The involvement of IL-17 during lymphocytic bronchiolitis in lung transplant patients

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Abstract

Background

IL-17 is involved in auto-immune and chronic pulmonary diseases and linked with neutrophilic inflammation. Azithromycin reduces and prevents BAL neutrophilia after lung transplantation (LTx). We wanted to study the involvement of IL-17 in different post-transplant complications in human LTx biopsies.

Methods

Immunohistochemical staining against IL-17A was performed on biopsies of LTx patients with either chronic rejection, acute A-grade rejection (A>2B0), lymphocytic bronchiolitis (LB), infection and stable patients. Biopsies of 7 patients with LB were stained before and after azithromycin treatment. IL-17⁺ cells were quantified as number/mm² lamina propria. Double staining with CD4/CD8 was performed to determine the origin of IL-17.

Results

In the LB group, biopsies showed significant presence of IL-17⁺ cells/mm² of lamina propria compared to stable, acute A-grade/chronic rejection and infection ($p<0.0001$). The number of IL-17⁺ cells on biopsy correlated with %BAL neutrophilia ($r=0.34$, $p=0.0056$). Azithromycin reduced both %BAL neutrophilia and IL-17⁺ cells (both $p=0.016$) in LB. CD8⁺ cells were the major source of IL-17.

Conclusion

IL-17⁺/CD8⁺ cells are present in LB after LTx but not in acute A-grade/chronic rejection nor during infection. Moreover, azithromycin significantly decreased the number of IL-17⁺ cells in the airway wall, which may further explain its effect on BAL neutrophilia.

Introduction

Accumulating data points to an important inflammatory role of IL-17A in auto-immune diseases (1), but also in inflammatory pulmonary diseases, like COPD and cystic fibrosis (2). There is already a lot of evidence supporting a role for IL-17 in primary graft dysfunction, acute/chronic rejection after lung transplantation (LTx) (3-7) but this is mainly mRNA and animal data: for instance, IL-17 antibodies inhibit the development of acute rejection and obliterative bronchiolitis in a mouse orthotopic LTx model (5). Few direct human evidence is available, although it was published that the cellular immune response to collagen V is mediated by IL-17A, contributing to BOS (6).

Azithromycin is increasingly being used to treat LTx patients with BOS because it reduces broncho alveolar lavage (BAL) neutrophilia and IL-8, although the exact mechanism remains unclear (8). *In vitro* data has demonstrated that azithromycin inhibits IL-17 induced IL-8 production in human airway epithelial and smooth muscle cells (9,10), which may explain the reduction in BAL neutrophilia.

We wanted to assess the presence of IL-17⁺ cells in biopsies obtained in different post-LTx conditions. We also wanted to identify the IL-17 producing T-cells and to investigate the effect of azithromycin on IL-17⁺ cells.

Material and methods

Patient population

Lung transplant patients were retrospectively selected according to their clinical situation based on biopsy examination, BAL differential cell count and FEV₁ evolution and were further subdivided into different groups: stable (n=10), chronic rejection (Bronchiolitis Obliterans Syndrome, BOS) (n=10), infection (n=14 of whom 7 cytomegalovirus (CMV) infection and 7 with *Pseudomonas aeruginosa*), A>2B0 (n=10), LB (n=34 of whom 24

patients experienced an episode of LB and were not yet treated with azithromycin, and 10 had a recurrence of LB whilst already on active azithromycin therapy for at least 2 months). The LB group with and without azithromycin are analyzed separately. Stable patients had a stable pulmonary function for at least 6 months after the index bronchoscopy/biopsy. BOS was diagnosed according to ISHLT criteria as a persistent obstructive, forced expiratory volume in 1 second (FEV₁) decline of at least 20% compared to baseline, with 2 measurements 3-6 weeks apart (11). None of the BOS patients had experienced an FEV₁ improvement upon treatment with azithromycin for at least 3 months, which was consistent with the previously described fBOS phenotype (12). Concurrent infection was excluded in all rejection (acute A-grade rejection, LB, BOS) groups. *Pseudomonas aeruginosa*/CMV infection was proven on biopsy or/and in BAL. To avoid selection bias, all cases were included in a sequential retrospective fashion, after a long enough follow up period.

In a small pilot study, we also investigated the effect of azithromycin treatment (250 mg, every other day) in 7 patients with LB. A follow-up BAL and biopsy was performed between 2 and 6 months after start of azithromycin. No other treatment or alteration in immunosuppressive dosage had been initiated during that period. Patient characteristics are displayed in table 1. The study was approved by the local hospitals ethical committee (S51577). All patients gave written informed consent.

Bronchoscopy and BAL

BAL was performed with 2 times 50 ml of saline as previously described (3). IL-6 and IL-8 in BAL were measured as described before (3). During each bronchoscopy, 5 biopsies are taken. Transbronchial biopsy (TBB) were fixed in formalin, embedded in paraffin and subsequently

5µm sections were obtained. Specimens were graded by one pathologist (EKV) according to international guidelines (13).

In 4 BOS patients, stainings were performed on explant lungs during retransplantation, as a consequence, no BAL data were available at that time.

Immunohistochemistry and immunofluorescent doublestaining

For immunohistochemistry, after pretreatment with TRIS-EDTA at 98°C and blocking of peroxidase, anti-IL-17A (317-ILB-050, R&D) was added to serial sections for 30 minutes followed by dual envision incubation (Dako, Heverlee, Belgium) and 10 minutes of incubation with diaminobenzidine (Dako). Slices were counterstained with Harris hematoxylin. Scoring was performed using stereology (14). Ten random grids of 6.25×10^{-4} mm² in the submucosa were counted and lymphocytes and IL-17+ cells were quantified as number of cells/mm² of lamina propria.

Immunofluorescent double staining was performed in a sequential manner. After pretreatment with TRIS-EDTA at 98°C and blocking of peroxidase, sections were incubated for 30 minutes with mouse antihuman-CD4 antibody (1/50, M7310, Dako) or mouse antihuman-CD8 antibody (1/50, M7103, Dako), followed by 30 minutes of Alexa Fluor® 488 goat anti-mouse IgG (A11029, Invitrogen, Merelbeke, Belgium). Sequentially, the sections were incubated with antibody against IL-17, followed by Alexa Fluor® 568 goat anti-mouse IgG (A11031, Invitrogen). Mounting was done with Prolong Gold Antifade Reagent (P36935, Invitrogen).

Statistics

Results are expressed as mean ($\pm$ standard error of mean). Significances between groups were tested by Kruskal-Wallis ANOVA in combination with Dunn's multiple comparison test or with one-way ANOVA combined with Bonferroni test when appropriate. Comparison between

2 groups was done with Mann Whitney test. Contingency tables were analyzed using Fisher's Exact test and correlations with Spearman Rank test (Prism 4.1 and SAS 9.3).

Results

Patient characteristics

There were no significant differences in age ($p=0.17$), gender ($p=0.89$), type of LTx ($p=0.88$), indication for LTx ($p=0.22$), CMV mismatch ($p=0.12$), type of immunosuppression ($p=0.78$) and postoperative day ($p=0.06$) between the different patient groups (table 1).

BAL cellularity and IL-17

ANOVA analysis shows a significant difference in % of neutrophils in the BAL of stable, BOS, infection, LB and acute rejection patients ($p<0.0001$). This was due to a higher % of neutrophils in the LB ($43.5\pm 5.9\%$), acute rejection ($28.7\pm 8.4\%$) and infection group ($31.2\pm 9.9\%$) compared to the stable group ($1.7\pm 0.3\%$) ($p<0.0001$, $p<0.05$ and $p<0.05$) (figure 1A). Similarly, there was a difference in % macrophages in BAL ($p=0.005$), which was due to a lower number in the LB ($48.6\pm 6.1\%$) ($p<0.001$) and acute rejection group ($45.7\pm 10.7\%$) ($p<0.01$) compared to stable patients ($91.1\pm 3.4\%$). There were no differences in % lymphocytes ($p=0.16$) and % eosinophils ($p=0.07$). There was a significant correlation in the total study group between the % of neutrophils and the IL-8 concentration in BAL ($R=0.36$, $p=0.0051$) (table2).

The number of IL-17⁺ cells was different in biopsies of stable, BOS, infection, LB and acute rejection patients ($p<0.0001$) due a higher number of IL-17⁺ cells in the LB group (283 ± 38 IL-17⁺ cells/mm² lamina propria) compared to the other groups (stable 16 ± 10 ; BOS 6 ± 4 ; infection 46 ± 23 ; AR 29 ± 16 ; all $p<0.0001$) (figure 1B). The number of IL-17⁺ cells correlated

with the % BAL neutrophilia ($R=0.34$, $p=0.0056$). More details are displayed in table 2. Despite the fact that A-grade rejection is characterized by a lymphocytic infiltrate around the blood vessels, IL-17⁺ cells could not be identified there. For representative pictures see figure 2.

Lymphocytic bronchiolitis

As the number of lymphocytes and IL-17⁺ cells is significantly different in the LB group compared to all other groups (as per definition of LB), we further subdivided this group based on the severity of the lymphocytic infiltrate present in the submucosa conform with international guidelines (13); B1R being a small to moderate infiltrate and B2R a severe to very severe lymphocytic infiltrate. The B2R group displays significant more IL-17⁺ cells compared to B1R ($p=0.005$) (402 ± 62 vs $198\pm32/\text{mm}^2$ lamina propria). Moreover, the ratio of %IL-17⁺cells/lymphocytes is significantly higher in the B2R ($44.1\pm2.9\%$) compared to B1R ($28.4\pm2.7\%$) ($p=0.0008$).

LB and azithromycin treatment

In only 7 patients of the previous group with LB (B1R, $n=6$; B2R, $n=1$), azithromycin was started at diagnosis, without any other modification of treatment. A control bronchoscopy with BAL and biopsy was performed at a mean of 85 days after initiation of azithromycin therapy. The FEV₁ at diagnosis of LB was decreased by a mean of $28\pm5\%$ and increased again with $25\pm14\%$ after treatment with azithromycin. Moreover, %BAL neutrophilia after therapy dropped from 62% to 20% ($p=0.016$) and LB could no longer be demonstrated. As a consequence, the number of IL-17⁺ cells/mm² of lamina propria decreased from 249 ± 66 to

16±13 (p=0.016) (figure 1C-D). IL-8 levels also significantly dropped from 109.2±32.9 to 3.3±3.3 pg/ml (p=0.016).

Patients who had recurrent LB whilst on active azithromycin therapy, displayed a higher % BAL neutrophilia (45.3±5.9%) compared to stable patients (1.7±0.3%) (p<0.0001) but not compared to patients with LB, not yet treated with azithromycin (65.3±11.2%) (figure 1E). Also the level of IL-8 in the BAL of these patients was very high (326.6±69.6 pg/ml), even higher compared to the LB without AZI (p=0.0026). There was no difference in the number of submucosal lymphocytes between patients with LB, whether treated or not with azithromycin (567±37 versus 766±59, p=0.96). In patients with recurrent LB under azithromycin therapy, however, the number of IL-17⁺ cells was significantly lower compared to LB before azithromycin treatment (26±24 versus 283±38, p<0.0001) and comparable to stable patients (16±10) (figure 1F). Also the IL-17⁺ cells/lymphocytes ratio was significantly lower in the recurrent LB versus the not yet treated LB patients (3.6±3.3 versus 34.9±2.5 %, p<0.0001) and again comparable to stable patients (5.0±2.7).

Doublestaining of IL-17 and CD4/CD8

In addition double staining was performed on patients with LB. Most IL-17 producing T-cells colocalized with CD8⁺ (figure 3). Very rarely CD4⁺ colocalization was found.

Discussion

The current study demonstrated that the number of IL-17⁺ cells is increased in the airway submucosa in LB, and correlates with %neutrophilia in BAL. After 3 months of azithromycin therapy, %BAL neutrophilia, IL-8 and IL-17⁺ cells significantly decreased. When patients developed recurrent LB with high BAL neutrophilia during azithromycin treatment, the number of IL-17⁺ cells did not differ from stable patients. T_c17 cells seem to be the major source of IL-17 during LB.

Presently, evidence is accumulating that LB is an independent risk factor for BOS (15). In fact, it is known that patients with a drop in FEV₁ due to isolated LB, do not respond well to high doses of IV steroids, in contrast to patients with isolated A-grade rejection (16). This is perfectly in agreement with our findings, suggesting another mechanism and another type of lymphocytic infiltration in acute A-grade rejection (non IL-17 related) versus LB, not yet treated with azithromycin (IL-17 related). Azithromycin was beneficial in increasing FEV₁ in patients with LB, while it decreased the IL-17⁺ lymphocytes and BAL IL-8 and neutrophil levels, suggesting an important action mechanism of azithromycin. This not only corroborates *in vitro* data showing that azithromycin decreases the IL-17 induced IL-8 production from airway smooth muscle cells (17), but may also further explain our previous finding that azithromycin reduces BAL neutrophilia in patients with neutrophilic BOS. Hence, it seems that azithromycin may be a valid and alternative treatment for patients with LB, although this needs further investigation.

Patients suffering from recurrent LB during azithromycin treatment did not show an increased number of submucosal IL-17⁺ cells, although the total number of lymphocytes, BAL IL-8 and neutrophilia was comparable to levels of patients with LB before azithromycin. This provides evidence for other, yet unknown, mechanisms leading to BAL neutrophilia, which are clearly IL-17 independent, and hence, no longer respond to azithromycin.

The exact source of IL-17⁺ cells seems to be CD8⁺ lymphocytes as demonstrated by double staining. There are (currently) five human types of IL-17 producing T- lymphocytes, namely CD4⁺ (T_H17) cells, CD8⁺ (T_C17) cells, $\gamma\delta$ T-cells, iNK T-cells and LTI cells (2). In our study, however, there was an almost 100% correlation between IL-17 and CD8 positivity, hence, we did not further explore the other IL-17 producing cell types. This corroborates previously published data showing a correlation between IL-17⁺ cells and CD8 cells, suggesting that not T_H17 cells but CD8⁺ cells are responsible for IL-17 production (18). Also in COPD, a predominant role for CD8 cells in the production of IL-17 has been described (19). A recent report also demonstrated IL-17⁺ neutrophils in the submucosa of cystic fibrosis patients (20), but this could not be corroborated in our study.

Although our study provides clear evidence for the involvement of IL-17⁺ cells in inflammatory-related complications after LTx, it has some drawbacks. Biopsies are small and this may lead to sampling error. It could be argued that the groups are small, but a higher number of biopsies or patients would probably not change the conclusions of this study. One could argue that the effect of azithromycin is very preliminary as its effect is only demonstrated in 7 patients with LB, but these were the only patients with a follow- up biopsy. Moreover the 10 patients with renewed LB under active azithromycin, did not show an increased number of IL-17⁺ cells in the submucosa, severely strengthening our observation that azithromycin may attenuate IL-17⁺ lymphocytes, by a yet unknown mechanism.

In conclusion our data demonstrate that LB is characterized by high numbers of IL-17/CD8⁺ cells in the airway wall. Moreover azithromycin is able to significantly decrease the number of IL-17⁺ cells and the concomitant BAL neutrophilia and IL-8 level. In patients with recurrent LB after azithromycin therapy, a low number of IL-17⁺ cells are present, although the BAL fluid displayed high neutrophilia/IL-8. This may be explained by a different

mechanism of neutrophil recruitment, which needs to be further investigated, and when unraveled, may pave the way for newer therapeutic interventions.

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None of the authors have anything to disclose

	Stable	BOS	Infection	AR	LB	LB on AZI
Number (n)	10	10	14	10	24	10
Age (years)	48±5	37±5	47±4	34±6	49±3	38±6
Sex (Male/Female)	6/4	5/5	9/5	4/6	13/11	5/5
Type of LTx (SSLTX/SLTX/HLTX)	9/1/0	9/1/0	10/3/1	8/1/1	19/4/1	9/0/1
Indication for Tx (n)						
Emphysema	4	1	8	3	13	2
Fibrosis	2	4	2	1	3	0
CF/Bronchiectasis	2/1	2/1	3/0	4/0	3/1	3/0
Eisenmenger/other	0/1	0/2	1/0	1/2	1/3	0/5
CMV (match/mis-/NA) (n)	5/1/4	7/3/0	8/5/1	7/1/2	14/9/1	7/1/2
Immunosuppression (n)						
Steroids	10	10	14	10	24	10
FK/CsA	8/2	9/1	14/0	10/0	20/4	10/0
AZA/MMF/none	9/1/0	7/1/1	10/3/1	6/3/1	13/8/3	4/2/4
Post-operative days	332±126	631±109	269±115	185±63	579±161	895±271

Table 1 Patient characteristics. Data are expressed as mean±SEM or as total values. Abbreviations: BOS: Bronchiolitis Obliterans Syndrome, AR= acute rejection; AZI: azithromycin, LTx: lung transplantation, SSLTX: sequential single LTX, SLTx, single LTx, HLTx: Heart-lung Tx, CF: cystic fibrosis, CMV: cytomegalovirus, FK: tacrolimus, cSA: cyclosporin A, MMF: mycophenolate mofetil, AZA: azathiopine,

	Stable	BOS	Infection	AR	LB	LB on AZI
BAL						
Total cell count (*10 ³ /ml)	0.14±0.05	0.15±0.07	0.48±0.20	0.67±0.29	0.76±0.18	1.47±0.33
% Macrophages	91.1±3.4	74.9±9.0	57.9±9.4	45.7±10.7	48.6±6.1	26.5±10.5
% Lymphocytes	7.1±3.3	5.3±1.8	9.8±2.7	16.5±8.0	5.3±1.1	3.7±1.2
% Neutrophilia	1.7±0.3	5.8±1.4	31.2±9.9	28.7±8.4	45.3±5.9	65.3±11.2
% Eosinophilia	0.0±0.0	0.4±0.2	1.1±0.8	9.1±6.0	0.8±0.3	4.5±4.2
IL-8 (pg/ml)	39.8±15.6	54.5±25.3	82.8±35.5	74.6±28.2	98.4±27.3	326.6±69.6
IL-6 (pg/ml)	0.8±0.7	0.2±0.1	0.9±0.4	54.3±35.9	4.4±2.6	7.1±3.3
cells/mm ² lamina propria						
Lymphocyte, n	110±47	228±81	305±77	189±58	766±59	567±37
IL-17 ⁺ cells, n	16±10	6±4	46±23	29±16	283±38	26±24
IL-17 ⁺ cells/lymphocytes, %	5.0±2.7	2.0±1.3	8.9±4.2	7.5±3.2	34.9±2.5	3.6±3.3

Table 2 BAL and immunohistochemistry results. Data are expressed as mean±SEM. Abbreviations: BOS: Bronchiolitis Obliterans Syndrome, AR: acute rejection, AZI: azithromycin, LTx: lung transplantation, BAL: bronchoalveolar lavage, IL: Interleukin,

Figure legends

Figure 1: BAL neutrophilia and number of IL-17⁺ cells during acute rejection, LB, infection and chronic rejection.

(*; p<0.05; **; p<0.01, ***; p<0.001)

A) The %BAL neutrophilia was significantly higher in LB (n=24), acute rejection (n=10) and infection (n=14) compared to stable patients (n=10) (p<0.0001 and p<0.05 and p<0.05).

B) Significant differences in the number of IL-17⁺ cells/mm² lamina propria were observed in LB compared to all other studied groups (all p<0.0001).

C) The %BAL neutrophilia dropped from 62% to 20% in patients treated with azithromycin for 3-6 months (n=7) (p=0.016). The red line shows the mean drop in neutrophilia.

D) The number of IL-17⁺ cells/mm² of lamina propria decreased from 249 to 16 IL-17⁺ cells/mm² lamina propria (p=0.016) in patients treated with azithromycin for 2-6 months. The red line shows the mean drop in IL-17⁺ cells.

E) The %BAL neutrophilia in LB (n=24) and recurrent LB (n=10) during ongoing azithromycin therapy (LB AZI) is not different, but is significantly higher compared to stable patients with (p<0.0001).

F) In patients with recurrent LB during ongoing azithromycin therapy (LB AZI), the number of IL-17⁺ cells was significantly lower compared to B1R rejection before azithromycin (p=0.0012) and comparable to control levels (p=0.95).

Figure 2: Representative H&E and IL-17 immunohistochemical images

A) Representative H&E image of a BOS biopsy displaying an OB lesion.

B) IL-17 staining in BOS biopsy which demonstrates an absence of IL-17⁺ cells in the proximity of an OB lesion.

C) Representative H&E image of lymphocytic infiltrate around the bloodvessel in an A3 acute rejection.

D) IL-17 staining around the bloodvessel showing the absence of IL-17 positivity in the lymphocytes surrounding the blood vessel.

E) Representative H&E of a B1R biopsy, where a mild infiltrate can be seen around the airway.

F) IL-17 staining in B1R biopsy. The arrows point to the presence of IL-17⁺ cells.

G) Representative H&E of a B2R biopsy showing a severe lymphocytic infiltrate in the airway submucosa.

H) IL-17 staining in B2R biopsy. The arrows show a dense lymphocytic infiltrate with a lot of IL-17⁺ cells.

Figure 3 double stainings:

A) Fluorescent staining against IL-17A. IL-17⁺ cells are shown in red

B) Fluorescent staining against CD8. CD-8⁺ cells are shown in green

C) DAPI staining. Cell nuclei are shown in blue.

D) Merge image of CD8/IL-17 and DAPI. Arrows show IL-17/CD8 double positive cells.

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