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Early View

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Impaired IgA Mucosal Immunity Following Lung Transplantation: A Potential Trigger for Bronchiolitis Obliterans Syndrome

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Take home message

The impairment of IgA-related mucosal immunity after lung transplantation occurs early in the development of bronchiolitis obliterans syndrome (BOS), preceding clinical diagnosis. This suggests its potential as a predictor for BOS onset.

Author contributions

F.M.C. performed experiments, most data analysis, co-supervised the experimental design and wrote the manuscript. B.D. performed IgA_1, IgA_2 and S-IgA ELISA. J.A. and A.V. helped with statistical analyses. N.H. helped with Multiplex experiments and analysis. T.P.-B. helped to write the manuscript. A.D. helped with immunohistochemistry and immunofluorescence quantifications. E.L. provided BOS biopsies and explants. L.F., M.R.-G., S.H., X.D., J.-F.M., A.T., J.L.P., V.B.-G. and D.L. participated in the collection of samples through COLT. M.P. co-supervised the experimental design. C.P. and O.B. co-supervised the experimental design and helped with manuscript writing and revision.

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ABSTRACT: 239 mots

Rationale. Bronchiolitis obliterans syndrome (BOS) limits long-term survival after lung transplantation (LuTx) and may be triggered by infections. As immunoglobulin (Ig)A is crucial to ensure adequate mucosal immunity, we explored whether IgA-related mucosal immunity is impaired in BOS.

Methods. Sixty LuTx recipients from the COLT cohort were retrospectively included. All participants were in stable condition within the first year post-transplant. At 3.5 years post-LuTx, 30 remained stable and 30 had developed BOS. Bronchoalveolar lavage fluid (BALF) and sera collected pre-transplant and at 6 (M6) and 12 months (M12) post-transplant were assessed for monomeric IgA, secretory (S)-IgA, secretory component (SC) and cytokine profiling. Second, bronchiolar polymeric Ig receptor (pIgR) expression and subepithelial IgA-producing B-cell numbers were compared across graft tissue samples from 54 LuTx recipients classified as stable, pre-BOS, BOS or end-stage BOS.

Results. S-IgA levels in BALF decreased between M6 and M12 ($p=0.0001$) and were reduced in BOS patients at M12 ($p=0.0018$). Patients with lower S-IgA levels had higher infection rates. BOS patients exhibited elevated SC levels in serum ($p<0.01$). Both reduced S-IgA in BALF and increased SC in serum were associated with higher risk of BOS. Lastly, a reduction in bronchiolar pIgR expression was observed in BOS patients ($p=0.0001$), that paralleled BOS severity.

Conclusions. This study demonstrates an early impairment of mucosal IgA immunity in LuTx patients, which was linked to the later development of BOS, suggesting that IgA-related markers may serve as early predictors of BOS onset.

INTRODUCTION

Over the last decades, lung transplantation (LuTx) has become the standard therapy for selected patients with end-stage lung diseases. Despite continuous improvement, clinical outcomes following LuTx remain disappointing, with a median survival of 6.5 years(1). The first cause of death beyond the first year following LuTx is chronic lung allograft dysfunction (CLAD), whose phenotypic expression comprises mainly bronchiolitis obliterans syndrome (BOS) (65%-70% of cases), or restrictive allograft syndrome (RAS) (10-35% of cases)(1-4). Risk factors for BOS development include acute cellular rejection and antibody-mediated rejection episodes, as well as pro-inflammatory triggers such as viral infections(4-12), gastro-oesophageal reflux(13, 14), or airborne pollutants(15-18), which induce direct injury to airway epithelial cells and local recruitment of immune cells(9).

Besides its physical barrier properties, normal airway epithelium displays active defence mechanisms, including the transcytosis of immunoglobulin (Ig)A towards the airway lumen(19, 20). IgA is the predominant Ig isotype in the airways (80%(21)), where it is mainly found as dimers, as opposed to serum, where monomeric IgA prevails(22). It can exist under two isoforms, namely IgA_1 and IgA_2, which differ by their hinge region, glycosylation and activity(23). Dimeric (d-)IgA is produced locally by subepithelial plasma cells following cognate interactions between T cells and dendritic cells (24, 25), then routed across the epithelium by its transporter, the polymeric Ig receptor (pIgR). Following the transcytosis of pIgR/d-IgA, the pIgR's ectodomain, called secretory component (SC), is cleaved. d-IgA/SC complexes are released, constituting secretory (S-)IgA. In the bronchial lumen, S-IgA ensures protective functions(19, 20, 26-28) through the so-called "immune exclusion" of mucosal antigens and pathogens(29), a phenomenon segregated from systemic immune response(30).

Previous studies have demonstrated that the pIgR/S-IgA system is impaired in several chronic respiratory diseases, such as COPD, asthma, cystic fibrosis, and idiopathic pulmonary fibrosis,

possibly promoting the increased respiratory infection rates observed in these diseases (20, 31-37). However, studies on IgA-related immunity following LuTx are lacking, with only one study reporting reduced S-IgA levels in bronchoalveolar lavage fluid (BALF) from LuTx patients during rejection episodes(38).

Based on the well-established link between repeated infections following LuTx and BOS occurrence(39), we hypothesised that the pIgR/IgA axis could be impaired in some LuTx patients, increasing their susceptibility to repeated respiratory infections, ultimately favouring BOS development. To investigate the determinants of mucosal immunity, we analysed two distinct cohorts of LuTx recipients. First, in 60 recipients from the COLT cohort(40), we assessed S-IgA, SC, IgA levels, and conducted cytokine profiling in BALF/serum samples at predefined time points. Of these, 30 recipients maintained stable allograft function at 3.5 years post-LuTx, while the remainder developed BOS during follow-up. Second, we conducted a cross-sectional immunohistochemical analysis to compare pIgR expression and the presence of IgA-producing plasma cells in allograft tissues from 54 patients at Foch Hospital, including both stable patients and those at various stages of BOS.

MATERIALS AND METHODS

A. The Cohort for lung transplantation (COLT)

Patients and study design

The Cohort for Lung Transplantation (COLT) was based on patients from 11 French LT centres (Programme transplantation 2008, PRTP-13, ClinicalTrials.gov NCT00980967 and Committee for the Protection of Patients Ouest 1-Tours, 2009-A00036-51), and conducted in accordance with recommendations of the Declaration of Helsinki. All patients gave their informed consent. Data from 60 LuTx recipients from the COLT cohort were retrospectively analysed. All patients had normal graft function at the pre-defined sampling time-points within the first year post-LuTx. Thus, 30 stable recipients (“stable” group) and 30 patients who had developed BOS

during the 3.5-years follow-up ("BOS" group), were compared (Figure 1a, flowchart). Patients with other forms of CLAD than BOS were excluded. These 60 patients were successively selected from the COLT cohort according to the availability of their samples (see supplementary methods). For all patients, BALF and serum were obtained at predefined time periods post-LuTx, i.e. 6 (M6) and 12 months post-LuTx (M12). Thus, 120 BALF and 120 serums were collected. For 30 stable and 28 BOS patients, sera collected prior to LuTx was also available and analysed (M0). More information on the study population is provided in the supplementary data. Demographic characteristics of the COLT study population are provided in Table 1.

ELISA for total IgA, IgA_1, IgA_2, S-IgA and SC.

Total IgA, IgA_1, IgA_2, S-IgA, and SC concentrations were assessed at M0, M6 and M12 using ELISA as previously described(41) (see supplementary data).

Multiplex Immunoassay quantification of cytokines in sera and BALF

A proliferation-inducing ligand (APRIL), B cell activating factor (BAFF), interleukin (IL)-4, IL-10, IL-21 and soluble CD40L levels were measured using Luminex in 120 BALF and 178 sera in the 60 patients of COLT, as they have been reported to induce class switch recombination towards IgA in B cells. Inflammation-related cytokines IL-6 and IL-8/CXCL-8 were also assessed. Samples were processed accordingly to the manufacturer's instructions (see supplementary data).

B. The Foch cohort

Patients and study design

Cross-sectional immunohistology study of IgA mucosal immunity was performed in allograft lung tissues samples (n=68) from 54 LuTx recipients from Foch hospital (Suresnes, France). Samples were retrospectively selected according to their functional status at the date of sampling (Foch Cohort) and included transbronchial biopsies (TBBx) or explanted grafts from re-LuTx. Samples were allocated to four groups: 1) TBBx from patients with stable allograft function up to 3 years post-TBBx ('stable' group), 2) TBBx from patients with functional stability at sampling, who developed BOS within the following year ('pre-BOS' group), 3) TBBx from patients with definite BOS at sampling ('BOS' group), and 4) explants from patients re-transplanted for end-stage BOS ('End-BOS' group). Of note, BOS samples were collected from patients included earlier in the pre-BOS group (see supplementary data and Flowchart, Figure 1b).

Immunohistochemistry staining for pIgR

pIgR expression at the bronchial epithelial level was explored in immunohistochemistry, performed on TBBx (n=53) and BOS explants samples (n=15) (see details in supplementary data).

Tyramide signal amplification immunofluorescence staining of subepithelial IgA^+ plasma cells

IgA^+ plasma cell numbers were counted by staining the samples for CD138 (positive in plasma cells and epithelial cells), CD79a (positive in B cells) and IgA in TBBx and BOS explant specimens using tyramide signal amplification immunofluorescence. This technique, previously described(36, 42), is detailed in the supplementary data. After manual delineation of the airway epithelium, quantification of staining intensity was selectively performed on these zones using Visiopharm®.

Statistical analysis

Continuous variables are presented as mean (± standard deviation) or median (± interquartile range) and compared by Student t-test or Mann-Whitney U-test depending on normality. Paired samples were analyzed using the Wilcoxon signed-rank test. Categorical variables were described with frequency and percentage and were compared by chi-square test or Fisher's exact test. Ordinary one-way ANOVA followed by Holm-Sidak's multiple comparison test was used to compare pIgR levels between groups.

Univariate and multivariate Cox proportional hazards models were employed to assess the association between exploratory variables and BOS-free survival, as per statistical analysis guidelines (43). Detailed information on Cox analysis is provided in the supplementary data. All analyses were two-tailed and p values inferior to 0.05 were considered statistically significant. Analyses involved the use of Stata v12 for Windows (StataCorp LP, College Station, TX), GraphPad Prism version 8.0.2 (GraphPad Software, La Jolla, CA) and R version 4.2.1.

RESULTS

A. COLT cohort analysis

1. The COLT population

Among the 60 recipients, 30 had stable allograft function throughout the study ("stable" group), while 30 developed BOS between 1 and 3.5 years post-LuTx ("BOS" group) (flowchart, Figure 1a). Patients' characteristics are summarized in Table 1. No difference was observed across the groups regarding recipients' characteristics (age at transplant, sex ratio, smoking exposure, initial respiratory disease), allograft characteristics (surgery type, donor age, CMV mismatch) or post-transplant events (induction therapy, extracorporeal life support use, grade 3 primary graft dysfunction occurrence, mean acute rejection score), except for a longer second lung ischaemia time before implantation in the BOS group (p=0.03).

2. pIgR/S-IgA system components in BALF/serum from COLT patients

2.1. Reduced S-IgA levels in BALF from BOS recipients

The functionality of the pIgR/S-IgA axis in BOS was explored by measuring S-IgA concentrations in BALF from stable and BOS patients, at 6 months (M6) and 12 months (M12) post-LuTx, before functional diagnosis of BOS in BOS patients. BALF S-IgA levels in BOS diminished between M6 and M12, while slightly increasing in stable recipients, with significant difference between both groups (p=0.0018, Figure 2a). A significant decrease in absolute S-IgA levels between M6 and M12 was observed in the BOS group (p=0.0004, Figure 2b). Among BOS patients, more pronounced reduction in S-IgA between M6 and M12 (r=0.32, p=0.12) and lower BALF S-IgA levels at M12 (r=0.44, p=0.01) were associated with a shorter time to BOS diagnosis in linear regression analyses (Figure 2d).

2.2. Increased SC levels in serum from BOS patients

Whereas similar SC levels in serum were observed before LuTx across the groups (M0), higher levels were measured in BOS patients both at M6 and M12, compared to stable ones (p=0.0085 and p=0.0062, respectively, Figure 2c).

2.3. Serum IgA levels in LuTx recipients

Total IgA, IgA_1, and IgA_2 levels in serum were similar between stable and BOS patients at each timepoint. A significant decrease of total serum IgA levels at M6 and M12 versus M0, was observed in both groups (Figure 3a, p<0.0001 for all four comparisons).

IgA subtype analysis revealed that only IgA_1 (and not IgA_2) was reduced after LuTx (Figure 3b, stable group: p<0.0001 for M6 vs M0, and p<0.0001 for M12 vs M0; BOS group, p=0.0066 for M6 vs M0, and p=0.0072 for M12 vs M0). Accordingly, a significant reduction of IgA_1/IgA_2 ratio was observed in both groups (stable group: p=0.021 for M6 vs M0, and p=0.0022 for M12 vs M0; BOS group, p=0.0041 for M6 vs M0, and p=0.0032 for M12 vs M0).

3. Cytokine profiling in BALF and serum from LuTx recipients

Whether impaired S-IgA levels in BALF from LuTx recipients could be influenced by cytokine profile modifications was assessed in serum and BALF from 60 LuTx recipients. No difference was observed between BOS and stable recipients, neither in serum (Figure S1) and BALF (Figure S2), except for IL-8 levels, which were increased in BALF from BOS recipients at M6 (p=0.016) and M12 (p=0.015). Of note, IL-4 was undetectable in most serum samples and is therefore not represented.

4. Determinants of BOS onset

The association of clinical and biological factors with BOS onset was then assessed using univariate analysis and multivariate Cox analyses (Table 2). Variables associated with BOS onset in univariate analysis included lower BALF IgA levels at M12 (p=0.001), BALF S-IgA

negative variation ($p<0.001$), and high serum SC levels (log_{10}) at M12 ($p<0.001$). In multivariable Cox model, BALF S-IgA level variation and serum SC at M12 were found as the sole significant variables in the model. Of note, S-IgA at M12 was intentionally left apart, as this variable is closely related with M12-M6 BALF S-IgA variation.

5. Freedom from BOS according to BALF S-IgA and serum SC levels post-LuTx

Whether BALF S-IgA and serum SC levels could be used to stratify the risk of subsequent BOS was then assessed by allocating patients to high- versus low-level groups, using the Youden index to determine cut-offs. These were set at 12.59ng/ml for BALF S-IgA at M12 and 90.99ng/ml for serum SC at M12. Additionally, patients were allocated to an "increase" or "decrease" group, depending on the evolution of S-IgA levels between M6 and M12 (ΔS-IgA).

Freedom from BOS at 3.5 years was reduced in the low S-IgA group compared to the high S-IgA group at M12 (log-rank test, $p<0.0001$, Figure 4a). In parallel, we observed lower BOS-free survival rates in patients with high SC in serum levels at M12 ($p=0.0002$, Figure 4b). Finally, lowered levels of S-IgA in BALF at M12 *versus* M6 (ΔS-IgA<0) were strongly associated with BOS onset ($p<0.0001$, Figure 4c).

6. Increased lung infections in LuTx patients with reduced S-IgA BALF levels.

To decipher the potential protective role of S-IgA levels in BALF against lung graft infections, the incidence of graft lung infections during follow-up until 3.5 years post-LuTx was compared between BOS and stable patients, including bacterial, fungal, and viral infections. An increased incidence of the total number of infections was observed in the BOS group compared with stable patients ($p=0.0049$, Figure 5a). Moreover, an increased incidence of infections was observed only in patients within the lower quartile of S-IgA BALF levels at M12 (Figure 5b).

B. Foch cohort: cross-sectional histological study

1. Foch cohort population

This cohort of 68 samples from 54 patients included 4 predefined groups: stable (n=20), pre-BOS (n=19), BOS (n=14), and end-stage BOS (End-BOS, n=15) (flowchart, Figure 1b). Patients' characteristics are summarised in Table 3. No demographic differences were observed across the four groups, notably for donor age and tobacco use.

2. Decreased expression of pIgR in the airway epithelium of BOS patients

First, a significant decrease in pIgR mean expression intensity was observed in BOS patients as compared to stable LuTx recipients ($p<0.0001$), that was even more present in end-stage BOS explants ($p<0.0001$). No difference was observed between pre-BOS and stable LuTx recipients (Figure 6a). When analysing the 14 pre-BOS/BOS paired samples, we observed a significant decrease in pIgR staining intensity in BOS *versus* pre-BOS TBBx ($p<0.01$, Figure 6b).

3. IgA^+ plasma cell numbers are similar in submucosa from stable, pre-BOS and BOS patients

Next, we assessed whether the local S-IgA deficiency observed in pre-BOS LuTx recipients could result from reduced local (i.e., submucosal) d-IgA production. No difference was observed across the groups, both in terms of absolute IgA^+ plasma cells per square millimetre (Figure 6c), and in terms of IgA^+ cell proportion among total plasma cells (Figure 6d).

DISCUSSION

In this study, we investigated whether IgA-related airway mucosal immunity is impaired in BOS and how this could play a role in the initiation and development of BOS following LuTx. We showed that the two main components of this system are impaired in BOS, with a reduced expression of pIgR in the airway epithelium and reduced levels of S-IgA in BALF from LuTx recipients, both observed before the diagnosis of BOS. Clinically, low S-IgA levels in BALF were associated with an increased risk of infection following LuTx. In addition, a decreased S-IgA level at 12 months was independently associated with an increased risk of subsequent BOS and graft failure at 3.5 years post-LuTx in Cox analyses. Additionally, we observed that the extracellular part of pIgR, known as the secretory component (SC), which is normally overwhelmingly a luminal product, was ectopically elevated in the serum from BOS patients, and that this increase was also correlated with the risk of BOS onset. Together, these findings highlight that IgA-related mucosal immunity is compromised early in BOS development.

To date, the impairment of IgA-related mucosal immunity has not been explored in LuTx patients. Only one prior study investigated S-IgA levels in BALF, comparing stable recipients, those with active infection, and those with clinically defined acute rejection episodes. It reported reduced S-IgA levels during rejection episodes(38), suggesting a protective role for mucosal immunity against rejection. Our findings suggest that mucosal immunity is dysregulated upstream of the functional diagnosis of BOS, and support the hypothesis that pathogenic mechanisms leading to BOS are initiated months before diagnosis criteria are met(8). Although the precise role this impairment plays in driving BOS remains unclear, we concurrently report increased infection rates in LuTx recipients with very low S-IgA levels in BALF, as well as increased IL-8/CXCL-8 levels in BOS patients. As inflammation has been shown to dysregulate epithelial properties, including the function of the pIgR/IgA system(34), these observations suggest a feedback loop, where repetitive infections and chronic

inflammation impair mucosal immunity, further promoting respiratory infections and sustained inflammation, ultimately leading to airway remodelling and BOS onset. In line with this, we observed elevated SC levels in the serum of pre-BOS recipients, likely indicating inflammation-driven epithelial barrier dysfunction, leading to the leakage of local proteins into the bloodstream such as SC or Collagen Type IV Alpha 5, a recently identified early BOS biomarker (44).

The reduced pIgR expression observed in the airway mucosa of BOS patients mirrors findings in COPD, which shares biological features with BOS, such as chronic neutrophilic inflammation and recurrent infection-induced exacerbations(45, 46). The progressive decline of pIgR expression across BOS severity stages parallels observations in COPD, where pIgR downregulation is thought to result from TGF-β-driven reprogramming of the bronchial epithelium(34, 36, 47). Additionally, pIgR-deficient mice spontaneously develop COPD-like disease with age, but this is prevented in germ-free conditions, highlighting the protective role of the pIgR/S-IgA axis(48). This suggests that reduced pIgR expression and low S-IgA levels may contribute to the progression of small airway remodeling during BOS, possibly influenced by the TGF-β-enriched microenvironment observed in BOS patients(49).

We also observed decreased IgA levels in serum from stable and BOS recipients. While this reduction is not unexpected given the immunosuppressive therapy associated with solid organ transplantation(50, 51), the decrease in serum IgA levels is similar in stable and BOS recipients, indirectly suggesting that systemic IgA immunity and mucosal S-IgA immunity are mostly independent, further highlighting the importance of focusing on local immune dysregulation when studying BOS mechanisms. The observation that only the IgA_1 subtype, which is more prominent in the serum(52), is reduced remains unclear, although IgA_2's stronger ability to induce pro-inflammatory activation of neutrophils(23) may be relevant to BOS pathogenesis.

Beyond immune exclusion, where S-IgA promotes the aggregation of microorganisms *in situ*(53), S-IgA also plays an important role in specific and adaptive immunity. Thus, FcαRI, an important receptor for S-IgA, is widely expressed on neutrophils, eosinophils, monocytes, and macrophages, facilitating antigen presentation(24, 25, 53, 54). A yet unexplored hypothesis is that the dysregulation of the pIgR/S-IgA system in BOS could lead to increased autoantibodies levels at mucosal surfaces, favouring autoimmunity through exposure to epithelial self-antigens, similar to what has been described in idiopathic pulmonary fibrosis (55, 56). Nevertheless, this hypothesis remains to be investigated in LuTx.

Our study has limitations. First, no BALF samples were available prior to LuTx in the COLT Cohort. While we observed differences in S-IgA concentrations between stable and BOS recipients, suggesting BOS-related mucosal immunity dysregulation, we did not directly assess the independent effect of immunosuppressive therapy on S-IgA levels. Second, elevated SC levels in the serum likely reflect increased epithelial permeability in the airways of BOS patients, though a digestive origin, less likely in the context of LuTx, cannot be ruled out. Third, details on the oral corticosteroid maintenance dosage were lacking, which could potentially bias the results. However, significant differences across groups are unlikely, as (i) no differences were observed in the occurrence of acute cellular rejection, and (ii) samples were collected before the BOS diagnosis. Lastly, while pIgR expression in control, pre-BOS, and BOS samples was assessed using TBBx, end-BOS samples were analyzed from explanted lungs. Consequently, we cannot exclude a potential bias, as end-BOS samples may originate from a more distal portion of the bronchial tree. However, given the already significant reduction in pIgR expression in BOS patients compared to controls and pre-BOS, we believe the further decline in end-BOS is more likely due to disease progression rather than sampling differences.

In conclusion, our results suggest that mucosal IgA-related immunity is impaired early in BOS, before the onset of functional decline. Both S-IgA mucosal levels and, in an unanticipated

manner, SC serum levels, were predictive of subsequent BOS onset in this corhort, which could suggest their role as predictive biomarker of BOS. Furthermore, the impairment of the pIgR/S-IgA axis following LuTx was associated with recurrent infections, which could trigger BOS development, while other hypothetical mechanisms such as a reduced immune exclusion of auto- and/or alloantigens warrant further dedicated studies. Other studies are also needed to draw conclusions on the importance of mucosal immunity impairment in BOS, keeping in mind that S-IgA inhalation therapy, currently under development, could represent an attractive – though still purely speculative – targeted strategy to prevent respiratory infections in LuTx patients with low S-IgA levels in the future.

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Competing interests. The authors have nothing to disclose.

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Tables

Table 1. Baseline Characteristics of patients from COLT, classified according to lung function at 3 years post-LuTx.

	Stable (n=30)	**BOS (n=30)**	**p-value**
Recipient sex (Female/Male)	16 / 14	11 / 19	0.19
Recipient age at transplant (years)	43.9 ± 16.0	41.5 ± 16.8	0.57
Donor sex (Female/Male)	12 / 18	12 / 18	0.79
Donor age (years)	44.5 ± 16.9	46.8 ± 15.8	0.58
Prior tobacco exposure	10 (34%)	9 (30%)	0.99
CMV status			
- Negative donor and recipient (D-/R-)	5	7	0.38
- Positive recipient (R+)	13	16	
- Mismatch (D+/R-)	12	7	
Type of surgery			
- Bilateral lung transplant	24 (80%)	25 (83%)	0.99
- Single lung transplant	6 (20%)	5 (17%)	
Surgical indication			
- CF	15 (50%)	12 (40%)	0.47
- COPD	11 (37%)	9 (30%)	
- ILD	3 (10%)	6 (20%)	
- Other	1 (3%)	3 (10%)	
High emergency lung transplant (n, %)	6 (20%)	4 (13%)	0.73
Cold ischaemia time (minutes)			
- First lung	281 ± 90	301 ± 65	0.31
- Second lung (if bilateral LuTx)	346 ± 91	404 ± 84	**0.03**
Extracorporeal life support (n, %)	6 (20%)	3 (10%)	0.47
Induction therapy	23 (77%)	22 (73%)	0.99
- Antithymocyte globulin	8 (35%)	11 (50%)	0.37
- Basiliximab	15 (65%)	11 (50%)	
Calcineurin inhibitor			
- Ciclosporin	2 (10%)	8 (29%)	0.09
- Tacrolimus	21 (90%)	20 (71%)	
Antimetabolite			
- Mycophenolate mofetil	25 (100%)	29 (100%)	0.99
- Azathioprine	0 (0%)	0 (0%)	
Comorbidities			
- Diabetes	5 (17%)	7 (23%)	0.75
Mean acute rejection score	3.94 ± 3.52	2.59 ± 1.58	0.16

Continuous values were analysed using an unpaired Student's t test and are expressed as means ± standard deviation. Categorial values were analysed using a Fisher's exact test. p values < 0.05 were considered significant.

BOS, bronchiolitis obliterans syndrome; CF, cystic fibrosis; COLT, Cohort for lung transplantation; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease.

Missing data: tobacco exposure was unknown for 1 recipient from the stable group, calcineurin inhibitor type was unknown for 7 patients in the stable group and for 2 in the BOS group. Antimetabolite type was unknown for 5 patients in the stable group and for 1 in the BOS group.

Table 2. Results of uni- and multivariable Cox regression analyses: clinical demographical and biological variables associated with BOS occurrence.

	Univariate analysis		Multivariate analysis	
	Hazard ratio [95% CI]	**p-value**	**Hazard ratio [95% CI]**	**p-value**
Donor sex (male *versus* female)	1.074 [0.507 – 2.275]	0.85	-	-
Age of the recipient	0.995 [0.973 – 1.018]	0.68	0.996 [0.971 – 1.023]	0.79
Tobacco smoking history (yes *versus* no)	1.165 [0.541 – 2.508]	0.70	1.076 [0.470 – 2.464]	0.86
Initial respiratory disease (*versus* cystic fibrosis)				
- COPD	1.030 [0.421 – 2.520]	0.95	-	-
- ILD	2.082 [0.779 – 5.568]	0.14	-	-
- Others	2.344 [0.658 – 8.347]	0.19	-	-
National emergency (yes *versus* no)	0.692 [0.241 – 1.991]	0.49	-	-
Diabetes	1.158 [0.494 – 2.715]	0.73	-	-
CMV mismatch (yes *versus* no)	0.652 [0.278 – 1.528]	0.32	-	-
First lung ischaemia time (minutes)	1.002 [0.998 – 1.007]	0.27	1.003 [0.998 – 1.007]	0.26
S-IgA (M6)	1.009 [0.993 – 1.024]	0.28	-	-
S-IgA (M12)	0.946 [0.916 – 0.978]	0.001	-	-
ΔS-IgA (M12-M6)	0.980 [0.969 – 0.991]	**<0.001**	0.982 [0.971 - 0.994]	**0.004**
Serum SC (M12)	7.01 [2.597 – 18.92]	**<0.001**	3.885 [1.155 - 13.07]	**0.03**

CI, confidence interval; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; SC, secretory component; S-IgA, secretory IgA.

Table 3. Baseline Characteristics of patients from the FOCH cohort, classified into 4 groups according to lung function at the date sampling (Stable, pre-BOS, BOS, and end-BOS groups).

	Stable (n=20)	Pre-BOS (n=19)	BOS (n=14)	End-BOS (n=15)	p-value
Recipient sex (Female/Male)	9/11	7/12	4/10	7/8	0.72
Recipient age at transplant (years)	41.9 ± 12.3	40.1 ± 16.1	41.4 ± 17.8	30.6 ± 8.9	0.14
Recipient age at sampling (years)	42.9 ± 12.2	41.2 ± 16.3	43.5 ± 17.4	38.8 ± 10.6	0.78
Donor sex (Female/Male)	7/12	10/9	7/7	7/8	0.78
Donor age (years)	50.7 ± 13.7	52.3 ± 14.0	52.8 ± 15.0	43.5 ± 22.0	0.71
Donor prior tobacco exposure (yes/no)	12/8	9/10	6/8	5/10	0.36
CMV status					
- Negative donor and recipient (D-/R-)	4	2	1	3	0.49
- Positive recipient (R+)	15	12	9	8	
- Mismatch (D+/R-)	1	5	4	4	
Surgical indication					
- CF	11	8	6	7	
- COPD	4	2	2	4	0.76
- ILD	1	4	1	1	
- Other	4	5	5	3	
Extracorporeal life support (n, %)	2 (10%)	2 (11%)	1 (8%)	2 (13%)	0.96
Induction therapy					
- None	1	5	4	2	0.50
- Antithymocyte globulin	5	5	4	5	
- Basiliximab	14	9	6	8	

Continuous values were analysed using an unpaired Student's t test and are expressed as means ± standard deviation. Categorial values were analysed using a Fisher's exact test. p values > 0.05 were considered non-significant and labelled "ns".

BOS, bronchiolitis obliterans syndrome; CF, cystic fibrosis; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; ns, not significant.

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Figures

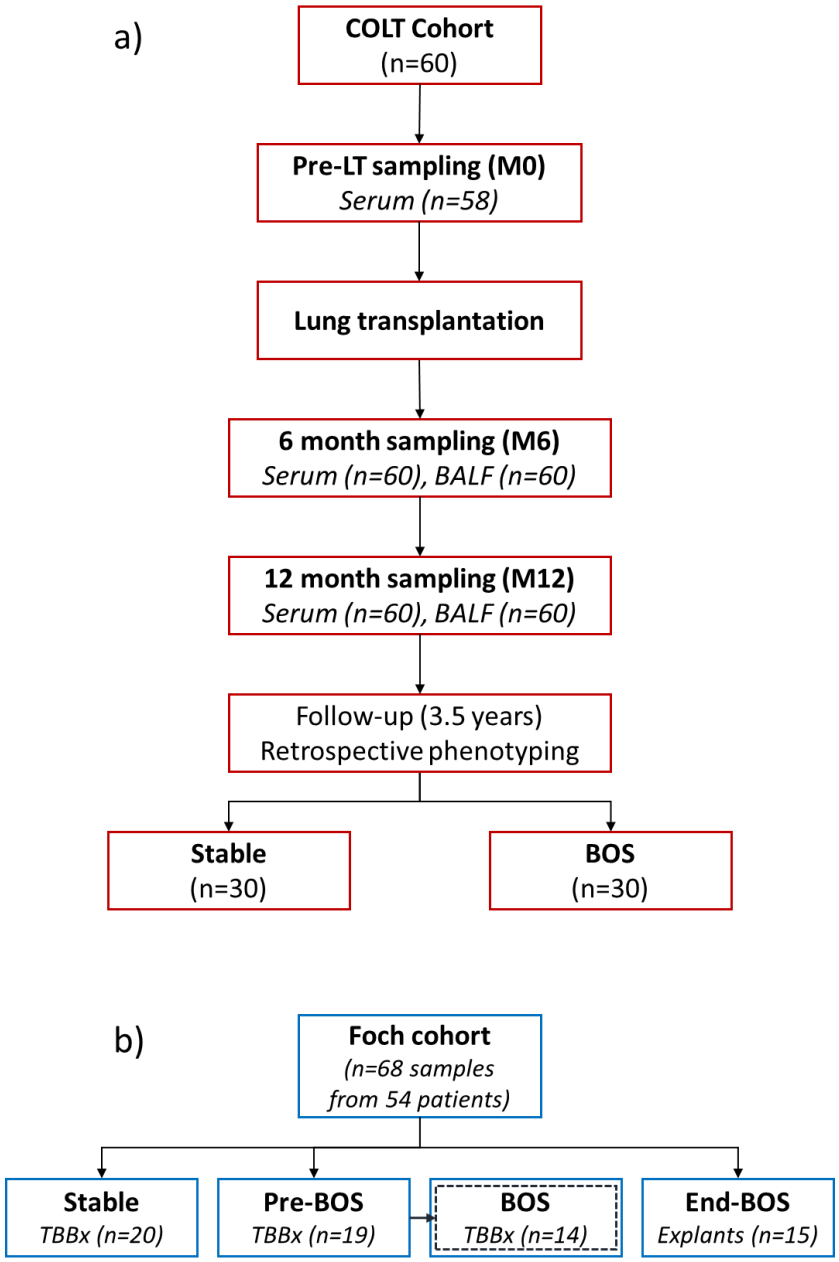


Figure 1. Study flowchart.

a) **COLT Study Population**: This cohort included patients with longitudinal follow-up. Samples were collected prior to LuTx (serum only, M0), as well as 6 and 12 months following LuTx (serum and BALF, M6 and M12, respectively). After a 3.5-year follow-up, patients were categorized into stable or BOS groups based on their functional outcome.

b) **Foch Cohort**: This cohort consisted of tissue samples from LuTx recipients with various post-transplant outcomes: patients with stable evolution (n=20), those who were stable at the time of transbronchial biopsy (TBBx) but later developed BOS within 1 year (pre-BOS, n=19), patients with definitive BOS > grade 1 (4) (n=14), and those with end-stage BOS (explants, n=15). Notably, the 14 BOS samples were from patients who had been part of the pre-BOS group.

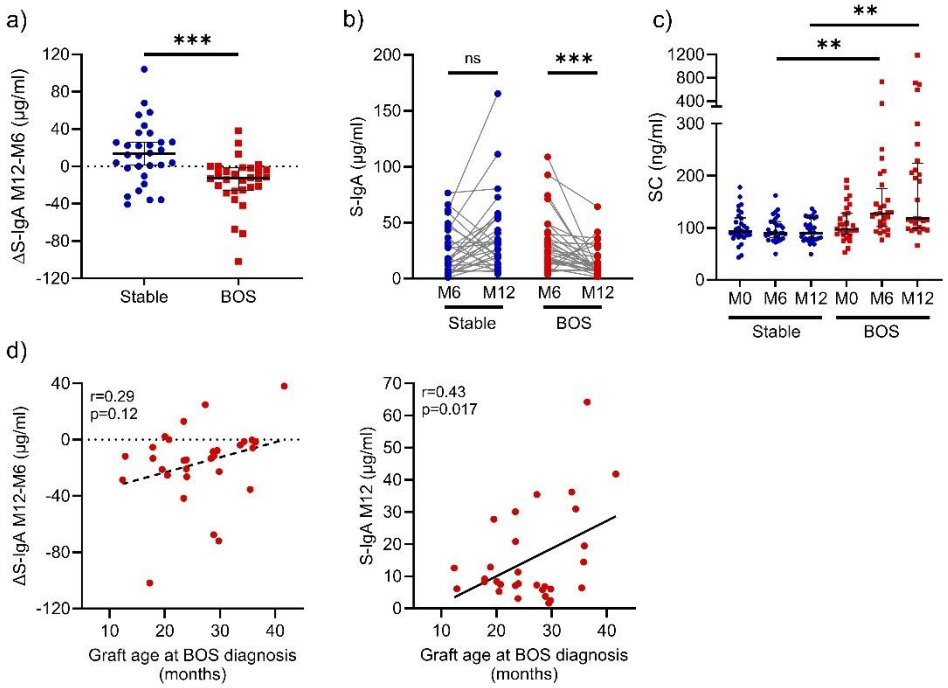


Figure 2. Concentrations of S-IgA in BALF and SC in serum from LuTx recipients

a) Absolute change in S-IgA levels in BALF from M6 to M12 in stable and BOS LuTx recipients. A significant decrease in S-IgA levels was observed from M6 to M12 in BOS patients, while a slight increase was observed in stable recipients, showing a statistically significant difference between the two groups (p=0.0018)

b) S-IgA levels in BALF at 6 months (M6) and 12 months (M12) post-LuTx in stable and BOS recipients. S-IgA levels were lower at M12 compared to M6 in BOS patients (p=0.0004), while stable recipients showed no significant decrease.

c) SC levels in serum pre-LuTx (M0), M6, and M12 in stable and BOS patients. BOS patients exhibited higher SC levels at M6 and M12 compared to stable recipients (p=0.0085 and p=0.0062, respectively). No significant difference in SC levels was observed between groups at M0 (pre-LuTx).

d) S-IgA levels at M12 (right), but not the S-IgA difference between M6 and M12 (left), directly correlated with an earlier occurrence of BOS.

, * and **** denote p-values less than 0.01, 0.001, and 0.0001, respectively.

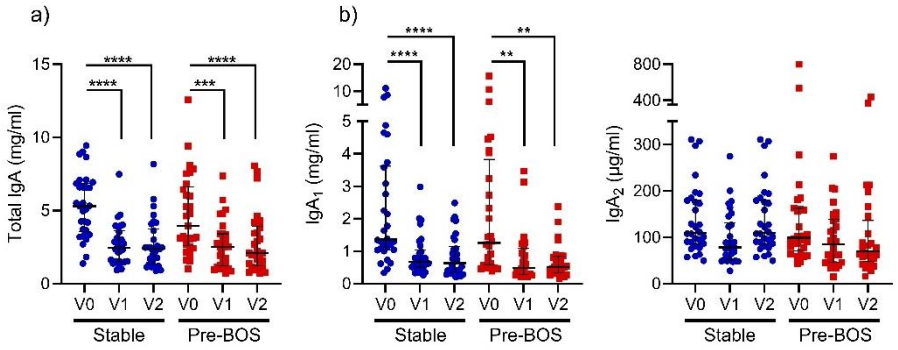


Figure 3. IgA, IgA_1 and IgA_2 levels in serum in stable and BOS patients (COLT cohort), Levels were measured prior to LuTx (M0), at 6 months (M6), and at 12 months (M12) post-LuTx.

a) Total IgA serum levels in stable and BOS LuTx recipients. Total IgA levels decreased similarly in both stable and BOS patients from M0 to M6 and from M0 to M12 ($p<0.0001$, for all four comparisons).

b) IgA_1 and IgA_2 serum levels in stable and BOS LuTx recipients. Both groups showed decreased IgA_1 levels after LuTx at M6 and M12 compared to M0 (stable group: $p=0.021$ for M6 vs M0, and $p=0.0022$ for M12 vs M0; BOS group, $p=0.0041$ for M6 vs M0, and $p=0.0032$ for M12 vs M0), while IgA_2 levels remained unchanged.

, ** highlight p values inferior to 0.01 and 0.0001, respectively.

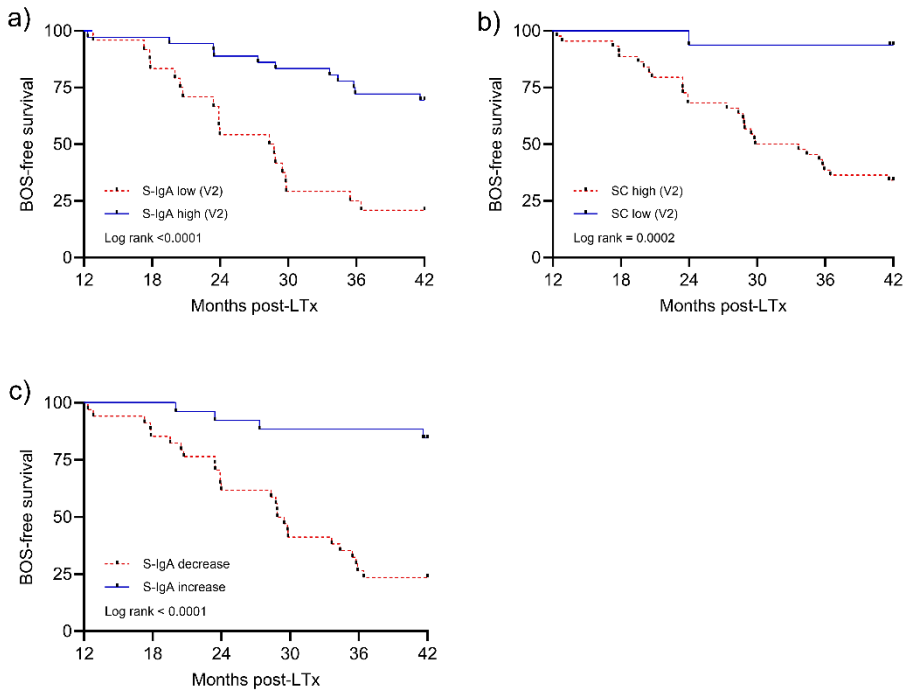


Figure 4. Freedom from BOS depending on BALF S-IgA and serum SC levels.

a) Freedom from BOS is higher for the high S-IgA groups as compared with the low S-IgA group, using a 12.59 µg/ml cut-off at M12 (Log-rank test, $p<0.0001$).

b) Freedom from BOS is higher for the low SC group as compared with the low SC group, using a 90.99 ng/ml cut-off at M12 (Log-rank test, $p=0.0002$).

c) Freedom from BOS is higher in LuTx recipients whose S-IgA levels increased at M12 versus M6, as compared to those who decreased (Log-rank test, $p<0.0001$).

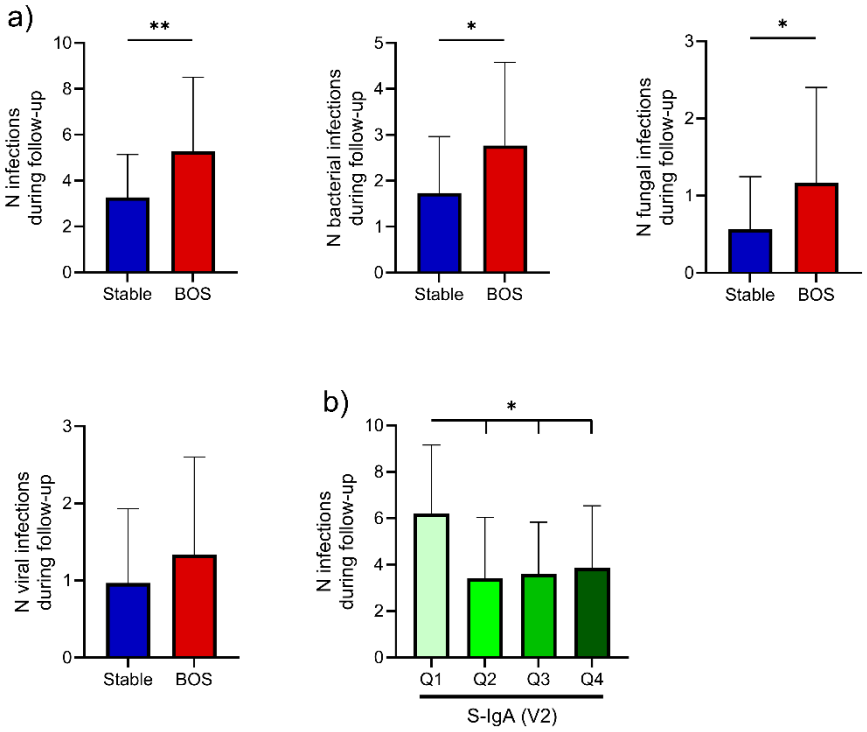


Figure 5. Infection rates in stable and BOS LuTx recipients

a) The total number of infections, bacterial infections, fungal infections, and viral infections recorded between 3 months and 3.5 years post-LuTx are presented. BOS recipients exhibited significantly higher infection rates across most categories compared to stable recipients (p=0.0049 for total infections, p=0.012 for bacterial infections, p=0.023 for fungal infections). b) LuTx recipients in the lowest S-IgA quartile at 12 months post-LuTx (M12) showed an increased risk of infections during the follow-up period. p=0.014 for Q1 *versus* Q2, p=0.025 for Q1 *versus* Q3, p=0.049 for Q1 *versus* Q4.

*, ** highlight p values inferior to 0.05 and 0.01, respectively.

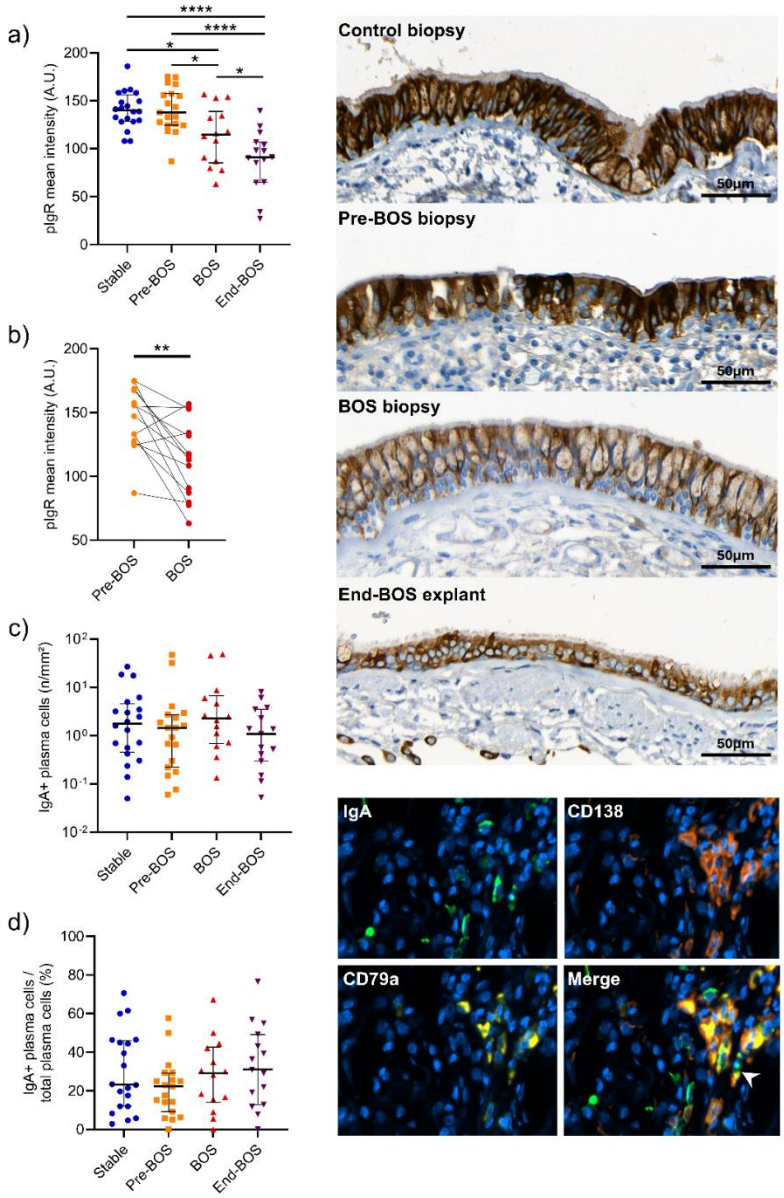


Figure 6. pIgR expression at the bronchiolar level is reduced in BOS patients.

a) Polymeric Ig receptor (pIgR) expression levels were reduced in the airway epithelium from BOS and End-BOS patients as compared to stable and Pre-BOS recipients (Stable vs BOS, p=0.012; Stable vs End-BOS, p<0.0001; Pre-BOS vs BOS, p=0.012; Pre-BOS vs End-BOS, p<0.0001; BOS vs End-BOS, p=0.0257).

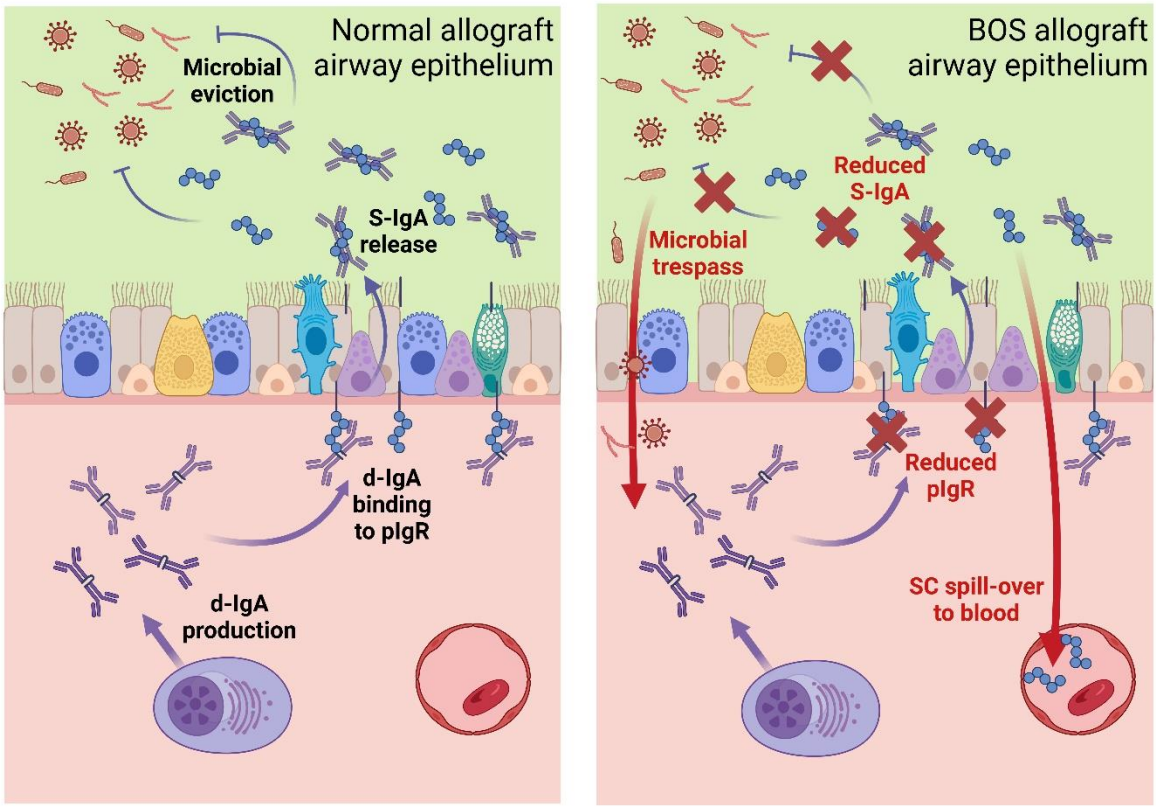
b) In the 14 paired TBBx (i.e., from the same 14 patients sampled both before and after BOS diagnosis), pIgR epithelial expression in the airway epithelium is decreased after BOS diagnosis (p=0.004).

c) Absolute IgA^+ plasma cell numbers (cells/mm²) in airway epithelium were similar in stable, pre-BOS, BOS and End-BOS LuTx recipients.

d) The proportion of IgA^+ plasma cells among total plasma cells were similar among the 4 groups.

BOS, bronchiolitis obliterans syndrome; pIgR, polymeric immunoglobulin receptor.

*, **, **** highlight p values inferior to 0.05, 0.01 and 0.0001, respectively.



Graphical abstract

SUPPLEMENTARY DATA.

Title: Impaired IgA Mucosal Immunity Following Lung Transplantation: A Potential Trigger for Bronchiolitis Obliterans Syndrome

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Supplementary methods

1. Study population from COLT

The Cohort for Lung Transplantation (COLT) was based on patients from 11 French LT centres (Programme transplantation 2008, PRTP-13, ClinicalTrials.gov NCT00980967, and Committee for the Protection of Patients Ouest 1-Tours, 2009-A00036-51), and conducted in accordance with recommendations of the Declaration of Helsinki. All patients gave their informed consent.

Starting with patients included in 2011, all subjects who (1) were labelled as "Stable" or "BOS" after a 3.5-year follow-up period and (2) had the required samples available per the study protocol (i.e. at least one available serum sample collected prior to LT [M0] and one BALF and one serum sample at six [M6] and twelve months [M12]) post-LT were consecutively selected to obtain 30 patients per group. No patients were excluded a priori from the study. The selection process was blinded (using anonymized data), and the investigators were not involved in patient selection.

Monitoring protocols of patients, immunosuppressive therapies, HLA typing, and detection of alloimmunization in LT candidates were left to the physician's discretion in each local LT centre. Pulmonary function tests, including spirometry and plethysmography when available, clinical data, and blood samples were collected at 1 month post-LT, then every 6 months within the 3 first years post-LT. CLAD was diagnosed according to the International Society for Heart and Lung Transplantation (ISHLT) criteria (1) for the presence of bronchiolitis obliterans syndrome (1, 2) or RAS onset (3-5). Criteria for diagnosis of primary graft dysfunction (PGD) (6), acute rejection (AR) episodes, and CLAD were applied as previously described (1, 3, 7). Stable graft function or CLAD status at 3 years post-LT were confirmed by repeated sessions of an ad-hoc adjudication multidisciplinary committee (1).

2. Study population from Foch hospital

Patients were selected and classified into four groups as follows: (1) LT recipients with stable lung function at 3.5 years post-LT (stable, n=20), with available transbronchial biopsies (TBBx) at 12 months ; (2) LT recipients with stable lung function at TBBx (performed between 8 and 47 months

after LT), who developed BOS within the 6 months following months (pre-BOS, n=19) ; (3) among these patients, 14 underwent TBBx within the 3-12 months after BOS diagnosis (BOS, n=14), allowing a paired analysis with a subpopulation of the pre-BOS group ; (4) explants from end-stage BOS patients (end-BOS, n=15) who had deceased from terminal BOS, with available graft explant.

3. ELISA for total IgA, IgA_1, IgA_2 in serum and SC, S-IgA in BALF

Total IgA, IgA_1 and IgA_2 concentrations in serum, as well as S-IgA and SC concentrations in BALF were assessed in samples of the 60 patients (at M0, M6, and M12) using ELISA using ELISA as previously described (8, 9). Mouse monoclonal antibodies to IgA1 (clone B3506) or IgA2 (clone A9603) at 1µg/ml for detection followed by sheep anti-mouse (m)IgG–horseradish peroxidase conjugate A6782 (Sigma-Aldrich, Saint-Louis, MO). S-IgA concentration was determined in the 120 BALF by sandwich ELISA, as previously described (8). SC concentration in the 178 sera was determined as previously reported (9).

4. Cytokine profiling by Multiplex Immunoassay quantification in sera and BALF (sample concentration)

Sample concentration. Prior to cytokine levels measurements, the 120 BALF and 178 sera were concentrated using Amicon® Ultra centrifugal filter 3K (UFC5003BK, Merck-Millipore, Cork, Ireland). Samples were processed accordingly to manufacturer's instruction. Samples were centrifugated at 14,000 g for 30 minutes, reaching 10- and 4-fold concentration factors for BALF and serum, respectively.

Cytokine assessment. Beads were analysed with Luminex MAGPIX (Merck Millipore, Molsheim, France) to measure the concentration in BALF and serum for BAFF, APRIL, IL-4, IL-6, IL-8/CXCL-8, IL-10, IL-21 and CD40L, using a tailor-made panel (ProcartaPlex™ Multiplex Immunoassay, tailor-made panel, Ref: PPX-08 ID MXRWFJ9 from ThermoFisher Scientific, Darmstadt, Germany).

5. Transbronchial biopsies sampling and processing

Transbronchial biopsies were obtained in a standardized manner, following established recommendations in the field (10), ensuring consistent subpleural sampling. To optimize tissue preservation, samples were paraffin-embedded, as this method has been shown to better maintain airway morphology compared to snap freezing while also ensuring reliable antigen preservation. (11).

6. Immunohistochemistry staining for pIgR and quantification

Samples were fixed in 4% formaldehyde and embedded in paraffin wax. Sections were deparaffinised in toluene and rehydrated through a graded series from methanol to water, then underwent antigen retrieval treatment using cooker pressure treatment at 15 pound-force per square inch (PSI) for 5 minutes. Endogenous peroxidase, biotin and streptavidin sites were inactivated by hydrogen peroxide and Bloxall (Vector Laboratories Inc., Burlingame, CA). Nonspecific protein binding sites were blocked with goat serum (Bio-Rad) for 1 h at room temperature. Anti-pIgR antibody (sc-374343, Santa Cruz Biotechnology, Dallas, TX) was incubated overnight at 4°C. Mouse IgG_1 (14-4714-82, ThermoFisher Scientific) was used as negative control. After washes with TBS 0,1% Tween 20, sections were incubated with anti-mouse polyHRP-conjugated IgG (B40961, ThermoFisher Scientific). Revelation was performed by using 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich). Sections were counterstained with Haematoxylin (Sigma-Aldrich) and coverslipped. Images were acquired with Leica SCN400 slide scanner (Leica Biosystems, Newcastle, United Kingdom).

Five micron-sections of lung tissue from transbronchial biopsies (n=53) and BOS explants (n=15), fixed in 4% formaldehyde and paraffin-embedded, were deparaffinised in toluene and rehydrated through a graded series from methanol to water. Antigen retrieval was performed in citrate buffer (pH 6,0 containing 0,1% of triton) using a pressure cooker at 15 PSI for 5 minutes. Sections were blocked for non-specific antigen binding by incubation in Bloxall (Vector Laboratories Inc.) for 15 min and then in 0,3% hydrogen peroxide with 5% goat serum (Bio-Rad) for 30 min.

pIgR staining quantification was performed by: (i) manually delineating the airway epithelium where both microscopic architecture was preserved and the reticular basement membrane was present (region of interest); (ii) measuring staining intensity for each pixel within the region of interest, normalized to background staining, using QuPath software (12), and (iii) calculating the mean pixel intensity by dividing the sum of all pixel intensities by the total number of pixels in the region of interest/

7. Tyramide signal amplification immunofluorescence staining for CD79a, IgA and CD138

Staining first included a 30 min protein blocking with 5% goat serum at room temperature, then the anti-IgA antibody (SA1-19258, ThermoFisher Scientific, 1:800 dilution) in 5% normal goat serum solution was applied for 1 hour at room temperature, then the SuperBoost™ goat anti-mouse, poly-HRP-conjugated secondary antibody (B40961, Thermo Fisher Scientific) was applied for 40 min at room temperature. HRP-conjugated polymer mediated the focal covalent binding of a Alexa Fluor 488 Tyramide Reagent (B40953, Invitrogen) using tyramide signal amplification. After a second stripping (similar conditions of antigen retrieval described above), a second round of staining was performed, using anti-CD79a (MA5-14556, Invitrogen, 1:2000 dilution) as primary antibody, then the SuperBoost™ goat anti-rabbit poly-HRP-conjugated (B40962, Thermo Fisher Scientific) as secondary antibody, and Alexa Fluor 555 Tyramide Reagent (B40955, Invitrogen) for revelation. Then, after a third antigen retrieval phase, a third-round staining was applied using anti-CD138 antibody (AM31984PU-M, Origene, 1:500 dilution) as primary antibody, then the SuperBoost™ goat anti-rabbit poly-HRP-conjugated secondary antibody (B40962, Thermo Fisher Scientific) as secondary antibody, and Alexa Fluor 647 Tyramide Reagent (B40958, Invitrogen) for revelation. Finally, sections were counterstained with Hoechst (Thermo Fisher Scientific) diluted at 10µg/ml in TBS-BSA 5% and mounted with Dako fluorescence mounting medium (Dako, Carpinteria, CA). For negative controls, rabbit (7074S, Cell signalling) and mouse (A6782, Sigma-Aldrich) isotype controls were used at the same concentration as the corresponding primary antibodies (diluted in 5% normal goat serum).

8. Statistical analysis

Covariates were selected among the variables available in the COLT database as those previously reported in the literature to be associated with CLAD/BOS and used for the univariate Cox model. Secondly, we constructed the multivariate model in two stages:

1. Essential Covariates (Face Validity): We included covariates with strong, established associations with CLAD/BOS in the literature. These included:
    - Recipient age (protective effect) (13);
    - Donor active tobacco use (risk factor) (14);
    - Longer ischemic times (risk factor) (13, 15);

These covariates were included regardless of their statistical significance in our current dataset to ensure the multivariate model's face validity.

2. Exploratory Covariates: We then considered a second set of covariates with less established associations:
    - Donor sex (male as a potential risk factor) (16);
    - Initial respiratory disease (potential influence) (17, 18);
    - Pre-transplant diabetes (potential risk via mortality and bronchiolitis) (19, 20)
    - CMV mismatch (potential role) (21).

Backward elimination was applied *solely* to this second set of covariates to refine the model, resulting in the final model presented in Table 2

Event-free survival curves were estimated using the Kaplan-Meier estimator and plotted using the ggsurvfit v.1.1.0 R package, using cut-offs determined by the Youden index (22). A log-rank test was employed to compare selected groups. S-IgA bronchoalveolar lavage fluid (BALF) levels and SC serum levels at 6 and 12 months post-LuTx, along with demographic and clinical patient characteristics, were included in the analysis.

Supplementary figures

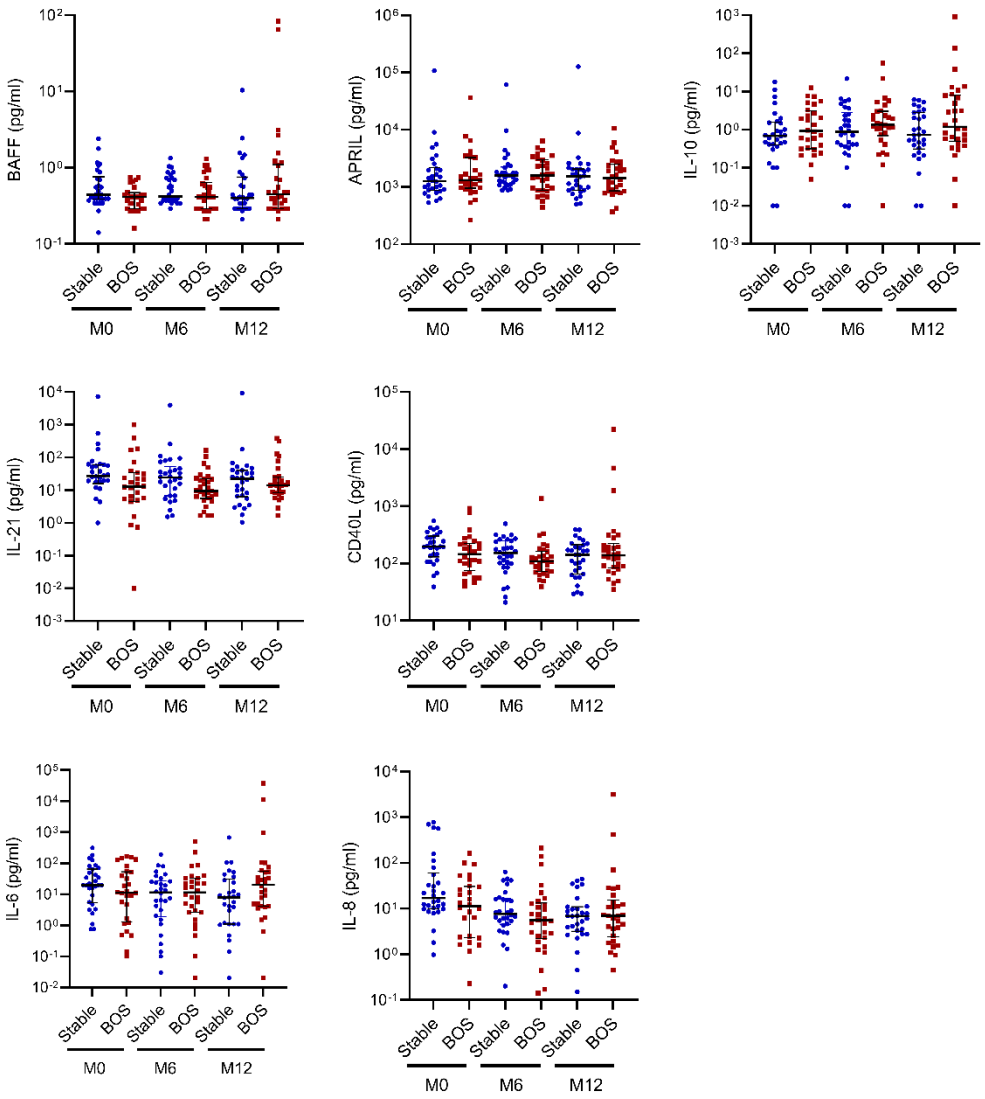

Figure S1. Cytokine profiling in serum from pre-BOS and stable LT recipients.
Using Luminex®, serum levels in BAFF, APRIL, IL-10, IL-21, CD40L as well as in IL-6 and IL-8 were assessed in stable (STA) and pre-BOS LT recipients at M1, M6, and M12. No difference was observed between groups or timepoints.

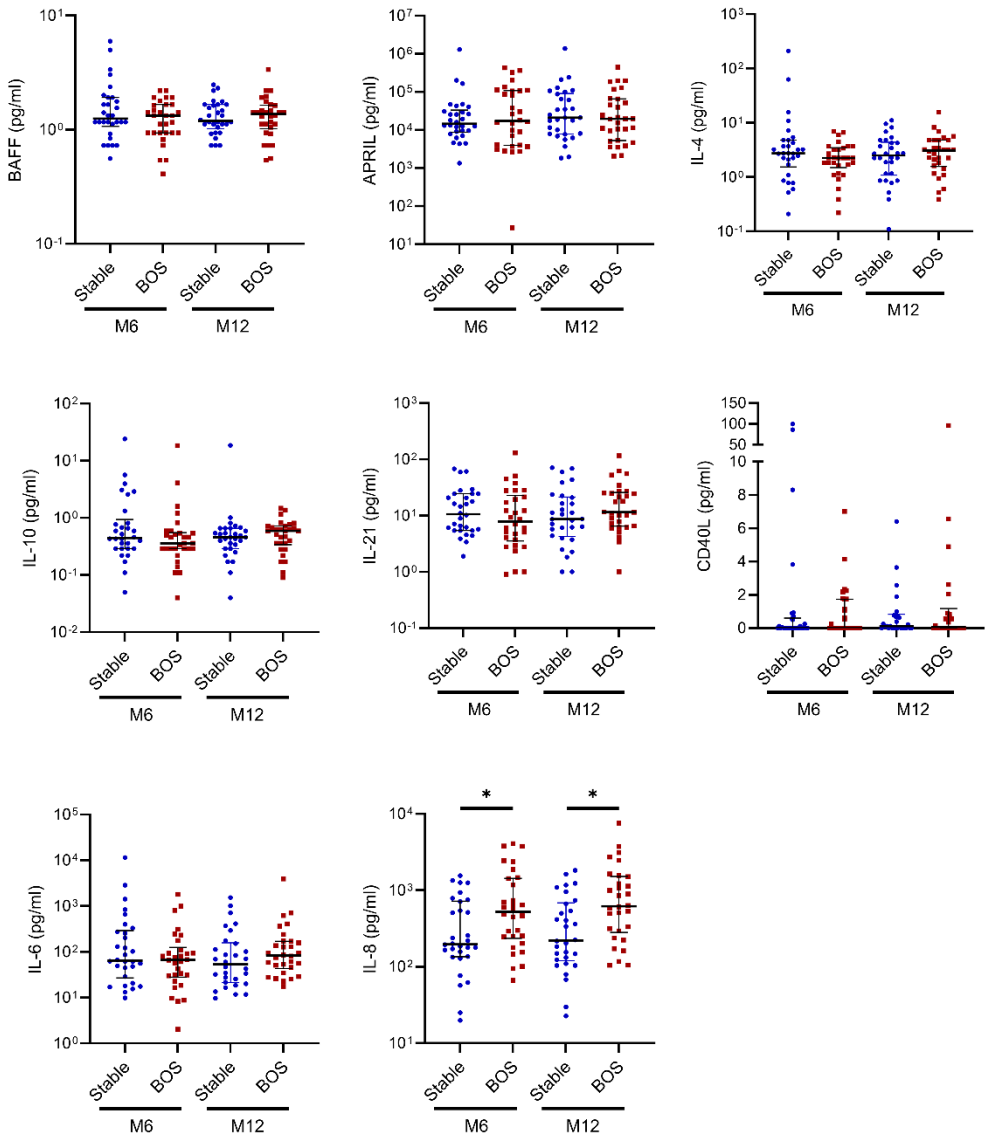

Figure S2. Cytokine profiling in BALF from pre-BOS and stable LT recipients.

Using Luminex®, levels in BAFF, APRIL, IL-10, IL-21, CD40L as well as in IL-6 and IL-8 were assessed in the BALF from stable (STA) and pre-BOS LT recipients at M6 and M12. No difference was observed between groups or timepoints, except for IL-8 whose levels were significantly increased in the BALF from pre-BOS LT recipients, both at M6 and M12.

STA, stable.

* highlights p values inferior to 0.05.

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