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Azithromycin and the Treatment of Lymphocytic Airway Inflammation After Lung Transplantation

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Lymphocytic airway inflammation is a major risk factor for chronic lung allograft dysfunction, for which there is no established treatment. We investigated whether azithromycin could control lymphocytic airway inflammation and improve allograft function. Fifteen lung transplant recipients demonstrating acute allograft dysfunction due to isolated lymphocytic airway inflammation were prospectively treated with azithromycin for at least 6 months (NCT01109160). Spirometry (FVC, FEV_1, $FEF_{25–75}$, Tiffeneau index) and FeNO were assessed before and up to 12 months after initiation of azithromycin. Radiologic features, local inflammation assessed on airway biopsy (rejection score, IL-17$^+$ cells/mm^2 lamina propria) and broncho-alveolar lavage fluid (total and differential cell counts, chemokine and cytokine levels); as well as systemic C-reactive protein levels were compared between baseline and after 3 months of treatment. Airflow improved and FeNO decreased to baseline levels after 1 month of azithromycin and were sustained thereafter. After 3 months of treatment, radiologic abnormalities, submucosal cellular inflammation, lavage protein levels of IL-1β, IL-8/CXCL-8, IP-10/CXCL-10, RANTES/CCL5, MIP1-α/CCL3, MIP-1β/CCL4, Eotaxin, PDGF-BB, total cell count, neutrophils and eosinophils, as well as plasma C-reactive protein levels all significantly decreased compared to baseline ($p < 0.05$). Administration of azithromycin was associated with suppression of posttransplant lymphocytic airway inflammation and clinical improvement in lung allograft function.

Abbreviations: APC, antigen presenting cells; ARAD, azithromycin-responsive allograft dysfunction; BAL, broncho-alveolar lavage; BO, bronchiolitis obliterans; CD, cluster of differentiation; CLAD, chronic lung allograft dysfunction; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; CT, computed tomography; EBB, endobronchial biopsies; ELISA, enzyme-linked immunosorbent assay; $FEF_{25–75}$, forced expiratory flow at 25% point to the 75% point of forced vital capacity; FeNO, exhaled nitric oxide; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; HLA, human leukocyte antigen; IL, interleukin; IP-10/CXCL10, interferon gamma-induced protein 10; ISHLT, International Society for Heart & Lung Transplantation; MHC, major histocompatibility complex; MIG/CXCL9, monokine induced by gamma interferon; MIP, macrophage inflammatory protein; NRAD, neutrophilic reversible allograft dysfunction; OB, obliterative bronchiolitis; PCR, polymerase chain reaction; RANTES/CCL5, regulated on activation, normal T cell expressed and secreted; TBB, transbronchial biopsies; Tc17, T effector 17 cell; Th17, T helper 17 cell; Treg, regulatory T cell

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Introduction

Lymphocytic airway inflammation is characterized by peribronchiolar and/or peribronchial submucosal infiltration of (predominantly CD8$^+$) T lymphocytes. It is one of the main risk factors for subsequent development of chronic lung allograft dysfunction (CLAD) after lung transplantation (1–3); which at its end-stage is characterized by small airways obstruction of the membranous, terminal and/or respiratory bronchioles. This is mainly due to ''constrictive''

bronchiolitis, also called obliterative bronchiolitis or bronchiolitis obliterans, characterized by peribronchiolar fibrosis resulting in extrinsic narrowing and obliteration of the bronchiolar lumen; or, less commonly, due to ''proliferative'' bronchiolitis, previously also called bronchiolitis obliterans organizing pneumonia, characterized by polypoid intraluminal plugs of proliferating fibroblasts and myofibroblasts within alveolar ducts and spaces with varying degrees of bronchiolar involvement (4,5). Posttransplant lymphocytic airway inflammation can be attributed both to alloantigen-dependent and/or alloantigen-independent factors (6). Following lung transplantation, local airway infiltration by antigen presenting cells (APC) and activated; alloreactive T-lymphocytes may be seen, similar to the context of graft-versus-host disease after hematopoietic stem cell transplantation (7–9). The source of these cells may either be of recipient or donor origin, as the latter can be retained within the reticulo-endothelial repositories (bronchus-associated lymphoid tissue) of the allograft. Donor lymphocytes may initially induce a limited graft-versus-host reaction, which may further trigger epithelial damage, expression of cryptic (non-HLA) epithelial epitopes, release of various cytokines/chemokines and subsequent attraction of recipient lymphocytes. Conversely, recipient lymphocytes can also directly or indirectly (i.e. after processing by recipient APC) recognize donor epithelial MHC class I or II antigens (8–10). Involvement of IL-17-producing lymphocytes, including $CD4^+$ T helper 17 cells (Th17) and $CD8^+$ T effector 17 cells (Tc17), is thought to be crucial for the evolution from acute to chronic allograft rejection (11–20). Th17 are characterized by production of IL-17, IL-22 and IL-21, whereas Tc17 are mainly characterized by IL-17 secretion and no IL-21 or IL-22 co-secretion (14,19). Particularly a Th17/Tc17-mediated immune response in the absence of an adequate counterbalance by regulatory T cells (Tregs) may lead to loss of immunologic tolerance against the allograft, as Tregs are known to inhibit effector T cells. However, Treg numbers are commonly reduced in the context of potent immunosuppression following lung transplantation (21). As such, the IL-17-mediated allograft inflammation; along with a favorable cytokine milieu, may induce a pro-fibrotic condition leading to small airways obliteration and finally chronic allograft dysfunction (13,16–18). Exposure of the allograft to the external milieu may also induce lymphocytic airway inflammation by alloantigen-independent stimuli, such as infection with respiratory pathogens or traffic-related air pollution (22). IL-17/IL-17 receptor signaling is thought to be critical for regulating the host innate immune response in this setting, since IL-17 induces the expression of antimicrobial proteins, cytokines and chemokines in nonimmune cells, such as respiratory epithelial cells (23,24).

In contrast to acute perivascular (grade A) rejection, treatment for lymphocytic bronchiolitis/bronchitis is currently not standardized due to the fact that adequate sampling and histologic grading of the affected airways is difficult, inconsistent and poorly reproducible, which obscures implementation of therapeutic guidelines (25). Inhaled budesonide might be useful in controlling posttransplant airway inflammation (26). Yet, the observed airflow limitation in lymphocytic airway inflammation is often refractory to treatment with systemic steroids or augmented immunosuppression (27), which is most likely due to the presence of steroid-resistant Th17/Tc17-driven inflammation (28). Given its beneficial effects in established CLAD, we hypothesized that azithromycin, a macrolide antibiotic known to modulate IL-17-mediated airway inflammation (29,30), could control posttransplant lymphocytic airway inflammation.

In the current prospective, open-label, proof of concept study we therefore investigated whether azithromycin could attenuate Th17/Tc17-mediated airway inflammation; and as such improve allograft function and associated radiologic features, in isolated lymphocytic bronchiolitis/bronchitis following lung transplantation.

Materials and Methods

Study design and population

Consecutive lung transplant recipients were prospectively included in the current study between July 2010 and August 2012 if isolated lymphocytic bronchiolitis/bronchitis (i.e. *without* concurrent acute perivascular [grade A] rejection) was diagnosed. Upon inclusion, azithromycin treatment was started, without any other therapeutic intervention (no antibiotic/antiviral/antifungal therapy, nor increase in steroids or immunosuppressives). The current study (NCT01109160) was approved by the local hospitals ethical committee (ML6342) and all patients gave informed consent before inclusion.

Additionally, we retrospectively identified 15 historical controls (Controls), which were matched with the studied patients for severity and postoperative time to diagnosis of lymphocytic bronchitis/bronchiolitis (interval of diagnosis between January 2010 and December 2013). Controls were treated with ''standard of care'' alone after diagnosis of isolated bronchitis/bronchiolitis, that is no azithromycin was started at the time of diagnosis (because of diagnosis before/after study-inclusion period or no informed consent during study-inclusion period). Controls' demographics and evolution of spirometry in comparison with the studied cohort is described in the Online Supplement.

Bronchoscopic procedures, microbiological and virological assessment, quantification of cell differentials and/or histology were performed as previously described (18,22). Briefly, *scheduled* broncho-alveolar lavage (BAL) was performed at 1, 3, 6, 12, 18 and 24 months posttransplantation. Surveillance transbronchial biopsies (TBB) together with endobronchial biopsies (EBB) were performed 1 and 3 months posttransplantation and thereafter surveillance EBB were performed at 6, 12, 18 and 24 months posttransplantation. *Diagnostic* bronchoscopy with BAL, TBB and EBB was performed whenever acute rejection, respiratory infection or chronic rejection was suspected, as previously described (18,22). According to the study protocol, patients included in the current study underwent control bronchoscopy with BAL and biopsies 3 months after initiation of azithromycin. Bronchoscopy, BAL and TBB/EBB biopsies were performed according to standard procedures and guidelines (31,32). Perivascular rejection (grade A) and lymphocytic bronchiolitis (grade B) were histologically

defined according to established criteria, large airway lymphocytic bronchitis was accordingly assessed (2,32,33).

Immunohistochemical staining of TBB/EBB for IL-17A and CD4 or CD8, with subsequent scoring using stereology, was performed as previously described (11). Lymphocytes and IL-17$^+$ cells were quantified as number of cells/mm^2 of lamina propria or as percentage of IL-17$^+$ cells per lymphocytes. BAL protein levels were determined using a multiplex immunoassay (Bio-Plex Pro^{TM} human cytokine 27-plex assay; Bio-Rad Laboratories N.V., Nazareth, Belgium) according to manufacturer's guidelines. Levels of human ENA-78/CXCL5, GCP-2/CXCL6, Mig/CXCL9 and MCP-3/CCL7 were quantified by sandwich enzyme-linked immunosorbent assay (ELISA), as previously described (34–37). Plasma C-reactive protein (CRP) levels were routinely measured at the University Hospital laboratory (Tina-quant CRP latex assay; Roche, Vilvoorde, Belgium) (22).

Spirometry (Masterscreen, Jaeger, Hoechberg, Germany) was performed according to established criteria (38). Exhaled nitric oxide (FeNO) was measured by means of a CLD 88sp chemiluminescence analyzer (Eco Medics AG, Dürnten, Switzerland), during a single breath exhalation according, as previously described (18,22). A FeNO value of 15 or greater parts per billion (ppb) was considered as the cutoff value of normal (39,40). CLAD, the clinical correlate of chronic allograft rejection which is defined as a persistent decrease in forced expiratory volume in 1 s (FEV_1) of at least 20% compared to baseline in the absence of other identifiable causes (41), was an exclusion criterion for the current study.

Therapeutic management

All patients, except one, received conventional triple-drug immunosuppression with methylprednisolone, a calcineurin inhibitor (cyclosporine A or tacrolimus) and a cytostatic agent (azathioprine or mycophenolate mofetil). Posttransplant drug choice and dosing adjustments were made according to a standardized protocol at the discretion of the treating clinician on the basis of blood leukocytosis, renal function, trough levels and postoperative time (42). One patient was on immunosuppressive bi-therapy with tacrolimus and methylprednisolone because of chronic leukopenia, which did not allow addition of a cytostatic agent. All patients received conventional prophylaxis for gastroesophageal reflux with proton pump inhibitors as well as immediate posttransplantation infectious prophylaxis for cytomegalovirus (CMV), *Aspergillus* spp. and *Pneumocystis jiroveci* (42). If needed, antibiotic, antiviral or antifungal treatment for posttransplant bacterial, viral or fungal infection was guided using cultures or specific polymerase chain reaction (PCR) assays.

When patients were diagnosed with isolated lymphocytic airway inflammation, treatment with azithromycin according to study protocol was started (i.e. 250 mg daily for 5 days followed by 250 mg thrice weekly) and continued for at least 6 months.

End points

Primary end points were evolution of histology, pulmonary function, radiologic features and BAL cellularity and protein levels after 3 months of treatment with azithromycin.

As a secondary outcome measure, the study protocol defined evolution of pulmonary function after 6 months, however, all patients were assessed up to 12 months after initiation of azithromycin.

Statistical analysis

Analyses were performed using Graphpad Prism 4.0 software (San Diego, CA). Results are expressed as mean (± standard error of mean) or median (interquartile range) where appropriate. Group means were compared using unpaired t-test and Mann–Whitney test or Wilcoxon signed rank test for normally and not-normally distributed variables, respectively. Chi-square test was used to compare proportions and linear regression for correlation analysis. All reported p-values are two-tailed and $p < 0.05$ was considered significant.

Results

Patients' characteristics

A total of 15 patients were included; all underwent double lung transplantation, most were male and chronic obstructive pulmonary disease (COPD) was the main underlying disease. Patient demographics are summarized in Table 1. Median time to diagnosis of lymphocytic bronchiolitis/bronchitis was 245 (86–651) days after transplantation (Table 2). Eight patients demonstrated lymphocytic airway inflammation diagnosed at surveillance biopsy (median time 92 [55–336] days posttransplant), whereas seven patients had a positive diagnostic biopsy in case of deteriorating lung function (421 [245–1101] days posttransplant; $p = 0.094$).

Intercurrent CMV-pneumonitis was excluded in all patients (negative BAL PCR, no viral inclusions on histology). BAL cultures at diagnosis of lymphocytic airway inflammation demonstrated no microorganisms in seven patients, whereas in five patients *Candida albicans* was present (two diagnostic vs. three surveillance bronchoscopies) and significant bacterial growth was seen in three patients: *Pseudomonas aeruginosa* n = 1 (diagnostic), *Serratia marcescens* n = 1 (surveillance), *Staphylococcus aureus* (MRSA) n = 1 (surveillance) (Table 1). Since baseline blood leukocytosis was within normal range and plasma CRP levels were low (Table 2), no specific antibiotic or antifungal therapy was initiated in these patients as per protocol. After 3 months of azithromycin, control BAL cultures demonstrated no microorganisms in 12 patients, *C. albicans* was present in 2 patients (one of which also demonstrated the presence of *Klebsiella pneumoniae*) and significant bacterial growth was seen in 2 patients (*K. pneumonia* n = 1, *Enterobacter cloacae* n = 1) (Table 1). Both patients with positive bacterial BAL cultures (*K. pneumoniae* and *E. cloacae*, respectively) after 3 months of azithromycin were not treated with additional and/or specific antibiotics (both were noninfectious and spontaneously cleared the identified microorganism, although in the patient with *K. pneumoniae* it was re-cultured after more than 1 year thereafter). The presence of *C. albicans* in BAL is commonly not treated (unless in case of clinical suspicion of Candida-pneumonitis in the intensive care unit).

Immunosuppressive regimen did not significantly differ between baseline and after 3 months of azithromycin, although tacrolimus trough levels tended to be somewhat lower after 3 months (Tables 1 and S1).

Table 1: Patient demographics

Patient	Recipient gender (M/F)	Recipient age (years)	Pre-LTx diagnosis	Year of LTx	Type of LTx (S/SS)	Donor gender (M/F)	Donor age (years)	Ischemic time of 1st/2nd lung (min)	CMV status of donor/receptor	Immunosuppressive at time of diagnosis	Regimen after 3 months AZI
1	M	54	COPD	2001	SS	M	41	225/360	D+/R+	FK/MPA/CS	FK/MPA/CS
2	M	58	COPD	2007	SS	M	40	275/392	D−/R−	CsA/AZA/CS	CsA/AZA/CS
3	M	61	COPD	2009	SS	M	27	185/302	D−/R−	FK/AZA/CS	FK/AZA/CS
4	F	22	CF	2009	SS	F	59	295/418	D−/R−	FK/AZA/CS	FK/AZA/CS
5	F	27	CF	2008	SS	F	38	281/411	D−/R+	FK/AZA/CS	FK/AZA/CS
6	M	57	COPD	2007	SS	M	44	305/451	D−/R−	FK/AZA/CS	FK/AZA/CS
7	F	59	COPD	2010	SS	F	53	208/341	D+/R−	CsA/AZA/CS	CsA/AZA/CS
8	M	63	COPD	2010	SS	M	71	211/336	D−/R+	CsA/AZA/CS	CsA/AZA/CS
9	F	57	COPD	2011	SS	F	48	267/424	D+/R+	CsA/AZA/CS	CsA/AZA/CS
10	F	33	CF	2011	SS	M	48	250/410	D−/R−	FK/MPA/CS	FK/MPA/CS
11	M	33	CLAD	2011	SS	M	44	232/429	D−/R+	FK/MPA/CS	FK/MPA/CS
12	M	16	Fibrosis	2010	SS	M	19	263/541	D−/R−	FK/CS	FK/CS
13	M	57	Fibrosis	2012	SS	M	45	234/402	D−/R+	FK/MPA/CS	FK/MPA/CS
14	F	17	CF	2011	SS	M	42	259/485	D−/R−	FK/AZA/CS	FK/AZA/CS
15	M	59	COPD	2012	SS	M	31	266/444	D−/R−	FK/MPA/CS	FK/MPA/CS

Patient	Surveillance (S) or indication (I) biopsy	Time of B-grade diagnosis (months post-LTx)	B-grade		BAL culture	
			At time of diagnosis	After 3 months AZI	At time of diagnosis	After 3 months AZI
1	I	90.1	2R	0	*P. aeruginosa*	Negative
2	S	23.7	1R	0	*C. albicans*	Negative
3	S	3.2	2R	0	*C. albicans*	Negative
4	S	2.8	2R	0	*C. albicans*	Negative
5	S	18.5	1R	0	*S. aureus*	*C. albicans*
6	I	35.5	1R	0	Negative	Negative
7	I	8.6	1R	0	*C. albicans*	Negative
8	I	7.9	1R	0	Negative	*K. pneumoniae*, *C. albicans*
9	S	0.7	1R	0	Negative	Negative
10	S	3.0	1R	0	Negative	Negative
11	S	0.8	1R	0	Negative	Negative
12	I	21.0	2R	1R	*C. albicans*	Negative
13	S	3.0	1R	0	*S. marescens*	Negative
14	I	13.6	2R	0	Negative	Negative
15	I	1.8	1R	0	Negative	*E. cloacae*

Data are expressed as mean ± SD, median (interquartile range) or as total values where appropriate. AZI, azithromycin; AZA, azathioprine; BAL, broncho-alveolar lavage; CMV, cytomegalovirus; CF, cystic fibrosis; CLAD, chronic lung allograft dysfunction; COPD, chronic obstructive pulmonary disease; CS, corticosteroids; CsA, cyclosporine A; FK, tacrolimus (FK506); LTx, lung transplantation; MPA, mycophenolate acid; S, single; SS, sequential single.

Histology

At baseline, the degree of lymphocytic airway inflammation was scored as ''high grade'' or grade B2R (B4 n = 1, B3 n = 4) in 5 patients (3 diagnostic vs. 2 surveillance) and as ''low grade'' or B1R (B2 n = 6, B1 n = 4) in 10 patients (4 diagnostic vs. 6 surveillance) (30). After 3 months of azithromycin, lymphocytic airway inflammation had disappeared in all but one patient, in whom it decreased from grade B2R (or B2) to grade B1R (or B1 according to the 1996 International Society for Heart & Lung Transplantation [ISHLT] grading (30)), with negative BAL culture (Table 2). There was a discrepancy in severity of lymphocytic airway inflammation between TBB (B1) and EBB (B2) at baseline in only one patient, in whom the highest score was used for further analyses.

Immunohistochemical staining for IL-17A demonstrated that more than half the submucosal lymphocytes detected at baseline were $CD8^+$ IL-17^+ T cells, which number significantly decreased after 3 months of azithromycin ($p = 0.0039$) (Table 2). A biopsy specimen stained for IL-17A at baseline and after 3 months of azithromycin therapy is shown in Figure 1.

At baseline, the number of submucosal IL-17^+ T cells correlated with the histologic severity (i.e. grade B0–B4 according to the 1996 ISHLT grading (32)) of lymphocytic airway inflammation ($r^2 = 0.38$, $p = 0.045$) and therefore was higher in high-grade (B2R) versus low-grade (B1R) inflammation (660.0 ± 184 vs. 406.7 ± 110.2 IL-17^+ T cells/mm^2 of lamina propria, $p = 0.024$).

Table 2: Bronchoscopic (biopsy, BAL) and blood characteristics

	Time of diagnosis	After 3 months AZI	p-Value
Biopsy			
Time of bronchoscopy (days after Tx)	245 (86–651)	358 (177–742)	**0.0001**
B-grade[1]	2.0 (1.0–3.0)	0.0 (0.0–0.0)	**0.0001**
Lymphocytes (n/mm^2 lamina propria)	896 (800–1120)	208 (125–380)	**0.0039**
IL-17$^+$ T-cells (n/mm^2 lamina propria)	448 (400–592)	36 (16–72)	**0.0039**
IL-17$^+$ T-cells/lymphocytes (%)	51.2 (42.1–57.0)	18.2 (6.7–22.9)	**0.0039**
BAL cellularity			
Total cells ($\times 10^5$/mL)	0.44 (0.19–0.76)	0.07 (0.04–0.22)	**0.0009**
Macrophages ($\times 10^5$/mL)	0.14 (0.08–0.25)	0.07 (0.03–0.15)	**0.008**
Macrophages (%)	34.8 (24.0–62.2)	83.2 (73.6–94.4)	**0.0002**
Neutrophils ($\times 10^5$/mL)	0.28 (0.04–0.44)	0.004 (0.001–0.008)	**0.0001**
Neutrophils (%)	54.6 (33.0–65.8)	3.2 (1.8–15.6)	**0.0001**
Lymphocytes ($\times 10^5$/mL)	0.02 (0.01–0.04)	0.004 (0.002–0.007)	**0.035**
Lymphocytes (%)	4.0 (1.0–11.6)	7.6 (2.4–15.6)	0.19
($\times 10^5$/mL)	0.001 (0.0–0.005)	0.0 (0.0–0.0)	**0.0078**
Eosinophils (%)	0.4 (0.0–1.0)	0.0 (0.0–0.2)	**0.0078**
BAL proteins (pg/mL)			
Th1-related			
MIG/CXCL9	21.5 (5.0–59.0)	5.0 (5.0–5.0)	0.094
IP-10/CXCL10	4608.0 (976.9–1350.0)	1039.0 (210.4–1656.0)	**0.012**
IL-12(p70)	8.1 (3.3–16.0)	8.1 (4.2–19.1)	0.54
IFN-γ	3.2 (3.2–12.8)	3.2 (3.2–3.2)	0.46
Th2-related			
IL-4	LOD	LOD	NA
IL-5	LOD	LOD	NA
IL-13	LOD	LOD	NA
Th17-related			
IL-1β	6.3 (2.3–42.5)	1.2 (0.3–4.8)	**0.011**
IL-6	8.5 (3.9–32.8)	6.7 (3.1–19.5)	0.36
IL-17	LOD	LOD	NA
Neutrophil-related			
ENA-78/CXCL5	7.5 (5.0–43.5)	18.0 (11.0–75.0)	0.11
GCP-2/CXCL6	853.0 (275.0–1835.0)	1132.0 (691.0–2735.0)	0.21
IL-8/CXCL8	448.0 (227.9–1307.0)	94.1 (35.4–282.2)	**0.0004**
Macrophage-related			
MCP-1/CCL2	81.0 (12.0–476.3)	50.5 (9.3–107.5)	0.31
MIP-1α/CCL3	6.9 (0.8–28.3)	0.8 (0.8–2.5)	**0.011**
MIP-1β/CCL4	168.7 (58.3–265.9)	31.1 (18.4–44.6)	**0.0008**
RANTES/CCL5	48.5 (3.9–102.0)	5.4 (0.9–35.0)	**0.040**
MCP-3/CCL7	LOD	LOD	NA
TNF-α	LOD	LOD	NA
Eosinophil-related			
Eotaxin/CCL11	30.1 (5.2–46.4)	1.3 (1.3–11.4)	**0.037**
Growth factors			
IL-7	5.1 (2.0–10.2)	3.6 (0.6–8.4)	0.46
G-CSF	61.1 (18.0–124.4)	24.5 (12.7–69.9)	0.085
GM-CSF	LOD	LOD	NA
FGF	LOD	LOD	NA
PDGF-BB	23.6 (11.7–85.5)	1.5 (1.5–13.8)	**0.004**
VEGF	157.5 (129.7–535.5)	175.0 (80.5–311.6)	0.33
Miscellaneous			
IL-1ra	109.1 (27.2–196.4)	34.2 (2.3–148.8)	0.19
IL-2	LOD	LOD	NA
IL-9	LOD	LOD	NA
IL-10	1.3 (0.2–2.6)	1.0 (0.2–3.5)	0.57
IL-15	LOD	LOD	NA
Blood cellularity			
Total leukocytes ($\times 10^9$/L)	7.6 ± 0.5	6.7 ± 0.4	0.073
Monocytes ($\times 10^9$/L)	0.6 ± 0.05	0.6 ± 0.04	0.34

Table 2: (*Continued*)

	Time of diagnosis	After 3 months AZI	p-Value
Monocytes (%)	9.0 ± 1.1	9.1 ± 0.5	0.56
Neutrophils ($\times 10^9$/L)	5.2 ± 0.4	4.3 ± 0.3	**0.050**
Neutrophils (%)	67.3 ± 3.6	64.4 ± 3.3	0.21
Lymphocytes ($\times 10^9$/L)	1.5 ± 0.2	1.6 ± 0.2	0.58
Lymphocytes (%)	20.1 ± 2.9	23.9 ± 3.0	**0.035**
Eosinophils ($\times 10^9$/L)	0.2 ± 0.05	0.1 ± 0.04	**0.008**
Eosinophils (%)	2.8 ± 0.9	2.1 ± 0.5	1.00
Basophils ($\times 10^9$/L)	0.03 ± 0.01	0.02 ± 0.01	1.00
Basophils (%)	0.5 ± 0.09	0.5 ± 0.06	0.64
Plasma CRP (mg/L)	4.7 (0.5–9.0)	0.6 (0.5–1.3)	**0.0049**

Data are expressed as mean ± SD or median (interquartile range) where appropriate.
A $p < 0.05$ is considered significant (values in bold).
IL, interleukin; BAL, broncho-alveolar lavage; CRP, C-reactive protein; NA, not applicable; LOD, below limit of detection: IL-1ra (5.5 pg/mL), IL-1β (0.6 pg/mL), IL-2 (1.6 pg/mL), IL-4 (0.7 pg/mL), IL-5 (0.6 pg/mL), IL-6 (2.6 pg/mL), IL-7 (1.1 pg/mL), IL-8/CXCL8 (1.0 pg/mL), IL-9 (2.5 pg/mL), IL-10 (0.3 pg/mL), IL-12(p70) (3.5 pg/mL), IL-13 (0.7 pg/mL), IL-15 (2.4 pg/mL), IL-17 (3.3 pg/mL), Eotaxin/CCL11 (2.5 pg/mL), FGF (1.9 pg/mL), G-CSF (1.7 pg/mL), GM-CSF (2.2 pg/mL), IFN-γ (6.4 pg/mL), IP-10 (6.1 pg/mL), MCP-1/CCL2 (1.1 pg/mL), MIP-1α/CCL3 (1.6 pg/mL), MIP-1β/CCL4 (2.4 pg/mL), PDGF-BB (2.9 pg/mL), RANTES/CCL5 (1.8 pg/mL), TNF-α (6.0 pg/mL), VEGF (3.1 pg/mL), ENA-78/CXCL5 (2.0 pg/mL), GCP-2/CXCL6 (20.0 pg/mL), MIG/CXCL9 (5.0 pg/mL) and MCP-3/CCL7 (20.0 pg/mL). If a protein concentration was under the limit of detection, a value of half the detection limit was accorded for further statistical analyses.
[1]B-grade according to 1996-ISHLT nomenclature (grade 0–4) (32).

Pulmonary function

An obstructive deterioration in spirometry was present in all patients (Figure 2): FEV_1, $FEF_{25–75}$ and Tiffeneau index were significantly lower at diagnosis of lymphocytic airway inflammation compared to the preceding months. Following initiation of azithromycin, FEV_1 and $FEF_{25–75}$ increased to prebaseline levels as soon as after 1 month of therapy, which even further improved thereafter and was sustained up to 12 months after initiation of azithromycin. Likewise, forced vital capacity (FVC) also significantly and sustainably increased. FeNO increased, although not significantly, upon the diagnosis of lymphocytic airway inflammation and after initiation of azithromycin FeNO tended to decrease again (Figure 2). Of note, FEV_1 and Tiffeneau index, but not FVC or $FEF_{25–75}$, tended to be higher over time after treatment in the studied patients compared to matched historical controls not treated with azithromycin (Figure S2, Table S2).

The number of submucosal IL-17$^+$ T cells at baseline correlated with the observed improvement in FEV_1 between baseline and 1 month ($r^2 = 0.37$, $p = 0.049$) as well as 3 months of azithromycin ($r^2 = 0.55$, $p = 0.0089$). A similar trend was seen with $FEF_{25–75}$ after 3 months ($r^2 = 0.35$, $p = 0.054$). The improvement in $FEF_{25–75}$ between baseline and 3 months of azithromycin also correlated with the total number of BAL cells at baseline ($r^2 = 0.45$, $p = 0.0064$) and with the degree of BAL neutrophilia at baseline ($r^2 = 0.41$, $p = 0.0097$ for total number of neutrophils and $r^2 = 0.30$, $p = 0.0340$ for % neutrophils).

Radiology

All patients were evaluated by chest computed tomography (CT) scan at diagnosis of lymphocytic airway inflammation, which was repeated after 3 months in all but two patients, who underwent a chest X-ray. CT at baseline demonstrated centrilobular nodules (tree-in-bud sign), ground glass opacities and bronchial wall thickening in 13 patients, whereas in 2 patients (both with B1R on histology) no focal parenchymal lesions, nor bronchial wall thickening was present and, therefore, only control chest X-rays were performed after 3 months in these patients. The observed CT anomalies were predominantly located in the lower lobes (12/13), and less frequently in upper (5/13) or middle (3/13) lobes; and mainly involved both lungs (6/13), the right lung only (6/13) or in 1/13 the left lung only. Compared to baseline; these observed radiologic abnormalities completely resolved after 3 months of azithromycin in 11 of the 13 patients with prior CT (illustrated in Figure 3). In one patient, there was remaining mild bronchial wall thickening (yet no centrilobular nodules or ground glass opacities anymore) and in another patient ground glass opacities persisted (though very much less pronounced compared to baseline). Control X-ray after 3 months was normal in both patients with no CT abnormalities at baseline.

BAL cellularity and BAL protein levels

Compared to baseline, a significant decrease in BAL total cell count ($p = 0.0009$), absolute and % BAL neutrophil numbers ($p = 0.0001$) as well as absolute and % BAL eosinophil

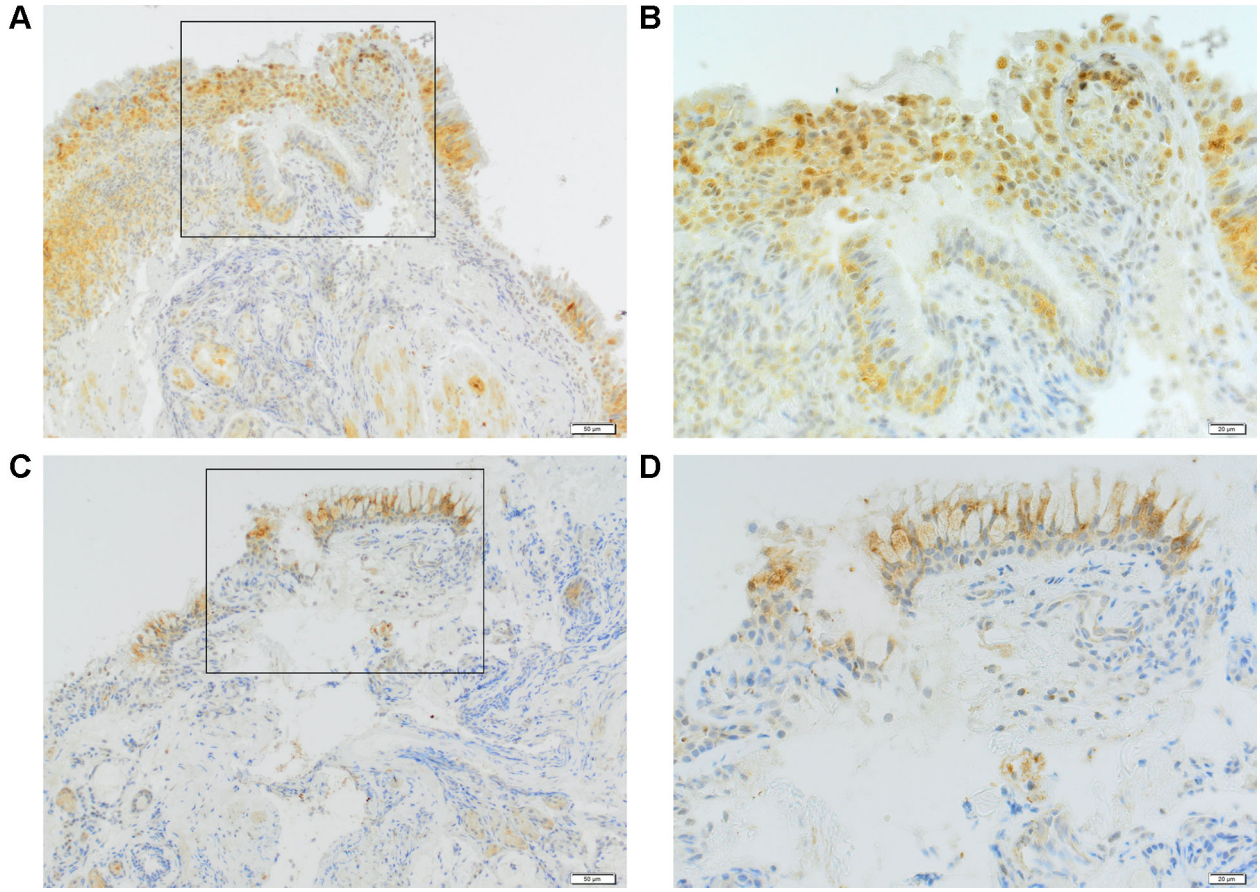

Figure 1: (A) 200× preazithromycin and (B) 400× preazithromycin (detail of panel A). IL-17A immunohistochemistry staining images demonstrating large-airway lymphocytic bronchitis with prominent sub-epithelial and occasional intraepithelial lymphocytes and some epithelial cell necrosis. Submucosal IL-17A-positive cells are brown-stained, which were also CD8-positive (not shown). (C) 200× postazithromycin, (D) 400× postazithromycin (detail of panel C). Control biopsy in the same patient after 3 months of azithromycin therapy reveals absence of lymphocytic airway inflammation with absent IL-17A staining.

numbers ($p = 0.0078$) was present after 3 months of azithromycin. Absolute BAL macrophage and lymphocyte numbers significantly decreased ($p = 0.008$ and 0.035, respectively), whereas % of BAL macrophages, but not % lymphocytes, increased after 3 months of azithromycin ($p = 0.0002$ and 0.19, respectively) (Table 2, Figure S1).

BAL protein levels of IL-1β ($p = 0.011$), IL-8/CXCL8 ($p = 0.0004$), IP-10/CXCL10 ($p = 0.012$), RANTES/CCL5 ($p = 0.040$), MIP-1α/CCL3 ($p = 0.011$), MIP-1β/CCL4 ($p = 0.0008$), Eotaxin/CCL11 ($p = 0.037$) and PDGF-BB ($p = 0.004$) significantly decreased after 3 months of azithromycin compared to baseline (Table 2). A similar trend was seen for BAL MIG/CXCL9 ($p = 0.094$) and G-CSF ($p = 0.084$) (Table 2, Figure S1).

The number of submucosal IL-17$^+$ T cells at baseline correlated with baseline BAL protein levels of IL-1ra ($r^2 = 0.46$, $p = 0.031$), IL-8/CXCL8 ($r^2 = 0.55$, $p = 0.014$), Eotaxin/CCL11 ($r^2 = 0.52$, $p = 0.018$), MIP-1α/CCL3 ($r^2 = 0.62$, $p = 0.0067$) and VEGF ($r^2 = 0.58$, $p = 0.010$). A similar trend was seen with IFN-γ ($r^2 = 0.38$, $p = 0.056$), PDGF-BB ($r^2 = 0.37$, $p = 0.060$) and IP-10/CXCL10 ($r^2 = 0.31$ $p = 0.091$).

Blood cellularity and CRP levels

Compared to baseline, a significant decrease in absolute number of both blood neutrophils ($p = 0.050$) and eosinophils ($p = 0.008$) was seen after 3 months of azithromycin. A similar trend was present for total leukocyte count ($p = 0.073$) (Table 2). The absolute number of blood lymphocytes did not change, despite the fact that the relative % of blood lymphocytes increased over time ($p = 0.035$). Monocyte and basophil numbers remained stable (Table 1). Plasma CRP levels significantly decreased after 3 months of azithromycin compared to baseline ($p = 0.0049$) (Table 2, Figure S1).

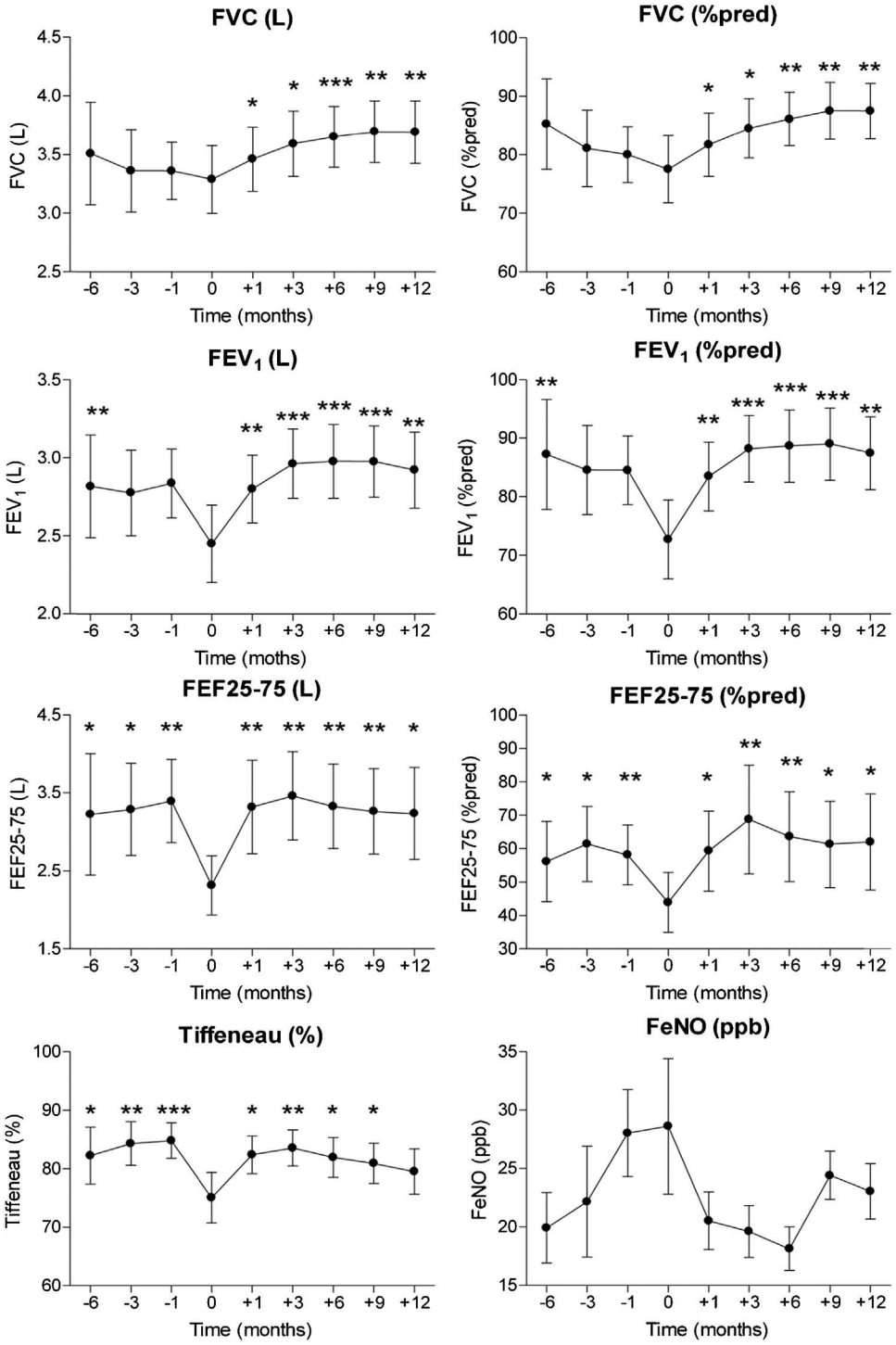

Figure 2: Evolution of spirometry and exhaled nitric oxide (FeNO) 6 months before, during (Time 0, start of azithromycin), 1, 3, 6, 9 and 12 months after diagnosis of isolated posttransplant lymphocytic bronchitis/bronchiolitis. FVC, forced vital capacity (liter or % predicted); FEV_1, forced expiratory volume in 1 s (liter or % predicted); $FEF_{25–75}$, forced expiratory flow at 25% point to the 75% point of forced vital capacity (liter or % predicted); Tiffeneau, $FEV_1/FVC \times 100$; FeNO, fractional exhaled nitric oxide (parts per billion [ppb]). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (vs. Time 0) (n = 8 patients at T-6, n = 11 at T-3, n = 12 at T-1, n = 15 at T0, T + 1, T + 3, T + 6, T + 9 and T + 12).

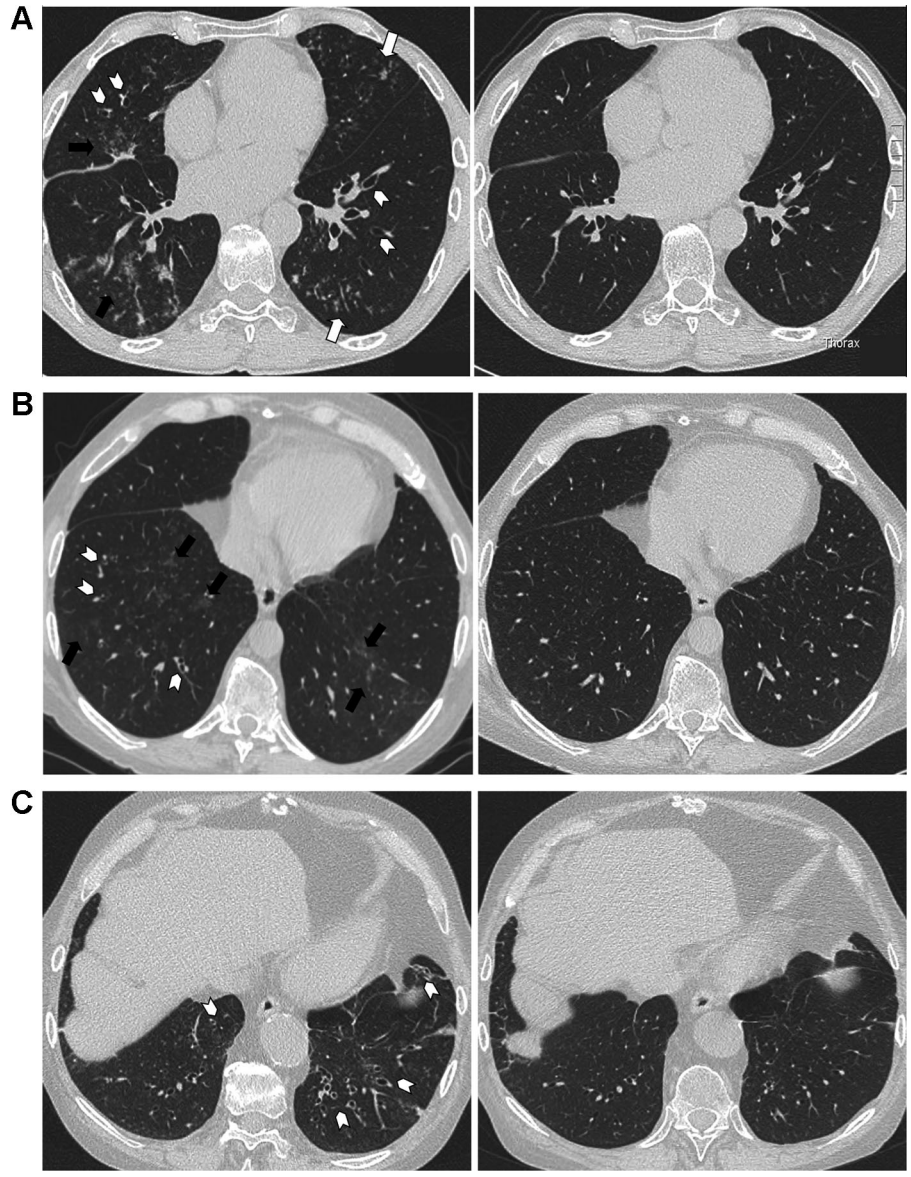

Figure 3: Radiologic features on chest computed tomography in three patients during and 3 months after diagnosis of posttransplant lymphocytic airway inflammation demonstrating regression of centrilobular nodules (tree-in-bud) (A; white full arrow), ground glass opacities (A and B; black full arrow) and bronchial wall thickening (A, B and C; white arrowheads).

The number of submucosal IL-17$^+$ T cells at baseline correlated with the number of circulating monocytes ($r^2 = 0.47$, $p = 0.019$ for total number of monocytes and $r^2 = 0.58$, $p = 0.0068$ for % monocytes) and with plasma CRP levels at baseline ($r^2 = 0.45$, $p = 0.024$). Interestingly, the improvement in FEV_1 between baseline and after 3 months of azithromycin did not only correlate with the number of IL-17$^+$ T cells at baseline as described above, but also with the % of circulating monocytes ($r^2 = 0.32$, $p = 0.027$) and with plasma CRP levels at baseline ($r^2 = 0.31$, $p = 0.032$). A similar finding was observed for the improvement in $FEF_{25–75}$ between baseline and after 3 months of azithromycin, which also correlated both with % of circulating monocytes ($r^2 = 0.28$, $p = 0.040$) and with plasma CRP levels at baseline ($r^2 = 0.39$, $p = 0.012$).

Discussion

Lymphocytic airway inflammation is a known cause of acute deterioration of lung allograft function and a precursor for subsequent chronic lung allograft dysfunction or so-called CLAD (1,2). In the current prospective study, we demonstrated that treatment with azithromycin was

associated with the restoration of allograft function in isolated posttransplant lymphocytic bronchiolitis/bronchitis. Moreover, our data confirm the involvement of $IL17^+$ T cell–mediated airway inflammation in this condition, which we believe was attenuated by azithromycin. The observed improvement in radiologic features, such as centrilobular nodules, ground glass opacities and bronchial wall thickening; and, particularly, reversal of the associated obstructive decline in spirometry is clinically remarkable given the fact that this condition is often refractory to treatment with systemic steroids or augmented immunosuppression (27), as may also be apparent when comparing the studied cohort to matched historical controls described in the Online Supplement. Corroborating the findings by De Soyza et al (26), we observed an increase in FeNO in lymphocytic airway inflammation, which decreased after initiation of azithromycin. Even more interesting is the fact that the improvement in pulmonary function with azithromycin is sustained for at least 12 months. Indeed, lymphocytic bronchiolitis/bronchitis may precede the onset of chronic allograft dysfunction during the subsequent year in some 30–50% of cases, depending on the severity (mainly B2R) and localization (mainly large airway) of the submucosal lymphocytic infiltrates (1,2). One can even question whether the improvement in airflow with azithromycin seen in some 40% of patients with established chronic allograft dysfunction, particularly in those patients demonstrating excess ($\geq$15%) BAL neutrophils (42,43) (denominated neutrophilic reversible allograft dysfunction or more recently azithromycin-responsive allograft dysfunction), might not have been due to some degree of (undiagnosed) lymphocytic bronchiolitis/bronchitis, especially as similar radiologic improvement was seen (44). Furthermore, prevention of chronic allograft dysfunction by prophylactic azithromycin administration might also be attributable to attenuation of $IL17^+$ T cell–mediated airway inflammation (42).

Mechanistically, our current, prospective data support previous work that demonstrated involvement of an $IL17^+$ T cell–mediated pathway in lymphocytic bronchiolitis/bronchitis, which induces elevated BAL neutrophilia through IL-17-induced IL-8/CXCL-8-mediated neutrophil chemotaxis (11,12,18,45). Another interesting finding is down-regulation of monokine induced by gamma interferon (MIG/CXCL9) and interferon gamma-induced protein 10 (IP-10/CXCL10) by azithromycin, as these CXCR3 chemokines play a critical role in the recruitment of mononuclear cells, including Th17/Tc17, into the lung, which is stated to be pivotal in the pathogenesis of CLAD (46,47). Besides involvement of CXC chemokines, which preferentially attract neutrophils (ELR CXC chemokines) and lymphocytes (non-ELR CXC chemokines), also CC chemokines, which attract lymphocytes, monocytes, eosinophils, basophils and natural killer cells with variable selectivity, seem to be involved in posttransplant lymphocytic airway inflammation. For instance, the CC chemokines macrophage inflammatory protein (MIP)-1α/CCL3 and MIP-1β/CCL4 were significantly down-regulated by azithromycin in the current study. These chemokines are shown to be involved in the development of bronchiolitis obliterans organizing pneumonia and fibrosis in various interstitial lung diseases. They are mainly secreted by activated macrophages and can induce chemotaxis of various types of leukocytes (48). Similar changes were observed with RANTES (regulated on activation, normal T cell expressed and secreted)/CCL5, a CC chemokine attracting T cells, eosinophils and basophils secreted by a variety of cells, including activated T cells, endothelial and epithelial cells; and which is known to be up-regulated in obliterative airway remodeling (49). The observed functional cytokine signature strongly suggests involvement of a distinct late memory (M3) $CD8^+$ T cell subset in posttransplant lymphocytic bronchiolitis/bronchitis (50). These cells predominantly produce MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5 without IL-2/IFN-γ coproduction (50). However, activated Tc17 effector cells can also secrete various chemokines, including MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, as well as MIG/CXCL9 and IP-10/CXCL10 (51), responsible for the subsequent attraction of type 1 lymphocytes (Th1 and Tc1). Of interest, these graft infiltrating Tc17 lymphocytes may in fact also be effector *memory* $CD8^+$ T cells (52,53). Given the documented infiltration of submucosal $CD8^+$ $IL17^+$ T cells in the current study, Tc17 lymphocytes are therefore the most likely source of the cytokine production in posttransplant lymphocytic bronchiolitis/bronchitis.

Remarkably, Eotaxin/CCL11 was also down-regulated by azithromycin. Eotaxin/CCL11 is another CC chemokine, mainly inducing eosinophil recruitment, but also chemotaxis of basophils, mast cells and Th2 lymphocytes. Eotaxin/CCL11 is mainly produced by lung epithelial cells, endothelial cells, fibroblasts and smooth muscle cells, for instance after IL-1β stimulation (54,55). The observed down-regulation of Eotaxin/CCL11 by azithromycin confirms previous reports of modulation of eosinophil chemotactic cytokine production by macrolide antibiotics (56). Consequently, a decrease in associated BAL eosinophilia—a predominant feature in lung allograft rejection (57)—was also seen. Of interest, also the number of circulating blood eosinophils and plasma CRP levels decreased with azithromycin, which may reflect systemic effects of macrolide therapy in lymphocytic airway inflammation, as was previously also shown in established CLAD (58). The observed correlation between submucosal $IL17^+$ T cell numbers and the number of circulating monocytes and plasma CRP levels in the current study indeed suggests a systemic proinflammatory status in this condition, which may warrant further investigation.

We acknowledge possible limitations of our study, such as the low number of included patients, which is inherent due to the single-center nature of this study and the very specific setting of isolated posttransplant lymphocytic airway inflammation. Also, inclusion of a control group would possibly have strengthened our findings, yet we

believe our data are supported by the prospective nature of the study in which all patients are their own controls over time. Moreover, considering the relatively low incidence of isolated posttransplant lymphocytic airway inflammation, conducting a proper randomized, placebo-controlled trial with enough subjects to obtain a sizable cohort of recipients would probably be very difficult to achieve in a single center. However, when we compared the studied patients to matched historical controls, treated with standard of care alone after diagnosis of bronchitis/bronchiolitis (i.e. no azithromycin was started at the time of diagnosis), FEV_1 and Tiffeneau index tended to be higher over time after treatment in the studied cohort, as described in the Online Supplement. These findings may indeed warrant confirmation in a randomized trial. Other limitations include operating characteristics of the grading system and potential confounding by other, untested variables, such as the influence of humoral (antibody-mediated) rejection (i.e. no C4d staining on biopsies). However, concurrent humoral rejection is unlikely, since circulating donor-specific anti-HLA antibodies were undetectable in all patients, both at baseline and after 3 months. Nevertheless, our study is the only one to date investigating the possible beneficial effect of azithromycin in biopsy-proven posttransplant lymphocytic airway inflammation. Moreover, we believe that the prospective nature of this study, together with the in-depth analysis of involved cells, chemokines and cytokines, which are shown to be potentially modifiable by azithromycin, not only makes it unique in its kind, but may also give rise to an exciting new therapeutic approach for this often therapy-refractory condition.

In conclusion, our findings demonstrate that azithromycin monotherapy is strongly associated with the reversal of posttransplant lymphocytic airway inflammation, which is most likely due to attenuation of the underlying $IL17^+$ T cell–mediated airway inflammation in this condition.

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Authors' Contributions

R.V. conceived the study, participated in the research cited in this manuscript and wrote the paper; S.E.V. and D.R. participated in the research cited in this manuscript, in writing the paper and its critical appraisal; E.V. and H.B. performed immunohistochemical staining and participated in the critical appraisal of this paper; E.M., A.M. and P.P. performed cytokine analyses and participated in the critical appraisal of this paper; D.S. performed multiplex analyses and participated in the critical appraisal of this paper; A.N. and D.E.V. participated in the critical appraisal of this paper; J.Y. performed most bronchoscopies and participated in the critical appraisal of this paper; L.J.D. participated in the critical appraisal of this paper; E.K.V. performed the histopathology cited in this manuscript and participated in the critical appraisal of this paper; B.C. performed the statistical analysis and participated in the critical appraisal of this paper; G.M.V. participated in the critical appraisal of this paper; B.M.V. participated in the research cited in this manuscript, in writing the paper and its critical appraisal.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: **Radar plots.**

Figure S2: **Spirometric evolution in historical controls.**

Table S1: Immunosuppressive regimen.

Table S2: Control patients demographics.