysis between control, BOS, and RAS. Staining reliability and quality was verified by a skilled pathologist (EKV). For reliability assessment, myeloid cell counting of nine patients (randomly three biopsies per group) was independently performed by another skilled observer (EV and EL). For lymphoid cell measurements, all control patients (n = 22) were similarly repeated by the second author (EL).

Statistical analysis

Results were expressed as the median and interquartile range (IQR) or mean (±SD). For discrete data, a chi-square test was used to assess significance. Significances between the three groups were tested by Kruskal–Wallis ANOVA in combination with Dunn's *post hoc* test correcting for multiple testing. Mann–Whitney U-test was performed when analyzing two groups. A P-value <0.05 was considered significant (GraphPad Prism 4.0, CA, USA). Intraobserver and interobserver reliability was evaluated by a Bland–Altman plot [16].

Results

Patient characteristics

Table 1 demonstrates that the nontransplant control patients were older ($P < 0.0001$), but that there were otherwise no differences in gender ($P = 0.51$), underlying disease ($P = 0.36$), type of transplantation ($P = 0.16$) between BOS and RAS. Following diagnosis of RAS, patients proceeded faster to retransplantation/death compared to BOS ($P = 0.035$, Fig. 1). No

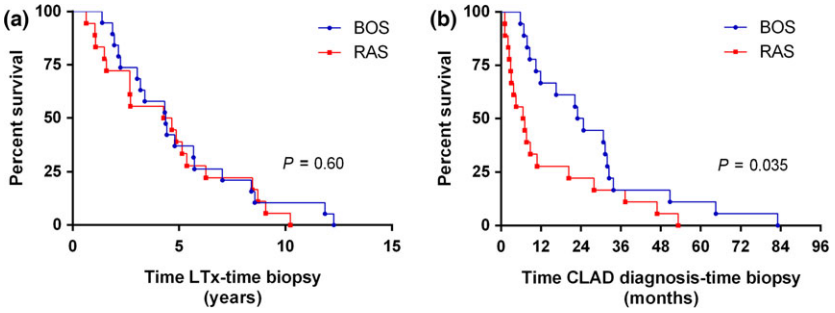

Figure 1 Mortality rate of BOS versus RAS. (a) Kaplan–Meier survival curve demonstrating the mortality rate of BOS versus RAS between moment of LTx and death. (b) Kaplan–Meier survival curve demonstrating the mortality rate of BOS versus RAS between diagnosis (BOS/RAS) and death. A P-value <0.05 was considered significant.

significant difference was seen for immunosuppressive therapy, except for higher daily steroid dose in RAS compared to BOS ($P = 0.039$). More RAS patients tended to receive montelukast compared to BOS ($P = 0.065$). No differences could be demonstrated for previous episodes of acute rejection, lymphocytic

Table 1. Patient characteristics.

	Control	BOS	RAS	*P*-value
Number of patients, *n*	22	19	18	
Female gender, *n* (%)	10 (45%)	11 (58%)	7 (39%)	0.51
Age (years)	61 (56–67)	41 (27–49)	38 (20–53)	**<0.0001**
Indication for LTx, *n* (%)				0.36
Emphysema, α-1ATD	NA	2 (11%)	6 (33%)	
Pulmonary fibrosis	NA	3 (15%)	2 (11%)	
CF/Bronchiectasis	NA	9 (47%)	4 (22%)	
Eisenmenger/PAH	NA	2 (11%)	3 (17%)	
Others (e.g. sarcoidosis, LAM)	NA	3 (16%)	3 (17%)	
Type LTx (SS-S), *n* (%)	NA	17 (89%)-2 (11%)	18 (100%)-0 (0%)	0.16
Pulmonary function at end-stage (absolute value)				
FEV_1 (%Pred)	NA	20 (17–26)	26 (19–36)	**0.021**
FEV_1 (L)	NA	0.60 (0.53–0.85)	0.85 (0.63–1.27)	**0.027**
FVC (%Pred)	NA	52 (42–59)	33 (25–38)	**<0.001**
FVC (L)	NA	1.88 (1.35–2.40)	1.42 (0.99–1.69)	**0.016**
FEV_1/FVC (%)	NA	33 (30–40)	76 (56–91)	**<0.0001**
TLC (%Pred)	NA	97 (87–110)	70 (58–93)	**0.006**
TLC (L)	NA	5.99 (4.78–6.42)	4.18 (3.48–4.99)	**0.019**
Pulmonary function at end-stage (relative to baseline)				
FEV_1 (%Best)	NA	22.2 (18.9–28.1)	28.1 (22.6–43.2)	**0.017**
FVC (%Best)	NA	57.6 (44.7–66.5)	32.1 (28.6–44.6)	**<0.001**
FEV_1/FVC (%Best)	NA	42.5 (35.6–50.9)	96.5 (69.2–107.2)	**<0.0001**
TLC (%Best)	NA	96.6 (80.1–100.0)	71.8 (60.8–98.2)	**0.021**
Treatment, *n* (%)				
AZA-MMF–everolimus–none	NA	5 (26%)-10 (53%)-0 (0%)-4 (21%)	7 (39%)-8 (44%)-2 (11%)-1(6%)	0.23
Tacrolimus–cyclosporine	NA	19 (100%)-0 (0%)	18 (100%)-0 (0%)	1.00
Steroid dose (mg)	NA	4.5 (4.0–12.0)	10.0 (7.0–27.5)	**0.039**
Azithromycin	NA	18 (95%)	18 (100%)	0.32
Montelukast	NA	8 (42%)	13 (72%)	0.065
Donor-specific anti-HLA antibodies, *n* (%)				0.039
Present	NA	1 (5%)	6 (33%)	
Absent	NA	13 (68%)	9 (50%)	
Not determined	NA	5	3	
Time between LTx and biopsy (years)	NA	5.1 (±0.7)	4.3 (±0.7)	0.43
Acute rejection history (*n*)				
Any acute rejection (AR)	NA	12 (67%)	10 (56%)	0.49
Severe AR(≥A2)	NA	4 (22%)	7 (39%)	0.28
Any lymphocytic bronchiolitis (LB)	NA	10 (56%)	12 (67%)	0.49
Severe LB (≥B2)	NA	5 (28%)	4 (22%)	0.38
Infection				
Respiratory	NA	11 (61%)	6 (33%)	0.10
Fungal	NA	2 (11%)	4 (22%)	0.31
Viral	NA	0	1	0.37
Colonization				
Pseudomonads	NA	4 (22%)	8 (44%)	0.15
Candida	NA	1 (6%)	0 (0%)	0.31
Aspergillus	NA	0 (0%)	0 (0%)	
GERD	NA	7 (39%)	4 (22%)	0.28

Table 1. Continued.

	Control	BOS	RAS	*P*-value
Cytomegalovirus status				0.25
Donor+/recipient+	NA	4 (22%)	4 (22%)	
Donor+/recipient−	NA	5 (28%)	4 (22%)	
Donor−/recipient+	NA	3 (17%)	0 (0%)	
Donor−/recipient−	NA	6 (33%)	10 (56%)	

LTx, lung transplantation; SS, sequential single-sided LTx; S, single-sided LTx; α-1ATD, alpha-1-antitrypsin deficiency; CF, cystic fibrosis; LAM, lymphangioleiomyomatosis; PAH, pulmonary arterial hypertension; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; TLC, total lung capacity; MMF, mycophenolate mofetil; AZA, azathioprine; NA, not applicable. Results are shown in numbers (percentage) or as mean ± SEM or as median (IQR). The *P*-value displayed on the right shows the results of the Kruskal–Wallis ANOVA or Mann–Whitney in case of continuous data. In case of discrete data, the results of the contingency table are shown. A *P*-value <0.05 was considered significant and is indicated in bold.

bronchiolitis, infection, and airway colonization (Table 1). While all patients demonstrated a decrease in FEV_1, RAS patients showed a higher FEV_1/FVC index ($P < 0.0001$) and lower TLC %predicted ($P = 0.006$) compared to BOS. Donor-specific anti-HLA antibodies were more prevalent in RAS compared to BOS ($P = 0.039$). Pathological reports in all RAS patients demonstrated extensive pleuroparenchymal fibrosis, septal thickening, and also obliterative bronchiolitis as demonstrated previously [17,18]. No signs of diffuse alveolar damage (DAD) could be demonstrated. Radiologically, BOS patients showed more severe airway wall thickening while RAS patients were characterized by more severe ground glass opacities, pleural thickening, reticular pattern, and volume loss (Table 2). A detailed description of every included patient is demonstrated in Tables S2 and S3.

Histological quantification and localization of myeloid cells on end-stage CLAD lung biopsies

Representative H&E stainings of control, BOS, and RAS are illustrated in Fig. 2 to demonstrate the histological characteristics. Separate results of the three compartments (airway, parenchyma, and blood vessels) and the average of the different compartments are summarized in Table 3. In general, myeloid cell types were more prevalent around the airways compared to parenchyma or around blood vessels (Table S5). Total neutrophils were increased in RAS compared to control, but were not increased in BOS versus control. Within the airway compartment, more neutrophils were present both in BOS ($P < 0.001$) and in RAS ($P < 0.001$) versus control. Total eosinophils were increased in RAS compared to control, but were not increased in BOS. The increased eosinophilia in RAS was primarily located in the parenchyma ($P < 0.05$) and around blood vessels ($P < 0.01$). Mast cells were increased in RAS versus control, specifically in the parenchyma ($P = 0.020$) and around blood vessels ($P = 0.031$, ANOVA).

Macrophages were more abundant in RAS compared to control ($P < 0.001$) and BOS ($P < 0.01$) in every compartment. More CD1a dendritic cells were present within the parenchyma in RAS versus control and BOS (respectively, $P < 0.001$ and $P < 0.05$). Resident mucosal (langerin positive) dendritic cells were increased in the parenchyma in RAS compared to control but, in contrast, were decreased around the airways in RAS compared to control ($P < 0.01$) (Table 3A and representative staining in Fig. 3a). Bland–Altman analysis demonstrated no differences regarding intra- and inter-observer reliability of myeloid cell quantification (Fig. S1).

Histological quantification and localization of lymphoid cells and structures

T helper ($CD4^+$) lymphocytes demonstrated significant variation between groups ($P = 0.032$); however, no individual differences between groups could be demonstrated. Cytotoxic ($CD8^+$) T lymphocytes were increased in both RAS and BOS compared to control ($P < 0.001$ and $P < 0.01$, respectively). Individual B cells were increased in RAS and BOS compared to control ($P < 0.001$ and $P < 0.01$) (Table 3B and Fig. 3b). Secondary lymphoid structures or follicles were almost exclusively present in RAS and not in BOS or control ($P < 0.05$ and $P < 0.001$, respectively) (Table 3B). These follicles were predominantly localized around the blood vessels and in the parenchyma and less frequent around the airways ($P < 0.001$). Bland–Altman analysis

Table 2. Radiologic characteristics. MDCT scans were scored for air-trapping, bronchial dilatation, centrilobular nodules, airway wall thickening, consolidation, ground glass opacities by an experienced radiologist blinded for the clinical histological data. Results were expressed as median (IQR). The *P*-value displayed on the right shows the results of the Mann–Whitney U-test. A *P*-value <0.05 was considered significant and is indicated in bold.

	BOS	RAS	*P*-value
Number of patients	19	18	
Expiratory *in vivo* CT			
Air-trapping (%)	77 (28–83)	39 (18–83.3)	0.36
Inspiratory *in vivo* CT			
Bronchus dilation (%)	31 (11–72)	28 (11–47)	0.38
Centrilobular nodules (%)	3 (0–17)	0 (0–3)	0.16
Airway wall thickening (%)	44 (17–64)	11 (6–33)	**0.004**
Consolidation (%)	0 (0–11)	36 (17–59)	**<0.0001**
Ground glass (%)	0 (0–17)	31 (14–44)	**0.002**
Pleural thickening (%)	11 (0–22)	64 (31–83)	**<0.0001**
Reticular pattern (%)	0 (0–9)	20 (9–47)	**<0.001**
Volume loss (%)	0 (0–14)	31 (9–50.2)	**<0.001**

demonstrated no differences regarding intra- and inter-observer reliability of lymphoid cell quantification (Fig. S1).

Discussion

Our findings demonstrate that the recently described RAS phenotype of CLAD displays a prominent cellular and humoral immune involvement. Although cellular adaptive immunity is present, it seems that the humoral component with presence of organized lymphoid follicles is more specific in RAS. These data demonstrate an immunological diversity within the two different phenotypes of chronic rejection.

In the current analysis, we focused on cells which are directly linked to innate and adaptive immunity. In general, effectors of the myeloid arm of the innate immune response being neutrophils, macrophages, eosinophils, and mast cells [2,9,19] were increased in RAS compared to control lungs or BOS. Historically, neutrophils are considered to be a predictive biomarker for BOS [20], and in this study, we showed that an increase in the number of neutrophils is more prominent in RAS lungs. Activated neutrophils release reactive oxygen species and proteolytic enzymes which cleaves elastin and collagen within the lung matrix, resulting in tissue damage. Recently, we showed that broncho-alveolar lavage eosinophilia ≥2% was associated with a higher incidence of CLAD and more specifically RAS and mortality [19]. Our current investigations confirm the presence of eosinophils in RAS and therefore increase the body of evidence that eosinophils play a role in the pathological progress of RAS. This can be because of the induction of pro- and anti-inflammatory mediators like in asthma [21]. A similar role is attributed to mast cells but their role in CLAD remains elusive although we did demonstrate a significant upregulation. In interstitial lung disease, mast cells can act in a profibrotic way via activating fibroblasts and inducing collagen synthesis which is probably equally important in RAS. Of interest, beside their function of antigen presenting cells, macrophages can also serve as effector cells by releasing lytic enzymes orchestrating antibody-dependent cell cytotoxicity (ADCC) [22]. Additionally, experimental animal models of lung transplant rejection demonstrated a reduction in allograft dysfunction after blockade of macrophage infiltration indicating a possible role of macrophages in the disease onset of rejection [23].

Adaptive immunity can be antibody-mediated, produced by B lymphocytes (humoral immunity) or cell-mediated by T lymphocytes. The adaptive cellular immune system consists of T helper ($CD4^+$) lymphocytes and cytotoxic T ($CD8^+$) lymphocytes recognizing alloantigens. Activation of T lymphocytes is the result of the interaction between an inactive lymphocyte and an antigen presenting cell (for example macrophages, dendritic cells, and B cells [2]. $CD8^+$ lymphocytes, increased in both BOS and RAS, release proteolytic enzymes contributing to cell death. Although $CD4^+$ lymphocytes tended to be increased in RAS, no statistical significant difference was seen.

Sato and colleagues were the first to demonstrate lymphoid cell neogenesis in lung allografts [24], showing that lymphoid tissue consisting of T and B cells was associated with active OB lesions while this was not the case in inactive OB lesions and in normal

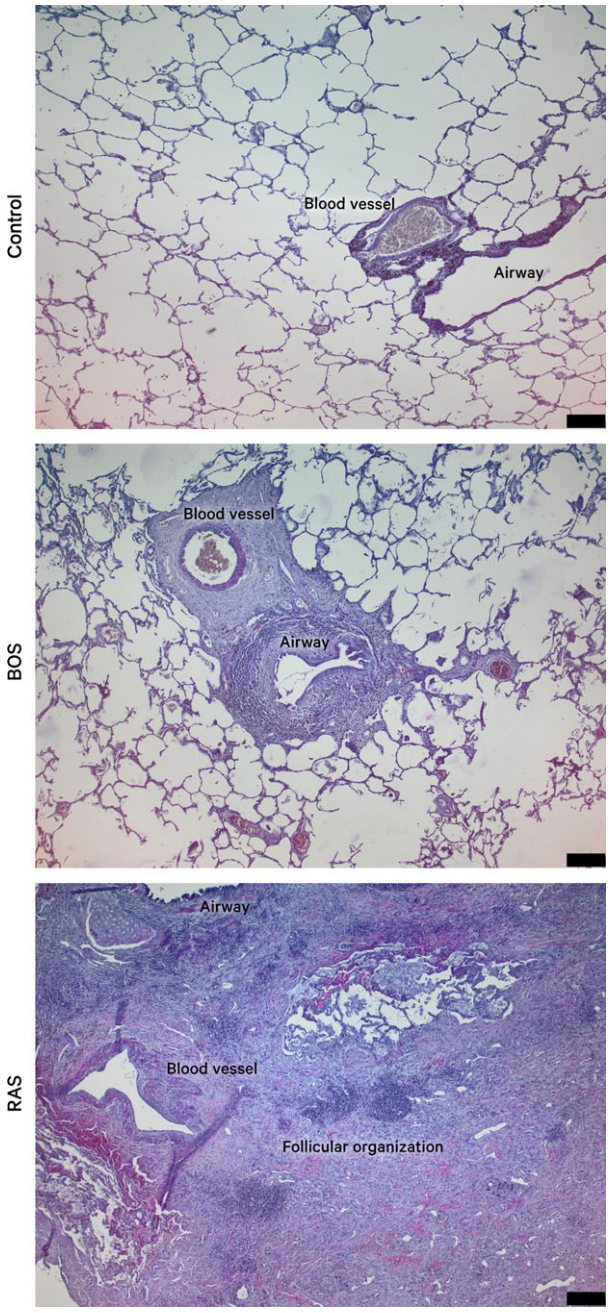

Figure 2 Representative H&E staining of control, BOS, and RAS. H&E staining of a control (upper), BOS (middle), and RAS (lower) patient. Control biopsies represent no abnormalities. In BOS, healthy looking parenchyma is accompanied by a narrowed fibrotic airway. In RAS, intensive tissue fibrosis is seen in parenchyma, blood vessel, and airways. Also, follicular organization is dominant in RAS (scale bar = 200 μm).

(nontransplant) control lungs. We were able to confirm this increase in lymphoid cells in BOS, but the increase was much more pronounced in RAS. At the time of this initial report by Sato, these different clinical phenotypes were not yet defined and therefore we can further refine this as we are the first to demonstrate structural lymphoid cell organization in RAS lungs suggesting an important role for humoral immunity in this phenotype. As we find such a prominent increase in B cells in the RAS phenotype, an important remaining question to address is whether this overlaps at least partly with antibody-medicated rejection (AMR). AMR is characterized by either the presence of donor-specific anti-HLA antibodies (DSA) in the context of vascular C4d deposition in combination with a mal functioning graft [25]. Lung transplant recipients who develop DSA have a worse CLAD-free and overall survival [11,26,27]. Interestingly, a recent study of Roux *et al.* [28] demonstrated that patients with confirmed DSA and C4d positivity evolved exclusively to a RAS phenotype of CLAD, which is in line with our current findings of the presence of B-cell follicles in RAS lungs. DSA were indeed more prevalent in RAS compared to BOS patients. Unfortunately, given the long time span to collect these lungs and the evolving techniques, data on C4d staining were not available in our patient group. Recently, our group demonstrated increased levels of immunoglobulins and complement proteins (C4d and C1q) in BAL fluid of RAS which strengthens our hypothesis that AMR might overlap with RAS [29].

Prior work demonstrated lung morphometric differences between RAS and BOS [18]. One of the major findings in RAS lungs was, next to extensive parenchymal fibrosis, the decrease in the absolute number of visible airways, terminal bronchioles and the presence of OB. This was also shown by Ofek and colleagues as they described the pathology of RAS lungs being characterized by pleuroparenchymal fibroelastosis and OB [17].

As RAS is only recently defined, we do not know a lot about the mechanisms, however, there might be some common ground between RAS and idiopathic pulmonary fibrosis (IPF), as these are both characterized by fibrotic lesions and thus might share pathophysiological mechanisms [30]. Indeed, B cells are also involved in IPF and are associated with patient outcomes [31]. Given the fact that BOS shows a pathologically less pronounced immune response, one might hypothesize that RAS represents a disrupted immune response, affecting the entire lung including the parenchyma, the blood vessels, and the airways, whereas in BOS, this immune response seems to be limited to the airways. This might also explain the limited survival in RAS patients [3,6].

Our study has some limitations as it is single centered and retrospective. Ideally, our control group would consist of stable lung transplant patients without

Table 3. End-stage immunohistochemical analysis (a) Quantification of myeloid cells in control, BOS, and RAS. The total average (Av.) and the subdivision in three compartments (airway, parenchyma, and blood vessel) are shown. Ten HPF per compartment per patient were used for analysis. (b) Quantification of total lymphoid cells and tissue in control, BOS, and RAS. The total number of cells was corrected for the area unit of the biopsy (mm²). Results are expressed as median (IQR).

	Control (n = 22)	BOS (n = 19)	RAS (n = 18)	ANOVA
(a) Subset of myeloid cells/HPF				
Neutrophils (MPO), Av.	8.9 (4.3–16.2)	13.7 (9.6–19.1)	15.9 (9.1–26.7)	
Airway	9.5 (6.4–15.4)	22.1 (17.6–35.0)$^{\#\#\#}$	21.8 (14.8–34.8)***	**<0.0001**
Parenchyma	10.2 (4.0–18.0)	14.8 (8.0–17.1)	17.8 (8.2–30.3)	0.11
Blood vessel	6.7 (2.7–12.9)	6.7 (4.6–12.8)	8.0 (4.8–15.7)	0.39
Eosinophils (EG-2), Av.	0.8 (0.3–1.7)	1.9 (0.4–8.0)	4.0 (0.8–7.4)	
Airway	0.8 (0.4–2.0)	2.5 (0.0–19.4)	4.0 (1.1–9.5)	**0.041**
Parenchyma	0.9 (0.4–1.8)	2.1 (0.3–5.6)	4.2 (0.8–11.2)*	**0.026**
Blood vessel	0.4 (0.2–1.6)	1.1 (0.1–2.2)$^{\#}$	2.8 (1.0–5.6)**,†	**0.004**
Mast cells (Tryptase), Av.	9.3 (7.0–12.9)	10.6 (7.1–13.9)	13.8 (9.6–19.7)	
Airway	19.4 (15.5–28.0)	18.9 (10.7–27.0)	21.0 (17.7–32.0)	0.33
Parenchyma	5.9 (3.8–8.7)	7.1 (6.0–10.3)	9.2 (6.9–14.9)*	**0.020**
Blood vessel	7.6 (7.1–9.3)	7.8 (5.9–11.6)	10.9 (8.5–14.3)*	**0.031**
Macrophages (CD68), Av.	13.9 (10.5–19.2)	16.5 (12.3–23.3)	32.0 (23.8–48.4)	
Airway	17.0 (11.5–19.5)	20.5 (13.6–28.4)	23.9 (20.5–40.6)**,†††	**0.002**
Parenchyma	15.5 (10.1–22.5)	15.9 (13.5–29.4)	41.8 (30.4–59.0)***,††	**<0.0001**
Blood vessel	11.3 (9.5–15.8)	11.9 (10.2–22.0)	26.7 (18.3–34.8)***	**<0.0001**
Dendritic cells (CD1a), Av.	0.4 (0.2–1.0)	0.5 (0.2–1.0)	0.7 (0.4–1.7)	
Airway	1.5 (0.6–2.7)	0.8 (0.3–2.0)	1.0 (0.3–2.7)	0.61
Parenchyma	0.1 (0.0–0.2)	0.2 (0.0–0.6)	0.6 (0.3–2.2)***,††	**0.002**
Blood vessel	0.3 (0.1–0.8)	0.3 (0.1–0.5)	0.8 (0.3–2.0)	0.086
Dendritic cells (CD207), Av.	0.7 (0.2–1.4)	0.2 (0.1–0.8)	0.2 (0.1–0.7)	
Airway	2.2 (1.3–4.4)	0.9 (0.3–2.3)	0.5 (0.2–1.2)**	**0.004**
Parenchyma	0.1 (0.0–0.3)	0.0 (0.0–0.2)	0.3 (0.1–1.2)	**0.050**
Blood vessel	0.1 (0.0–0.4)	0.0 (0.0–0.4)	0.1 (0.0–0.2)	0.10
(b) Subset of lymphoid cells/mm² area				
T helper (CD4)	1.1 (0.3–2.1)	1.0 (0.1–2.8)	2.4 (0.8–7.0)	**0.032**
T cytotoxic (CD8)	3.0 (0.4–4.8)	10.6 (2.5–14.7)$^{\#\#}$	20.9 (11.8–39.4)***	**<0.0001**
B cell (CD20)	0.1 (0.0–0.5)	1.1 (0.3–3.5)$^{\#\#}$	2.3 (0.5–10.8)***	**<0.0001**
Lymphoid follicles	0.00 (0.00–0.00)	0.00 (0.00–0.01)	0.01 (0.00–0.08)***,†	**<0.0001**

The P-values displayed on the right show the results of the Kruskal–Wallis ANOVA. Significances of the Dunn's *post hoc* test are presented as: $^{\#}$(BOS versus control); *(RAS versus control); †(BOS versus RAS) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). HPF, high-power field. A P-value <0.05 was considered to be significant and is indicated in bold.

evidence for CLAD; however, only transbronchial biopsies can be retrieved from these patients, which are too small in size for adequate staining of differential cell involvement. Moreover, in our center, autopsies are not routinely performed in lung transplant patients who die from nonpulmonary causes which explains why we were not able to include such a control group. Therefore, biopsies were used from nontransplanted lungs from patients without lung disease and/or a normal pulmonary function representing a valuable control group. The included sample sizes seem small at first, but in this, field it can be regarded as very large and very unique as most reports establishing diagnostic criteria for RAS were only to include less than 50 patients with RAS as this disease is still considered rather rare. Nevertheless, we were able to demonstrate a variety and diversity of the immune response between BOS and RAS patients.

In conclusion, the adaptive cellular immune regulation was present in both BOS and RAS, whereas the innate and humoral component was more pronounced in RAS. Revealing the immunological mechanisms in different phenotypes of CLAD may lead to a more personalized treatment which may improve patients'

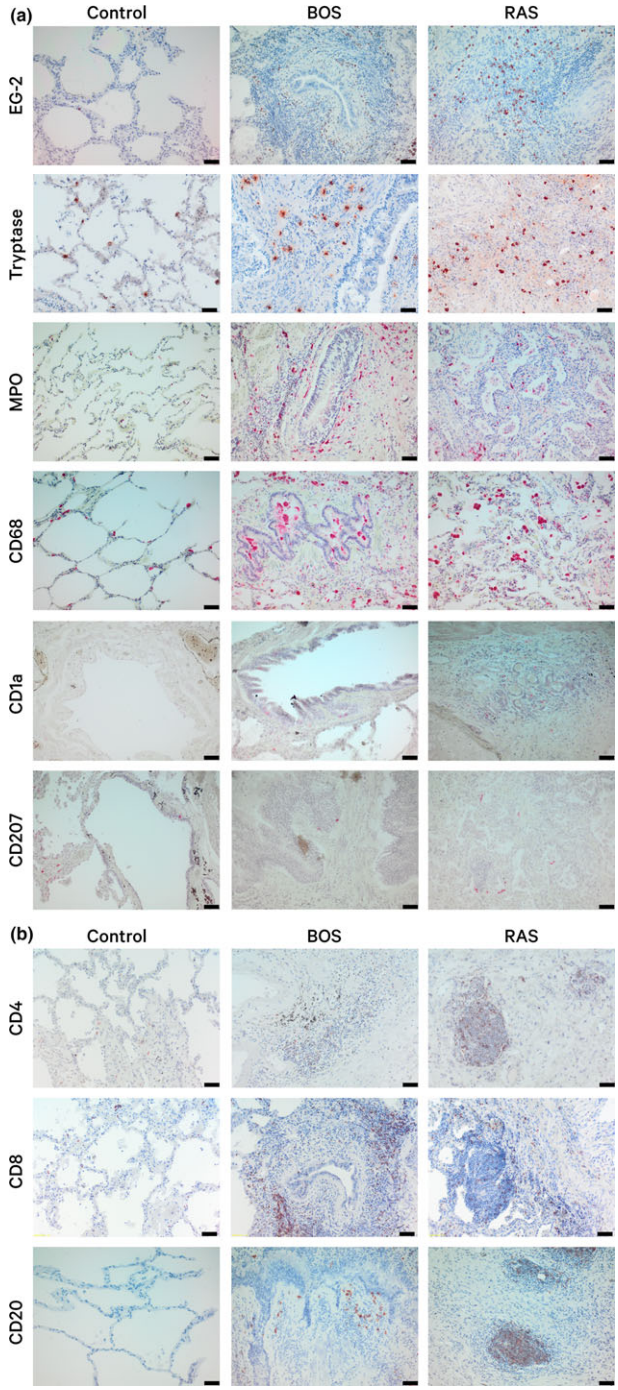

Figure 3 Immunohistochemistry of end-stage biopsies and control. Formalin-fixed paraffin-embedded human lung tissue was stained for (a) eosinophils (EG-2), mast cells (tryptase), neutrophils (MPO), macrophages (CD68), dendritic cells (CD1a and CD207), and (b) T helper cells (CD4), cytotoxic T cells (CD8), and B cells (CD20). Scale bar = 50 μm.

outcome after transplantation. Our findings, regarding the immunological diversity of BOS and RAS, lead us to speculate about different treatment options. If humoral immunity plays a pathogenic role, therapies that specifically target B cells could perhaps benefit RAS patients.

Authorship

EV: involved in data acquisition, writing of the manuscript, and data analysis. EL and EKV: involved in data acquisition and critical appraisal of the manuscript. SEV: participated in the writing of the manuscript and critical appraisal of the manuscript. DR, HB, JS, TT, GGB and DEVR: involved in critical appraisal of the manuscript. MR, KRB, KVDE, JV and M-PE: involved in data acquisition. GMV, RV and BV: participated in research design, participated in the writing of the paper, and involved in critical appraisal of the manuscript.

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Conflict of interest

The authors of this manuscript have no conflicts of interest to disclose.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Characteristics of non-transplant control patients (immunohistochemistry).

Table S2. Characteristics of end-stage BOS patients.

Table S3. Characteristics of end-stage RAS patients.

Table S4. Immunohistochemical reagents.

Table S5. Quantification of myeloid cell types and lymphoid follicles according to localization.

Figure S1. Inter- and intra-observer reliability.

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