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CHRONIC LUNG ALLOGRAFT DYSFUNCTION: CLINICAL AND EXPERIMENTAL STUDY

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**A Paolo, Andrea e Luca
mia linfa vitale.**

**A mamma, papà e fratelli
che credono in me.**

**Alla Prof.ssa Calabrese
che trova il risvolto positivo sempre
anche nelle avversità.**

**A Francesca
la cui volontà supera ogni difficoltà.**

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ABSTRACT

INTRODUCTION

Transplantation is the only effective treatment for several end-stage lung diseases. Remarkable progress has been made in improving outcomes, although the 5-year graft survival is still less than 50% primarily because of the development of chronic lung allograft dysfunction (CLAD). CLAD has been now recognized as a heterogeneous condition that includes an obstructive form (bronchiolitis obliterans syndrome, BOS) and a restrictive allograft dysfunction (restrictive allograft syndrome, RAS). BOS, and its histological correlate obliterative bronchiolitis -OB- represents the principal form of CLAD (~75%). The specific etiology and pathogenesis of BOS/OB are not fully understood. Multiple immune mechanisms seem to contribute to the development of BOS/OB, thus it is thought to represent a final common pathway of a process triggered by both alloantigen dependent and independent mechanisms. While the role of alloimmunity has long been established more recent studies have begun to demonstrate the role of autoimmunity in the development of BOS. A few experimental and clinical studies have demonstrated that collagen V and K- α 1 tubulin, modified during ischemia reperfusion injury, may trigger autoimmune response, both humoral and cell mediated. Interleukin 17 (IL17), a proinflammatory cytokine involved in autoimmune and infectious diseases, has recently been suggested to play a key role in the development of CLAD.

The development of animal models, mimicking the human transplantation procedure, is of great importance to elucidate the pathogenetic mechanisms leading to BOS/OB, to identify important biomarkers of OB and finally to test the effectiveness of new target therapies.

However up to today two important issues are largely discussed in rodent orthotopic models: 1) the reproducibility of the surgical procedure 2) the identification of the best

genetic strain (inbred versus outbred rats) for the development of immunological lesions similar to those in humans.

AIM OF THE RESEARCH

The main goals of the present PhD research project were:

- 1) development of a reproducible orthotopic lung transplant animal model, obtaining immunological lesions, particularly CLAD, similar to those of humans;
- 2) evaluation of IL17/IL23 pathway, crucial in autoimmune response, through a careful investigation in preclinical models and in clinical index cases of CLAD.

MATERIALS AND METHODS

Two different animal models were used to perform orthotopic lung transplantation (OLT): outbred rat strain (20 CD SPF left lungs were transplanted into VAF) and inbred rat strain (32 Lewis left lung rats were transplanted into Fisher 344). Only the long term survival animals (sacrificed 30 and 90 days after LT) were subjected to a full immunological evaluation as follows: a) detection of donor-specific antibodies (DSA) testing serum samples with the flow cross match technique b) morphological and immunophenotype evaluation of acute and chronic immunological lesions developed in the graft c) immunohistochemical and molecular (RT-qPCR) analysis of IL17/IL23 pathway in the graft and bronchoalveolar lavage (BAL) of animals and in all scheduled transbronchial biopsies of two index cases that developed CLAD.

RESULTS

Surgical mortality and early graft failure (within 24 hours) was higher in the **outbred** than inbred group (only 2 of a total 20 outbred rats survived). The two survival OLT outbred rats (sacrificed 13 and 14 days after OLT) developed well evident immunological

disorders: one showed acute cellular rejection (ACR) with coexistent early OB and the other late OB.

Immunological disorders (only minimal ACR: A1B1) were rare (only 1/11; 9%) in the first 15 days of OLT **inbred** rats. In this period the inbred grafts showed ischemia/reperfusion or infections. ACR (\geq A2B1) developed in 2/6 (33%) inbred grafts at 30 days.

Ninety days after OLT was the best time point for the development of immunological disorders: ACR (\geq A2B1) and OB (both early and late) were detected in 7/15 (46%) and 8/15 (53%) animals respectively, regardless of immunosuppressive treatment.

DSA IgG showed higher median levels in those with ACR or OB than those without (70% and 34%, respectively vs 13%).

A strong IL17 immunostaining was detected in inbred grafts that developed ACR and OB. IL 17 was equally expressed in inflammatory cells (macrophages and lymphocytes) of inbred grafts with ACR and OB while it was more expressed in epithelial and endothelial cells of inbred grafts with OB. No staining was detected in grafts of animals without any sign of rejection. IL23 expression was high in grafts with both absence and presence of rejection. Molecular analysis of IL17 and IL23 expression in BAL fluids showed higher levels of mRNA in grafts with ACR than OB. All scheduled transbronchial biopsies of the two index cases with ACR and OB showed IL17 overexpression with the same pattern detected in the preclinical model.

CONCLUSIONS

Outbred rodents that could have been more similar to humans due to high genetic diversity can not be used as a reliable OLT model because of the high rate of dramatic early graft failure.

A reproducible model of both ACR and OB was developed in inbred rats (Lewis to Fisher 344) and 90 days post-transplantation was the optimal endpoint established. IL17, overexpressed in ACR and overall in OB lesions, is a crucial mediator in post-transplant immunological lesions and could be considered a potential therapeutic target in clinical transplantation.

RIASSUNTO

INTRODUZIONE

Il trapianto di polmone è l'unica opzione terapeutica per alcune patologie polmonari terminali. Notevoli progressi sono stati fatti in questo ambito, tuttavia la sopravvivenza dell'organo dopo 5 anni è inferiore al 50%, principalmente a causa dello sviluppo del rigetto cronico. Il rigetto cronico si presenta in modo eterogeneo, in quanto può essere caratterizzato da una forma ostruttiva (sindrome della bronchiolite obliterante, BOS) o da una restrittiva (RAS). La BOS e il suo corrispondente aspetto istopatologico, la bronchiolite obliterante (BO), rappresentano la principale forma di rigetto cronico (~75%). L'eziologia e l'esatta patogenesi della BOS/BO non sono ancora state completamente chiarite in quanto diversi meccanismi immunitari sembrano essere coinvolti nel suo sviluppo e sembra essere la conseguenza di un processo indotto da meccanismi dipendenti/indipendenti dagli alloantigeni. Infatti, il ruolo dell'alloimmunità nello sviluppo della BOS/BO è stato dimostrato da tempo, mentre quello dell'autoimmunità è emerso solo recentemente.

Pochi lavori sperimentali e clinici hanno dimostrato che il collagene V e la tubulina K- α 1, modificati nel danno da ischemia e riperfusione, possono indurre la risposta autoimmune, sia umorale che cellulo-mediata. L'interleuchina17 (IL17), una citochina proinfiammatoria coinvolta in patologie autoimmuni ed infettive, è stata proposta recentemente come fattore cruciale nello sviluppo del rigetto cronico. Lo sviluppo di modelli animali, che subiscono una procedura trapiantologica analoga all'umana, risulta di grande importanza al fine di chiarire i meccanismi patogenetici legati allo sviluppo della BOS/BO, di identificare biomarcatori precoci e di provare l'efficacia di nuove terapie. Attualmente, due importanti aspetti vengono largamente discussi nei modelli di

trapianto ortotopico nei roditori: 1) la riproducibilità della procedura chirurgica e 2) l'identificazione del migliore genotipo (inbred o outbred) per lo sviluppo di lesioni immunologiche simili a quelle umane.

SCOPO DELLA RICERCA

I principali obiettivi di questa ricerca sono stati:

- 1) sviluppo di un modello animale di trapianto ortotopico di polmone riproducibile con lesioni immunologiche simili a quelle umane, in particolare quelle tipiche del rigetto cronico;
- 2) verificare l'ipotesi che IL17/IL23 giochi un ruolo chiave nello sviluppo del rigetto cronico mediante uno studio scrupoloso nei modelli preclinici e in casi clinici emblematici.

MATERIALI E METODI

Due modelli animali sono stati utilizzati per eseguire il trapianto ortotopico di polmone (LT): il modello outbred (20 polmoni sinistri CD SPF sono stati trapiantati in VAF) e il modello inbred (32 polmoni sinistri di ratti Lewis sono stati trapiantati in Fisher 344). Esclusivamente i ratti con sopravvivenza a lungo termine (sacrificati 30 e 90 giorni dopo LT) sono stati studiati in modo approfondito dal punto di vista immunologico mediante:

- a) ricerca di anticorpi anti-donatore (DSA) mediante citometria a flusso sui campioni ematici;
- b) valutazione morfologica ed immunofenotipica di lesioni immunologiche acute e croniche sviluppatesi nel polmone trapiantato;
- c) analisi immunoistochimica e molecolare (PCR semiquantitativa) del meccanismo IL17/IL23 nell'organo trapiantato e nel BAL dei modelli animali e nelle biopsie transbronchiali di monitoraggio di due casi clinici emblematici di pazienti che hanno sviluppato la BO.

RISULTATI

La mortalità perioperatoria e la disfunzione precoce dell'organo trapiantato (entro le 24 ore) erano più elevate nel gruppo di animali **outbred** rispetto agli inbred (solo 2/20 ratti outbred sono sopravvissuti): uno presentava rigetto cellulare acuto (ACR) con coesistente BO precoce, l'altro un rigetto cronico tardivo. Nei primi 15 giorni dopo LT i topi inbred presentavano raramente lesioni immunologiche (solo 1/11: 9%) e si trattava di ACR lieve (A1B1). In questo periodo i polmoni trapiantati inbred mostravano danno da ischemia/riperfusionazione o infezioni.

In 2/6 (33%) dei polmoni trapiantati inbred è stato riscontrato un importante ACR (\geq A2B1) 30 giorni dopo LT. Il sacrificio a 90 giorni è risultato ottimale per lo sviluppo di lesioni immunologiche: ACR (\geq A2B1) e BO (lesioni precoci e tardive) sono state riscontrate in 7/15 (46%) e 8/15 (53%) animali rispettivamente, indipendentemente dal trattamento di immunosoppressione.

Gli animali con ACR o BO presentavano livelli di Ig DSA maggiori rispetto a quelli che non presentavano alcun segno di rigetto (rispettivamente 70% e 34% vs 13%).

Una forte positività immunoistochimica per IL17 è stata riscontrata nei polmoni trapiantati dei topi inbred che avevano sviluppato ACR e BO. Non erano evidenti differenze significative nell'espressione di IL17 nelle cellule infiammatorie (macrofagi e linfociti) di polmoni inbred con ACR e BO, mentre è risultata maggiore nelle cellule epiteliali ed endoteliali di polmoni inbred con BO rispetto a quelli con ACR. Non è stata riscontrata positività nei polmoni di animali senza alcun segno di rigetto. L'espressione di IL23 era elevata sia in assenza che in presenza di rigetto. L'analisi molecolare dell'espressione di IL17 e IL23 nel BAL ha dimostrato maggiori livelli di mRNA nei polmoni trapiantati con ACR rispetto a quelli con BO. Tutte le biopsie di monitoraggio

dei due casi emblematici caratterizzate da ACR e BO hanno mostrato un'elevata espressione di IL17 con lo stesso pattern riscontrato nel modello preclinico.

CONCLUSIONI

I ratti outbred, che potrebbero essere considerati più simili all'uomo data la loro diversità genetica, non possono essere considerati un modello riproducibile di LT a causa dell'elevata mortalità precoce. E' stato sviluppato un modello riproducibile di rigetto acuto cellulare e cronico nei ratti inbred (da Lewis a Fisher 344) e il sacrificio 90 giorni dopo il trapianto è risultata la tempistica ottimale. IL17, notevolmente espressa nell'ACR e nella BO, è un mediatore cruciale nelle lesioni immunologiche post-trapianto e potrebbe rappresentare un importante target terapeutico nella trapiantologia clinica.

1) INTRODUCTION

1.1 Lung allograft dysfunction: clinical and pathological aspects

Lung transplantation represents the only therapeutic option for many incurable pulmonary diseases, such as cystic fibrosis, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease. Remarkable progress has been made in improving outcomes, although the 5-year graft survival is still less than 50% primarily because of the development of chronic allograft dysfunction (CLAD) [1]. Acute lung allograft dysfunction is sustained by different pathological processes whose specific treatment is crucial for their impact on CLAD.

1.2 Acute lung allograft dysfunction (primary graft dysfunction, infection, acute rejection)

Lung allograft dysfunction may be an acute phenomenon (acute lung allograft dysfunction, ALAD), leading to an acute decline in forced expiratory volume, FEV1 (with or without forced vital capacity decline) and may be due to various conditions that affect the graft, such as primary graft dysfunction (PGD), respiratory infections and acute rejection. In some of the conditions, spirometry will not be available, but ALAD may be diagnosed by other tools such as radiology, oxygenation status, and biopsy specimen.

PGD after lung transplantation represents a multifactorial injury to the transplanted lung that develops in the first 72 hours after transplantation; it is variously referred as “ischemia-reperfusion injury”, “early graft dysfunction” and “reimplantation edema”. PGD is characterized by severe hypoxia, lung edema and diffuse pulmonary opacities at radiography without other identifiable cause. The typical pathological pattern of PGD is diffuse alveolar damage (DAD). The incidence of PGD is reported to be in the range of 10

to 25%. Despite significant advantages in organ preservation, surgical technique, and post-operative care, PGD is up today an important cause of morbidity and mortality [2,3,4].

Infections are very important and common complications of lung transplantation. Bacterial pneumonias are the major infection complications in the early, intermediate, and late post-operative periods. Most of the infections occur in the first 11 months after transplantation. The underlying native lung may predispose to infection as occur in end-stage suppurative disease such as cystic fibrosis and bronchiectasis, in the late post-operative period the major predisposing factor is the presence of CLAD. The diagnostic approach to suspected pneumonia at any time period post-transplant includes sputum, blood cultures and often bronchoscopy with bronchoalveolar lavage (BAL), sterile brush and sometimes biopsy. The role of new biomarkers such as procalcitonin for diagnosis or follow-up has not been well established. Viral infection after lung transplantation is common and the most frequent are caused by cytomegalovirus (CMV) or caused by other community-acquired respiratory viruses. CMV is the second most frequent cause of pneumonia. Seronegative organ recipients are more susceptible to the infection. The lowest risk occurs in donor-negative/recipient negative patients [5]. The majority of CMV episodes occurred within the first 3 months following lung transplant, while the majority of the later infections were due to influenza and occurred after 1 year. CMV is the most prevalent and most important despite significant advances in both diagnosis and management. As well as contributing directly to both morbidity and mortality, mounting evidence suggests a relationship between CMV pneumonitis and chronic rejection in the form of bronchiolitis obliterans syndrome (BOS) and decreased survival despite treatment [6]. The incidence of CMV infection has been reported to range from 30% to 86%, with a

mortality of 2–12% [7]. CMV may coexist with rejection. Both of these individual processes induce a cytokine cascade that in essence promotes the development of the other. Tumour necrosis factor-alpha, a key signal in the reactivation of CMV from latency, is released during allograft rejection, thereby facilitating the onset of viral replication and subsequent infection. Conversely, infection of the vascular endothelium and smooth muscle by CMV leads to an upregulation of adhesion molecules promoting an increase in the quantity of inflammatory cells in the graft and subsequent development of rejection. Additionally, molecular mimicry and the production of anti-endothelial antibodies with CMV may also play a role in the development of rejection [8]. Recent diagnostic tools have effected a shift in the diagnosis of CMV infection and disease. The previous method of diagnosis, pp65 antigen detection, has been replaced by quantitative nucleic acid-based amplification testing via polymerase chain reaction (PCR) for the recognition of viraemia by most centres, with 85% of institutions using this method for monitoring and diagnosis [9]. There are no universally accepted viral load cut-offs for positive and negative results, and that reported values may be dissimilar between different laboratories. Despite this, current guidelines on the management of CMV in solid organ transplant patients do not clearly favor one test over the other and cite both as acceptable options for diagnosis. There are two accepted approaches to the prevention of disease from CMV, universal prophylaxis and pre-emptive therapy, and although there are no randomized trials comparing one strategy versus the other, most centres favour the former or may sometimes employ both [9]. The first, universal prophylaxis, involves administration of antivirals to all transplant patients deemed to be at high risk by serostatus. The second, pre-emptive therapy, is comprised of monitoring at-risk patients for viral replication and administering antivirals at a predetermined level of replication in the hopes of treating patients prior to the onset of disease. A Cochrane Review comparing

prophylaxis in different groups of solid organ transplant patients with antivirals versus placebo or no treatment showed a significant reduction in disease (relative risk 0.42), infection (relative risk 0.61), mortality from CMV disease (relative risk 0.26) and all-cause mortality (relative risk 0.63). Interestingly, the review also found a decrease in the risk of developing herpes-simplex virus, varicella-zoster virus and bacterial infections [10]. Prophylaxis may not only be beneficial in decreasing direct morbidity and mortality from CMV disease but may also have secondary effects by decreasing the morbidity and mortality of both acute and chronic rejection. The Cochrane Review previously mentioned failed to show a difference in acute rejection episodes, but other small studies have shown statistically significant differences in lung transplant recipient specifically and it is generally believed that prevention of CMV decreases the risk for acute rejection [11-15]. Fungal infections are a common complication after lung transplant with an estimated incidence of 15–35% and an overall mortality of 80% [16]. Complications at the site of the anastomosis (i.e. stenosis or necrosis) create the ideal environment for these infections. Other risk factors include the immunomodulatory effect of coexistent infections (i.e. viral) and neutropenia [17,18]. Pretransplant fungal colonization is common, especially in patients with cystic fibrosis and chronic obstructive pulmonary disease, and it has been associated with post-transplant fungal infection and BOS although not all colonized patients develop active/invasive infection [19]. The most common fungal pathogens are *Candida* and *Aspergillus* species, while Zygomycetes, *Scedosporium*, *Fusarium*, *Cryptococcus* species, histoplasmosis and coccidiomycosis occur less commonly. These infections, more prevalent during the first few months after transplantation, can manifest as invasive disease with a reported 1-year cumulative incidence of 8.6%. Regarding the diagnosis there are limited data on the role of minimally invasive tests such galactomannan, PCR and 1,3-β-D-glucan assay for the diagnosis of

invasive aspergillosis [20]. Diagnosis of invasive aspergillosis may require aggressive procedures (i.e. biopsy) to verify tissue involvement; however, this is not always possible, and often, the diagnosis is reached on evaluation of computed tomography chest findings and fungal staining/culture from bronchoscopy (i.e. BAL).

Lung allograft rejection can be a hyperacute, acute or chronic process and it occurs through immunologic mechanisms that include the innate and the adaptive immune systems [21]. The innate immune system can cause hyperacute rejection after transplantation, such as of an ABO mismatched donor even if donor and recipient are normally blood crossmatched [21].

When considering pathological aspects, acute rejection is characterized by perivascular mononuclear cell infiltrates, which may be accompanied by sub-endothelial infiltration, so-called endothelialitis or intimitis, and also by lymphocytic bronchitis and bronchiolitis.

Histological pulmonary allograft rejection is now graded according the revised working formulation for classification and grading of pulmonary allograft rejection as described in the following table [22].

Table 1. Revised Working Formulation for Classification and Grading of Pulmonary Allograft Rejection

A:	Acute rejection
	Grade 0—none
	Grade 1—minimal
	Grade 2—mild
	Grade 3—moderate
	Grade 4—Severe
B:	Airway inflammation
	Grade 0—none
	Grade 1R—low grade
	Grade 2R—high grade
	Grade X—ungradeable
C:	Chronic airway rejection—obliterative bronchiolitis
	0—absent
	1—present
D:	Chronic vascular rejection—accelerated graft vascular sclerosis

"R" denotes revised grade to avoid confusion with 1996 scheme.

The intensity of the perivascular mononuclear cell cuffs and the distribution of the mononuclear cells, including extension beyond the vascular adventitia into adjacent alveolar septa, form the basis of the histological grade. Acute rejection usually affects more than one vessel (particularly in adequate transbronchial biopsy samples) but is occasionally seen as a solitary perivascular infiltrate [22].

Grade A0 (No Acute Rejection): normal pulmonary parenchyma is present without evidence of mononuclear cell infiltration, hemorrhage or necrosis.

Grade A1 (Minimal Acute Rejection): there are scattered, infrequent perivascular mononuclear infiltrates in alveolated lung parenchyma.

Grade A2 (Mild Acute Rejection): more frequent perivascular mononuclear infiltrates are seen surrounding venules and arterioles and are readily recognizable at low magnification.

Grade A3 (Moderate Acute Rejection): easily recognizable cuffing of venules and arterioles by dense perivascular mononuclear cell infiltrates, which are commonly associated with endothelialitis.

Grade A4 (Severe Acute Rejection): diffuse perivascular, interstitial and air-space infiltrates of mononuclear cells with prominent alveolar pneumocyte damage and endothelialitis.

The revised working formulation allowed airway inflammation to be graded from B0 (no inflammation) to B2R (high grade small airway inflammation) and main histological features are summarized below [22].

Grade B0 (No Airway Inflammation): no evidence of bronchiolar inflammation.

Grade B1R (Low-grade Small Airway Inflammation): mononuclear cells within the submucosa of the bronchioles, which can be infrequent and scattered or forming a circumferential band.

Grade B2R (High-grade Small Airway Inflammation): the mononuclear cells in the submucosa appear larger and activated, with greater numbers of eosinophils and plasmacytoid cells.

Grade BX (Ungradeable Small Airways Inflammation): the changes are ungradeable due to sampling problems, infection, tangential cutting, artifact, etc.

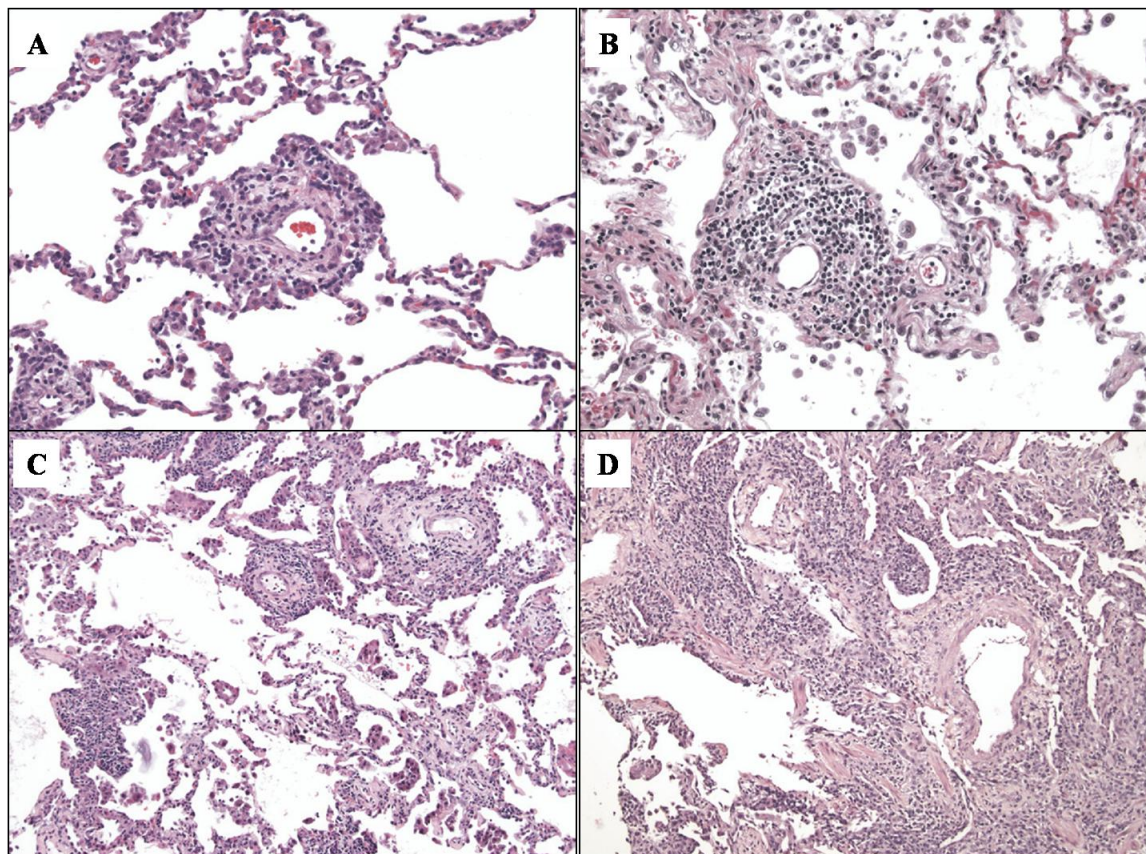


Figure 1.1 Emblematic cases of parenchymal acute rejection graded as A1 (A), A2 (B), A3 (C) and A4 (D). From Stewart S. et al., *J Heart Lung Transplant* 2007;26:1229–42.

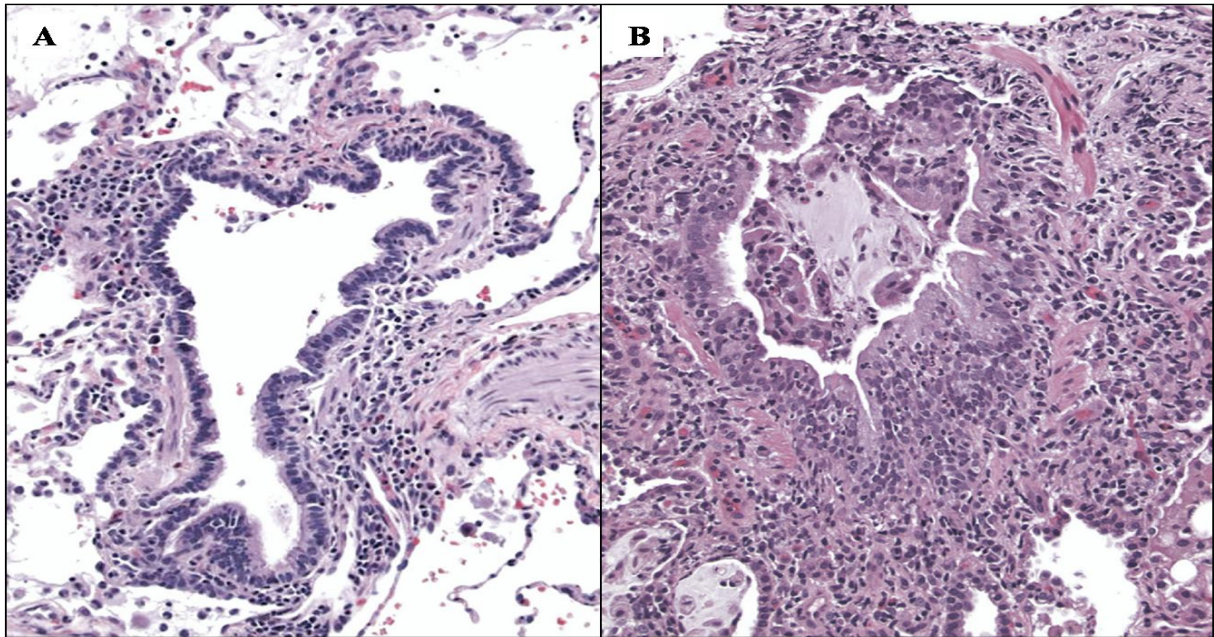


Figure 1.2. Emblematic cases of bronchiolar acute rejection graded as B1R (A) and B2R (B). From Stewart S. et al., *J Heart Lung Transplant* 2007;26:1229–42.

1.4 Chronic lung allograft dysfunction – clinical aspects

When the pulmonary function decline is persistent and not restored to 90% of baseline, chronic lung allograft dysfunction (CLAD) may be suspected [23]. CLAD following lung transplantation is a heterogeneous condition that includes an obstructive form (bronchiolitis obliterans syndrome, BOS) and a restrictive allograft dysfunction (restrictive allograft syndrome, RAS). Although BOS, characterized clinically by irreversible obstructive deficits in pulmonary function tests, remains the major cause of late mortality, RAS accounts for 25–35% of CLAD [24]. The term “chronic” implies a certain duration of time, and in analogy with the BOS definition, it has been suggested a minimum of 3 weeks as a sufficiently prolonged period to label allograft dysfunction as “chronic.” This interval of at least 3 weeks is chosen arbitrarily but is inspired by the BOS definition [23]. Diagnostic criteria of the two different types of CLAD are summarized in the following Table.

Entity	Classic BOS	RAS
Pulmonary function	Obstructive ($FEV_1 \leq 80\%$ of stable baseline value)	Restrictive ($TLC \leq 90\%$ of stable baseline value) and/or FEV_1/FVC normal or increased (with FEV_1 and/or FVC decline $\leq 80\%$ of stable baseline value)
HRCT thoracic imaging	Air trapping usually present No/minimal infiltrates With/without bronchiectasis	Infiltrates usually present With/without bronchiectasis With/without air trapping
Histopathology	OB (difficult to diagnose by transbronchial biopsy specimen)	Parenchymal/pleural fibrosis with/without OB
Clinical course	Typically progressive but may stabilize Recipients may have coexistent chronic bacterial infection May evolve to RAS	Tends to be relentlessly progressive May start as or coincide with BOS
Other	Usually responds poorly to pharmacologic therapies	Correlates with the presence of early DAD post-transplant

Table 1.1 From Verleden GM, *J Heart Lung Transplant* 2014; 33:127–133

BOS is clinically characterized by progressive (often fatal) airflow obstruction (FEV1 falls below 80% of the best value they achieved after transplantation), the absence of parenchymal infiltrates on chest radiographs, a mosaic pattern of perfusion on high-resolution computed tomographic scan, poor responsiveness to therapy, and high mortality rates [25]. On the basis of these criteria, BOS affects 48% of recipients at 5 years and 76% at 10 years after lung transplantation. Although treatment with azithromycin can sometimes stabilize and even reverse the progressive decline in lung function associated with CLAD, frequently this treatment fails, leaving retransplantation as the only treatment option.

RAS is defined as CLAD with an irreversible decline in total lung capacity (TLC) to < 90% of baseline as determined using the method explained later. BOS was strictly defined as CLAD without restrictive changes of RAS. Thus, the diagnosis of RAS was not made until FEV1 dropped to meet the criteria of CLAD, even if TLC had already declined to meet the threshold. The diagnosis of BOS was not made until a valid TLC measurement was done to rule out RAS, even if spirometry showed a decline in FEV1 meeting the criteria of CLAD [26].

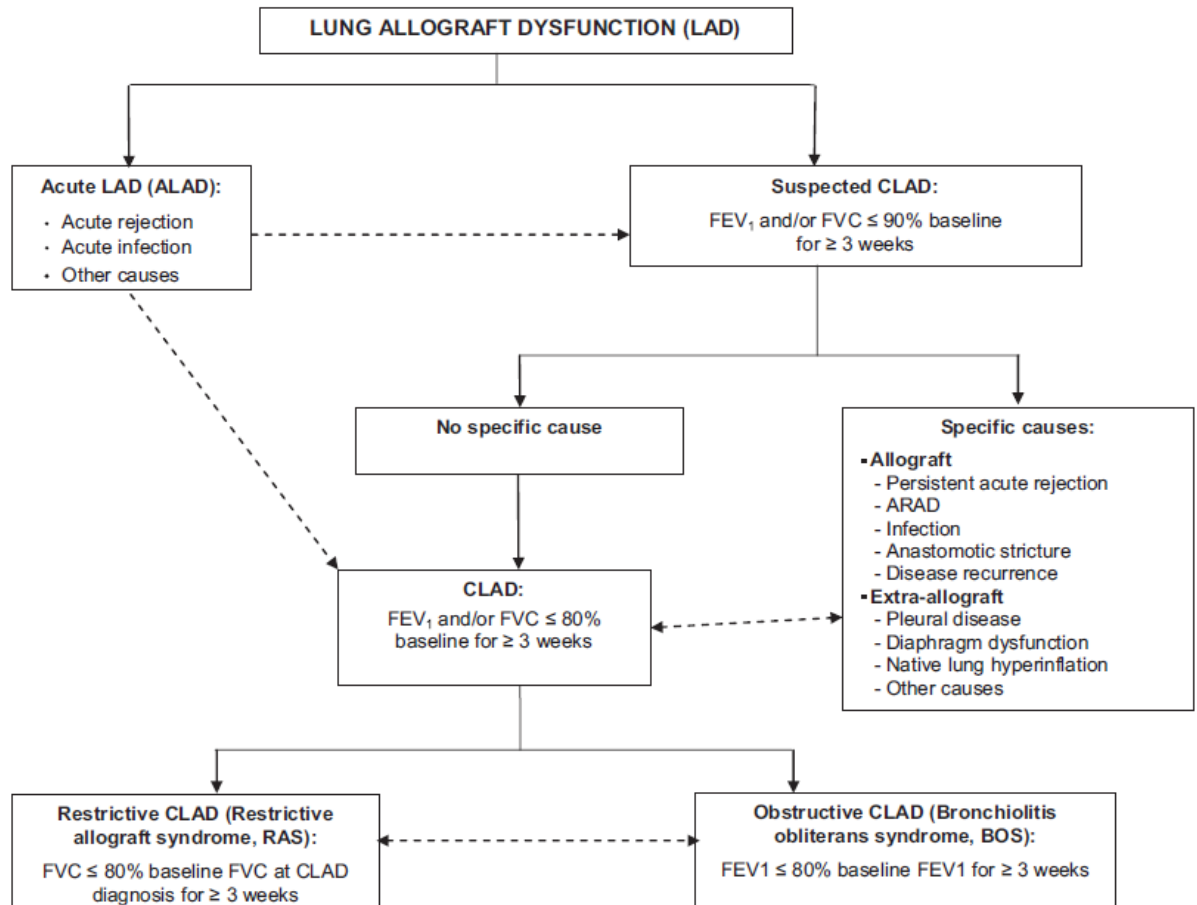


Figure 1.3. This flow chart suggests an approach that can be used to evaluate a lung transplant recipient's decline in post-bronchodilator forced expiratory volume in 1 second (FEV₁) with or without a decline in forced vital capacity (FVC) of $\geq 10\%$. This may be acute lung allograft dysfunction (ALAD) and may normalize with treatment. When the lung function decline, however, persists for at least 3 weeks without the FEV₁ and/or FVC returning to $\geq 90\%$ of the post-operative best values, it is suggested this is chronic, and chronic lung allograft dysfunction (CLAD) is suspected. Extended pulmonary function tests (PFT), including spirometry and lung volumes, high-resolution computed tomography (HRCT) of the thorax, and bronchoscopy with bronchoalveolar lavage (BAL) and transbronchial biopsy specimens may identify a cause or causes of suspected CLAD that may still be (completely) reversible upon specific treatment. If the FEV₁ and/or FVC declines further to $\leq 80\%$ of the post-operative best values, despite treatment or without identifying a clear cause, a specific CLAD phenotype should be identified. (Suspected) CLAD could also be a consequence of ALAD if the lung function decline persists. Some patients never develop suspected CLAD but may already have CLAD when they are diagnosed. BOS: bronchiolitis obliterans syndrome; CXR: routine chest X-ray; FEV₁: forced expiratory volume in 1 second; SLT: single lung transplant; ARAD: azithromycin-responsive allograft dysfunction; RAS: restrictive allograft syndrome. From Verleden GM, *J Heart Lung Transplant* 2014; 33:127–133.

1.5 Chronic lung allograft dysfunction – pathological aspects

1.5.1 Obliterative bronchiolitis

The pathological term obliterative bronchiolitis (OB) was introduced in 1984 to describe airway lesions observed in five patients, suffering from BOS after lung transplantation, and these findings have been confirmed by others [27]. OB describes dense eosinophilic hyaline fibrosis in the sub-mucosa of membranous and respiratory bronchioles, resulting in partial or complete luminal occlusion. This tissue can be concentric or eccentric and may be associated with fragmentation and destruction of the smooth muscle and elastica of the airway wall. It may extend into the peri-bronchiolar interstitium. Mucostasis and/or foamy histiocytes in the distal air spaces are commonly associated with obliterative bronchiolitis and may be observed in transbronchial biopsies in the absence of bronchiolar occlusion or any bronchiolar tissue [22]. The consensus in 2006 was that the distinction between active and inactive obliterative bronchiolitis is no longer useful and the condition should be designated merely as C0, indicating a biopsy with no evidence of obliterative bronchiolitis, and C1, indicating that obliterative bronchiolitis is present in the biopsy. Histological OB is graded as described in the following table [22].

Table 1. Revised Working Formulation for Classification and Grading of Pulmonary Allograft Rejection

A: Acute rejection
Grade 0—none
Grade 1—minimal
Grade 2—mild
Grade 3—moderate
Grade 4—Severe
B: Airway inflammation
Grade 0—none
Grade 1R—low grade
Grade 2R—high grade
Grade X—ungradeable
C: Chronic airway rejection—obliterative bronchiolitis
1—present
D: Chronic vascular rejection—accelerated graft vascular sclerosis

"R" denotes revised grade to avoid confusion with 1996 scheme.

Transbronchial biopsy is an insensitive method for detecting OB and the clinical use of BOS with its functional grading is the preferred means of diagnosing and monitoring CLAD [22]. A very recent work by Verleden *et al.* using micro-computed tomography, demonstrated that the constrictive bronchiolitis targets conducting airways while sparing larger airways as well as terminal bronchioles and the alveolar surface [28].

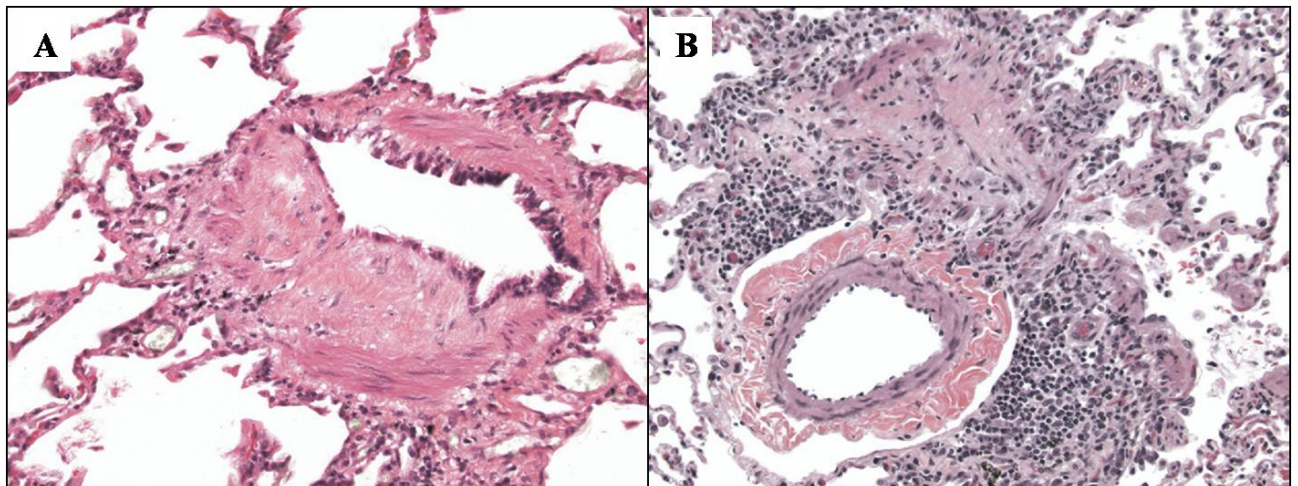


Figure 1.4. A) This small bronchiole shows eccentric scarring of the submucosa of the small airway associated with an inconspicuous peribronchiolar mononuclear infiltrate. The overlying epithelium appears attenuated, while the lumen of the airway is distorted. Such partial occlusion of the small airways may be responsible for significant increases in airflow resistance. H&E. B) The hint to underlying obliterative bronchiolitis in this case is the interrupted cords of smooth muscle forming a tubular structure associated

with dense scar tissue in a position adjacent to a pulmonary artery. H&E. From Stewart S. et al., J Heart Lung Transplant 2007;26:1229–42.

1.5.2 RAS

RAS is characterized by restrictive changes in pulmonary function tests that may correlate with inflammatory and fibroproliferative processes in peripheral lung tissue: extensive pulmonary interstitial fibrosis is dominant in the upper lobes of transplanted lungs and was initially reported based on radiographic and histological evidences [26]. A recent study represents the first reporting pleuroparenchymal fibroelastosis as a major histopathologic correlate of RAS in the largest single series of pleuroparenchymal fibroelastosis cases to date [24]. It is characterized radiologically by features suggestive of a chronic interstitial pneumonia with upper lobe predominance, and histologically by pleural fibrosis and parenchymal fibroelastosis in a predominantly subpleural distribution, with a sharp demarcation between fibroelastotic and unaffected lung parenchyma, and with the presence of fibroblastic foci at this interface. A limited number of cases with similar radiologic and pathologic features have also been reported, including a very recent article by Reddy et al, suggesting a broader spectrum of histopathologic findings [24]. Consistent with the recent finding that onset of RAS is often preceded by the presence of DAD in biopsies, it has been found that pleuroparenchymal fibroelastosis in RAS patients was very often present concurrently with features of DAD. Specimens obtained 1 year after clinical onset of CLAD typically demonstrated features of DAD, whereas those obtained at intervals of a year or more after CLAD onset showed DAD less frequently. These findings, together with the finding in some cases of DAD appearing to merge into areas of pleuroparenchymal fibroelastosis, support a temporal sequence of DAD preceding the development of pleuroparenchymal fibroelastosis in the natural history of RAS [24].

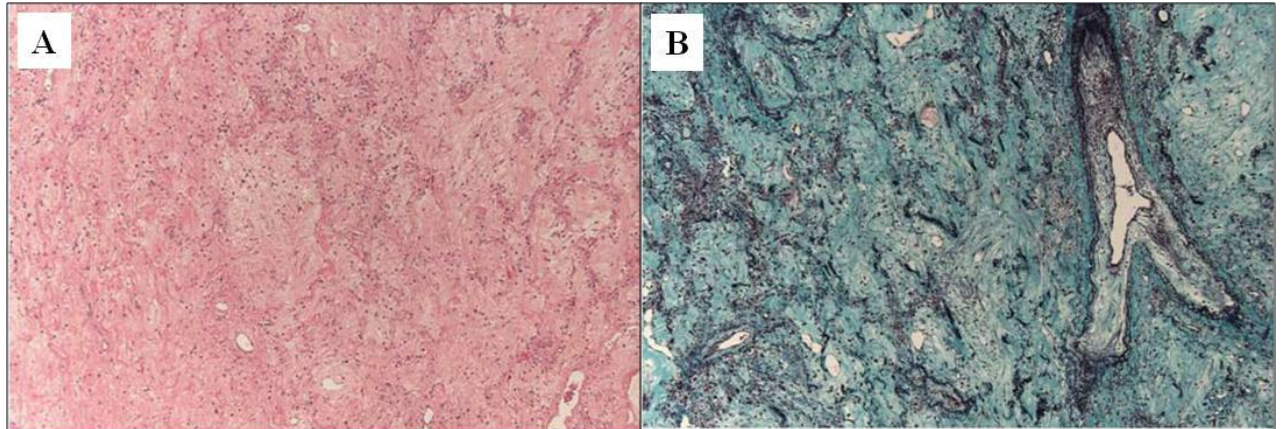


Figure 1.5. *Pleuroparenchymal fibroelastosis: areas of pleuroparenchymal fibroelastosis characterized by confluent areas of hypocellular collagen deposition with preservation and thickening of the alveolar septal elastic framework. (A) Hematoxylin and eosin stain, original magnification X50; (B) Elastic trichrome stain, original magnification X50. From Ofek E. et al., Modern Pathology (2013) 26, 350–356.*

1.6 Etiopathogenesis of bronchiolitis obliterans syndrome

The main factors that seem to be etiologically related to BOS are both immunological and non immunological: prolonged ischemia time, PGD, CMV pneumonitis, aspergillus colonization, respiratory virus infection and gastro-esophageal reflux.

Although the pathogenesis of this progressive airway obstruction is unknown, different immunological mechanisms seem to be involved in the development of BOS. Thus it is thought to represent a final common pathway of a process triggered by both alloantigen dependent and independent mechanisms.

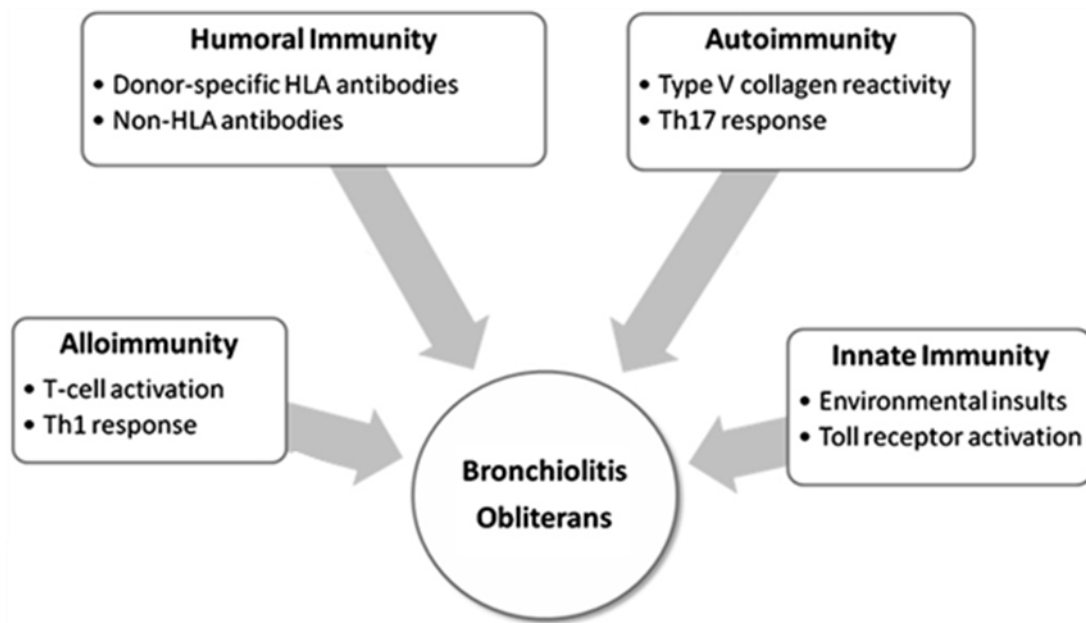


Figure 1.6. Multiple immune mechanisms contribute to the development of OB. Potential therapeutic targets are highlighted. HLA 5 human leukocyte antigen; IVIG 5 IV immunoglobulin; PAMP 5 pathogen-associated molecular pattern; Th 5 T helper. Modified from Todd J et al., *Chest* 2011; 140(2): 502 – 508.

1.6.1 Innate Immunity and Response to Environmental Insults

In recent years, the central importance of innate immunity in host defense has been recognized, particularly with the identification of toll-like receptors (TLRs) in humans. Innate immunity relies on recognition of highly conserved microbial pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by innate pattern recognition receptors (PRRs). TLRs, the prototypic family of innate PRRs, are found on pulmonary antigen presenting cells and lung epithelium where they regulate the pulmonary response to inhaled toxins and infections. Both exogenous and endogenous ligands for these receptors have been described, including lipopolysaccharide (LPS), high-mobility group box 1, and hyaluronan fragments. In the context of lung transplantation, genetic studies support the importance of innate immunity and TLRs in the pathobiology of BOS. Taken together, published data suggest a constant interplay among environmental stimuli, the innate immune response, recipient genetic susceptibilities, and adaptive immunity. In fact, many of the previously identified and emerging clinical risk factors for BOS are factors that would be likely to activate pulmonary innate immunity [29].

1.6.2 Alloimmune T-Cell Reactivity

The rarity of OB in patients without transplantation emphasizes the fundamental role of alloimmune

T-cell reactivity in the development of this condition. Acute cellular rejection is the most consistently described risk factor for BOS. Specifically, both acute vascular (A-grade) rejection, especially if histologically severe, and lymphocytic bronchiolitis (B-grade) rejection are associated with a significantly increased risk of BOS. In animal models using tracheal transplantation, the initial alloimmune response is of the T helper (Th) 1

type, with interferon- γ being the predominant cytokine. Interferon- γ upregulates the expression of adhesion and costimulatory molecules by airway epithelial cells, thus further augmenting the alloimmune response by stimulating lymphocyte infiltration and priming T-cell responses. The airway epithelial cell itself, once activated, generates a profibrotic milieu, producing growth factors that ultimately result in tracheal obliteration. The presence of obliterative disease in allogeneic, but not syngeneic, tracheal transplantations supports the importance of alloimmunity in airway fibrosis [29].

Despite clinical and basic evidence supporting a central role for alloimmune reactivity in the development of BOS, the failure of T-cell-based immunosuppressive regimens to prevent the onset of BOS or stabilize lung function after its onset supports the importance of other mechanisms of disease pathogenesis. Several additional immune- and nonimmune-related mechanisms that likely contribute to the high burden of OB after lung transplantation. Clearly, increased understanding of these factors is critical to the development of improved approaches to prevent and treat BOS [29].

1.6.3 Humoral Immunity

Laboratory advancements in the detection and characterization of human leukocyte antigen (HLA) antibodies by flow cytometry in conjunction with tissue immunostaining for complement fixation have provided clinical evidence that antibody-mediated rejection occurs in lung transplantation. The development of post-transplant HLA antibodies in lung transplant recipients is correlated with an increased risk for BOS and worse overall survival. Recognition of the role of humoral, or antibody mediated, processes in the pathogenesis of BOS has had a substantive impact on the clinical approach to its prevention and treatment [29]. Indeed, B-cell-modulating therapies are now being used to reduce the humoral immune response in lung transplant recipients who develop donor-

specific HLA antibodies in an effort to decrease the occurrence or progression of BOS. This treatment can have the benefit of being preemptive when given prior to the onset of acute rejection or BOS in patients with donor-specific antibody (DSA) [29].

1.6.4 Autoimmunity

The discovery of autoimmunity as a mediator of BOS is one of the most exciting novel cellular mechanisms recently described. Sumpter and Wilkes have developed the concept that rejection is biphasic, with the first phase representing tissue injury and the second representing autoimmunity. Tissue injury (from immune or nonimmune insults) exposes normally sequestered self-antigens, and their fragments are released into the lung, acting as triggers for autoreactive T-cell proliferation and autoantibody production [30]. The exposed self-antigens can thus sustain rejection even in the absence of persistent alloimmunity [29].

Type 5 collagen [col(V)], which resides beneath the basement membrane in the perivascular and peribronchiolar tissues of the lung, was the first described potential self-antigen.

A fivefold to 10-fold increased risk of high-grade BOS in those patients with elevated col(V)-specific cell-mediated immunity.

Further investigation suggested that autoreactive Th17 cells, known to be associated with chronic fibrotic autoimmune diseases in humans, in part mediate this response. In added support of this concept, a separate study found several cytokines important in Th17 cell development to be present in increased amounts in BAL fluid from patients with BOS compared with control subjects. Recently, other novel autoimmune targets on the epithelial cell surface, including K- α 1 tubulin, have been identified, and binding of autoantibodies to these targets has been shown to promote fibroproliferative events in

vitro. The concept of inducible immune tolerance to col(V) or other self-antigens is highly intriguing, and exploitation of this idea may represent a future novel approach to the prevention or treatment of BOS [29].

Th17 cells, a subset of T helper cells distinct from Th1 and Th2 cells, play a key role in the production of several cytokines such as IL17, IL21 and IL22. First described in 1983, IL17a is the first member of the IL17 family that is comprised of six isoforms. Produced by T lymphocytes, these cells promote neutrophil growth and activation in the lungs, joint space, central nervous system, and intestines. IL17a and IL17f specifically have been shown to play an important role in host defense and autoimmunity [31].

When Verleden et al. looked at biopsies and BAL specimens from lung transplant recipients undergoing acute rejection, higher IL17 were correlated with increased neutrophils and lymphocytes, demonstrating the potential role of IL17 in acute rejection. Additionally, this group then demonstrated that higher IL17 mRNA and protein levels in BALs from transplant recipients were associated with the development of BOS [32]. Another mechanism by which IL17 may contribute to rejection was postulated with IL17 inducing iBALT, which may contribute to autoimmune reaction in allograft lungs. Finally, data in a murine orthotopic lung transplantation model demonstrated that neutralizing IL17 prevented OB, down regulated acute rejection, and upregulated systemic IL-10. These studies offer multiple roles by which IL17 may mediate immune responses and rejection [31].

IL17 has also been implicated in the development of immune responses to self-antigens. Autoantibodies to col(V) from lung transplant recipients were IL17 dependent and associated with the development of OB after transplantation. Interestingly, the adoptive transfer of lymph node cells reactive against col(V) from immunized donors into isograft recipients induced OB without an alloimmune response. IL17 has been found to

contribute to the autoimmune response to K- α 1 tubulin as well. Among mice who were administered antibodies to donor MHC class I antigens, inhibition of IL17 resulted in decreased levels of autoantibodies to col(V) and K- α 1 tubulin. Combined, these results propose a key role for IL17 in the development of autoimmunity. However, recent work has highlighted that not all Th17 cells are pathogenic and that other key cytokines such as IL23 is necessary to induce autoimmune disease. Thus, the connection between IL17 and autoimmunity in human studies needs to be further and deeply investigated [31].

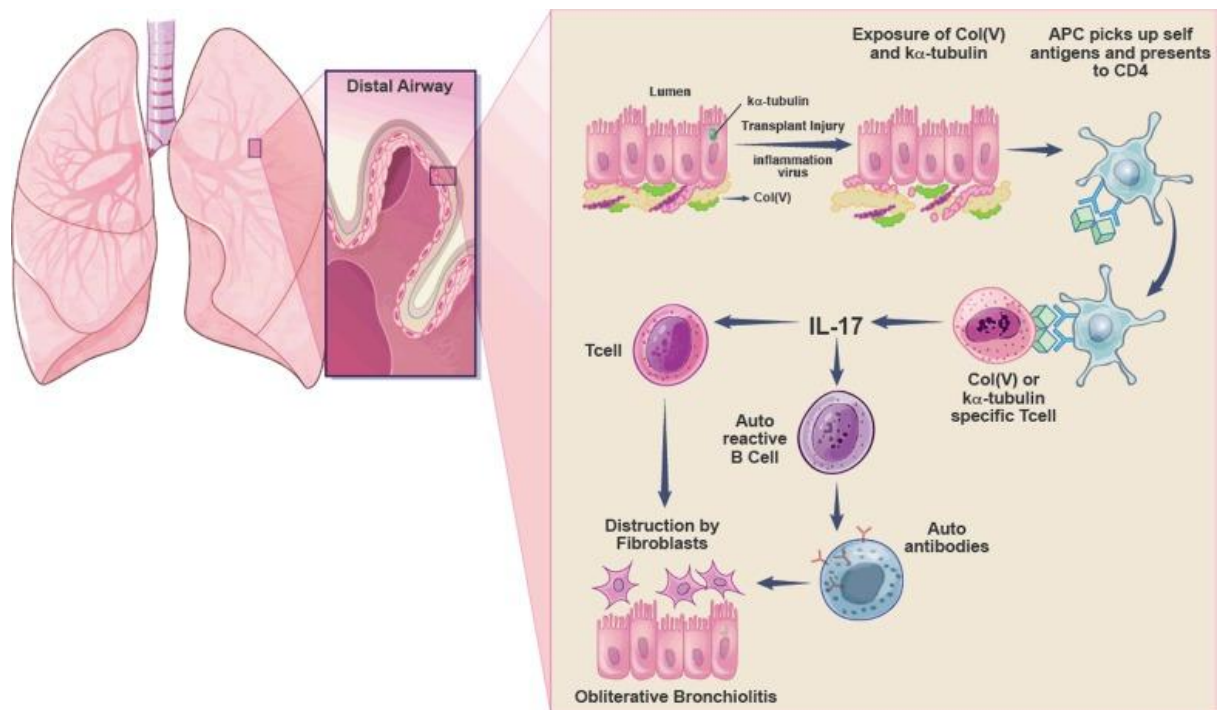


Figure 1.7. Autoimmunity in lung transplantation. After transplantation, exposure of collagen type V [col(V)] and K- α 1 tubulin triggers autoimmune responses, both humoral and cell mediated, which contribute to chronic rejection and obliterative bronchiolitis. APC, antigen presenting cell. From Weber DJ and Wilkes DS. *Am J Physiol Lung Cell Mol Physiol.* 2013;304(5):L307-11.

1.7 Animal models of BOS

To better understand the underlying mechanisms of OB development, a research model that mimicked the phenomenon of pulmonary chronic rejection was introduced in 1993 [33]. In this model, tracheal rings were heterotopically implanted under the skin or into the abdominal cavity of rats or mice, and these rodents developed the typical features of OB histology. This model possesses the advantages of reproducibility, and is simple to perform. However, researchers argued that this model did not sufficiently reflect the complexity of clinical OB. In search of more physiological models, various other techniques were introduced in rodents, among them the orthotopic tracheal segment interposition and the implantation of a donor trachea into a recipient lung [34]. With the introduction of the model of orthotopic single-lung transplantation in the mouse, the physiological ventilation and perfusion that equal the human transplantation condition can be provided. Preclinical models, such as the miniature swine transplantation model in which OB lesions develop, were proposed, using minor histocompatibility complex (MiHC) antigen–mismatched combinations. Although this model provides the obvious advantages of being closest to human OB, of the anatomic similarities of the transplant, of the ability to monitor individual animals continuously by repeated biopsy, and of the option for bronchoalveolar lavage and computed tomography imaging, these models are limited in their availability, their need for special breeding facilities, and their high cost. The main characteristics of rat and murine models of OB are summarized in the following table [34].

Model	Technical Demand	Reproducibility	Ventilation	Perfusion	OB Pathology
Heterotopic tracheal Tx	+	+++	No	No	+++
Orthotopic tracheal Tx	+	++	Yes	No	++
Intrapulmonary tracheal implantation	+	++	No	Restricted	++
Orthotopic lung Tx, rat	++	++	Yes	Yes	++
Orthotopic lung Tx, murine	+++	+	Yes	Yes	+

Definition of abbreviations: OB, obliterative bronchiolitis; Tx, transplant.
Plus signs indicate graduation: +, low/mild; ++, moderate; +++, high/severe.

Table 1.2. From Jungraithmayr W et al., *Am J Respir Cell Mol Biol.* 2013;48(6):675-84.

1.7.1 Heterotopic tracheal transplantation

The invention of heterotopic tracheal transplantation model in 1993 enabled the reproduction of the phenomenon of OB after transplantation for the first time. Tracheal segments were either implanted into a subcutaneous pouch in the neck, or placed intraperitoneally. This approach is technically effortless, reproduces representative results in vivo, and simulates the identical histopathological changes of human OB. The majority of published studies so far were performed on the basis of this model. However, its shortcomings are not negligible. Implanted tracheal segments undergo severe initial ischemia, relying only on diffusion from the surrounding tissue. Furthermore, physiological ventilation as a central functional aspect in lung transplantation does not occur, and large airways instead of bronchioles are investigated, thereby not reflecting the pathological hallmarks of OB. Finally, the observed changes occur within a short span of time, in contrast with the slowly developing OB in human lung-transplant recipients [34].

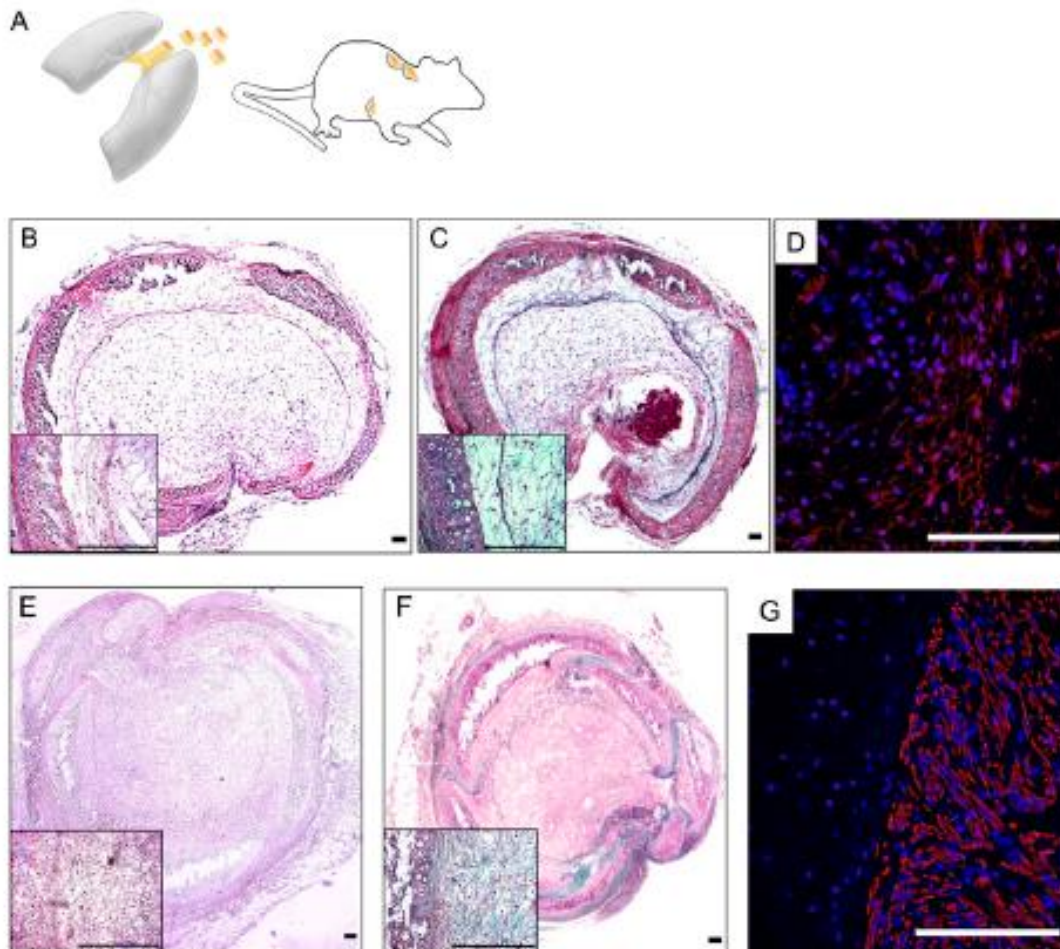


Figure 1.8. (A) Model of heterotopic tracheal transplantation. Segments of a donor trachea, consisting of one or two cartilaginous segments, are harvested from a donor. These are implanted heterotopically into either a subcutaneous pouch in the neck, or into the greater omentum via a small laparotomy. Histologic sections from murine heterotopic tracheal segment allografts are delineated according to hematoxylin-and-eosin (B), trichrome (C), and α -smooth muscle actin (α -SMA) staining (D; blue, nuclei; red, myofibroblasts). Histological sections from rat allografts are also shown according to hematoxylin-and-eosin (E), trichrome (F), and α -SMA staining (G; blue, nuclei; red, myofibroblasts). The obliterated lumen was characterized by a dense accumulation of proliferated fibroblasts and inflammatory cells (insets) with a strong intraluminal staining of smooth muscle cells (D and G), and the absence of respiratory epithelium. Scale bars, 100 μ m. From Jungraithmayr W et al. *Am J Respir Cell Mol Biol.* 2013;48(6):675-84.

1.7.2 Orthotopic tracheal transplantation

The disadvantages of the heterotopic tracheal model motivated the search for a more physiological setting. A new model of orthotopic tracheal transplantation was introduced by Ikonen and colleagues in 2000, and was later refined by Schrepfer and colleagues, in which a segment of a donor trachea was interposed into a recipient trachea to provide physiological ventilation [35,36]. These authors described a long-term patency of fully histoincompatible allografts in nonimmunosuppressed rats, as was also observed in mice. Despite acute alloimmune injury and the induction of myofibroblast proliferation, epithelial regrowth from the host limited the progression of OB, thus emphasizing the role of the epithelium in the control of airway obliteration [34].

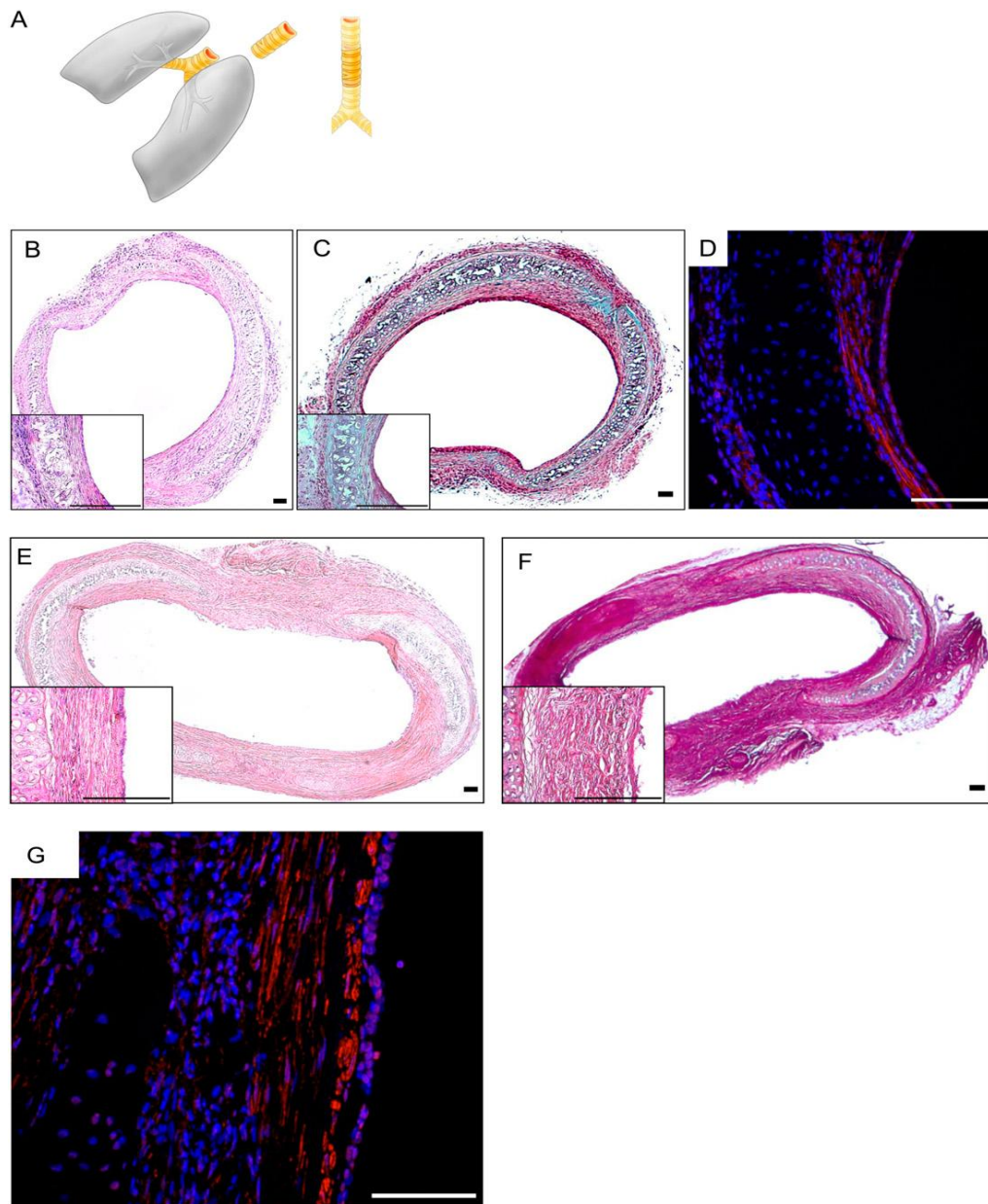


Figure 1.9. (A) Model of orthotopic tracheal transplantation. The whole trachea is harvested from a donor and orthotopically implanted by suturing the trachea at the cranial and caudal lumen by an end-to-end running suture into the recipient. Histologic sections from murine orthotopic tracheal allografts are delineated according to hematoxylin-and-eosin (B), trichrome (C), and α -SMA staining (D; blue, nuclei; red, myofibroblasts). Histological sections from rat allografts are also shown according to hematoxylin-and-eosin (E), trichrome (F), and α -SMA staining (G; blue, nuclei; red, myofibroblasts). On Day 60 after implantation, orthotopic allografts did not obliterate, but show a mild epithelial regrowth (insets in B and C) and a proliferation of myofibroblasts and smooth muscle cells (insets in E and F), with strong staining of smooth muscle cells (G). Scale bars, 100 μ m. From Jungrathmayr W et al. *Am J Respir Cell Mol Biol.* 2013;48(6):675-84.

1.7.2 Orthotopic lung transplantation

The model of orthotopic lung transplantation has the advantage not only of being a transplantation model of physiologic ventilation and perfusion, but it also best reproduces the surgical procedure in humans. In this model, the recipient's artery, vein, and main bronchus are cuff-anastomosed, or alternatively sutured to the respective vessels and bronchus of a single donor graft, thus mimicking the human transplantation procedure [34].

Orthotopic rat lung transplants have been used to investigate ischemia–reperfusion injury and acute rejection. However, in using rat orthotopic lung transplantation as a model of OB, control of the immune response is a major challenge. Without immunosuppression, major histocompatibility

(MHC)-fully mismatched lung allografts (e.g. Brown Norway to Lewis rats) are acutely rejected and become necrotic within several days, while only short-term immunosuppression (e.g. cyclosporine for the first few days) was found to enable long-term acceptance of allografts. A commonly used orthotopic rat lung transplant model of 'chronic rejection' is a moderately histoincompatible strain combination, from Fisher 344 (MHC type, RT1lv1) rats to Wistar Kyoto (RT1l) rats without immunosuppression [37]. Importantly, however, many reports have indicated that the chronic lesions in orthotopically transplanted rat lungs are not typical OB lesions [38].

Two important issues are up today largely discussed in rodent orthotopic lung models: a) the reproducibility of surgical procedure; b) the identification of the best genetic strain that develop immunological lesions similar to those in humans. Although the orthotopic lung transplantation model is technically demanding, it holds great promise for boosting clinically relevant research. To reach this goal, a wider use of this model must be achieved, because only a few centers worldwide can successfully implement this model at

present. On the basis of this research model, the development of reagents will be promoted through systematic exploration and meticulous analyses in therapeutic proof-of-concept studies [34].

Inbred strains are generated by 20 generations or more of brother-sister mating. Thus they show a more homogeneous genetic background than their outbred counterparts. Historically, inbred mice have been utilized for such studies as their limited genetic variability removes much of the inter-subject variability, making more reproducible results and simpler data interpretation. Recent studies have suggested that such genetically similar mice may not serve as an accurate model for the human conditions (which are genetically outbred) particularly when considered the immune responses occurring in the transplant field. A murine orthotopic lung transplant model has been reported recently, which shows new promise to use small animals for chronic investigation [40]. The possibility of using transgenic mice to explore the molecular mechanisms of OB is attractive, while reproduction of OB-like lesions might be challenging as in rat orthotopic lung transplantation [37].

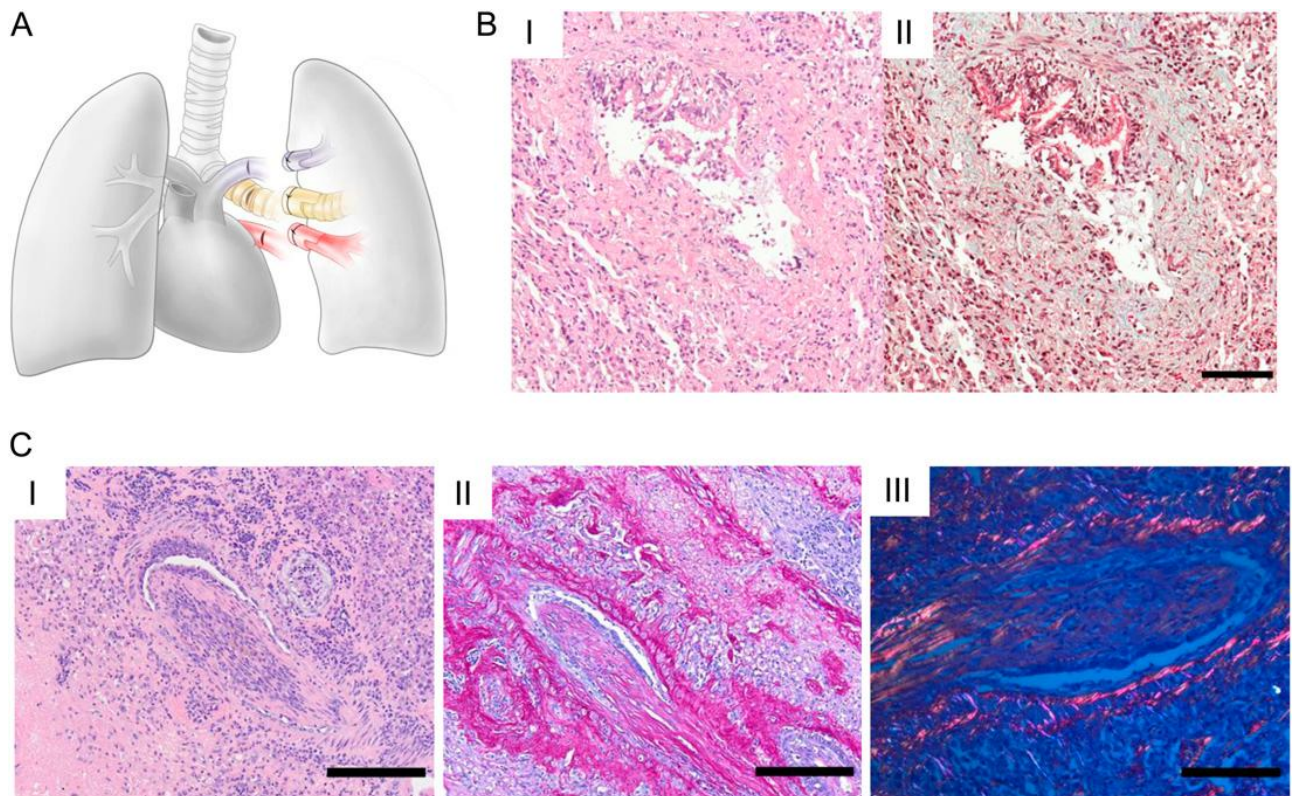


Figure 1.10. (A) Orthotopic single lung transplantation. The left lung is removed from a donor animal, the artery (blue), bronchus (yellow), and vein (red) are separated, and each is equipped with a specially designed plastic cuff. The lung is then introduced into the recipient's respective bronchus and vessels, to obtain a perfused and ventilated transplant. (B) Histologic sections from rat orthotopic lung allografts show typical obliterative bronchiolitis lesions on day 60 after transplant. Only small parts of the respiratory epithelium are intact, and the smooth muscle layer has vanished. Instead, increasing amounts of fibrous tissue have obliterated the bronchial lumen (I, hematoxylin-and-eosin; II, Trichrome). (C) Histological sections from murine orthotopic lung allografts show obliterative bronchiolitis, 70 days after transplantation. Mononuclear cells are prominently present within fibrotic plugs that protrude into the airway lumen (I, hematoxylin-and-eosin stain), with intense staining of collagen within the plugs (II, Sirius red). The polarized Sirius red light clearly indicates the difference in younger, rather reddish collagen and older, whiter collagen (III, Sirius red polarized). From Jungraithmayr W et al. *Am J Respir Cell Mol Biol.* 2013;48(6):675-84.

1.8 From bench to bedside

Each of the proposed models which provided interesting insights for the interpretation of the pathogenesis of OB. For almost 20 years, the technique of heterotopic tracheal transplantation was the leading experimental model in OB research. Important insights about how and when OB changes occur were achieved soon after the introduction of the heterotopic tracheal transplantation model. Hertz and colleagues demonstrated that within 21 days after transplantation, murine allografts developed airway fibroproliferation, whereas isografts showed normal respiratory epithelia. Boehler and colleagues then proved that the development of OB was alloantigen-dependent. Only allogeneic grafts showed typical OB lesions, whereas isografts were reconstituted with a normal epithelial lining after recovery from ischemia [41]. Hertz and colleagues later showed that OB lesions progressed if the initial period of alloimmune injury was sufficient, even if the alloimmune stimulus was removed [42]. To address the need for a more physiologic experimental setup, a variety of small animal models have been proposed during the past two decades, such as the orthotopic tracheal transplantation model or the intrapulmonary trachea implantation model. Answers to remaining questions could be determined via the newly introduced model of orthotopic murine lung transplantation, which not only reflects the full physiology of a transplanted graft, but also allows for the investigation of the influence from other factors that are most relevant in the evolution of OB, such as acid aspiration or other nonimmunologic stimuli. Beyond the possibility of genetic modifications in the mouse through which human diseases can be explored, transplant-related complications such as PGD or ischemia–reperfusion injury and their potential therapy options could be investigated in this model. Moreover, the investigation of non-heart-beating donor organs as well as the *ex vivo* reconditioning of potentially

transplantable organs, which plays an increasing role in the retrieval of organs, can be performed in this model. Moreover, the establishment of OB in minor mismatched recipients could also provide the last opportunity for testing novel therapeutic interventions such as inhibition of crucial mediators involved in EMT development.

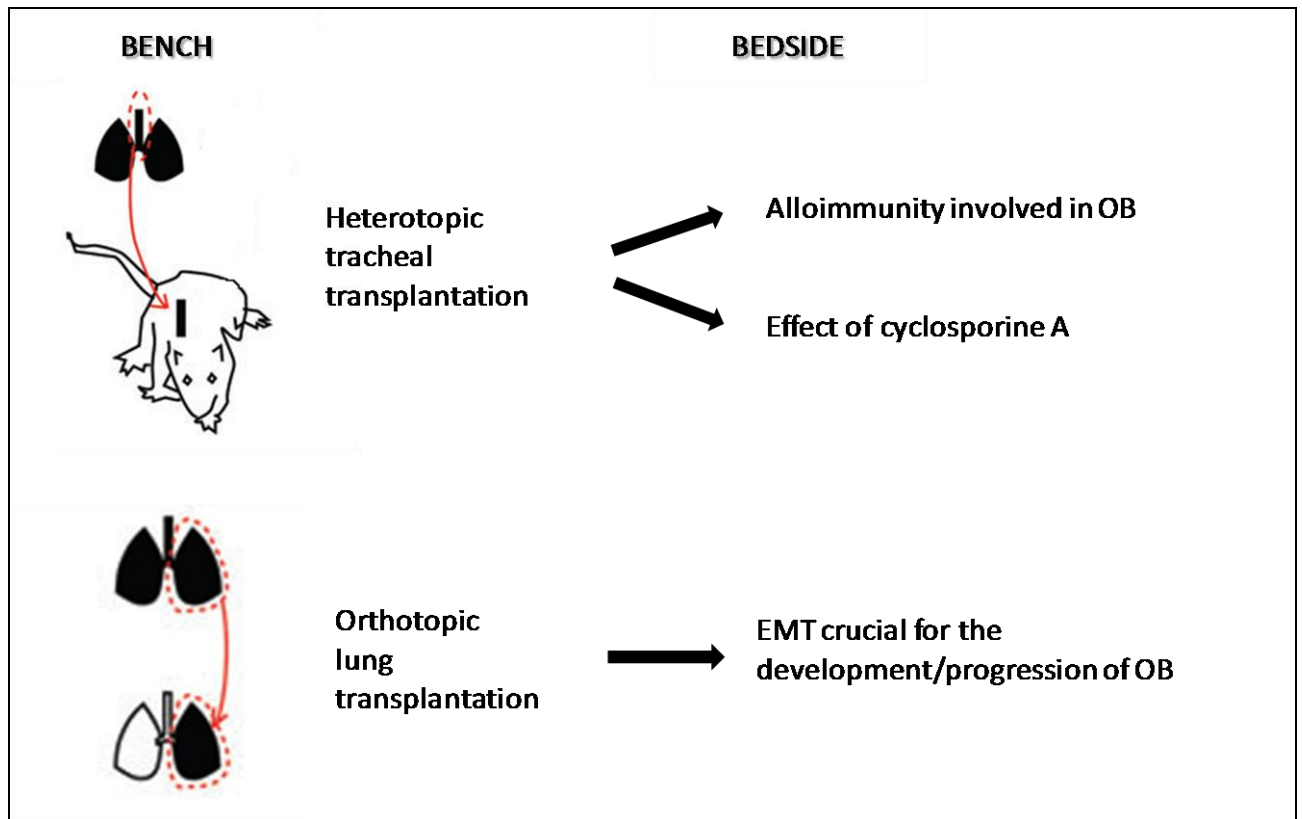


Figure 1.11. A cartoon describing the principal evidences in clinical lung transplantation derived from animal studies.

2) AIM OF THE RESEARCH

The main goals of the present PhD research project were:

- 3) Development of reproducible orthotopic lung transplant animal model, with immunological lesions, particularly CLAD, similar to those of humans (*first year of PhD*);
- 4) Test the hypothesis that IL17/IL23 plays a key role in the development of CLAD through a careful investigation in preclinical models and clinical index cases (*second/third year of PhD*);

All the activities were performed following the Gantt.

PHASES	ACTIVITIES	ACTIVITY DESCRIPTION												FIRST YEAR												SECOND YEAR												THIRD YEAR											
		MONTHS												1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
Development of CLAD experimental model	Activity 1	Study of literature																																															
	Activity 2	Development of experimental model																																															
	Activity 3	Macroscopic evaluation of rat lung tissues																																															
	Activity 4	Microscopic evaluation of rat lung tissues																																															
	Activity 5	Serum testing of donor-specific antibodies																																															
	Activity 6	Data presented at national and international conferences																																															
Evaluation of IL17/IL23 in experimental model	Activity 7	Study of literature																																															
	Activity 8	Immunohistochemistry for IL17 and IL23																																															
	Activity 9	Semi-quantitative RT-PCR for IL17 and IL23 expression																																															
	Activity 10	Data presented at national and international conferences																																															
IL17 expression in 2 index cases developing OB	Activity 11	Study of literature																																															
	Activity 12	Immunohistochemistry for IL17																																															
Identification of an adhesion molecule as carrier for targeted therapies	Activity 14	Study of literature																																															
	Activity 15	Immunohistochemistry for CD44																																															
Data analysis and writing of thesis/papers	Activity 14	Data analysis																																															
	Activity 15	Writing of thesis/papers																																															

3) MATERIALS AND METHODS

3.1 Rat orthotopic lung transplantation

3.1.1 Animal housing

Transplantation was performed pathogen-free in rats weighing 220–280 g. Animal care and animal experiments were performed according to the guidelines in force in Italy (DDL 116, 21/02/92 and subsequent addenda). Experimental protocols and appropriate animal care procedures were authorized by special Decrees of Italian authorities. All efforts were made to minimize animal suffering and animal care was supervised by veterinarians and animal technicians skilled in the healthcare and housing of transplanted rats. All animals were housed under standard environmental conditions (12-hour light-dark cycle, temperature: $22 \pm 1^\circ\text{C}$ and humidity: 50%) with free access to food and water.

3.1.2 Animal model #1: Outbred rat strain.

CD SPF left lungs were transplanted orthotopically into VAF recipients using non-suture cuff technique.

3.1.3 Animal model #2: Inbred rat strain.

Lewis (Lew) (RT-11) left lungs were transplanted orthotopically into Fisher 344 (F344; RT-1v) recipients using non-suture cuff technique.

11 rats died in the early post-operative period (from day 0 to 15th), and represent the “short-term survival” group, while the remaining 21 represent the “long-term survival” group.

The “long-term survival” group was divided in the following experimental subgroups:

1) without any suppressive treatment

GROUP A (n=6) sacrificed at 30 post- operative day;

GROUP B (n=6) sacrificed at 90 post- operative day;

2) with suppressive treatment

GROUP C (n=5) sacrificed at 90 post- operative day (cyclosporin A, 1.5 mg/kg from day 1 to 7)

GROUP D (n=4) sacrificed at 90 post- operative, treated (cyclosporin A, 1.5 mg/kg from day 7 to 14)

The transplantations were performed under clean, nonsterile conditions by a single surgeon. All the microsurgical procedures were done under the magnification between 6 to 20x of a stereoscopic microscope.

3.2 Organ harvesting

The donor animal was preanaesthetized in the glass chamber inhaling 2% Isoflurane. Five hundred units of heparin were administered via the inferior vena cava. A tracheostomy was performed and the animal was ventilated through the tube (14 GA iv. Catheter) with $FiO_2=1.0$, $f=100/\text{min}$, $TV=2.5-8,5 \text{ ml/kg}$ by a RodentVentilator. The lungs were flushed with 10 ml of cold (4 °C) preservation solution through the main pulmonary artery. Subsequently, the heart-lung block was removed at end-tidal volume and the left lung was separated ex-vivo from the heart and right lung. The inflated left lung was placed into preservation solution at 4°C until transplantation. Cuffs were prepared for vascular and bronchial anastomoses. We used 16-gauge intravenous catheters to make cuffs for each pulmonary artery (PA) and 14-gauge catheters for the pulmonary veins (PV) and bronchi.

3.3 Lung transplantation

Recipient animals were anesthetized, orotracheally intubated and ventilated with a 2% isoflurane and 100% oxygen mixture for adequate sedation at a rate of 70 breaths/min, with a tidal volume of 1 mL/100 gr body weight and a positive end-expiratory pressure of 2 cmH₂O. Rats were placed on right decubitus and a left posterolateral thoracotomy through the fourth intercostal space was made. The lung was removed from the chest cavity. The hilum of the lung was dissected and the pulmonary artery, pulmonary vein, and bronchus were identified. Ligatures and microvascular clamps were placed on each of these structures next to the heart. A small incision was made on the ventral part of pulmonary artery, vein and bronchus, then each element was anastomosed by placing cuffs inside each of the corresponding structures. A 7-0 polifilament ligatures were put around the cuffs to fix it to each element. Once the lung was implanted, the native lung was excised. The ventilation and then the perfusion of the graft were restored by removing clips from left bronchus, PV and PA respectively. The thoracotomy was then closed and a pleural drainage tube connected to a syringe was introduced. The drainage tube was aspirated to return the pleural cavity to negative pressure and, when the animal was breathing spontaneously, the drainage tube was removed.

3.4 Broncoalveolar lavage (BAL)

At definite time points lung recipients have been sacrificed. After opening the abdominal cavity and exsanguination via the aorta abdominalis, the lungs were exposed and a cannula (1.2 mm internal diameter) fixed in the trachea. The lungs were lavaged gently, via this cannula, to avoid tissue rupture. Lavage fluid (2 mL) was slowly injected into the lung using a syringe and then sucked out again. The operation was repeated 2 times with

fresh, cold (4°C) saline. BAL from either right and left lung have been obtained by specifically clamping the controlateral bronchus.

3.5 Serological analysis

The humoral immune response in the rat model of chronic lung rejection have also been evaluated. At early and late time points following transplantation, serum collected from recipient animals have been assessed for the presence of donor-specific IgG antibodies by flow cytometry. Donor cells have been incubated with recipient serum and specific antibody binding determined by the use of secondary anti-IgG antibodies.

To define donor-specific allo-antibodies responses, we harvested sera from F344 recipients at day 8, 30, 60 or 90, and tested by two-color flow cytometry to detect circulating IgG allo-Ab binding to LEW splenocytes as target cells. In recipients administered a suboptimal dosage of cyclosporin A, allo-antibodies have been evaluated at 90 days following transplantation.

Donor-specific antibodies were detected using testing serum samples with the Flow cross match technique. At sacrifice, serum samples were obtained from centrifugation of blood taken from the abdominal aorta, then immediately stored at -20 °C until analysis. Donor splenocytes, separated by density gradient centrifugation (Hystopaque, Sigma, St Louis, USA), were used as target cells. After measuring the concentration of Lew splenocytes, we incubated 1×10^5 cells with recipient serum samples (1:256 diluted, 50 μ l) for 30 min at 4 °C, then washed the samples in PBS containing 0.2% sodium azide three times. Negative controls were incubated with normal LEW rat serum. After that, samples were stained by 30 min incubation in the dark on ice with FITC-conjugated anti-rat IgG Fc γ Fragment specific or anti-rat IgM (Jackson Immuno Research laboratories, Inc.) (50 μ l) diluted to 1:100 used as second Abs. Phycoerythrin-conjugated anti-CD3 monoclonal Ab

(Biolegend, San Diego, CA, USA) were used to identify T cells. Target cell samples were then washed twice with PBS and tested with flow cytometry. We performed two-color FCXM analyses on 10 000- recorded cells with FACS Calibur (Becton Dickinson, San Jose, CA). CELL QUEST software (Becton Dickinson) was used for data processing. The signal events of lymphocytes gated by forward and side scatter parameters were recorded. T cells were selected by positive gating with phycoerythrin-conjugated anti-CD3. Fluorescence data were plotted using logarithmic amplification

3.6 Macroscopic and microscopic evaluations

The animal was sacrificed and the entire heart-lung block was explanted and carefully examined. In particular the graft was measured, weighed, photographed, cut and formalin-fixed paraffin-embedded. After 24 hours samples were sectioned and stained with haematoxylin-eosin for the routine diagnostic approach: specimens were examined for the presence or absence of ACR by using the nomenclature revised by the Lung Rejection Study Group of the ISHLT [22]. The presence of airway inflammation was determined based on evidence of lymphocytic bronchiolitis (LB) in all biopsy specimens [22].

3.7 Immunohistochemistry

Tissue samples were processed for sectioning and, after dehydration, embedded in paraffin wax. Five µm-thick sections were processed for immunohistochemical analysis of IL17, IL23 and CD44. In all experimental and clinical samples, immunohistochemistry (IHC) was carried out by using the following antibody panel: rabbit polyclonal anti-IL17 (1:100, Abcam, Cambridge, UK); rabbit polyclonal anti-IL23 (1:100, Bioss, Woburn, Massachusetts, USA); rabbit polyclonal anti-CD44 (1:100, Abbotec, San Diego, CA, USA). For all immunohistochemical experiments, negative controls were obtained by

incubation of the sections with the omission of primary antibody and using the antibody diluents alone or the appropriate non-immune IgG in each case. Briefly, after dewaxing and hydration, sections were incubated in citrate buffer 5 mM at pH 6.0 in a microwave oven for 30 minutes, for antigen retrieval. Afterwards, sections were treated for 60 min with the primary monoclonal antibodies and subsequently incubated with Ultravision Quanto Detection System HRP-polymer (Runcorn, UK). Immunoreactivity was visualized with diaminobenzidine (DAB, Dako, Glostrup, Denmark). Finally, the sections were counterstained with Mayer's haematoxylin. Data were expressed using a score system from 0 to 3 (0: no staining, 1: staining in up to 30% of cells, 2: staining in 30-50% cells, 3: staining in more than 50% of cells).

3.8 Semiquantitative RT-PCR

Molecular analysis for IL17 and IL23 expression was performed in all BAL samples. Total RNA was extracted from BAL fluids by a modified RNeasy method, as previously described [43]. The RNA pellets were re-dissolved in 20 µl sterile DEPC-treated water and incubated with 5U of deoxyribonuclease I (Sigma Aldrich, Milan, Italy) for 15 min at room temperature. 1 µg of extracted total RNA was used for the first complementary DNA (cDNA) synthesis and conventional RT-PCR was used. The PCR mix was made up to a volume of 50µl using 1X PCR Buffer II, 2mM MgCl₂ solution, 200 µM each of dATP, dCTP, dGTP, dUTP, 400 nM of each primer, 1.25 Units of AmpliTaq Gold and 6µl of cDNA. After the initial denaturation at 95°C for 10 min, the cDNA was amplified by 35 three-step cycles (30 sec at 95°C, 30 sec at annealing temperature, 1 min at 72°C). The appropriate number of cycles of PCR was determined so that the amount of PCR product versus the intensity of the ethidium bromide-stained product on Agarose gel were within a linear range. All samples were analyzed using glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) primers to verify adequate nucleic acid extraction. The sequences of primers for GAPDH, IL17 and IL23 and annealing temperature conditions are listed in Table 3.1. All samples were processed with simultaneous positive and negative controls (reaction mixture without RNA and cDNA templates). Precautions were taken to avoid false positives as a result of contamination by PCR product carry over, by strictly following the guidelines for the general handling of the PCR procedure, such as separation of rooms, boards, and lab benches (i.e. extraction of nucleic acids, PCR amplification and gene sequencing performed in different rooms with separate equipment and pipettes). Following PCR amplification, PCR products (15 µl) were subjected to electrophoresis on 3% ultrapure Agarose gel (Invitrogen, Italy) in 1X TAE buffer (Tris-acetate 0.04 M and EDTA 0.001 M) containing 0.03 µg/ml ethidium bromide. The gels were visualized by UV transillumination and photographed with a Alliance 2.7 (UVITEC, Cambridge, UK). The optical density of each band was quantified by densitometry using 1D gel analysis (UVITEC, Cambridge, UK) and levels of mRNA expression were normalized by calculating them as a percentage of GAPDH mRNA expression levels.

Primer	Sequence	Annealing temperature
GAPDH Fw	AATCCCATCACCATCTTCC	57°C
GAPDH Rv	GGCAGTGATGGCATGGACTG	
IL17 Fw	ACAGTGAAGGCAGCGGTACT	60°C
IL17 Rv	GCTCAGAGTCCAGGGTGAAG	
IL23 Fw	TCACAGGGGAGCCTTCTCTA	60°C
IL23 Rv	GGCACTAAGGGCTCAGTCAG	

Table 3.1 Sequences and annealing temperatures of the three different primer sets.

3.9 Immunofluorescence

Formalin-fixed samples were processed for paraffin embedding. Serial 5 µm-thick slices were cut using a microtome, mounted onto clean slides and stored at room temperature. Thus obtained slices were then dewaxed in xylene and rehydrated through serial alcohol's (100%, 95%, 70%; two changes of 3 min each) and distilled water (twice for 5 min each) and then air-dried for thirty minutes. Afterwards, unmasked the antigens and epitopes with 5Mm sodium citrate buffer at pH 6.0 in a microwave oven for 30 minutes. Tissue samples were processed for immunophenotypization of inflammatory cell infiltrate by immunofluorescence. In all samples, immunofluorescence was carried out by using the following antibody panel: anti-rat-CD4 fluorescein isothiocyanate (FITC) conjugated, anti-rat-CD8a allophycocyanin (APC) conjugated and anti-rat-CD45RA phycoerythrin (PE) conjugated (Biolegend, San Diego, CA, USA). All the antibodies were diluted 1:20 in phosphate buffered (PBS). Non-specific binding interaction were identified using specific isotype-matched, non-immune antibody or secondary antibody alone. Slides were stored at 4°C and analysed within 24 h. The analysis of samples were performed with a fluorescence microscope Leica DMI6000CS (Leica Microsystem CMS, Germany). FITC and PE fluorescence were visualized by excitation at 488nm and emission at 570 nm. APC fluorescence was visualized by excitation at 633 nm and emission at 660 nm. All samples were analysed by differential interference contrast (DIC) objective and images were viewed and captured at 40x magnification. Images were acquired using a DFC365FX camera and analysed with Leica LAS-AF 3.1. software.

3.10 Statistical analysis

All cases were coded and the measurements were made blindly. Data were expressed as mean \pm standard deviation (SD), or as median, Q1-Q3 when appropriate. Differences between groups were analysed using Kruskal-Wallis test and the Mann-Whitney U test.

4) RESULTS

4.1 Development of CLAD experimental models with morphologic characterization of both acute and chronic immunologic lesions and serological screening of DSA

ACTIVITY DESCRIPTION	FIRST YEAR												SECOND YEAR												THIRD YEAR											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
Study of literature	■	■	■																																	
Development of experimental model				■	■	■	■	■	■	■	■	■																								
Macroscopic evaluation of rat lung tissues				■	■	■	■	■	■	■	■	■																								
Microscopic evaluation of rat lung tissues				■	■	■	■	■	■	■	■	■																								
Serum testing of donor-specific antibodies																																				
Data presented at national and international conferences																																				

4.1.1 Animal model #1: outbred rat strain.

CD SPF left lungs were transplanted orthotopically into 20 VAF recipients using non-suture cuff technique. Seventeen rats died of early severe ischemia/reperfusion injury and only 3 rats out of 20 survived. One of them developed a severe PGD and died 24 hours after lung transplantation. The other two died at days 13rd and 14th post-transplant and the explanted grafts were carefully studied according the previous described methodology.

The gross examination of these grafts displayed a slight increase in volume and consistence. At histology: one showed severe acute cellular rejection graded A3B2 with coexistent initial OB (Figure 4.1 A and B). The other had severe OB with diffuse fibrosis, bronchiectasies and complete obliteration of the airways (“vanishing bronchiolitis”) (Figure 4.1 C and D).

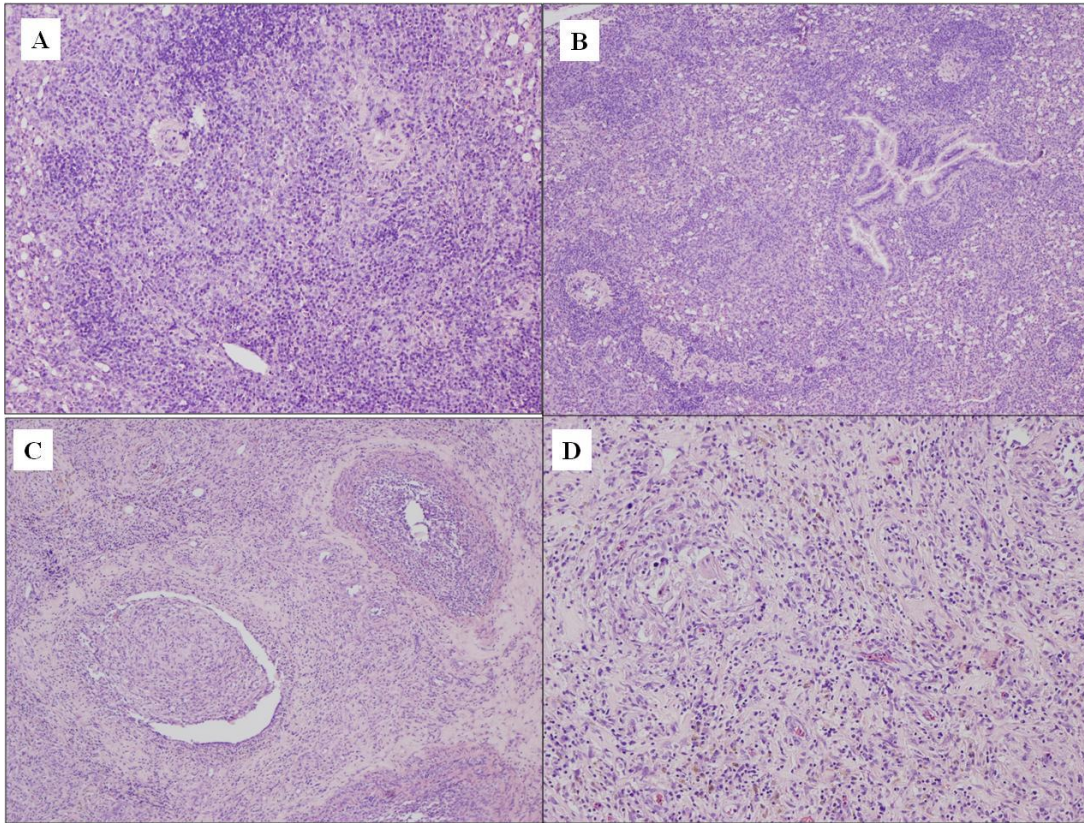


Figure 4.1. Emblematic image of severe ACR graded A3B2 detected in the first outbred graft (A, B) and OB, diffuse fibrosis with complete obliteration of the airways (“vanishing bronchiolitis”) detected in the second outbred graft (C, D).

4.1.2 Experimental model #2: Inbred rat strain.

Lewis (Lew) (RT-11) left lungs were transplanted orthotopically into 32 Fisher 344 (F344; RT-1v) recipients using non-suture cuff technique.

Rats with a short term graft survival:

11/32 rats (34.4%) died of PGD in the early post-transplant period (from day 0 to 15th, with a mean \pm SD survival of 3 \pm 4 days). The histology showed diffuse hemorrhage and alveolar damage, typical aspects of ischemia/reperfusion injury (Figure 4.2).

Rats with a long term graft survival:

Survived animals (65.6%) were divided into 2 experimental groups:

1) without any suppressive treatment

GROUP A (n=6), sacrificed at 30 post-operative day;

GROUP B (n=6), sacrificed at 90 post-operative day;

2) with suppressive treatment;

GROUP C (n=5), sacrificed at 90 post-operative day (cyclosporin A, 1.5 mg/kg, from day 1 to 7);

GROUP D (n=4), sacrificed at 90 post-operative day (cyclosporin A, 1.5 mg/kg, from day 7 to 14).

Main histological aspects are summarized in the following tables.

	Group	Animal	Survival (days)	Histological diagnosis
		F008	2	A0B0/ ischemia/reperfusion injury
		F026	3	A0B0/ ischemia/reperfusion injury
		F027	0	A0B0/ ischemia/reperfusion injury
		F062	2	A0B0/ ischemia/reperfusion injury
		F064	2	A0B0/ ischemia/reperfusion injury
		F065	1	A0B0/ ischemia/reperfusion injury
		F063	4	A0B0/ infection
		F069	1	A0B0/ ischemia/reperfusion injury
		F072	0	A0B0/ ischemia/reperfusion injury
		F045	8	A1B1/ ischemia/reperfusion injury
		F070	15	A0B0/ infection
Without immunosuppression	A	F007	30	OB
		F009	30	A0B0/ infection
		F011	30	A3B2
		F013	30	A0B0/BALT hyperplasia
		F014	30	A2/3 B1
		F015	30	A0B0/ infection
	B	F019	90	A0B0/ BALT hyperplasia
		F025	90	Severe OB
		F030	90	Severe OB
		F035	90	Aspergillus bronchitis/A2BX
		F049	90	A3B2
F051		90	A3B2/early OB	
With immunosuppression	C	F058	90	Severe OB (vanishing bronchiolitis syndrome)
		F060	90	A2B1
		F067	90	Severe OB (vanishing bronchiolitis syndrome)
		F073	90	Severe OB (vanishing bronchiolitis syndrome)
		F078	90	A3B2
	D	F053	90	Severe OB (vanishing bronchiolitis syndrome)
		F054	90	A0B0/ infection
		F055	90	A3B2/ early OB
		F079	90	A3B2

Group	POD	IS	n	% Acute rejection	% Chronic rejection	Other histological findings
	0-15	/	1	9%	0	Ischemic damage (82%) Infection (18%)
A	30	/	6	33%	17%	Infection (33%) BALT hyperplasia (17%)
B	90	/	6	50%	50%	BALT hyperplasia (17%) Aspergillosis (17%)
C	90	DAY 1 to 7	5	40%	60%	-
D	90	DAY 7 to 14	4	50%*	50%*	Infection (25%)

* *In one animal there was a coexistence of acute and chronic rejection.*

POD: post-operative death/sacrifice; BALT: bronchus-associated lymphoid tissue.

After 30 days, acute cellular rejection and OB were found in 2/6 (33%) and 1/6 (17%) inbred grafts respectively. After 90 days, OB was found in 8/15 (53%) rats, with or without suppressive treatment. Considering animals with suppressive treatment, group C developed ACR in 2/5 (40%) and OB in 3/5 (60%), while group D developed ACR in 2/4 (50%) and OB in 2/4 (50%) (emblematic histological aspects in Figure 4.3-4.6).

Immunofluorescence showed a prevalent CD4 and CD8 positive lymphocytes in ACR, minimal B lymphocytes and no NK cells (Figure 4.7).

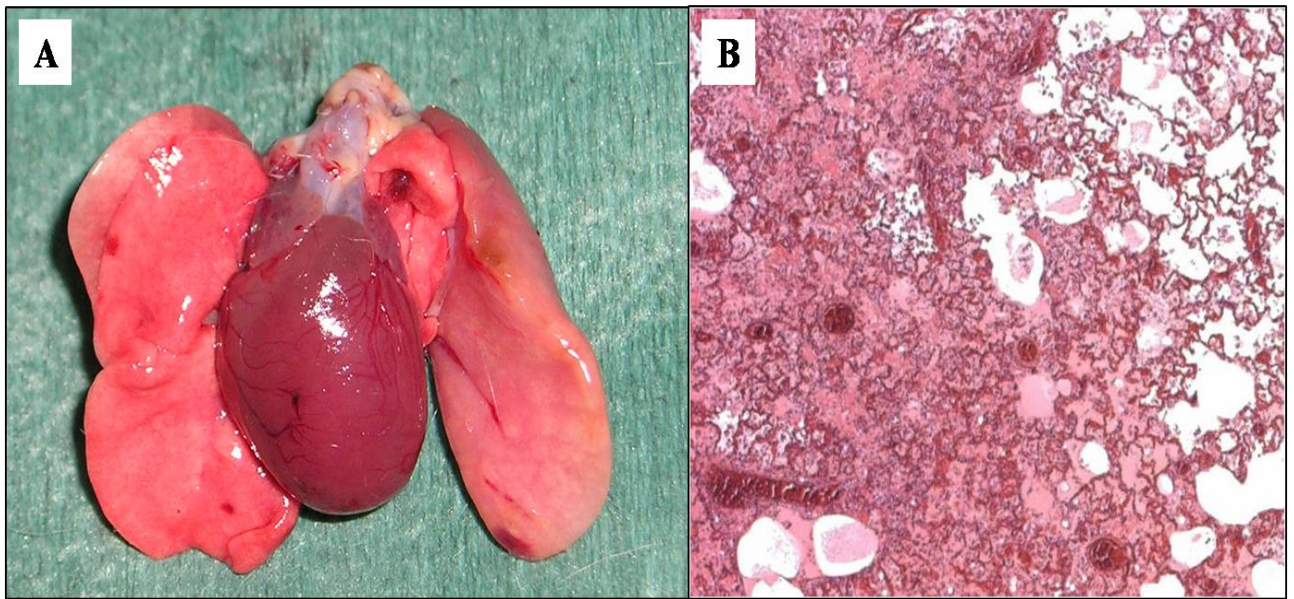


Figure 4.2. An emblematic image of ischemic injury, edema and blood extravasations typical of ischemia-reperfusion injury detected in the short term inbred graft survival.

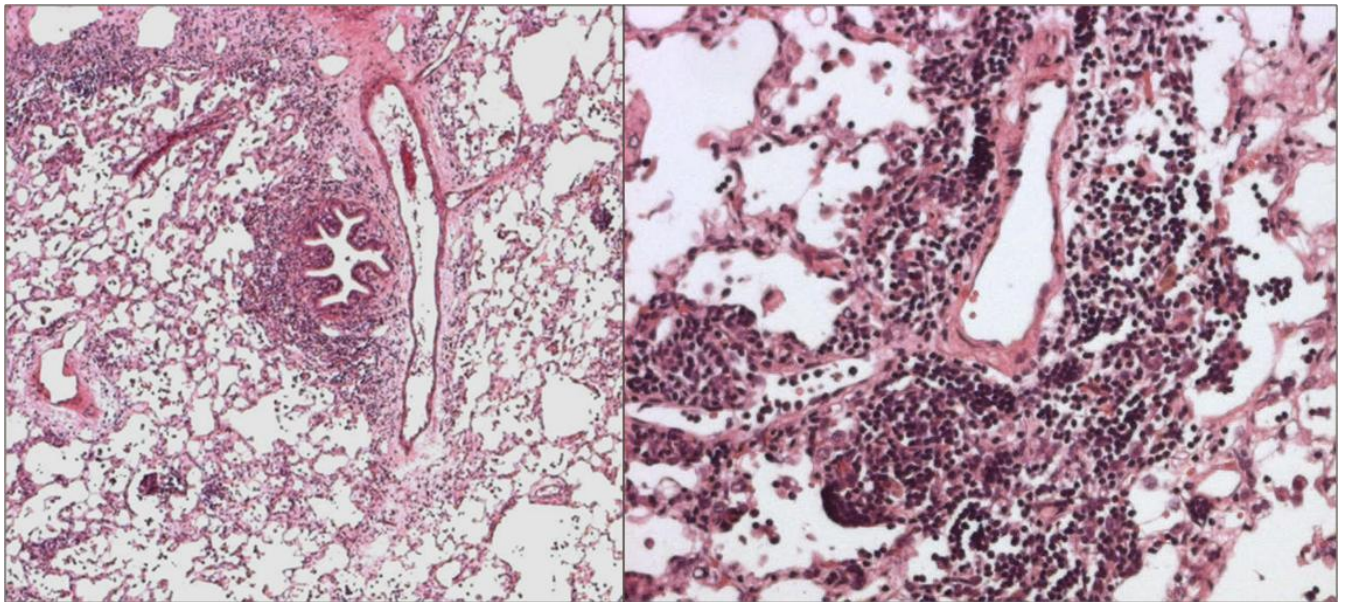


Figure 4.3. Emblematic aspects of severe acute cellular rejection (A3B1) 30 days after orthotopic lung transplantation.

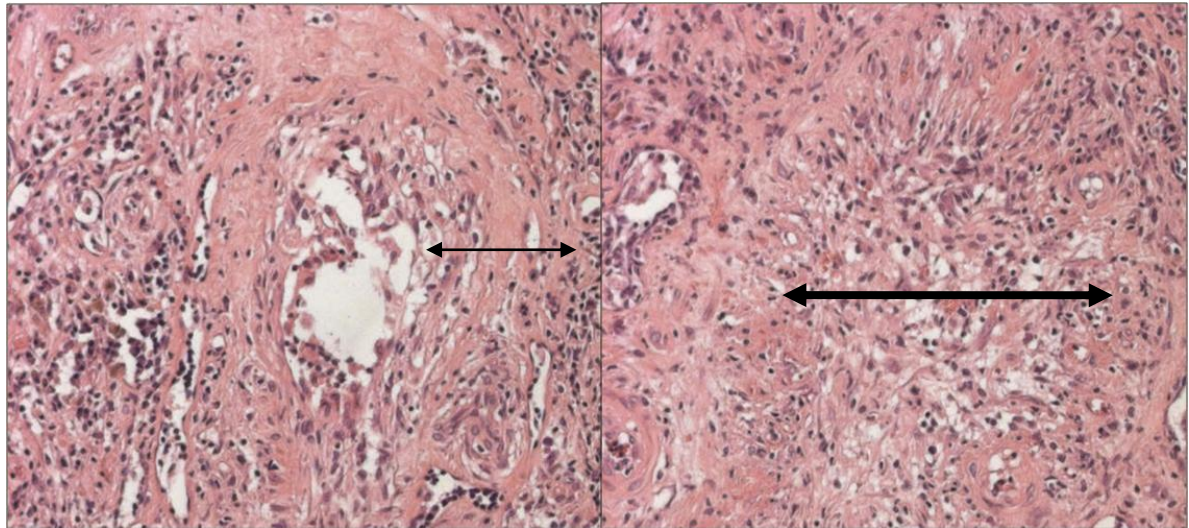


Figure 4.4. Emblematic images of chronic rejection: OB (C1) 90 days after lung transplantation: eccentric(arrow) and concentric (thick arrow) obliteration are well visible.

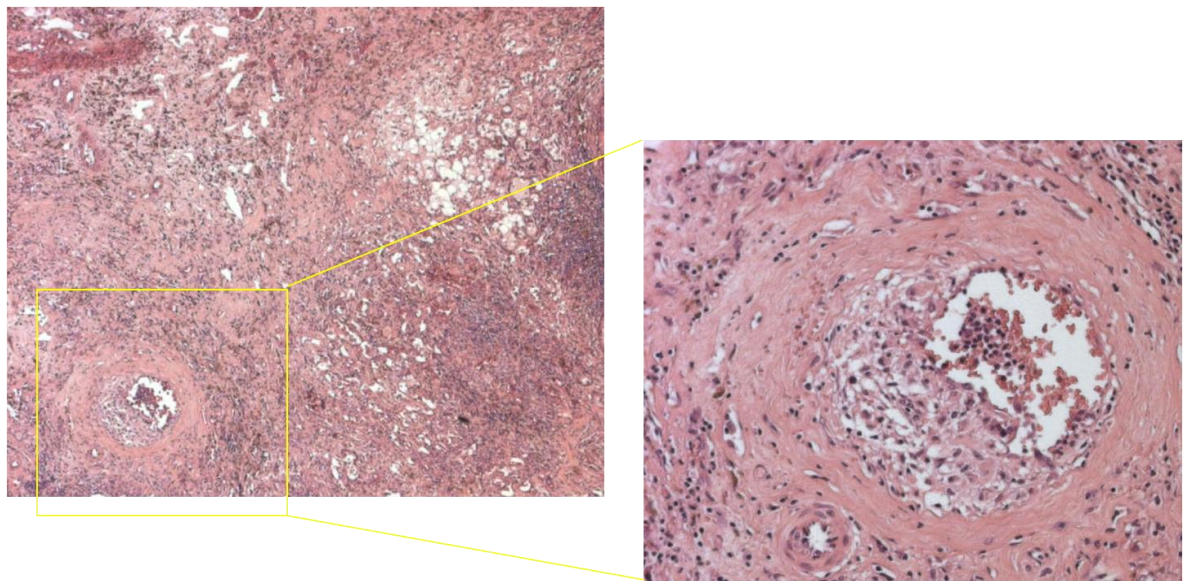


Figure 4.5. An emblematic image of chronic vascular rejection.

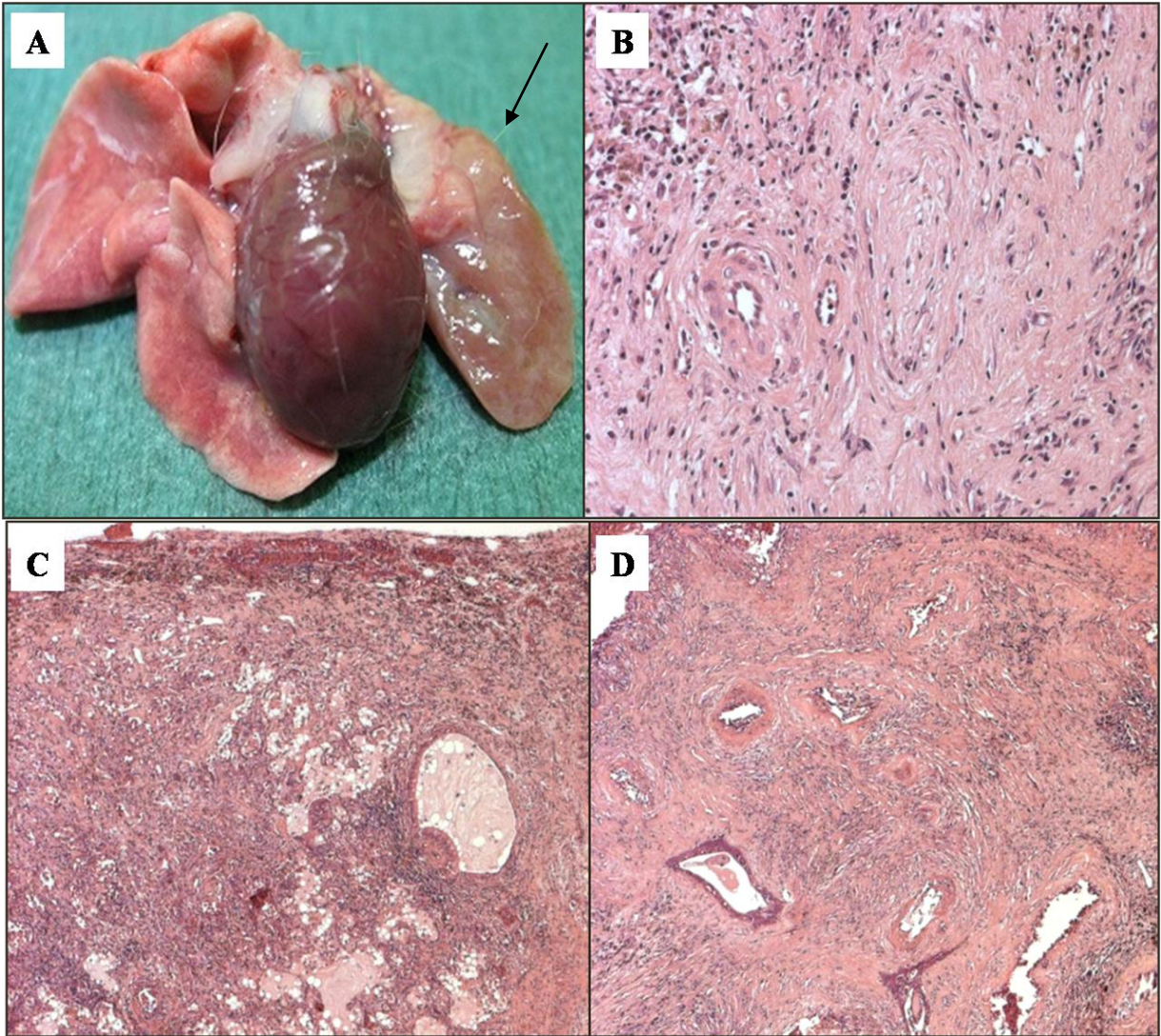


Figure 4.6. Indirect signs of severe chronic rejection: gross appearance showed a graft with white-grey color (A, arrow) while histology showed extensive fibrosis (B), bronchiectasies (C) and fibrosis with honeycomb (D), the so-called “vanishing bronchiolitis syndrome”.

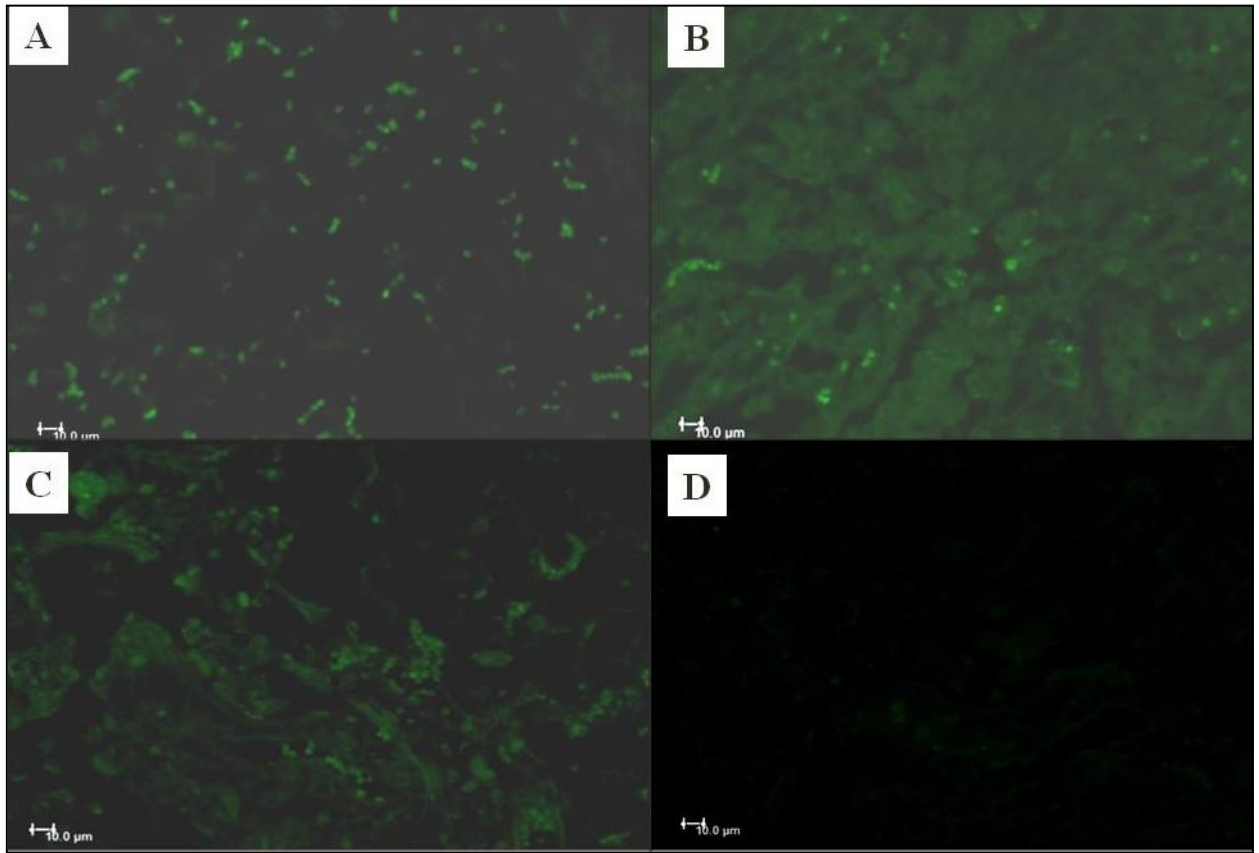


Figure 4.7. Emblematic images of immunofluorescence using specific antibodies to characterize $CD4^+$ T lymphocytes (A), $CD8^+$ T lymphocytes (B), B lymphocytes (C) and NK cells (D).

4.1.3 DSA detection

No donor specific IgG response can be detected at day 8 post transplantation and systemic IgG allo-Ab responses can be detected starting from day 30 following transplantation. At this time point IgG antibodies were significantly higher compared to that of normal LEW rat serum in most of the animal tested. Low levels or no antibodies were detected in animals without ACR (median values, Q1-Q3: 13.2, 9.3-18.4) (Figure 4.8). Higher levels of anti-IgG antibodies have been obtained in animals with ACR and OB (median values, Q1-Q3: 70.1, 56.5-74.5 and 34.3, 29.4-75 respectively) (Figure 4.8). Reduced levels of donor specific antibodies were detected in recipients treated with cyclosporine A and higher values of DSA were found in more severe ACR.

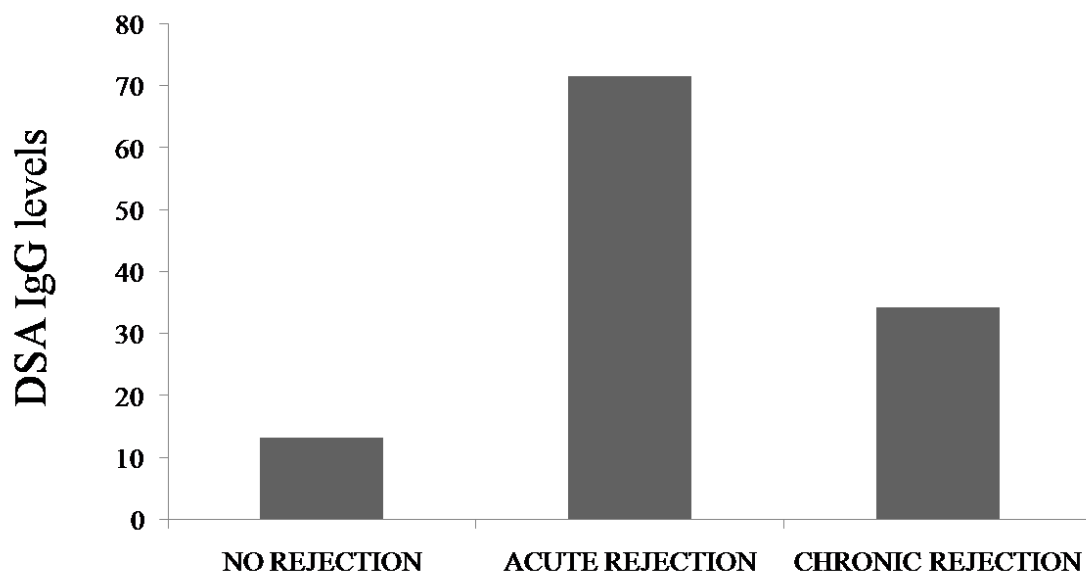


Figure 4.8. Histogram showing median DSA IgG levels in animals with or without acute/chronic rejection.

4.2 Evaluation of IL17/IL23 pathway (immunohistochemical and molecular analyses) in experimental model.

ACTIVITY DESCRIPTION	FIRST YEAR												SECOND YEAR												THIRD YEAR											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
Study of literature																																				
Immunohistochemistry for IL17 and IL23																																				
Semiquantitative RT-PCR for IL17 and IL23 expression																																				
Data presented at national and international conferences																																				

4.2.1 Immunohistochemistry

A strong IL17 immunostaining was detected in grafts of rats that developed ACR, both in inflammatory and epithelial cells (median, Q1-Q3: 3, 2.5-3 in macrophages, 2, 1.5-2.5 in lymphocytes, 2, 1-2.5 in epithelial cells, 2, 0.5-2.5 in endothelial cells) and OB (median, Q1-Q3: 3, 3-3 in macrophages, 2.5, 1.75-3 in lymphocytes, 3, 2.25-3 in epithelial cells and 3, 0.75-3 in endothelial cells) (Figure 4.9 and 4.10). No staining was detected in grafts of animals without any sign of rejection. Although not statistically significant, IL17 expression was higher in OB, mainly produced by macrophages, epithelial and endothelial cells. IL23 expression was high both in absence and in presence of rejection, mainly in macrophages and endothelium (Figure 4.9).

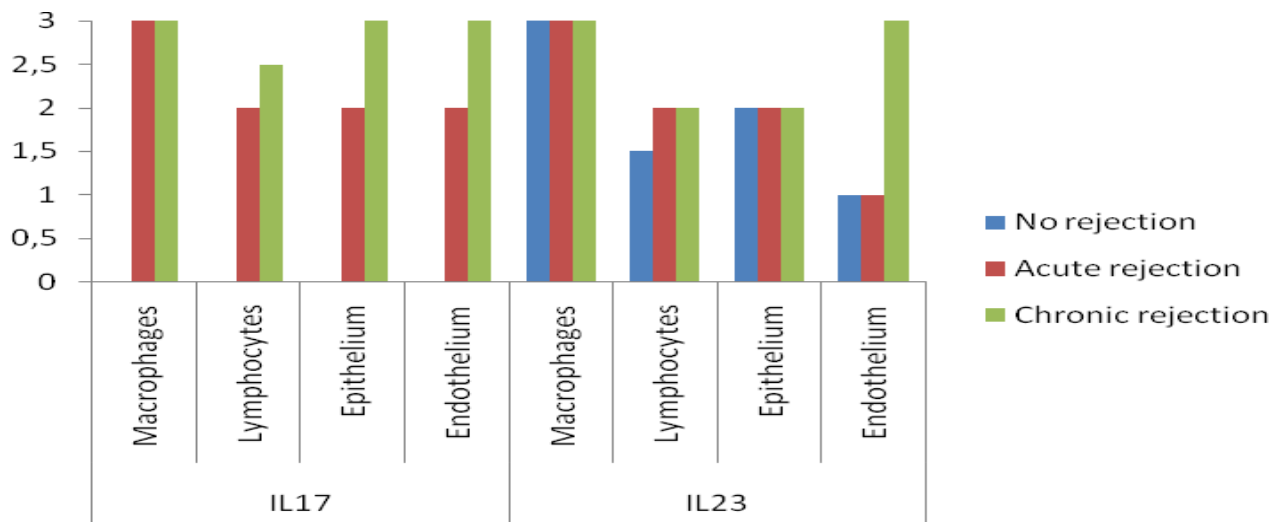


Figure 4.9. Histogram showing IL17 and IL23 expression median score in the absence or presence of rejection distinguishing the different cell types.

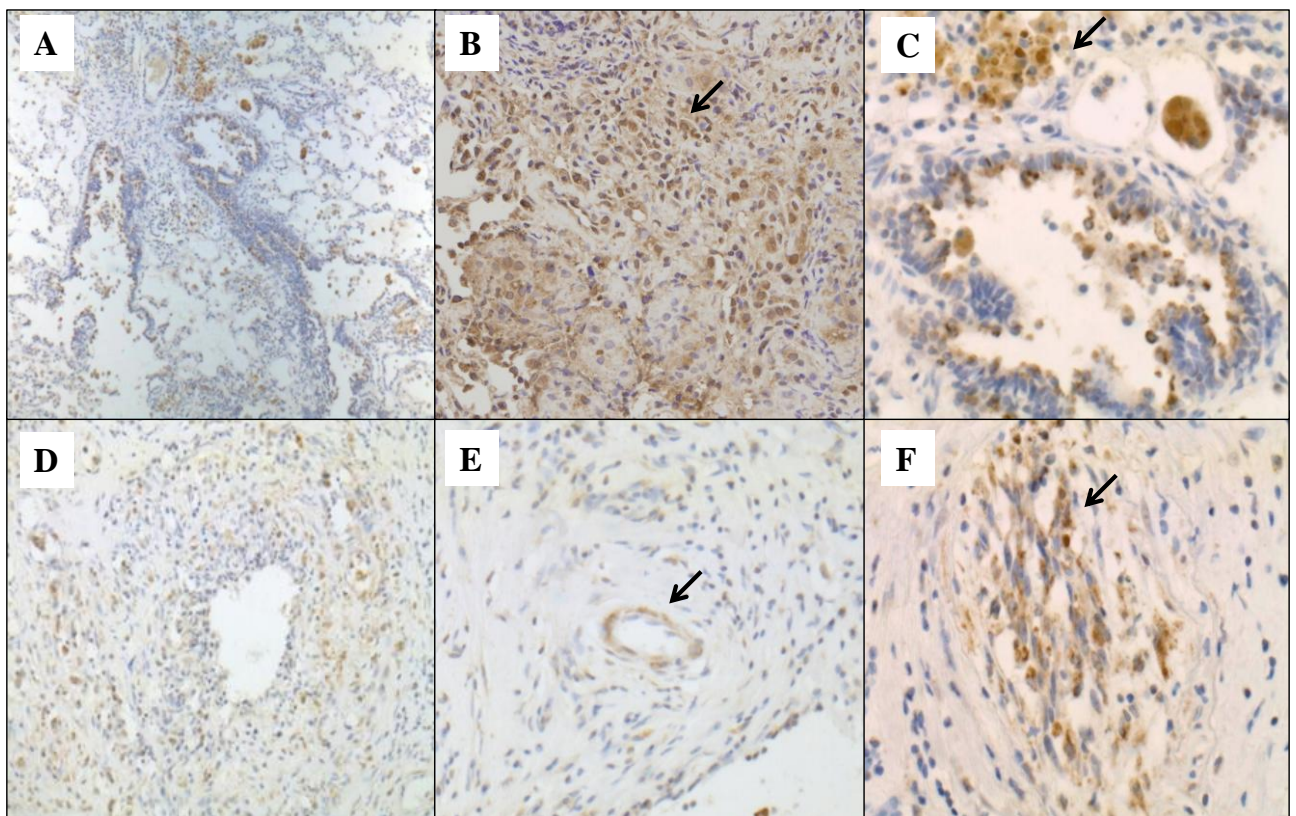


Figure 4.10. Immunohistochemistry for IL17 showed a high expression in all cell types. Staining is well visible in lymphocytes (B), macrophages and epithelium (C, arrow and thick arrow respectively), endothelial cells (D) and fibroblasts (F).

4.2.2 Molecular analysis

GAPDH was amplifiable in all BAL samples (some emblematic cases in the Figure 4.11). Molecular analysis of IL17 and IL23 expression in BAL fluids showed higher levels of mRNA in animals with acute rejection than chronic rejection (IL17/GAPDH mRNA ratio: 35.8% vs 15.1% and IL23/GAPDH mRNA ratio: 47.5% vs 21.2%) (Figure 4.12).

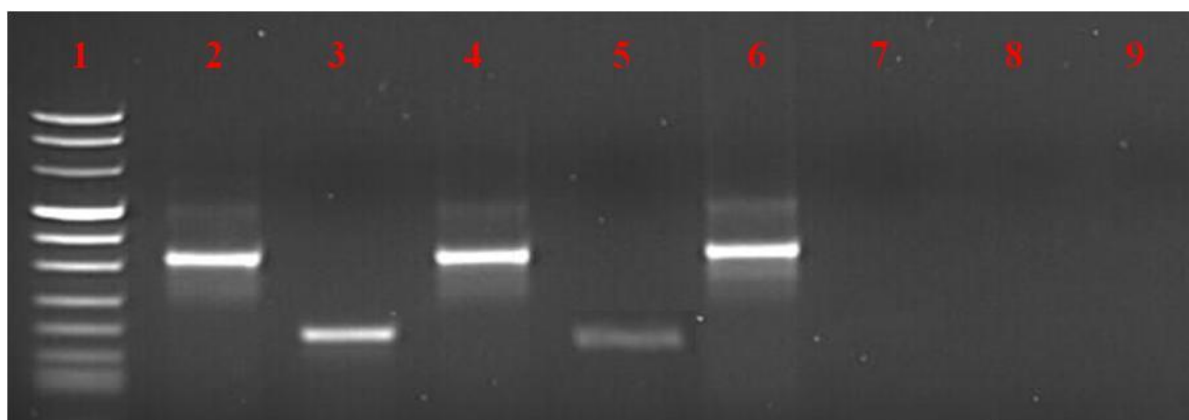


Figure 4.11 Agarose gel electrophoresis of PCR amplicons of GAPDH (lanes 2, 4, 6, 8) and IL17 (lanes 3, 5, 7, 9). Emblematic amplicons of animals with ACR (lanes 2 and 3), OB (lanes 4 and 5) and without any sign of rejection (lanes 6 and 7). Molecular weight marker VIII and negative controls are in lanes 1, 8 and 9 respectively).

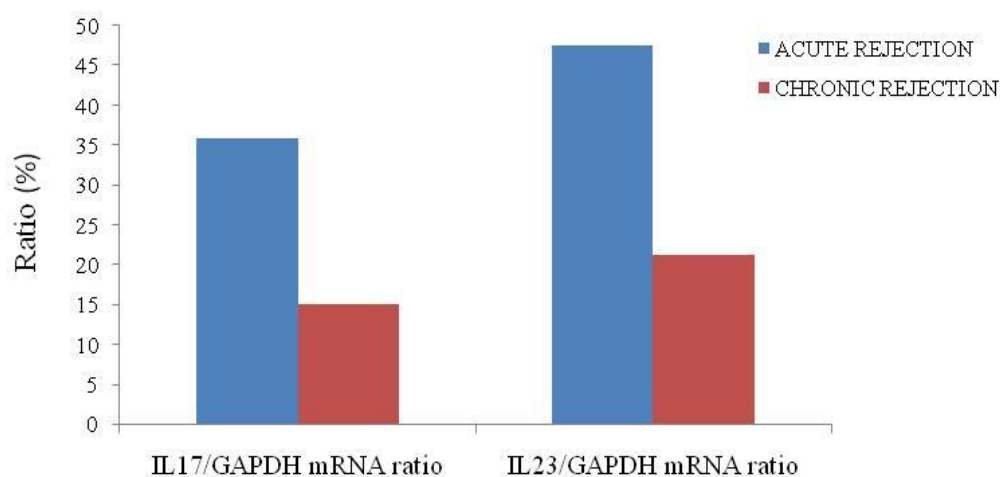


Figure 4.12 Histogram showing IL17 and IL23/GAPDH mRNA ratio in BAL of rats with acute or chronic rejection.

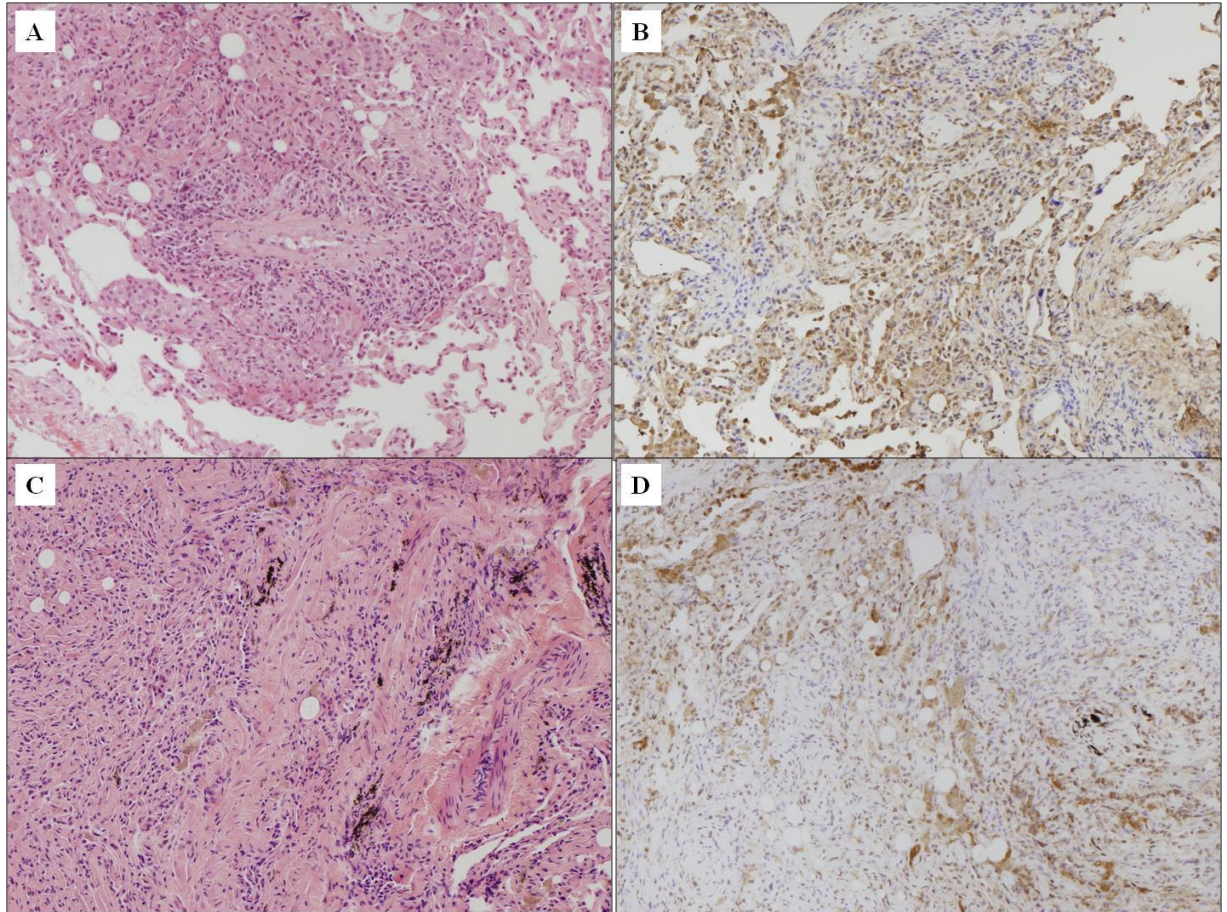


Figure 4.13. Hematoxylin and eosin stain showing typical aspects of severe ACR (A) and OB (C): a strong immunohistochemical IL17 staining was detected in the same fields (B and D respectively).

4.3.2 Index case #2

C.Z., female, 61 years old, lung transplanted for idiopathic pulmonary fibrosis.

12 scheduled transbronchial biopsies were analyzed and showed an overexpression of IL17 in all of them. In particular:

Scheduled TBB	Post-transplant time	Histologic diagnosis	IL17 expression (score 0-3)			
			Lymphocytes	Macrophages	Epithelial cells	Endothelial cells
1	22 days	A3B1	3	3	3	3
2	2 months	A2B0	3	3	3	3
3	3 months	A0BX	2	3	3	2
4	6 months	A3B0	3	3	3	3
5	9 months	A1B0	2	3	3	2
6	14 months	A0C1	3	3	3	3
7	21 months	A1/2BX	3	3	3	3
8	23 months	A0B0	2	3	3	2
9	30 months	A2BX	3	3	3	3
10	32 months	A0B0	2	3	3	2
11	39 months	A0B0	2	3	3	2
12	46 months	A0B0	2	3	2	2

4.3.3 Control case #3

G.P., male, 55 years old, lung transplanted for idiopathic pulmonary fibrosis.

6 scheduled transbronchial biopsies were analyzed and showed no rejection nor expression of IL17 in all of them.

5) DISCUSSION

Transplantation is the only effective treatment for several end-stage lung diseases. Although immunosuppressive regimens efficiently control ACR [43], two main problems impact recipient survival. On one hand, PGD, occurring in the immediate postoperative period, is caused by ischemia-reperfusion injury and when severe is associated with up to 40% mortality within 30 days after transplantation. On the other hand, CLAD, in particular OB, leads to an irreversible decline in lung function and accounts for more than 50% of lung allograft failures occurring within 5 years following transplantation. Due to no effective therapies for OB there has been an extensive search for preclinical models that replicate OB. The use of animal models is critical to investigate the pathological mechanisms behind OB and to develop therapeutic strategies. The introduction of the orthotopic lung transplantation model in rodents has been of great demand for this technique due to the obvious experimental advantages the rodent offers over other animal (both large and small) models. These include the availability of rodent-specific reagents as well as knockout and transgenic technology. Nevertheless the orthotopic lung transplant procedure in rodents is challenging due to microsurgical difficulty and extreme fragility of tissues. Introduction of the cuff technique has allowed for the widespread use of orthotopic lung transplantation in rats [44]. In mice and rats, unlike humans, the left lung contains only one lobe and makes up only 25% of the total lung mass. This makes left-single lung transplantation feasible in the murine model without the need for a circulatory support system. Thus a left-single lung transplantation using the cuff technique was the basis for the development of the orthotopic lung transplant model in the present research study.

Historically, inbred mouse/rat models have been utilized in the transplant field as their limited genetic variability removes much of the inter-subject variability from the experimental results, making results more reproducible and data interpretation simpler. However, recent studies have suggested that such genetically identical rodents may not serve as an accurate model for the human condition (which are generally outbred) in terms of their immune responses [45].

Thus the first goal of this research study was to evaluate the utility of the inbred versus outbred rat model for obtaining reproducible immunological lesions, in particular CLAD. Only 2 out of 20 outbred rats developed immunological lesions (severe ACR and CLAD) 13 and 14 days post-transplant, while 18 (90%) died within 24 hours due to severe PGD. These data are similar to the few studies reported in the literature: in these studies, animals presented high mortality rate in the early peri-operative period which could be due to a hyperacute/severe acute rejection related to the high genetic variability and the development of different comorbidities related to the absence of an appropriate strain selection [46]. However this model, as underlined by other authors, could give some interesting insights in transplant pathology such as providing an opportunity to gain better understanding of the inflammatory events that lead to early graft injury [46].

The OLT of inbred rats (Lewis to Fisher 344) was definitely the most successful model for the development of reproducible immunological lesions (ACR and OB) allowing the first objective of the research to be achieved. Severe ACR and/or OB was obtained in all long term (30 and 90 days after OLT) surviving inbred rats. Morphological findings of OB in the rats were extremely similar to those detected in clinical samples: the same early and late OB stigmata were found (e.g. eccentric and/or concentric fibro-proliferative lesion and vanishing airway bronchiolitis). Some authors have reported variable results in the OLT grafts in the Fisher 344→Wister Kyoto obtaining OB in only a few cases [47].

In this work the Authors detected true OB lesions in only a few grafts; the majority were characterized by extensive tissue remodeling due to atelectasis. The same Authors using another strain (supplied by Charles River) obtained different results with a irreversible destruction of grafts within 14 days post-transplant [47]. These data emphasize the importance of genetic differences between rat sub-strains which can significantly influence the findings. In the present research the best time point was 90 days, obtaining OB in 60% or 50%, respectively, of animals with (from day 1-to 7 days after OLT) or without immunosuppressive treatment. Animals with a delayed immunosuppressive treatment (from 7 to 14 days after OLT) showed a higher frequency of ACR than those treated earlier (from 1 to 7 days) (50% vs 40%); in these grafts ACR was present even in coexistence with OB. The same ACR frequency (50%) was observed in the grafts of animals without immunosuppressive treatment.

Even if only small number of animals were included in the study the data obtained point out two important aspects: 1) the greater effectiveness of early immunosuppressive treatment with cyclosporine (from 1 to 7 days after OLT) on frequency of ACR, and 2) the limited impact of cyclosporine treatment on the development of OB.

Regarding the first aspect, the finding is not surprising because, as in humans, the immediate post-transplant time is the period at higher risk of ACR, thus immunosuppressive treatment is extremely important and effective in this period.

The limited impact of cyclosporine treatment on the development of OB in our inbred grafts supports the concept that ACR only partially influences the development of OB. Although the pathogenesis of progressive airway obstruction is unknown, different immunological mechanisms seem to be involved in the development of OB. The presence in our model of high levels of DSA (IgG) in grafts with ACR or OB (72% and 34%), regardless of cyclosporine treatment supports the evidence that humoral response can

play a key role in this process. In the human lung transplant field increasingly emerging data suggest that humoral immunity with HLA and not-HLA DSA, may have an important role in the development of CLAD [48]. In terms of the types of antibodies involved in autoimmune reactions, IgG has been the primary isotype identified. When looking at human lung allograft recipients, the anti-col (V) antibodies were all of the IgG type without evidence of any IgM [49].

The cytokine IL17 has been implicated in the development of immune response to self-antigens.

In the inbred grafts of this research there was a high IL17 expression both in those with ACR and OB. The higher IL17 expression in graft with OB could be explained by important IL17 levels in epithelial and endothelial cells rather than in inflammatory cells (lymphocytes and macrophages) that are a common source of this cytokine. Based on a literature review, no previous studies in the transplantation setting have analytically evaluated IL17 expression distinguishing the different cellular sources. There is only one previous longitudinal study that examined IL17 expression in BAL and bronchial biopsy focusing on lymphomonocytes. The Authors detected a significant high IL17 level linked with the presence of non-specific airway CD8 T cell infiltration; IL17 expression was low in CD4 and granulocytes and fell with time post-lung transplantation [50]. The discrepancies with the present findings showing a more evident IL17 expression in chronic lesions (OB), may be related to two principal factors: first of all bronchial biopsies and not transbronchial biopsies were used. BOS lesions are predominantly located in small airway rather than in larger airways which were biopsied in the study. In other words the best tool to investigate BOS lesions was not used. Secondly, the focus was primarily on inflammatory cells (CD4, CD8 and granulocytes) leaving out other important cells such as macrophages, epithelial, and endothelial cells. Interestingly a high

IL17 level was found in epithelial and endothelial cells of grafts with OB. Although lymphomonocytes are the principal source of this cytokine, IL17 overexpression can occur in mucosal and cutaneous epithelial cells in response to stimulation with infective agents or other proinflammatory mediators [51]. Thus it could be hypothesized that injured/activated epithelium acts in an autocrine way leading to the secretion of different cytokines such as IL17 which can influence epithelial modification favoring epithelial-mesenchymal transition (EMT). IL17 endothelial expression has also been demonstrated as an important source in other inflammatory processes such as synovitis and rheumatoid arthritis [52].

A few studies have detected IL17 overexpression in BAL from patients with CLAD [53; 32]. In the present animal model, molecular analysis in BAL is not a mirror of IL17 tissue data: BAL IL17 mRNA levels were higher in grafts with ACR than those with OB. This contradictory result could be explained by the fact that epithelial and endothelial cells, two important sources of this cytokine in grafts with OB (see above), are rarely detected in BAL. Moreover, BAL cellularity was not characterized before molecular investigations due to the small quantity. This could significantly influence the data and could explain the discrepancies with immunohistochemical findings.

An interesting finding of this research study was that the same IL17 expression pattern was detected in the transbronchial biopsies of the index cases that developed OB. All these data confirm the importance of this cytokine in lung immunological disorders, particularly CLAD, suggesting innovative opportunities for target therapies.

6) SUMMARY

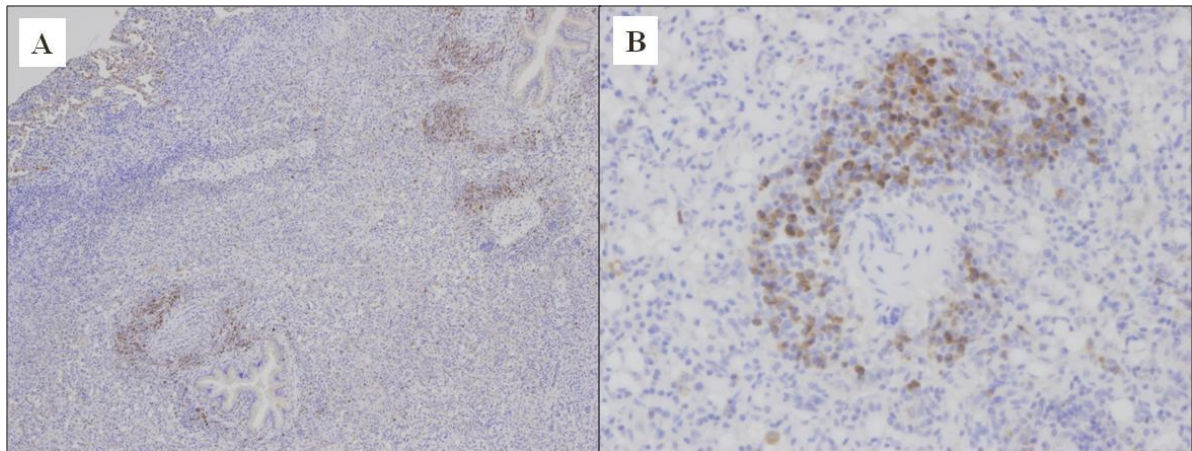
The main results of this PhD study can be summarized as follows:

- 1) a reproducible inbred OLT animal model with immunological lesions, particularly ACR and OB, was obtained;
- 2) morphological lesions of OB detected in rats are similar to those typically detected in human lung transplant, and reproduce both early and late stages of OB, up to the “vanishing bronchiolitis syndrome”;
- 3) humoral immunological response (increased DSA and IL17 expression) plays a key role in ACR and CLAD;
- 4) the cytokine IL17 mediated pathway needs to be further investigated because it can represent an important therapeutic target in the prevention of CLAD.

7) FUTURE RESEARCH BASED ON PHD RESULTS

On the basis of in vitro studies performed by our partner of Pavia, CD44 has been identified as key adhesion molecule to develop targeted therapies to prevent CLAD and we are studying CD44 expression in our OLT model in order to assess biological activity of aerosolized nanoparticles (granted by CARIPO Foundation). To this purpose, gold nanoparticles with a IL17 antagonist containing core and an anti-CD44 monoclonal antibody on the external surface, will be provided.

In this early phase, immunohistochemistry using antibody anti-CD44 showed a strong positivity in inflammatory cells and fibroblasts. No staining was detected in epithelial cells.



Emblematic images showing strong CD44 staining in fibroblasts and inflammatory cells in chronic (A) and acute (B) rejection.

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9) CURRICULUM VITAE

FORMATO EUROPEO PER IL CURRICULUM VITAE



INFORMAZIONI PERSONALI

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ESPERIENZA LAVORATIVA

- Date (da – a) **Gennaio 2011 ad oggi**
- Nome e indirizzo del datore di lavoro Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari, Sezione di Anatomia Patologica, Università degli Studi di Padova
- Tipo di impiego Dottorando di Ricerca e Assegnista di Ricerca

- Date (da – a) **Novembre 2010-Gennaio 2011**
- Nome e indirizzo del datore di lavoro Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari, Sezione di Anatomia Patologica, Università degli Studi di Padova
- Tipo di impiego Borsista di Ricerca

- Date (da – a) **2004-2010**
- Nome e indirizzo del datore di lavoro Università di Modena e Reggio Emilia
- Tipo di impiego Specializzando in Anatomia Patologica

Principali mansioni e responsabilità

Ambiti di ricerca: patologia polmonare e patologia trapiantologica.

Attività scientifica: 17 lavori per estenso (h Index: 4; 37 citazioni totali), 23 lavori brevi ed abstracts.

- Studi morfologici e molecolari di patologie toraciche neoplastiche (soprattutto carcinoma non a piccole cellule del polmone, tumori neuroendocrini, timomi) e non neoplastiche (soprattutto Fibrosi Polmonare Idiopatica e Broncopneumopatia Cronica Ostruttiva).

- Partecipazione attiva nel Gruppo di studio multidisciplinare sul Trapianto di Polmone di Padova.

- Collaborazione con l'Istituto Oncologico Veneto, il Dipartimento di Pediatria, di Medicina Clinica e Sperimentale dell'Università degli Studi di Padova e con il Consorzio per la Ricerca sul Trapianto di Organi.

- 11 relazioni a corsi e congressi nazionali o internazionali.

- Attività di tutoraggio a studenti laureandi nel CDL in Tecniche di Laboratorio Biomedico e a studenti stranieri per stage organizzato dal Servizio Internazionale per gli Studenti di Medicina.

ISTRUZIONE E FORMAZIONE

- Date (da – a) 2004-2010
- Nome e tipo di istituto di istruzione o formazione Scuola di Specializzazione in Anatomia Patologica. Università degli Studi di Modena e Reggio Emilia.
- Principali materie / abilità professionali oggetto dello studio
- Qualifica conseguita Specializzazione in Anatomia Patologica

- Date (da – a) 1996-2004
- Nome e tipo di istituto di istruzione o formazione Corso di Laurea specialistica in Medicina e Chirurgia. Università degli Studi di Bologna.

<ul style="list-style-type: none"> • Principali materie / abilità professionali oggetto dello studio • Qualifica conseguita 	<p>Laurea Magistrale in Medicina e Chirurgia con votazione 108/110.</p>
<ul style="list-style-type: none"> • Date (da – a) 	<p>1990-1995</p>
<ul style="list-style-type: none"> • Nome e tipo di istituto di istruzione o formazione 	<p>Liceo Scientifico “N. Copernico” (Bologna)</p>
<ul style="list-style-type: none"> • Principali materie / abilità professionali oggetto dello studio • Qualifica conseguita 	<p>Maturità scientifica</p>
<p>CAPACITÀ E COMPETENZE PERSONALI <i>Acquisite nel corso della vita e della carriera ma non necessariamente riconosciute da certificati e diplomi ufficiali.</i></p>	
<p>MADRELINGUA</p>	<p>ITALIANO</p>
<p>ALTRE LINGUA</p>	<p>INGLESE</p>
<ul style="list-style-type: none"> • Capacità di lettura • Capacità di scrittura • Capacità di espressione orale 	<p>OTTIMO OTTIMO OTTIMO</p>
<p>ALTRE CAPACITÀ E COMPETENZE</p> <p><i>Competenze non precedentemente indicate.</i></p>	<p>Ottima conoscenza di Windows XP e VISTA (Word, Power Point, Excell), Internet, Software bioinformatici e database scientifici.</p>

10) PRODUCTS OF THE RESEARCH

A. Papers

1st year (2012)

- Calabrese F, Lunardi F, Balestro E, Marulli G, Perissinotto E, Loy M, **Nannini N**, Valente M, Saetta M, Agostini C, Rea F. "Serpine B4 isoform overexpression is associated with aberrant epithelial proliferation and lung cancer in idiopathic pulmonary fibrosis". Pathology. 2012 Apr;44(3):192-8.
- Schirosi L, **Nannini N**, Nicoli D, Cavazza A, Valli R, Buti S, Garagnani L, Sartori G, Calabrese F, Marchetti A, Buttitta F, Felicioni L, Migaldi M, Rea F, Di Chiara F, Mengoli MC, Rossi G. "Activating c-KIT mutations in a subset of thymic carcinoma and response to different c-KIT inhibitors". Ann Oncol. 2012 Sep;23(9):2409-14.

2nd year (2013)

- Herpes virus infection is associated with vascular remodeling and pulmonary hypertension in idiopathic pulmonary fibrosis. Calabrese F, Kipar A, Lunardi F, Balestro E, Perissinotto E, Rossi E, **Nannini N**, Marulli G, Stewart JP, Rea F. PLoS One. 2013;8(2):e55715.
- Localized pleuropulmonary crystal-storing histiocytosis: 5 cases of a rare histiocytic disorder with variable clinicoradiologic features. Rossi G, De Rosa N, Cavazza A, Mengoli MC, Della Casa G, **Nannini N**, Colby TV. Am J Surg Pathol. 2013 Jun;37(6):906-12.
- The mitochondrial chaperone TRAP1 promotes neoplastic growth by inhibiting succinate dehydrogenase. Sciacovelli M, Guzzo G, Morello V, Frezza C, Zheng L, **Nannini N**, Calabrese F, Laudiero G, Esposito F, Landriscina M, Defilippi P, Bernardi P, Rasola A. Cell Metab. 2013 Jun 4;17(6):988-99.

3rd year (2014)

- Necrotizing sarcoid granulomatosis with an uncommon manifestation: clinicopathological features and review of literature. Giraud C, **Nannini N**, Balestro E, Meneghin A, Lunardi F, Polverosi R, Calabrese F. *Respir Care*. 2014 Sep;59(9):e132-6.
- Pasello G, Urso L, Silic-Benussi M, Schiavon M, Cavallari I, Marulli G, **Nannini N**, Rea F, Ciminale V, Favaretto A. "Synergistic antitumor activity of recombinant human Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in combination with carboplatin and pemetrexed in malignant pleural mesothelioma." *J Thorac Oncol*. 2014 Jul;9(7):1008-17.
- Elena Scagliori, Laura Evangelista, Annalori Panunzio, Fiorella Calabrese, **Nazarena Nannini**, Roberta Polverosi, Fabio Pomerri . Conflicting or complementary role of computed tomography (CT) and positron emission tomography (PET)/CT in the assessment of thymic cancer and thymoma: our experience and literature review. *Thoracic Cancer* ISSN 1759-7706 *Accepted*
- Asymptomatic pulmonary Kaposi's sarcoma in a patient submitted to lung transplant. **N. Nannini**, A. Rebusso, F. Calabrese, L. Battistella, M. Schiavon, M. Loy, , F. Lunardi, F. Calabrese, F. Rea *Submitted to Transplantation Proceedings*

B. Abstracts

1st year (2012)

- E. Rossi, A. Floriani, C. Rinaldo, **N. Nannini**, G. Marulli, M. Loy, F. Rea, F. Calabrese, E. Balestro. "Idiopathic pulmonary fibrosis and silent microaspiration: the role of videolarinoscopy". *Eur Respir J* 2012; 40: Suppl. 56, XXXs.

- **N. Nannini**, F. Lunardi, M. Vadori, A. Dedja, E. Cozzi, F. Rea, F. Calabrese. “A new experimental model of orthotopic lung transplantation: Padova experience”. *Virchows Arch* (2012) 461 (Suppl 1): S1-S332.
- **N. Nannini**, F. Lunardi, E. Balestro, E. Rossi, M. Loy, M. Saetta, F. Rea, F. Calabrese. “Epithelial dysplasia and lung cancer in end-stage idiopathic pulmonary fibrosis: Padova experience”. *Virchows Arch* (2012) 461 (Suppl 1): S1-S332.
- F. Calabrese, **N. Nannini**, F. Lunardi, M. Vadori, E. Cozzi, F. Rea. “Interleukin-17 tissue expression correlates with severity of acute and chronic allograft rejection in rat orthotopic lung transplantation”. Sottomesso all’ ISHLT 33rd Annual Meeting and Scientific Session.

2nd year (2013)

- F. Calabrese, M. Schiavon, F. Lunardi, **N. Nannini**, G. Marulli, S. Nicotra, P. Feltracco, G. Di Gregorio, M. Loy, F. Rea. Tissue Evaluation of Donor Lungs Preserved with the Organ Care System (OCS) Device: Experience of Padova. *J Heart Lung Transplant* 2013, 32 (4S): S155. ISHLT 2013.
- F. Calabrese, **N. Nannini**, F. Lunardi, M. Vadori, E. Cozzi, F. Rea. Interleukin-17 Tissue Expression Correlates with Severity of Acute and Chronic Allograft Rejection in Rat Orthotopic Lung Transplantation. *J Heart Lung Transplant* 2013, 32 (4S): S263. ISHLT 2013.
- **N. Nannini**, F. Lunardi, M. Schiavon, S. Baraldo, E. Balestro, M. Saetta, F. Rea, F. Calabrese. Morphological and molecular characterization of adenocarcinoma associated with chronic obstructive pulmonary disease (COPD). PPS 2013.
- F. Lunardi; **N. Nannini**; E. Balestro; E. Rossi; F. Rea; M. Saetta; F. Calabrese. Clinical and morphological characterization of IPF phenotypes. PPS 2013.

- F. Lunardi, **N. Nannini**, B. Montini, F. Cinetto, M. Gnoato, M. Facco, C. Agostini, F. Calabrese. Evaluation of matrix-metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in bleomycin (BLM) – induced pulmonary fibrosis before and after GSK-3 treatment. PPS 2013.
- F. Calabrese, **N. Nannini**, F. Lunardi, M. Vadori, E. Cozzi, F. Rea. “Overexpression of interleukin-17 pathway in allograft rejection of rat orthotopic lung transplantation.” *Virchows Arch* (2013) 463: 101-352.
- F. Calabrese, **N. Nannini**, F. Lunardi, S. Baraldo, E. Bazzan, E. Balestro, M. Schiavon, F. Rea, M. Saetta. “Adaptive immune response in COPD patients with and without $\alpha 1$ antitrypsin deficiency.” *Virchows Arch* (2013) 463: 101-352.
- Lunardi F, **Nannini N**, Balestro E, Rossi E, Loy M, Rea F, Calabrese F. Impact of combined CMV prophylaxis on short term complications after lung transplantation. AMIT 2013.
- Balestro E., Calabrese F., Rea F., Rossi E., Lunardi F., Schiavon M., Bazzan E., Loy M., Marulli G., **Nannini N.**, Turato G., Baraldo S., Cosio M., Saetta M. Distinct clinical and pathological phenotypes in IPF. European Respiratory Society Congress 2013, Barcelona Spain, 6-11 September 2013, *Eur Respir J* 2013 42: Suppl 57. P465.
- Baraldo S., Bazzan E., Turato G., Molena B., Lunardi F., **Nannini N**, Damin M., Schiavon M., Balestro E., Loy M., Rea F., Cosio M., Calabrese F., Saetta M. Similar adaptive immune response in COPD patients with and without $\alpha 1$ antitrypsin deficiency. European Respiratory Society Congress 2013, Barcelona Spain, 6-11 September 2013, *Eur Respir J* 2013 42: Suppl 57: P599.
- Calabrese F., Lunardi F, **Nannini N**, Balestro E, Andriolo L, Loy M, Rea F, Calabrese F. Trattamento profilattico combinato anti-CMV: impatto sulle complicanze a breve termine dopo trapianto di polmone. NIT 2013.

- Rebusso A, Loy M, Calabrese F, Battistella L, Schiavon M, Lunardi F, **Nannini N**, Marulli G, Nicotra S, Breda C, Rea F. Elevata incidenza di rigetto acuto nel trapianto polmonare per fibrosi cistica o bronchi ectasie. NIT 2013.
- Schiavon M, Marulli G, Zuin A, Calabrese F, **Nannini N**, Comacchio GM, Rossi E, Battistella L, Rebusso A, Rea F. Morphological and molecular characterization of cancer phenotypes associated with chronic obstructive pulmonary disease (COPD). XXI° European Congress ESTS, Birmingham 2013.

3rd year (2014)

- F. Calabrese, M. Schiavon, **N. Nannini**, F. Lunardi, G. Marulli, G. Di Gregorio, A. Rebusso, E. Balestro, M. Loy, F. Rea Apoptosis and Expression of Inducible Nitric Oxide Synthase in Normothermic Lung Perfused With Organ Care System (OCS) Compared To Standard Cold Show Donor Lungs. ISHLT, 34th Annual Meeting and Scientific Sessions April 10 – 13, 2014, San Diego.
- **Nannini N**, Lunardi F, Sadeghpour S, Schiavon M, Rebusso A, Loy M, Di Gregorio G, Rea F, Calabrese F. Apoptosis and expression of inducible nitric oxide synthase in normothermic perfused lung compared to standard cold storage donor lungs. Virch Arch 2014; 465:S47
- **Nannini N**, Lunardi F, Loy M, Balestro E, Rea F, Calabrese F. Idiopathic pulmonary fibrosis: overexpression of transforming growth factor beta in native lungs is associated with vessel remodeling and pulmonary hypertension. Virch Arch 2014; 465:S47.
- Balestro E, Turato G, Polverosi R, Barbiero G, Zagallo S, Floriani A, Rea F, Calabrese F, Bazzan E, Schiavon M, **Nannini N**, Baraldo S, Saetta M, Cosio M. High resolution computed tomography (HRCT) can differentiate rapid from slow progressing Idiopathic pulmonary fibrosis (IPF). Eur Respir J 2014; 44: Suppl 58, P758.

- Turato G, Floriani A, Baraldo S, Balestro E, Bazzan E, Schiavon M, Cazzuffi R, Damin M, **Nannini N**, Calabrese F, Rea F, Saetta M, Cosio MG. “Mucus production, lung inflammation and functional decay in IPF”. European Respiratory Society Congress, Munich Germany, 6-10 September 2014. Eur Respir J 2014; 44: Suppl 58, P3503.
- Schiavon M, Marulli G, Verderi E, **Nannini N**, Feltracco P, Gregori D, Breda C, Rea F. Right sleeve pneumonectomy and right extended pneumonectomy: early and long term outcomes comparison. XXI° European Congress ESTS, Copenhagen 2014.
- F. Lunardi, **N. Nannini**, E. Balestro, M. Loy, E. Perissinotto, F. Rea, F. Calabrese. Elevato rischio di disfunzione primaria del graft e di infezione ricorrente in pazienti trapiantati per fibrosi polmonare idiopatica con associata infezione virale. SITO 2014.
- F. Calabrese, M. Seveso, M. Loy, **N. Nannini**, E. Ruffoni, D. Sgarabotto, P. Feltracco, E. Cozzi, F. Rea. Trattamento del rigetto anticorpo-mediato nel trapianto di polmone: esperienza del centro trapianti di padova. SITO 2014.
- A. Rebusso, F. Calabrese, L. Battistella, M. Schiavon, M. Loy, **N. Nannini**, F. Lunardi, F. Calabrese, F. Rea. Sarcoma di Kaposi polmonare asintomatico in paziente sottoposto a trapianto polmonare. SITO 2014:
- **N. Nannini**, M. Schiavon, F. Lunardi, G. Marulli, G. Di Gregorio, A. Rebusso, E. Balestro, M. Loy, F. Calabrese, F. Rea. Ridotto danno apoptotico e minor espressione di i-nos nei polmoni preservati con ocs in comparazione alla preservazione standard. SITO 2014.

C. Book chapters

2nd year (2013)

- Fiorella Calabrese, Francesca Lunardi, **Nazarena Nannini**. Biobanks in rare disease. NEW INSIGHTS ON BIOBANKS. October 2013 CLEUP
- Fiorella Calabrese, **Nazarena Nannini**, Massimo Ruge. Classificazione patologica: evoluzione, luci e ombre. Tumori Neuroendocrini Polmonari: i carcinoidi. Ed med stage 2013

D. Speaker at National/International conferences

1st year (2012)

- Pneumo Under 40. Il respiro giovane della pneumologia. Aspetti anatomopatologici di interesse pneumologico, Milano 10 febbraio 2012 **(Speaker)**.
- NSCLC dall'esame istologico alla terapia: opinioni a confronto, Padova 14 maggio 2012 **(Speaker)**.
- ECP 2012. 24th European Congress of Pathology. Praga 8 – 12 September 2012 **(Speaker)**.
- "Il mesotelioma pleurico: aspetti clinico-patologici e giuridici, Padova 8 novembre 2012. **(Scientific Committee member)**

2nd year (2013)

- AIR MEETING ITALIA 2013- Milano, 28/29 Giugno 2013. **(Speaker)**
- ECP 2013. 25th European Congress of Pathology. Lisbon 31 August– 4 September 2013. **(Speaker)**

3rd year (2014)

- ECP 2014. 26th European Congress of Pathology. London 30 August– 3 September 2014. (**Speaker**)
- SITO 2014. XXXVIII Congresso Nazionale della Società Italiana Trapianti d'Organo. 24-26 settembre 2014. (**Speaker**)

E. Other

Awards:

- Award “Il sogno di Valter” of the “Unione Trapiantati Polmone Padova” (UTPP), Padova 21 March 2012.
- ECP 2014. 26th European Congress of Pathology. London 30 August– 3 September 2014: bursary for oral presentation

Tutorial activity:

- Tutor of students attending a bachelor's degree in Biomedical Techniques “Analisi mutazionale dell'oncogene KRAS da tessuti neoplastici polmonari processati con differenti metodologie” (2011-2012).
- Tutor of 1 foreign student during their training in our lab, organized by SISM, with the project “Molecular approaches to chronic inflammatory lung diseases” (2012-2013).