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AIRWAY INFLAMMATION AND REMODELLING POST HUMAN LUNG TRANSPLANTATION

Thesis submitted in fulfillment of the requirement for the degree of

Doctor of Philosophy in Medicine

by

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TABLE OF CONTENTS

ABSTRACT

Background: "Airway remodelling" refers to the airway structural changes seen in airway diseases such as Asthma and Chronic Obstructive Pulmonary Disease (COPD). It includes subepthelial, mucosal collagen deposition, epithelial injury and denudation, and changes in airway vascularity, mucous glands, and smooth muscle that occur pathologically in the airway walls. These structural changes are most likely the consequence of ongoing chronic airway inflammation, and have as yet poorly determined effects on patient symptoms, lung function, airway reactivity, and outcomes from acute exacerbations of the diseases. In the last decade, by using endobronchial biopsy (EBB) technique, numerous aspects of airway remodelling have been investigated in Asthma and COPD. These studies have allowed a much greater understanding of the pathogenesis of these airway diseases.

Although Bronchiolitis Obliterans Syndrome (BOS) is the major cause of late death in lung transplant recipients, there has been little attention given to the investigation of airway wall inflammation, architectural damage and its repair post lung transplantation. Most previous studies have been done primarily using bronchoalveolar lavage (BAL) samples or on transbronchial lung biopsy (TBB) specimens, which provide only indirect information on airway inflammation. In these studies, it was hypothesised that BOS results from tissue inflammation, damage and remodelling in response to graft injury caused by a number of potential alloantigen-dependent and -independent insults to the transplanted lung. Airway inflammation (whatever the offending insult) leading to fibrosis of the airway is likely to be the hallmark of BOS. Therefore, EBB from both lung transplant recipients (LTR) with and without BOS were examined: (1) to investigate the cellular inflammation, vascular changes,

and scar collagen deposition directly in the airway walls of human lung allografts; (2) to characterise the features of airway inflammation and remodelling in LTR who develop BOS; (3) to determine the relationship between indices of the cellular and structural changes and clinical parameters in BOS patients; and (4) to evaluate the potential role for TGF-β_1 and TNF-α in the pathogenic process of BOS post lung transplant.

Methods: A total of 41 LTR (24 clinically stable, 17 with BOS) and 28 normal healthy controls were recruited for the four parts of the study presented in this thesis. All the subjects underwent bronchoscopy, with BAL and EBB procedures, as well as lung function testing. Lung transplant patients also had TBB procedure. Immunohistochemical staining (ABC method) was performed on EBB tissue sections for neutrophils, macrophages (CD68), total leukocytes (CD45), TGF-β_1 TNF-α expression, and collagen subtypes for examination of scar collagen deposition (type I, III and V collagens) and to identify bronchial vessels (type IV collagen). BAL IL-8, TGF-β_1 and TNF-α levels were measured in unconcentrated BAL supernatants with ELISA and chemiluminescent immunoassays using commercial available kits. To determine whether alveolar macrophages (AM) from LTR were likely to be responsible for BAL TGF-β_1 levels, AM were isolated from BAL cells and cultured with and without added stimuli (LPS 1 μg/ml) for evaluating TGF-β_1 production by AM *in vitro*. Competitive reverse transcription polymerase chain reaction (RT-PCR) was used to quantify the expression of TGF-β_1 mRNA in both non-cultured BAL cells and cultured AM. In addition, BAL total and differential cell counts were performed.

Results: The novel features of airway inflammation and remodelling in LTR observed in these studies were prominent airway wall neutrophilia, markedly increased airway wall

vascularity, and excessive scar collagen (collagen type III) deposition, the latter was only demonstrated in airway biopsy specimens from LTR who had developed BOS.

In accordance with previous studies, highly increased BAL IL-8 levels and BAL neutrophilia in LTR were confirmed by the study. These changes were more significant in LTR with BOS compared with clinically stable LTR. A significant correlation between BAL IL-8 and BAL neutrophilia was found in patients with BOS.

BAL TGF-β_1 protein levels, but not TNF-α, increased significantly in both LTR with and without BOS. However, no significant up-regulation of TGF-β_1 protein expression was found in airway biopsy specimens, nor in AM culture supernatants from the LTR studied.

Despite the significant increase in BAL TGF-β_1 protein levels in LTR *in vivo*, no evidence of up-regulation of TGF-β_1 mRNA levels in non-cultured BAL cells, nor in AM after culture was found from the same group of patients.

Lastly, significant inverse relationships between lung function parameters and airway neutrophilia, increased airway vascularity, collagen deposition, and up-regulated BAL TGF-β_1 protein levels were found in patients with BOS.

Conclusions: These studies have advanced the characterisations of the features of chronic airway inflammation and remodelling that occur in human lung allografts post transplant. These features may lead to airway wall thickening and stiffness that would have a profound effect on airway physiological characteristics, and contribute to the development of BOS.

Cross-sectionally, both BAL TGF-β_1 and IL-8 protein levels increased not only in LTR with BOS, but also in those who were clinically stable, although the elevation of BAL IL-8 and BAL neutrophils were more predominant in BOS patients. If longitudinal studies could confirm the up-regulation of BAL TGF-β_1 preceding the development of BOS, then monitoring BAL TGF-β_1 levels routinely in LTR could be of practical value in predicting the development of airway fibrosis in lung allografts. If a causal link between airway neutrophils and/or increased IL-8 levels and airway damage/remodelling in BOS exists, it could create novel potential targets for further therapeutic interventions.

Bronchial biopsy, by directly sampling airway wall subepthelial mucosa, has provided much valuable data to help better characterise the airway remodelling process that occurs in chronic lung rejection, and its physiological implications. EBB may be of greater utility than the traditional TBB in long term follow up of lung transplant patients.

DECLARATION

I hereby certify that, except as acknowledged, this thesis contains only the original work of the candidate, and contains no material which has been accepted for the award of any other degree or diploma in any university or other institution. To the best of my knowledge and belief, this thesis contains no material previously written or published by another person, except where due reference is made in context of the thesis.

[redacted]

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PUBLICATIONS ARISING FROM OR RELATED TO THIS THESIS

Papers:

1. **Ling Zheng,** E Haydn Walters, Chris Ward, Ning Wang, Bernadette Orsida, Helen Whitford, Trevor J Williams, Tom Kotsimbos, Gregory I Snell. Airway neutrophilia in stable and Bronchiolitis Obliterans Syndrome patients following lung transplant. *Thorax 2000; 55: 53-59.*

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LIST OF ABBREVIATIONS

ABC	Avidin-Biotin-Enzyme-Complex
AM	Alveolar Microphage
AMP	Adenosine-5-monophosphate
ALG	Antilymphocyte Globulin
APC	Antigen-Presenting Cells
ARDS	Adult Respiratory Distress Syndrome
ASM	Airway Smooth Muscle
ATG	Antithymocyte globulin
BAL	Bronchoalveolar Lavage
BAR	Bronchial Artery Revascularization
bFGF	basic Fibroblast Growth Factor
BHR	Bronchial Hyperresponsiveness
BM	Basement Membrane
BO	Bronchiolitis Obliterans
BOOP	Bronchiolitis Obliterans Organising Pneumonia
BOS	Bronchiolitis Obliterans Syndrome
BSLTx	Bilateral Lung Transplantation
cDNA	complementary Deoxyribonucleic Acid
CF	Cystic Fibrosis
CMV	Cytomegalovirus
COPD	Chronic Obstructive Pulmonary Disease
CsA	Cyclosporine A

DC	Dendritic cells
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
EBB	Endobronchial Biopsy
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetracetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
GCs	Gluco-Corticosteroids
$FEF_{25-75\%}$	Mean Forced Expiratory Flow between 25-75% of the FVC
$FEV_{1.0}$	Forced Expiratory Volume in 1 Second
FVC	Forced Vital Capacity
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HLA	Human Leucocyte Antigen
HLTx	Heart-Lung Transplantation
HRP	Horseradish Peroxidase
ICAM-1	Intercellular Adhesion Molecule
IFNγ	Interferon-γ
IL-	Interleukin
IPF	Idiopathic Pulmonary Fibrosis
ISHLT	International Society of Heart and Lung Transplantation
OKT3	Monoclonal antibody to CD3 complex of T-cell receptor
LPS	Lipopolysaccharide
LTR	Lung Transplant Recipients
LTx	Lung Transplantation

MHC	Major Histocompatibility Complex
MMF	Mycophenolate Mofetil
mRNA	messager Ribonucleic Acid
NK cells	Natural Killer cells
OCT	Ornithine Carbamyl Transferase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PPH	Primary Pulmonary Hypertension
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
RLU	Relative Light Unit
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SLTx	Single Lung Transplantation
TBB	Transbronchial Biopsy
TBS	Tris-Buffered Saline
TGF	Transforming Growth Factor
TMB	3, 3', 5, 5'-tetramethylbenidine
TNF-α	Tumour Necrosis Factor-α
VCAM-1	Vascular Cell Adhesion Molecule-1
VHD	Valvular Heart Disease
VLA-1	Very Late Antigen-1

CHAPTER I. LITERATURE REVIEW

1.1 Chronic Rejection in Human Lung Allografts

1.1.1 Introduction

In human organ transplantation, graft rejection is the single most important limitation to better medium and long-term survival, regardless of organ type. According to histopathological pattern, graft rejection in lung transplant has been traditionally classified as hyperacute, acute, and chronic. Hyperacute rejection has virtually been eliminated in allotransplantation by prescreening the recipient's serum for antibodies against a standard panel of cells (1). Acute rejection, under the current immunosuppressive regimens, is infrequently fatal, although with high incidence (the majority of lung transplant recipients experience at least one episode of acute rejection regardless of immunosuppressive regimen) (2-11). Chronic rejection has a disturbingly high prevalence, bodes a poor prognosis, and is the major cause of late death in the recipients (1, 7, 12, 13).

The first human lung transplant operation was performed in 1963 (14), but successful lung transplantation was not achieved until the early 1980s (15-17), when the introduction of the immunosuppressive drug, Cyclosporine A, ushered in the era of practicable solid organ transplantation. Within less than three decades, lung transplantation has become an accepted therapy in selected patients with end-stage pulmonary parenchymal or vascular diseases. The results have progressively improved secondary to refinements in tissue typing, greater understanding of patient physiology, better surgical and preoperative techniques, and more effective immunosuppressive modalities with coincident appreciation of their risks. Table 1 summaries the actuarial survival rate following lung transplantation from the U.S. Scientific

Registry (1): overall, the 1-year survival rate was 72%, 63% at 2-year, 56% at three-year, and 46% at 5-year (1, 18). However, at the same time, it has become clear that a significant proportion of lung allografts falter and fail within the first months or years after placement, primarily because of progressive and irreversible chronic rejection despite continued administration of maintenance immuno-suppression. Thus, as a long-term answer to irreversible severe lung damage, lung transplantation has not yet lived up to its hoped-for potential; chronic rejection, that enigmatic process leading to the bulk of late graft failures, remains largely undefined, and as yet uncontrollable.

Table 1. Recipient Survival after Lung Transplantation, by Diagnosis*

		Actuarial Survival, %			
Diagnosis	**N**	**1 Mo**	**1 Yr**	**2 Yr**	**3 Yr**
Chronic obstructive pulmonary diseases	617	94	79	72	62
α1-antitrypsin deficiency emphysema	277	87	72	65	58
Cystic fibrosis	249	91	73	63	57
Idiopathic pulmonary fibrosis	245	86	66	55	51
Primary pulmonary hypertension	204	77	67	61	51
Retransplantation	58	60	39	31	28
Overall	**1,936**	**87**	**72**	**63**	**56**

*Data from U.S. Scientific Registry; for the cohort of transplants performed from October 1987 through December 1993 (Ref. 1).

1.1.2 Lung Transplant Bronchiolitis Obliterans (BO) and Bronchiolitis Obliterans Syndrome (BOS)

"Bronchiolitis Obliterans" (BO) (without organisation) in the non-transplant setting, refers to a rare clinical syndrome characterised by progressive airflow obstruction (19), which is classically associated with toxic gas exposure, viral infection, or systemic connective-tissue diseases (20-23). The most common histological findings is constrictive bronchiolitis with bronchiole inflammation, fibrosis, scarring, and stenosis but without the intraluminal plugs or polyps that are found in bronchiolitis obliterans organising pneumonia (BOOP).

More recently, a similar process has been also described in bone marrow transplant recipients (24, 25). The first lung transplant BO was reported in 1984 by Burke and colleague at Stanford (26). In 14 long-term survivors after lung transplant, 5 developed obstructive airway disease and none of these five patients showed a tendency for spontaneous improvement of their flow rates, but histological evidence of BO on lung biopsies was found in three of them (26). Since then, lung transplant BO has being increasingly recognised as the airway pathological manifestation of chronic lung rejection in human lung transplants (27-31). The term "Bronchiolitis Obliterans Syndrome (BOS)" has been applied to clinical presentation of lung allograft dysfunction with obstructive physiology, where histology proof of BO is not thought necessary when other causes of airflow obstruction can be excluded (1).

The incidence of BOS has been in the range of 35-50% reported by multiple centres throughout the world (7, 11-13). The main clinical feature of BOS is deteriorating lung allograft function that develops 3 months or more after transplantation, and the mean time to onset or diagnosis of BOS has been 16 to 20 months, but the range has been very broad (12,

10). BOS can present quite acutely and imitate a respiratory infection, or can manifest as an insidious but progressive decline in lung function. The physical examination may be unremarkable, but patients may have basal inspiratory crackles or squeaks. Likewise, the chest radiograph is generally normal or has minor, nonspecific changes only (32, 33).

The diagnosis of BOS is ultimately made by bronchoscopy and spirometry (1, 31, 34). Bronchoscopy has been routinely performed in lung transplant patients who develop pulmonary function changes or symptoms. By means of bronchoscopy, bronchoalveolar lavage (BAL) and transbronchial biopsy (TBB), other causes of declining graft function, such as acute rejection, infection, or an airway complications, for example, airway dehiscence, stenosis, and bronchomalacia, can be largely excluded (1). Although biopsy evidence of BO is not a criterion for BOS (35), it is very supportive (1). The sensitivity of TBB for detecting BO has been variable from centre to centre, ranging from 15 to 59% (36-38), and relatively low at most centres around the world (34, 36, 37, 39, 40). The specificity ranges from 75 to 93% (36, 38), because of the difficulty of sampling bronchioles. Spirometric criteria for the diagnosis and stages of BOS have been standardized by the International Society for Heart and Lung Transplantation in 1993 (35), and updated in 2001 (Table 2). The stages are based on fractional decrements in the $FEV_{1.0}$ from its baseline value (defined as best post-operative $FEV_{1.0}$), each stage has a designator to indicate the absence or presence of demonstrated BO pathologically (41).

Table 2. Staging System for Bronchiolitis Obliterans Syndrome*

BOS Stage†	$FEV_{1.0}$, % of Baseline§	FEF_{25-75}, % of Baseline
BOS 0	>90%	>75%
BOS Potential	81-90%	≤75%
BOS 1	66-80%	--
BOS 2	51-65%	--
BOS 3	≤50%	--

* Estenne M, et al. 2001. J. Heart Lung Transplant (in press). (Ref. 41).
§ Baseline $FEV_{1.0}$ is the average of the two highest $FEV_{1.0}$ obtained over > 4 weeks.
† Subcategories a and b, without and with bronchiolitis obliterans, respectively.

Unfortunately, BO/BOS is essentially untreatable at the present, as no good medical remedy (indeed a complete lack of radomised control trials) for this problem exists. It has been treated by augmenting the immunosuppressive regimen with azathioprine (42), corticosteriods, and antilymphocyte antibody preparations (OKT3, ATGAM) (43), total lymphoid irradiation (44), and aerosolized cyclosporine (45), but the responses are often transient, and the progress is rarely fully arrested by such therapy once it has begun (1). Prevention of BOS seems to the most important direction for further therapeutic intervention.

Several new immunosuppressants have emerged that show promise. Rapamycin and SDZ RAD attack the fibroproliferative response that may lead to the BO lesion (46, 47). Rapamycin, a structural analogue of tacrolimus, seems to downregulate not only the response of T-cells to IL-2, but also to inhibit growth factor-induced mesenchymal cell proliferation (46). It has been shown in animal studies to reduce arterial intimal thickening caused by allograft rejection, decrease infiltration of T-cells and macrophages into the arterial graft, and decrease mRNAs for growth factors such as PDGF, bFGF, and TGF (46). SDZ RAD is a new

Rapamycin analogue with potent immunosuppressive activity (47). Combinations of SDZ RAD and cyclosporine have been noted to prolong allograft survival in animal models of heart and kidney transplantation (47).

Similarly, Mycophenolate Mofetil (MMF) is an antimetablite that has clinical immunosuppressive benefit in solid organ transplantation (48-54). MMF is the morpholinylethyl ester of mycophenolic acid that inhibits inosine monophosphate dehydrogenase, a key enzyme in the de novo purine synthesis of lymphocytes, crucially important for proliferation of human T and B lymphocytes (48-54). It was hoped these antiproliferative effects would protect against chronic rejection. In three large multicentre international trials with 1500 renal transplant patients, MMF was shown to result in a significant reduction of biopsy-proven acute rejection from 40.8% in the azathioprine/placebo-treated patients to 19.8% and 16.5% in MMF 2 g/d- and MMF 3 g/d-treated patients, respectively (51-53). Although the available studies with MMF prophylaxis were not powered for assessing the effect of this drug on long-term survival, the reduction in acute rejection in renal transplant trials suggested that MMF might be protective against chronic rejection. In the Tricontinental study (53), compared with azathioprine, MMF reduced graft loss due to rejection from 9.9% to 5.8% (MMF 2 g/d) and 3% (MMF 3 g/d) respectively at 3 years after transplantation. Whether this simply relates to changes in the incidence of acute rejection or represents a true reduction in chronic rejection is unclear. In addition, the role for MMF in lung transplantation remains controversial (54). One recent randomized prospective, open-label, multicentre study showed a similar acute rejection rate and overall survival at 6 months in lung transplant recipients whether treated with either MMF or azathioprine (54).

The causes of death after transplantation are shown in Table 3 (55). BOS has been the major cause of late death in human lung transplant programs (1, 2, 3, 11, 13, 55-58). BOS onset conferred increased risk of death with a hazard ratio of 5.96 (95% confidence interval [CI]: 3.61, 9.83) (58). Mean survival rates after the diagnosis of BOS were 74% at 1 year, 50% at 3 years, and 43% at 5 years in the series of heart-lung and lung transplant recipients reported by Stanford University (38). Heng and his colleague (58) have also reported similar findings on the actuarial probability of survival after onset of BOS in their study (Fig. 1). The reported case fatality rates of 25 to 29% in the lung transplant recipients who developed BOS (1, 7, 12) is much higher than the 7% mortality in a contemporaneous subgroup without BOS (12).

Table 3. Causes of Death after Lung Transplantation*

	Percent of Death	
	Time Period after Transplantation	
Cause	***<=90 days (n=848)***	***>90 days (n=932)***
Rejection	5%	29%
Infection, other than CMV	29%	24%
CMV infection	5%	5%
Primary graft failure	13%	
Airway complication	5%	
Neoplasm		6%
Cardiac failure	9%	2%
Multiorgan failure	6%	3%
Hemorrhage	6%	4%
Other	21%	26%

*Data from the St. Louis International Lung Transplant Registry (Ref. 55).

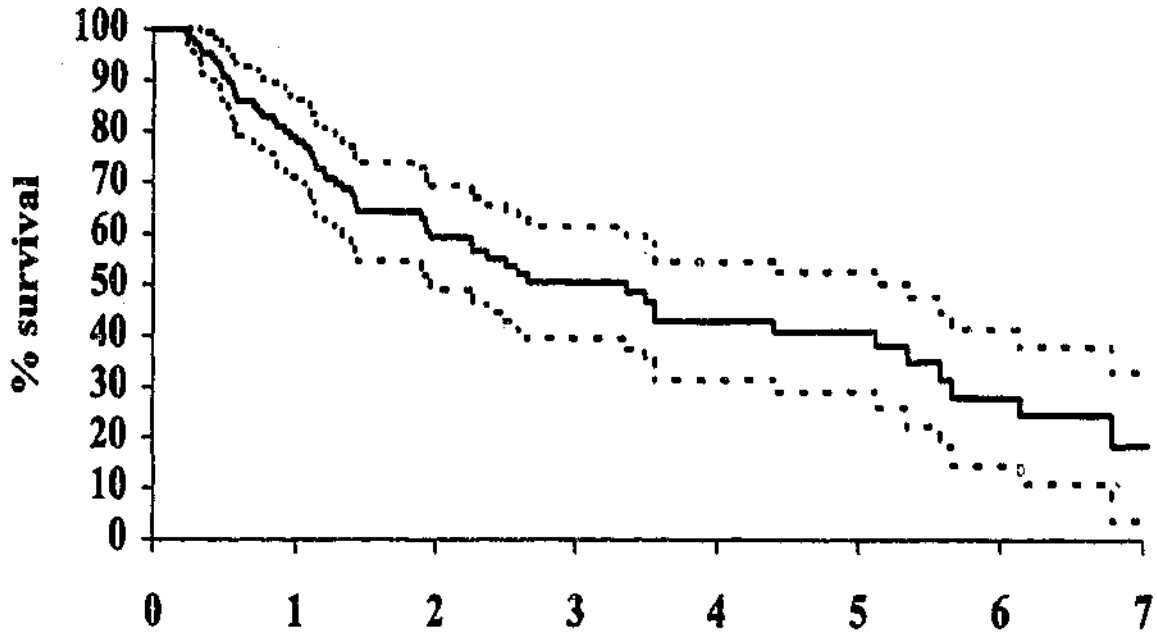


Fig. 1. Actuarial probability of survival after onset of BOS with 95% CI. (Data from Heng, et al 1998 J Heart Lung Transplant 17:1255-1263. [Ref. 58])

1.1.3 Pathology of BO

Pathologically, the trademark of chronic rejection in all solid organs has been fibrous obliteration of endothelialized or epithelialized luminal structure (59-61). Similarly, chronic rejection in the lung is separated into two components - an airway component and a vascular component (30, 31). Bronchiolitis obliterans (BO) is likely to be the pathologic hallmark of the airway component (27, 28, 31).

Lung transplant BO, by definition, is restricted to the membranous and respiratory bronchioles, and involves fibrous scarring in the small airway walls (29). This airway wall mucosal scarring may be eccentric, concentric or may completely obliterate the lumen of the airways (29-31). It is subclassified into active and inactive phases (29-31). In active phases, there is an infiltrate of mononuclear inflammatory cells, which accompanies the dense hyaline fibrous scarring of the airways; inactive BO is characterised by dense fibrous scarring without cellular infiltrates (30, 31). Therefore, the active form of BO is the most likely to respond to augmented immunosuppression therapy (42, 62), while the inactive BO is clearly irreversible with current therapies (31). BO is usually associated with obstructive lung function changes, and may present as subtle, diffuse interstitial or peribronchial infiltrates on chest radiographs (30).

Although the pathological diagnosis of BO is restricted to the injury of small airways, where it has been thought that the alloreactive lymphoid infiltrate is more characteristically centred, the entire bronchial tree may be involved (29). Large airway inflammation and scarring frequently accompany the distal BO (30, 31, 63-65), and pathologically commonly manifest as bronchitis and bronchiectasis (30, 31), where airway smooth muscle may be replaced by

fibrous tissue. The obliterative fibrosing process may also extend from bronchioles to involve proximal small cartilage containing-airways, thus giving the appearance of obliterative bronchitis (30, 31).

Chronic vascular pathology may occur concomitantly with BO (27-31). The pathologic appearance of this condition is essentially similar to the proliferative vascular sclerosis of other solid organ grafts and involves large elastic and smaller muscular vessels (59, 60, 66, 67). The impact of these vascular changes on the outcome after lung transplantation has not been fully estimated, because the chronic airway changes are the dominant factor affecting survival (31, 46).

1.1.4 Risk Factors for BO/BOS

Several risk factors for the development of BO/BOS post lung transplant have been reported in the literature (Table 4).

Table 4. Rick Factors for BO/BOS post Lung Transplant

Risk factors	References
Histoincompatibility	68-73
Frequency and intensity of acute rejection episodes	10-13, 34, 58, 61, 73-76, 78
Infection (particularly CMV)	1 2, 21, 31, 34, 75, 77-87
Airway ischaemia	1, 2, 8, 75, 88-100
Immunosuppressive drugs?	1, 34, 59, 61, 75, 101-114

1.1.4.1 Histoincompatibility

Histoincompatibility is obviously of prime importance. Antigens encoded by genes of the major histocompatibility complex (MHC) play a singular role in acting as major stimulants and targets of transplant rejection. Historically, this has been well documented by experimental animal studies and clinical studies in different organ transplantation showing that donor and recipient disparity at the MHC is primarily responsible for allograft rejection (68-71). Transplants between genetically identical monozygotic twins were found not to be rejected, whereas genetically disparate dizygotic twins or siblings sharing both Human Leucocyte Antigen (HLA) chromosomal segments (haplotypes) had better graft survival than those sharing only one haplotype, who in turn had better outcome than those sharing neither (72). Moreover, with unrelated cadaveric donor grafts, the risk of rejection was also found to be associated with the number of mismatched donor MHC antigens (70). One recent study has reported data from a single institute, examining the influence of donor and recipient HLA locus (HLA –A, –B and –DR) mismatching on the development of BO (73). By univariate and multivariable analysis of risk factor for BO in 152 lung transplant recipients, they found that HLA-A mismatching was one of the most significant risk factors for BO, in contrast, mismatches between donors and recipients at the HLA-DR and HLA-B loci were not important variables determining BO (73).

1.1.4.2 Acute Rejection

Frequent and severe acute rejection episodes have regularly been identified as the most significant risk factor for BO/BOS (7, 8, 10, 11, 13, 34, 58, 73-75). Three or more episodes of acute rejection have been strongly associated with the subsequent development of BO/BOS

(7, 11). For example, in the Pittsburgh experience, 95% of recipients who had three or more episodes of acute rejection (grade A 2) developed BOS, whereas only 18% of those with less th[illegible] episodes had BOS during 5 yr of follow-up (7). In Cambridge, UK, the group with esta[illegible]ned BOS had an average of one episode of acute rejection every 14 weeks in the first year, whereas the corresponding figure for the patients who remained clinically stable was only one episode every 93 weeks (8). Heng and his colleagues (58) investigated risk factors for BO in 230 lung transplant recipients, and found that compared with patients with 0 to 2 episodes, the hazard ratio (HR) for 3 or more acute rejection episodes was 3.4 (95% CI: 3.35, 4.94). Table 5 shows the hazard ratios, confidence intervals and P values for different episodes of acute rejection foun[illegible] by Heng and his colleague (58). In addition, data from the most recent study (73) have further suggested that any episode of Grade A2 or A3 acute rejection was the most significant risk factor for BO, but episode of Grade A1 acute rejection showed no significant association with BO. By 1 year after transplant, 27% of recipients with at least one episode of Grade A2 or A3 acute rejection developed BO, compared with 11% of recipients with no episodes of Grade A2 or A3 acute rejection (Fig. 2) (73). Acute rejection was also suggested to affect BOS progression once present (HR: 1.28/episode; 95% CI: 1.12, 1.45) and survival (HR: 1.20/episode; 95% CI: 1.05, 1.37) (58).

Th[illegible] are two hypotheses that seem to explain the influence of acute rejection episodes on chronic rejection. First, frequent and/or severe acute rejection episodes may allow indirect presentation of donor MHC peptides (namely, allogeneic MHC molecules are processed and presented by recipient antigen-presenting cell [APC], rather than by donor APC) (76) to gain importance over the time; and in turn, initiate and/or amplify a long-term host alloresponsiveness. Alternatively, ongoing immunological injury from repeated acute

rejection episodes may progressively reduce the amount of functional parenchyma, and this progressive functional deficit may contribute to the later gradual organ deterioration in an "alloantigen-independent" fashion (61).

Table 5. Multiple Variable Analysis of Risk Factor for BOS Onset

Risk Factor	unadjusted HR	adjusted HR	95% CI	*P*
Acute rejection within 6 months post-Tx				
0 episode	baseline	baseline		
1 episode	0.54	0.52	0.24, 1.13	
2 episodes	0.80	0.86	0.43, 1.73	
3 episodes	2.69	3.29	1.58, 6.84	
4 episodes	2.30	2.65	1.22, 5.73	
5 episodes	1.35	2.17	0.74, 6.40	
6 episodes	4.32	6.61	2.46, 17.8	
7+ episodes	2.87	3.20	1.23, 8.31	*<0.001*
Comparison with linear model				***0.001***

HR: Hazard ratio
Data from Heng, et al. 1998. J Heart Lung Transplant 17:1255-1263. (Ref. 58).

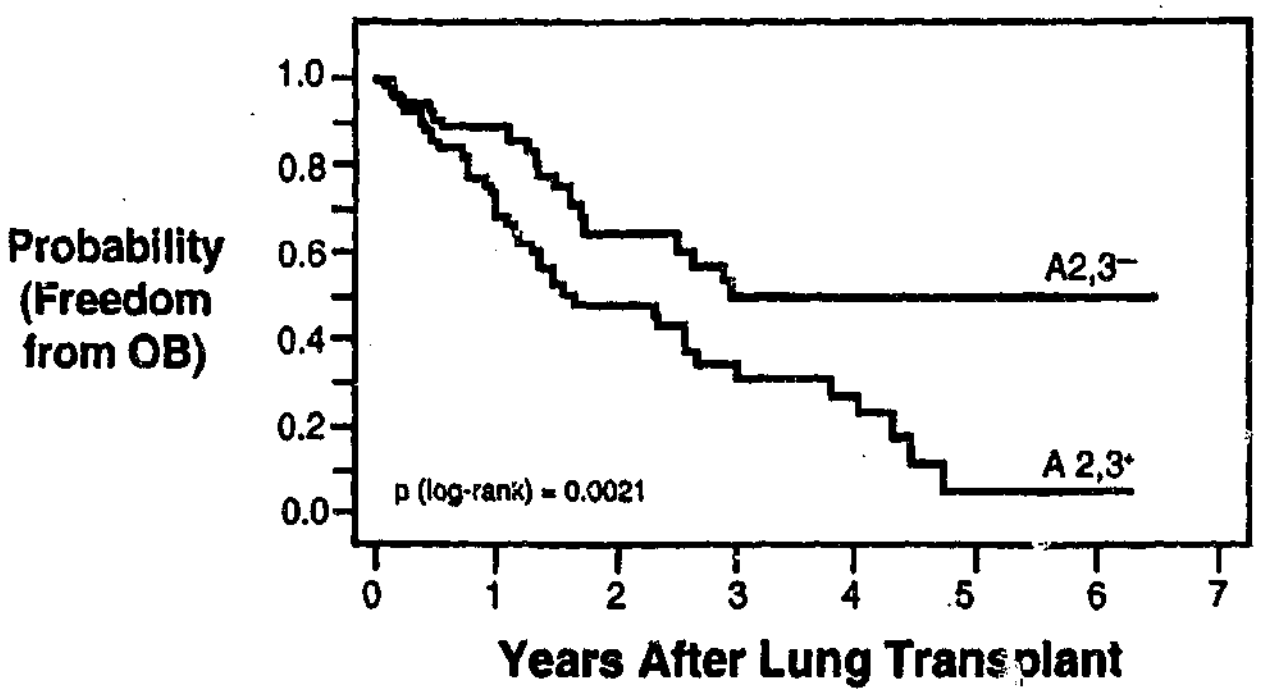


Fig. 2. Influence of Grade A2 or A3 acute rejection on the developmet of BO after transplantation. Recipients with no episodes of Grade A2 or Grade 3 acute rejection (*upper curve*) have higher probabilities (p[log-rank]=0.0021) of freedom from BO as compared with at least one episode of Grade A2 or A3 acute rejection (*lower curve*). (Data from Schulman, et al. 2001. Am J respir Crit Care Med 163: 437-442. [Ref 73])

1.1.4.3 Infection

A third, widely accepted contributing factor, is infection (34, 75, 77-79). Certain infectious agents have been implicated in the development of BO in the nontransplanted population, particularly viruses, including respiratory syncytial virus, adenovirus, rhinovirus, coronavirus, influenza, and parainfluenza, measles, mumps, and cytomegalovirus (CMV) (21, 80-82). In addition, bacterial infection has been also reported in association with BO in a nontransplant setting (82). Lung transplant recipients have a greatly increased susceptibility to infection by both common pathogens and opportunistic organisms (1, 2, 57, 83), not only because of their immunosuppressant regimens, but also because lung denervation that may damage local defence mechanisms including the cough reflex and mucociliary clearance. Moreover, loss of bronchial circulation and disruption of lymphatic drainage may also breach lung defences. Local recurrent infections may either directly injure the epithelium or have a trigger function in augmenting immunological attack, such as up-regulation of donor MHC antigen on airway epithelium (75, 78, 79).

CMV infection (with or without pneumonitis) is one of the most problematic infections causing both morbidity and mortality to lung transplant recipients (1, 31) and may be a predisposition to BO/BOS, but this association remains controversial (7, 10, 13, 34, 84-87). However, CMV infection could contribute to allograft injury and the development of BO/BOS by other routes such as cytokine-induced up-regulation of donor MHC antigens or immunological cross-reactivity between CMV antigen and donor MHC antigens (77). Two recent studies on risk factors for BO by univariate and multivariable analyses have shown the similar finding that besides acute rejection, CMV infection or/and CMV pneumonitis was the second most significant risk factor for BO (58, 73).

1.1.4.4 Airway Ischaemia

The lung allograft is unique among all the transplanted organs in that the donor airways experience not only the early injury secondary to prolonged ischaemia and re-perfusion, but also potential post-operative ischaemia due to the loss of the bronchial artery, which is not re-connected in the operative procedure (1). Potentially increased ischaemic time and the loss of blood supply may lead to scarring and eventual obliteration of the bronchial lumen (2, 75, 88-90), because epithelium injured by ischaemia might express more MHC class II surface antigens, which, in turn, would initiate a secondary immune-mediated injury against epithelial cells (91). Additional ischaemia might be caused by the primary immune response, if directed against endothelial antigens. (92). Immune-mediated endothelial cell injury may then lead to occlusive arteriopathy resulting in epithelial ischaemia (92). A poorer graft survival has been demonstrated to correlate with the pre-operative ischaemia time in both kidney (93) and liver transplants (94). However, in lung transplant, although some studies have shown an effect of ischaemia on later BOS development (2, 7, 88-90), other studies have not found a significant relationship (8, 95).

Although the long-term functional capacity of airways deprived of their bronchial arterial supply is unknown, post-operative bronchial ischaemia appears to be the most important factor influencing the process of airway healing (88). Some groups have, therefore, performed direct bronchial artery revascularization (BAR) for a number of lung transplant recipients, and investigated the effects of BAR on BOS/BO post transplant (96-100). Preliminary data have suggested that good BAR may postpone the onset of BOS/BO with survival rates of 85, 81, 61, and 69% respectively in patients with BAR after 1, 2, 3, 4 and 5 years (99), which seems better than the reported rate in the patients without BAR (1). Another study from the same

group has also shown that the total incidence of BOS in the patients with BAR at three years was 33% and 1, 2 and 3-year probability of remaining free from BOS was, respectively, 85%, 82%, and 67% (100). However, longer follow-up with controls in a large number of patients is needed to verify the effect of BAR on the development of BOS.

1.1.4.5 Immunosuppressive drugs

The influence of various immunosuppressive drugs on chronic rejection is conjectural (59, 61, 75, 101-104). The commonly used agents, their mechanisms of action and their side effects are summarized in Table 6. As immunologic monitoring of the effects of those drugs is not yet clinical applicable, therapy is only regulated by signs of drug toxicity and by physiologic or histologic evidence of rejection, and to a lesser extent blood drug levels (1). The mainstay of solid organ transplantation has been the immunosuppressive drug regimen, which also predisposes the recipient to infection (1), and may contribute to the development of BOS as described above. However, inadequate or fluctuating levels of immunosuppression may allow subclinical rejection, which may ultimately worsen the chronic rejection (34, 102). Additionally, high-doses of immunosuppressive drugs such as CsA and tacrolimus clearly results in other significant side effects such as nephro-toxicity, hypertension, hyperlipidemia, marrow suppression, and neuro-toxicity (59, 61, 105-107).

Although widespread introduction of CsA in the early 1980s greatly improved first year cadaveric allograft survival rates, the rate of graft loss after the first year has not changed (105-107). Indeed, despite its efficacy as an immunosuppressive agent, the nephrotoxic effects of CsA have been difficult to define and differentiate from renal chronic rejection (59,

61). Histopathologically, both entities are characterised by interstitial fibrosis, tubular atrophy and vascular changes (105). Experimental data also showed that CsA-treated aortic allografts in rats developed drug-related early subendothelial inflammation followed by proliferation of smooth muscle cell into the intima, with intimal thickening and accelerated arteriosclerosis (108). However, no difference in incidence of transplant related coronary artery disease has been found in human heart transplant recipients treated with CsA (101-103), and little data are available from the human lung transplant field. Recent studies have suggested that CsA enhances the production of TGF-β_1 protein, as well as the expression of its receptor and mRNA both *in vitro* and *in vivo* (109-114). The dual action (immunosuppressive and profibrogenic) of CsA might explain why this drug, although revolutionary in organ transplantation, might not be optimal for long-term graft survival.

Table 6. Immunosuppressive Drugs: Mechanisms of Action and Toxicities

Drug	Mechanisms of action	Major Toxicities
Cyclosporine (Sandimmune; Neoral)	Inhibits IL-2 gene transcription; blunts T-lymphocyte activation and proliferation	Nephrotoxicity, hypertension, neurologic problems (tremor, headache, etc.) hirsutism, gingival hyperplasia
Tacrolimus (Prograf)	Inhibits IL-2 gene transcription; blunts T-lymphocyte activation and proliferation	Nephrotoxicity, hypertension, hyperglycemia, neurotoxicity
Azathioprine (Imuran)	Inhibits purine salvage and biosynthesis; blocks lymphocyte proliferation	Leukopenia, macrocytic anaemia, cholestatic hepatitis, pancreatitis
Mycophenolate Mofetil (CellCept)	Inhibits purine biosynthesis; blocks lymphocyte proliferation	Diarrhoea, dyspepsia, leukopenia, anaemia
Corticosteroids	Block cytokine gene transcription and secretion from mononuclear phagocytes; possible lyse T lymphocytes	Hyperglycemia, hypertension, osteoporosis, cataracts, myopathy, dyslipidemia, mood disturbances
ATG or ALG (ATGAM)	Depletes lymphocytes by lysis or by opsonization and phagocytosis; possible modulates immune response inn other ways	Leukopenia, thrombocytopenia, fever, allergic reaction to horse serum, serum sickness
OKT3 (Orthoclone OKT3)	Depletes CD3 lymphocytes by opsonization and phagocytosis; modulates T-cell interaction with antigen presenting cells	First-dose, cytokine-release syndrome, including hypotension or pulmonary edema, transient azotemia, leukopenia, aseptic meningitis

Definition of abbreviations: ATG=antithymocyte globulin; ALG=antilymphocyte globulin; OKT3=monoclonal antibody to CD3 complex of T-cell receptor.
Data from Trulock EP. 1997. Am J Respir Crit Care Med 155: 789-818 (Ref. 1)

1.1.5 Pathogenesis of BO/BOS

How "rejection" leads to BO/BOS remains poorly understood. The hypothesis of "response to injury" is perhaps the most tenable explanation, with BO/BOS resulting from tissue damage and remodelling in response to graft injury caused by a series of alloantigen-dependent and -independent insults to the transplanted lung. Airway inflammation (whatever the offending insults) leading to fibrosis of the airway is the likely to be hallmark of BO/BOS. Inflammatory mediators including growth factors are believed to have an important role in the generation of tissue fibrotic response.

1.1.5.1 The Participation of Graft Cells

Antigen presenting cells are the pivotal cells that initiate alloantigen-dependent inflammation in the transplanted organ. Both bronchial epithelium and vascular endothelium of the donor lung allograft may present donor antigens to host lymphocytes, thereby initiating an immune response against the graft, as they bear donor class II MHC antigens (66, 79, 92, 115-117). For the same reason, donor epithelial and endothelial cells also represent direct targets for recipient alloreactive cells, and in turn, the initiated immune attacks and inflammation up-regulate the expression of donor MHC antigens, leading to amplification of the ongoing airway inflammation and injury in a vicious cycle (66, 71, 79, 92, 118).

Bronchial vascular endothelium, particularly, is uniquely positioned to regulate the process of cellular infiltration into the graft, because the endothelium provides an allogeneic barrier between the recipient circulating cells and the donor lung (66, 92). To gain entry from blood

into the graft tissue, recipient leukocytes must adhere to and penetrate the vascular endothelium. Exposure of the endothelium to circulating cytokines, especially TNF-α and IFNγ, increases the expression of endothelial adhesion molecules and enhances the cascade of inflammatory cellular infiltration into the airways (59, 71). In contrast, bronchial epithelial cells are abundant, and constantly exposed to extrinsic infectious agents, therefore, are also likely to often the target for alloantigen independent insults following the transplant, such as viral and bacterial infections (1).

Moreover, both bronchial epithelial as well as vascular endothelial cells can also amplify local inflammatory events, via synthesis of interleukins, chemokines, colony stimulating factors and growth factors in response to immune and non-immune attacks (66, 67, 92, 95, 119-121). For example, endothelial cells have been shown to produce IL-1, bFGF, and PDGF-like protein that are all implicated in chronic transplant rejection (66). Airway epithelial cells may represent an important source of IL-8, the most potent chemoattractant for neutrophils (121-123), and can produce TGF-β (121, 123, 124), a growth factor that may play a key role in the later airway repair phase followed the inflammatory injuries (125-128)

1.1.5.2 The Participation of Recipient Cells

Generally, it has been thought that in response to the allograft, a recipient's T helper lymphocytes (CD4+ T-cells) are activated first by recognizing donor MHC class II antigen, with release of lymphokines, such as interleukin-2 (IL-2), IL-4, IL-5, and IFNγ, which are all necessary for initiating the subsequent rejection cascade (71, 93). IL-2 is involved in the activation of T cytotoxic cells (CD8+ T-cells), and natural killer (NK) cells. IL-4 and IL-5 are

required for the differentiation of B cells to produce anti-donor antibodies (71, 93). IFNγ increases the antigen presenting activity of macrophages, and up-regulates the expression of MHC antigen in the graft (71, 93). However, a persistent and preponderant CD8+ T cell sub-population, but not CD4+ T cells, have been found in the airway wall and BAL fluid of lung allografts from both clinically stable recipients and the recipients with BOS (129-132). It is possible that CD4+ T cells are both necessary and sufficient to cause rejection, but it is likely that rather than causing damage directly, they recruit other cells, such as CD8+ T cells, NK cells, and macrophages into the process. CD8+ T cells, by recognizing and binding to the donor MHC class I antigen, can directly injury the graft by lysing graft endothelial, epithelial and other parenchymal cells (1, 71, 93).

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) of the immune system, which display an extraordinary capacity to stimulate native T cells and initiate primary immune responses (133, 134). Once primed, these lymphocytes undergo blastogenesis and clonally restricted expansion, and induce the effector arm of the immune response. Human lung DC are localised within airway epithelium, alveolar septa, and in the connective tissue surrounding pulmonary vessels (135). In the context of lung transplantation, however, despite a potential important role for DC in the development of BOS, there are only few studies on the role of DC in human lung allografts (136-138). In 1990, Yousem and co-workers showed increased numbers of DC in the tracheal and bronchial epithelium and submucosa of patients with BO in particular (136). In 1998, Milne and co-workers looked at mononuclear phagocyte population in the transplanted human lung, using unused donor lungs, open lung biopsies and transbronchial biopsies (TBB) from transplanted lungs (137). They found a marked depletion of CD1a+ DC in lung allografts compared with unused donor lungs

(137). Most recently, Leonard and his co-workers found an increased numbers of DC in both TBB and endobronchial biopsies (EBB) of patients with BO/BOS compared with clinical stable patients, and a markedly greater number of DC in EBB than in TBB (138). They suggest that Milne and co-workers may have underestimated the number of DC by not using another marker of DC such as high Class II MHC expression (138). Further studies of the role for DC in chronic lung rejection are needed.

Macrophage accumulation has been also demonstrated in the airway walls of lung allograft post transplant (129, 130), indicating that a recruitment of recipient monocytes from the circulation into lung allograft also occurs after transplantation. Macrophages are equipped with numerous mediators, such as active enzymes, arachidonic acid metabolites, free radicals, nitrous oxide, and cytokines, and are capable of functioning as antigen presenting cells and participating in immune reactions (139). Within the graft, recipient macrophages are faced with an overwhelming stimulus, become activated and discharge their chemical armamentarium and a variety of pro-inflammatory cytokines into the surrounding tissues of the graft, causing inflammation and damage to the tissues (59, 61, 71, 140). Moreover, in the later repair phase of graft injury, macrophages have been suggested to be a potential key cell contributing to the development of BO/BOS, through their production of growth factors, including TGF-β, and PDGF (124, 141-143).

Neutrophils may be another important particip[illegible]t involved in the development of BO/BOS post lung transplant. BAL neutrophilia have been reported in lung transplant recipients, but especially marked in patients with BOS, together with highly increased levels of IL-8 in BAL fluid (130, 145-147). Neutrophils are rarely present in large numbers in normal airway and

lung tissue (144). Neutrophil influx is an important secondary event in the armamentarium of the body's Innate Immune System in response to bacterial and viral infection. But some neutrophil products, such as proteases, matrix metalloproteinases, acid hydrolases, low-molecular-weight cationic protein, lipid secretory products, and reactive oxygen metabolities, can also induce tissue injury at the site of neutrophil-dominated inflammation, thereby, facilitating the development of pulmonary fibrosis (148-151). There is significant clinical and experimental evidence of persistent neutrophil alveolitis preceding and playing an important role in the pathogenesis of pulmonary fibrosis such as IPF (144) and ARDS (152). Thus, it is possible that recipient neutrophils may at least add a contribution to promoting airway fibrosis in the development of BO/BOS. Little is known about the role of other leukocytes, such as mast cells and eosinophils, in the pathogenesis of BOS post-transplant.

1.1.5.3 Cytokines and Growth Factors in the Development of BO/BOS

It is become increasing clear that many aspects of chronic lung rejection are mediated by cytokines and growth factors released by recipient alloreactive cells, and injured or activated graft endothelial and epithelial cells. Several of these may contribute to bronchial obliteration, or airway fibrotic process, such as TNF-α, TGF-β, and PDGF.

TNF-α is generally considered to be a pro-inflammatory cytokine (142). Many cells including monocytes/macrophages, T lymphocytes, mast cells, and epithelial cells produce TNF-α, but the principle source is the monocytes/macrophage (142). TNFα exerts a variety of biologic effects, that directly target the cells possessing TNF-α receptors, and induces secondary cytokine production and other inflammatory mediators to orchestrate acute and chronic

inflammation (142). It activates neutrophils, lymphocytes, and monocytes, and promotes the emigration of all these inflammatory leucocytes to the inflamed tissues via up-regulation of endothelial leucocyte adhesion molecules such as ICAM-1, and VCAM-1 (142, 153, 154). TNF-α can potently stimulate secretion of pro-inflammatory and chemotactic cytokines, including IL-8, IL-1, RANTES and GM-CSF, from epithelium, endothelium, mesenchymal cells, and fibroblasts (142, 155-157). Systemic elevation in circulating TNF-α and intratracheal injection of TNF-α can cause pulmonary inflammation and injury in animals (158, 159). TNF-α also stimulates fibroblast proliferation (160), and can induce the biosynthesis of PDGF and TGF-β (161).

In the transplanted lung, several studies have demonstrated an early increase of TNF-α production by BAL cells after an ischaemia/reperfusion injury (162), and increased BAL TNF-α levels in transplant recipients with acute lung rejection, or with CMV pneumonitis (163). However, during chronic lung rejection, or in recipients with BO, TNF-α, produced by alveolar macrophages, has been found to be reduced (163, 164). This may suggest that, as a pro-inflammatory cytokine, TNF-α is most involved in the tissue injury phase during the development of BOS, rather than in the tissue repair phase, which are more likely mediated by growth factors, such as TGF-β, and PDGF.

TGF-β belongs to a family of multifunctional polypeptides that regulates normal cell growth, development, and tissue remodelling following injury (124). Monocytes express TGF-β mRNA constitutively but only release the protein when activated (165, 166). Pulmonary macrophages may store large amount of TGF-β during pulmonary inflammation (167), and

lung fibroblasts themselves may be a source of TGF-β (168). TGF-β can be produced also by inflammatory cells including neutrophils (169), eosinophils (170), as well as structural cells such as airway smooth muscle cells and airway epithelial cells (171). The TGF-β family of proteins has the most stimulatory effect on extracellular matrix deposition of any cytokines so far examined (124, 172-175). In the lung, TGF-β_1 regulates the accumulation of extracellular matrix, and the generation of tissue fibrosis (124). *In vitro* studies have shown that TGF-β_1 increases the expression of the major extracellular matrix protein, fibronectin and also collagen by human lung fibroblasts (172, 173). In animal models of pulmonary fibrosis, enhanced TGF-β_1 gene expression is temporally and specifically related to increased collagen expression and deposition (174), and anti-TGF-β_1 antibodies reduce collagen deposition in a murine bleomycin-induced lung fibrosis model (175). *In vivo* studies have further demonstrated that TGF-β_1 immunoreactivity and mRNA expression are increased in asthmatic and COPD airways (176-178), and alveolar macrophages from patients with asthma and chronic bronchitis produce more TGF-β_1 than normal controls (179-181).

Various lines of evidence also exist supporting the role of TGF-β_1 in the development of BO/BOS post lung transplant (125-128). Preliminary data from our group has demonstrated that BAL TGF-β_1 levels were significantly increased in clinically stable lung transplant recipients, and even more so in recipients with development of BOS (128). Magnan and colleagues observed similar findings, i.e. that alveolar macrophages produced more TGF-β_1 at the onset of acute rejection, CMV infection and in lung transplant recipients who developed BOS (141). One recent study showed that patients with chronic lung rejection showed marked peaks of TGF-β_1 mRNA expression by BAL cells, and these peaks preceded the diagnosis of chronic rejection by several months (125). El-Gamel and his colleagues demonstrated a

positive association between the level of tissue expression of TGF-β_1 and the grade of lung allograft fibrosis (126). A study from the same group showed that lung and heart-lung transplant recipients who developed allograft fibrosis had a higher frequency of a TGF-β_1 polymorphism i.e. arginine/arginine homozygous in codon 25, compared with patients without allograft fibrosis (127). Hence, they believe that this TGF-β_1 genotype has prognostic significance in transplant recipients (126, 127).

PDGF is a potent mitogen and chemotactic agent, a cationic glycoprotein named subsequent to its discovery in α granules of platelets (143). This name later proved inappropriately restrictive, as it was recognised that PDGFs are synthesised by a wide variety of cells such as endothelial and epithelial cells, activated monocytes and macrophages (143). The cellular targets of PDGF action in the lung include fibroblasts, vascular smooth-muscle cells, and related mesenchymal cells, as well as epithelial and endothelial cells (143). PDGF may activate fibroblast to proliferate and secrete collagen (182). Several studies suggest that PDGF has a pivotal role in fibroproliferative disorders of lung, including Idiopathic Pulmonary Fibrosis (IPF) (183), Adult Respiratory Distress Syndrome (ARDS) (184), and Bronchiolitis Obliterans-Organising Pneumonia (BOOP) (185). Immunostaining has confirmed the presence of PDGF-like proteins in fibrotic lung and its absence in normal lung samples (143).

A number of investigators have examined the role of PDGF in the pathogenesis of transplant BOS (186-188). Hertz and colleagues observed that BAL fluid from BOS patients significantly stimulated fibroblast migration, whereas fluid from controls did not, and demonstrated increased concentrations of PDGF in BAL fluid from BOS patients (188). Prospective evaluation of sequential BAL fluid samples from a patient who developed BOS

demonstrated markedly increased PDGF concentration before the onset of irreversible airflow obstruction (188). Experimentally, obliterative lesions have also been produced in heterotopic tracheal isografts that were injected with PDGF (186). In addition, an increase in PDGF receptor mRNA expression and PDGF immunoreactivity were demonstrated during progressive loss of respiratory epithelium and airway occlusion in experimental BO, and treatment with inhibitor of PDGF receptor (CGP 53716) significantly reduced myofibroproliferation and airway occlusion (187). Moreover, PDGF is up-regulated during the development of chronic rejection of renal (189) and cardiac (190) allografts. Those findings are supportive of a role for PDGF in the fibroproliferative changes observed in lung transplant BOS.

1.2 Airway Remodelling

1.2.1 Introduction

"Airway remodelling" is quite a new concept and refers to the structural changes seen in the airways of airway diseases, such as Asthma and Chronic Obstructive Pulmonary Disease (COPD) (191-193). These include subepthelial collagen deposition, mucosal collagen deposition, epithelial injury and denudation, and changes in airway vascularity, mucous glands, and smooth muscle (191-198). These structural changes are most likely the consequence of ongoing chronic airway inflammation, but have as yet poorly determined effects on patient symptoms, lung function, airway reactivity, symptoms, frequency and outcome of acute exacerbations of these diseases (191, 192, 199, 200).

Numerous aspects of airway remodelling have been investigated in Asthma and to a lesser extent in COPD in the last decade (Table 7) (191-193). Those observations have suggested that airway remodelling is a crucial factor in the long-term outcome of those diseases. However, little information is available in the airway remodelling that occurs in human transplanted lung, which may yield new insights into the development of BOS.

1.2.2 Airway Inflammation and Remodelling in Asthma

Asthma used to be thought of as an entirely reversible airway disorder. However, this assumption has recently undergone reevaluation, because of newer understanding of the structural changes that occur in asthmatic airways (192-197). A number of studies have demonstrated that many asthmatics experience an accelerated rate of respiratory function deterioration (201, 202). This has been nicely illustrated in a study of 17,506 people followed over a 15-year period, where 1,095 asthmatic patients experienced a greater decline in lung function ($FEV_{1.0}$) than did controls (38 ml/year vs. 22 ml/year) (202).

Airway inflammation is a central feature of asthma, (203-206), and it appears to be far more complex than a simple eosinophilic inflammation alone. All cells of the airways-inflammatory and structural- are involved and become activated, including T-cells, eosinophils, mast cells, macrophages, epithelial cells, fibroblasts and bronchial smooth muscle cells. However, eosinophils, mast cells, and CD4+ T-lymphocytes seem to be most central (191, 203-206). Those cells play an effector role by the release of pro-inflammatory mediators, cytotoxic mediators and cytokines resulting in vascular leakage, hypersecretion of mucus, smooth muscle contraction, epithelial shedding, and bronchial hyperresponsiveness (199, 207). In

addition, they are also involved in the process of tissue remodelling and fibrosis by the release of growth factors (199, 207). The development, progression and/or resolution of airway inflammation in asthma appear critical to the presence and development of symptoms, pulmonary function abnormalities, disease severity, and is the central target of treatment (191, 204, 208).

Blood vessel proliferation, and expansion of the vascular bed may occur within the airway walls in asthma when airway inflammation becomes chronic (191, 197, 198). There may be two types of vascular remodelling, sprouting angiogenesis and microvascular enlargement. In sprouting angiogenesis, endothelial cells proliferate and migrate to form new vessels (191). In microvascular enlargement, vessels can be enlarged circumferentially as a results of the proliferation of endothelial cells and other elements of the vessel wall, and thus may appear to be dilated or congested (191). It has long been recognized that in fatal asthma, the airway mucosa is edematous and contains dilated, congested blood vessels (209), and increased number of large blood vessels have been reported in autopsy lung specimens from patients dying of severe acute asthma (210). More recent morphometric studies have confirmed these early observations, and have shown that enlarged, congested mucosal blood vessels, and increase in mucosal vessel numbers contribute to the increased airway wall thickness even in mild asthma (191, 198). The changes in the number or caliber of mucosal blood vessels are likely to be functionally important in asthma, because even modest increases in airway wall thickness can potentially amplify the narrowing of the airway lumen produced by a fixed amount of bronchial muscle contraction (211).

Airway wall fibrosis or scar collagen deposition is another major structural change demonstrated recently in asthma (193-195). Histopathological and immunohistochemistry studies in asthma using light and electron microscopy have shown thickening of the basement membrane (BM), and a significant accumulation of collagen proteins (i.e. collagen type III and V) in the mucosa of the airway walls (193-195, 204). In patients who have died of asthma, thickening of the BM region is nonuniform, with a median thickness of 17.5 μm (212) compared to 7 μm in nonasthmatic airways (199). In young asthmatics and even in those with very mild asthma, increased subepithelial collagen deposition also occurs (204). Collagen deposition within the airway wall is thought to result in the irreversible stiffness of the airways in asthma (193-195), which could, in tern, lead to poorer response to bronchodilator therapy, especially during severe attacks, and more severe airflow obstruction when in addition submucosal vasculature (197, 198, 211), and airway smooth muscle mass increase (213).

An increase in airway smooth muscle mass caused by varying degrees of hypertrophy and hyperplasia has been well described in the asthmatic airway (196, 213-217). Autopsy has revealed an increased smooth muscle area and thickness in the asthmatic airways (216, 217). The ability of smooth muscle hyperplasia and hypertrophy to cause exaggerated physiologic response to contractile agonists and to play a role in the generation of airway hyperresponsiveness is being increasingly appreciated (213, 214). However, very recent studies have demonstrated that airway smooth muscle is phenotypically and functionally "plastic" and has the capacity to produce multiple inflammatory mediators, such as GM-CSF, IL-8, Eotaxin, to contribute to the persistence of chronic inflammation in asthma, and smooth

muscle cells may also be an important source of extracellular matrix in the asthmatic airway (213, 218).

Accumulating evidence suggests that epithelial damage and shedding are also important features of asthmatic airways and may, at least in part, be a consequence of inflammation. Epithelial desquamation and sloughing are characteristic findings in bronchial tissue obtained at autopsy (204), in airway biopsy (219), and BAL from the patients with asthma (220). Epithelial injury may contribute to bronchial hyperresponsiveness in asthma, because it could make it easier for irritant factors to access the nerve endings of the mucosa, enhance the penetration of allergen particles to mediator secreting cells in the mucosa, and could decrease production of epithelium-derived bronchodilator substances (204, 221, 222). In addition, mucous gland and goblet cell hyperplasia are also a frequent pathological findings in asthmatic airways (Table 7) (191, 226, 223, 224). Increased production of viscid mucus causing bronchial plugging is a common pathologic feature of death from asthma (204), and increased secretions may also contribute to airflow limitation in patients with stable asthma (204).

1.2.3 Airway Inflammation and Remodelling in COPD

COPD is a disorder characterized physiologically by the presence of chronic airflow obstruction and embraces three main conditions-chronic bronchitis, emphysema (usually smoking-related), and chronic unremitting asthma with fixed airflow obstruction. The airways in smoking-related COPD are also inflamed, but, in contrast to asthma, the predominant types of inflammatory cells are neutrophils, macrophages and CD8+ lymphocytes (200, 225-229).

Table 7 summarizes the main distinctions between COPD and asthma in terms of the predominant inflammatory cell phenotypes, some structure changes of the airway wall, and functional consequences (192, 200, 225-229). However, my review of the literature has shown that little information is available about mucosa collagen or vascular changes in COPD airways.

1.2.4 Airway Inflammation and Remodelling in the Transplanted Lung

Although lung transplant BO/BOS is the major cause of late death in recipients, there has been surprisingly little attention given to the investigation of airway architectural damage and its repair post lung transplantation. The major publications available in the literature in this area are those arising from this Ph.D. work (230-232), and related studies from the same group (129, 130). In 1990, a transbronchial biopsy study demonstrated airway basement membrane thickening in BO patients (233), suggesting that subepithelial collagen deposition occurs in the process of BO/BOS development. In 1992, an open lung biopsy and postmortem study showed reduplication and crenation of the subepithelial basement membrane with irregular plaque-like thickening changes in the recipients with BO (234). This paper also described that pathological changes are seen invariable in the large airways (a site easily sampled with EBB) of patients with BOS, whereas small airways will be found only in 40% of TBB specimens (234). This reinforces the potential role for EBB over TBB in monitoring airway remodeling in lung transplant patients. Snell and his colleagues, in 1997, for the first time, reported that despite the conventional triple immunosupressive regimen, a significant CD8+ T-lymphocyte and macrophage infiltrate were seen within the large airway walls even in clinically stable lung transplant recipients (129). Since then, a series of publications in this

field have emerged from the current Ph. D. project, which have addressed several aspects of airway inflammation and remodelling in human lung allografts, particularly, the potential role of neutrophils in the development of BOS, airway vascular changes, and submucosa collagen deposition (230-232). The details are described in Chapter III.

1.2.5 Clinical Consequences of Airway Inflammation and Remodelling

Bronchial Hyperresponsiveness: Bronchial hyperresponsiveness (BHR) is the exaggerated response of airways to a wide variety of nonspecific stimuli that narrow the airways (235-240). It is an important pathophysiological characteristic of bronchial asthma (235-238), and also often presents in patients with COPD (235, 239, 240). As derived from several publications, Table 8 shows the prevalence of BHR in asthma and COPD with different stimuli used (241-243). In asthma, more severe hyperresponsiveness is associated with worsening of the symptoms and a steeper fall in $FEV_{1.0}$ (202). In COPD, the severity of airway hyperresponsiveness predicted subsequent decline of $FEV_{1.0}$ (244).

Table 8. Prevalence (%) of BHR to Different Stimuli in Asthma and COPD*

Stimuli	Asthma	COPD
Acetylcholine	75	64
Methacholine	80	70
Histamine	82	21
Propranolol	67	21
SO_2	95	30
Adenosine-5-monophosphate (AMP)	90	90/39[#]
Hyperventilation[†]	96	11
Fog	30	81

[#] 90% in smokers, 39% in nonsmokers. [†] Hyperventilation of cold air.
* data from references of (231-233).

Airway inflammation and structural abnormalities have been proposed in the pathogenesis of BHR (235-239), at least in asthmatic patients, although it is yet unknown which factors within the airways of individuals are responsible. For example, indirect stimuli (e.g. AMP, tachykinis, bradykinin, and propranolol) are thought to cause airway constriction by activating either inflammatory cells or neural pathway (235-239). In asthma, eosinophilic inflammation has been demonstrated to be associated with BHR induced by ultrasonically nebulized distilled water (237). COPD subjects with BHR had significant higher number of airway mucosal CD8+ T-lymphocytes and higher percentage of sputum eosinophils than those without BHR (239). Increased airway wall thickness in asthma involving an increase in muscle mass, mucous glands, and in vessel area lead to markedly reduced airway caliber (193-196, 198). Those features could also potentially contribute to BHR by different mechanisms. For example, thickening of the inner wall can amplify the effects of airway smooth muscle and an increase in muscle may increase the force-generating capacity of the muscle, allowing it to overcome the elastic loads provided by lung recoil, airway mucosal folding, and radial constraint (236, 238).

Many lung or heart-lung transplant recipients also demonstrate marked BHR (245-248). Although not universally accepted, studies from our group and others showed no association between cellular airway inflammation and BHR of lung allografts (245, 247). These studies may suggest an alternative mechanism for BHR following lung transplantation from that usually assumed in asthma.

Increased Airway Resistance: Changes in airway carliber and airway thickness are functionally important because they can amplify airway resistance markedly, as airway

resistance is inversely proportional to the fourth power of airway radius. The wall of the airway can be divided into three areas (249): (1) the inner wall, consisting of epithelium, basement membrane, lamina propria, and loose connective tissue between the lamina propria and the airway smooth muscle; (2) the smooth muscle layer in between; and (3) the outer wall, with loose connective tissue between the muscle layer and the surrounding parenchyma (the adventitia).

Figure 3 shows the effect of inner airway wall thickening on airway resistance. Normal airways have an inner wall area of 20% (209). When 30% airway smooth muscle (ASM) shortening occurs, the airway resistance increases by 7.6-fold (209). If the inner wall thickness, e.g. occupying 40% of the airway walls instead of 20%, this will increase the airway resistance to some extent (1.8-fold). However, with the same magnitude of smooth muscle shortening (30%), airway resistance can be increased to a large extent (80-fold of the baseline) (209). Therefore, increased airway wall thickness also has the potential to contribute to the development of increased airflow resistance in airway diseases.

Irreversible Component of the Airways Obstruction: For decades, asthma has been considered as a condition of reversible airflow obstruction. However, some patients have been shown to have irreversible changes in lung function, despite appropriate and aggressive anti-inflammatory therapy (250, 251), and some patients have an enhanced decline in lung function over time (202, 252). It is now increasingly recognised that abnormalities in structure in the airways of asthmatics may be irreversible, and account for the persistent airway obstruction in asthma. Fibrosis within the airway walls (together with other features of

remodelling) could lead to several adverse consequences such as stiffness of airway walls, poorer response to bronchodilator therapy and worsening of airflow obstruction.

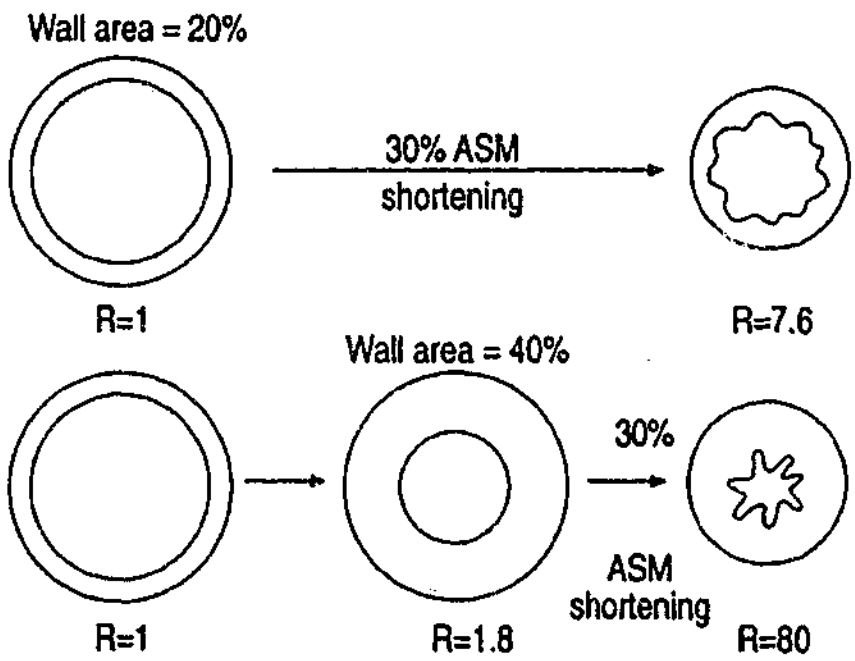


Fig. 3. The effect of inner wall thickening. In this example, the normal airways have an inner wall area of 20%. When 30% airway smmoth muscle (ASM) shortening occures, the airway resistance (R) increases from an arbitrary valune of 1 to 7.6. An increase in wall area to 40% produces a small increase in resistance to 1.8 × baseline, but with the thicker wall, 30% ASM shortneing increases resistance to 80 × baseline. (Data from Kuwano A, et al. 1993. Am Rev Respir Dis 148: 1220-1225. [Ref. 209]).

Table 7. Comparison of COPD and Asthma

	COPD	**Asthma**
Inflammatory cells	Neutrophils, CD8+ cells (Tc), Macrophages++	Mast cells, Eosinophils, CD4+ cells (Th2), Macrophages+, Neutrophils
Inflammatory mediators	LTB_4, TNF-α, IL-8, Matrix metalloproteinases (MMP-1, -9, and -12), Oxidative stress+++	LTB_4, histamine, IL-4, IL-5, IL-13, Eotaxin, RANTES, TNF-α, TGF-β, bFGF, Oxidative stress+
Surface epithelium	Fragility undetermined	Fragility/loss
Reticular basement membrane	Variable or normal	Homogeneously thickened
Bronchial mucous cells	Metaplasia/hyperplasia	Mucous metaplasia is debated
Bronchial glands	Enlarged mass (increased acidic glycoprotein)	Enlarged mass (no change in mucin histochemistry)
Bronchial smooth muscle	Enlarged mass (small airways)	Enlarged mass (large and small airways)
Congestion/oedema	Variable	Present
Bronchial blood vessels	unknown	Increased and enlarged
ECM components	unknown	Collagen III and V increased
BHR	Yes	Yes
Response to bronchodilator	Variable	Yes
Response to corticosteroid	Variable	Yes

*Th2 = T-helper type, Tc =T-cytotoxic cells; LTB4 = Leukotriene-B_4.

1.3 Summary and Aims of These Studies

"Airway remodelling" refers to the alterations in structural cells and tissues in airway diseases such as Asthma and COPD, which include subepthelial and mucosal collagen deposition, epithelial injury and denudation, and changes in airway vascularity, mucous glands, and smooth muscle (191-198). These changes are most likely the consequence of ongoing chronic airway inflammation, but have as yet poorly determined effects on patient symptom control, lung function, airway reactivity, and outcome from acute exacerbations of the diseases (191, 192, 199, 200). In the last decade, studies of airway remodelling in chronic asthmatics and to a lesser extent in COPD by bronchoscopic techniques (i.e. endobronchial biopsy and BAL) have provided much helpful and insightful data (Table 7) (191-193), and led to a better understanding in pathogenesis of those airway diseases.

Although it has been more than thirty years since the first human lung transplantation, the pathogenesis of BOS remains poorly understood. Airway wall inflammation leading to fibrosis is the likely hallmark of BOS. However, most of previous studies have been done primarily in BAL or on transbronchial lung biopsy specimens, which provide only indirect information on airway inflammation. As a results, little is known about airway wall inflammation and structural changes that occur in the airways of human transplanted lung,

Therefore, the aims of these studies were to employ endobronchial biopsy specimens, to investigate the cellular inflammation, vascular changes, and scar collagen deposition directly in the airway walls of human lung allografts, to characterise the features of airway inflammation and remodelling in lung transplant recipients who develop BOS, to determine

the relationship between the cellular and structural change indices and clinical parameters in BOS patients, and, finally, to evaluate the potential role of TGF-β_1 and TNF-α in the pathogenic process of BOS post lung transplant.

CHAPTER II. MATERIALS AND METHODS

2.1 Reagents, Buffers, Solutions

2.1.1 Reagents and Chemicals

Reagents and Chemicals	Supplier	Cat. No
2-Mercaptoethanol	Sigma Biology, USA	M 3148
AmpliTaq Gold®, 250U, with GeneAmp 10× PCR Buffer II and $MgCl_2$ solution	Roche Molecular System	N 808-0241
3-Aminopropyltriethoxysilane	Sigma Chemical Co. USA	A3648
Acetone	APS, NewZealand	TECH 00000008
Biomount-X	Bio-Chroma, Australia	240
Biotinylated Horse Anti-mouse Immunoglobulins	Vector Laboratories	BA-2000
Biotinylated Rabbit Anti-Goat Immunoglobulins	DAKO, Denmark	E 466
DAB Chromogen	DAKO, Denmark	S3000
Deoxyribonucleoside Triphosphate	Perkin Elmer, USA	N808-0007
Diethyl Pyrocarbonate (DEPC)	Sigma, USA	D 5758
Di-sodium Hydrogen Orthophosphate	FSE, Australia	S/3760/53

Eosin	Sigma Chemical Co, USA	E4382
EDTA	BDH, Australia	10093.5V
Ethanol	APS, NewZealand	--
Formalin Solution, Neutral Buffered	Sigma Chemical Co. USA	HT5014
GeneAmp®RNA PCR Kit Components	Perkin Elmer, USA	
MuLV Reverse Transcriptase	Perkin Elmer, USA	N808-0018
RNase Inhibitor	Perkin Elmer, USA	N808-0119
Oligo $d(T)_{16}$	Perkin Elmer, USA	N808-0128
Glacial Acetic Acid	APS, NewZealand	2789
Goat Anti-type V Collagen	Southern Biotechnology, USA	1350-1
Goat Anti-type I Collagen	Southern Biotechnology, USA	1310-10
Hydrochloride Acid (HCl)	BHD, Australia	101252F
Heamatoxylin	Sigma Chemical Co. USA	H9627
Histopure BP	Australian Biostain P/L, Australia	AHP.T
Human IL-8 ELISA Assay Kit	Amersham, UK	RPN 2764
Human TGF-β_1 ELISA Assay Kit	Amersham, UK	RPN 2763
Human TGF-β_1 PCR Mimic	Clontech Laboratories, Inc., USA	5890-1

Hydrogen Peroxide, 30% H_2O_2	MERCK, Germany	107209
L-glutamin (100×), lyophilized	GIBCO, USA	12403
Lipopolysaccharide from Escherichia (LPS)	Sigma Chemical Co.	L9023
Magnesium Sulphate	APS, NewZealand	Univar 302
Metal enhanced diaminobenzidine	Pierce, Illinois, USA	34065
Methanol	Merck Pty Limited, Germany	10158
Monoclonal Mouse Anti-human Collagen Type III	Biogenex Laboratories, USA	MU-167-UC
Monoclonal Mouse Anti-human Collagen Type IV	DAKO, Denmark	M 0785
Monoclonal Mouse Anti-human Leukocyte Common Antigen, CD45	DAKO, Denmark	M 0701
Monoclonal Mouse Anti-human Macrophage CD68 Antigen, CD68	DAKO, Denmark	M 814
Monoclonal Mouse Anti-human Neutrophil Elastase	DAKO, Denmark	M 0752
Monoclonal Mouse Anti-human TGF-β_1	Serotec Ltd, England	MCA797
Monoclonal Mouse Anti-human TNF-α	Sanbio, Netherlands	MON 5006
Molecular Biology Grade Agarose	Progen Industries, Australia	200-0010
Mouse IgG1 (negative control)	DAKO, Denmark	X 0931
Mouse IgG2a (negative control)	DAKO, Denmark	X 943

Normal Horse Serum	Vector Laboratories, USA	S-2000
Normal Rabbit Serum	DAKO, Demark	X 902
Penicillin-Streptomycin Solution	Sigma Aldrich Pty., Ltd., USA	P4333
Peroxidase-Conjugated Streptavidin	DAKO, Denmark	P 0397
Phosphate Buffered Saline Tablet	Sigma Bio Sciences, USA	P-4417
Protease XIV, 0.025%	Sigma, USA	P 5147
QuantiGlo™ Human TNF-α Immunoassay kit	R&D, USA	QTA00
Rapid Dip Fixative	Australian Biotain, Australia	ARDF
Rapid Diff. Kit Solution 1	Australian Biotain, Australia	ARD1
Rapid Diff. Kit Solution 2	Australian Biotain, Australia	ARD2
Rneasy Mini Kit (250)	Qiagen, USA	74106
RPMI 1640 Medium	Sigma, USA	R-5507
Sodium Bicarbonate	Sigma Chemical Co., USA	S8875
Sodium Chloride	Merck, Germany	10241
Sodium Dihydrogen Orthophsphate	Merck, Germany	019320
Streptavidin	DAKO, Denmark	P 0397

TGF-β_1 Primers U: GCCCTGGACACCAACTATTG L: AGGCTCCAAATGTAGGG	Clontech, USA	--
Trizma Base	Sigma Chemical, Co. USA	T 1503
Trypan Blue Solution	Sigma Chemical Co. USA	T8154
VECTASTAIN ABC Kit	Vector Laboratories, USA	PK-6100
VECTASTAIN ABC Kit (Mouse IgG)	Vector Laboratories, USA	PK-6102

2.1.2 Solutions and Buffers

2.1.2.1 Aminopropyltrietheoxysilane Solution, 20 %

Stock aminopropyltrietheoxysilane	10 ml
Acetone	500ml

This solution must be made freshly prior to use to treat glass slides before histochemical and immunohistochemical stain.

2.1.2.2 Acid Alcohol, 0.1%

Stock solution	10 ml of concentrated HCL
	990 ml of 70% ethanol
Working solution	10 ml of stock solution
	990 ml of distilled water

2.1.2.3 Bouins Solution

Glacial acetic acid	5 ml
Formalin, 40%	25 ml
Picric acid	75 ml

2.1.2.4 DEPC Water, 0.1%

DEPC	1 ml
MilliQ water	1000 ml

The DEPC water was stirred at least 30 min in the fume hood, and autoclaved.

2.1.2.5 Heamatoxylin/Eosin

Harris' Haematoxylin:

Haematoxylin	6 g
Absolute ethanol	60 ml
Alumininum ammonium sulphate	120 g
MilliQ water	1200 ml
Mercuric oxide	3 g

1. Dissolve the Alumininum Ammonium Sulphate in hot MilliQ water
2. Dissolve the Haematoxylin in the ethanol
3. Add 2. To 1. And bring rapidly to the boil
4. Add the Mercuric Oxide (the solution will turn dark purple and frothing may occur)
5. Cool quickly in "bath" of running tap water.
6. Filter before use and add the Acetic Acid
7. Determine the optimum staining time for each new batch of stain. As a general rule, tissues fixed in PLP require 5 minute while those fixed in acetone need 10 minutes. This is with one dip in acid alcohol.

Eosin:

Stock solution, 1%	1 g of Eosin (Chroma, Alcohol and water soluble)
	MilliQ water 100 ml
Working solution, 01%	100 ml of stock solution
	900 ml of MilliQ water
	2 g of Calcium Chloride (3-8 mesh)

2.1.2.6 Phosphate Buffered Saline (PBS), 10×

MilliQ water	1000 ml
NaCl	85 g
Sodium dihydrogen orthophsphate	3.9 g
Di-sodium hydrogen orthophosphate	10.7 g

This solution should be diluted 10 times with MilliQ water and the pH should be adjusted to 7.2 prior to use.

2.1.2.7 Scott's tap water Substitute

Sodium Bicarbonate	3.5 g
Magnesium sulphate	20 g
MilliQ water	1000 ml

Note: MilliQ water refers to deionnised water, and purified using a MilliQ water purification system.

2.1.2.8 TAE Buffer, 10×

Tris OH	96.4 g
Glacial acetic acid	22.85 ml
500 mM EDTA pH 8	40.0 ml
MilliQ water	up to 2000 ml

TAE buffer diluted to 1× in MilliQ water before use.

2.1.2.9 Tris-Buffered Saline (TBS), 10×

MilliQ water	1000 ml
Tris	120 g
NaCl	85 g

This solution must be stirred for overnight, diluted 10 times with MilliQ water, and the pH should be adjusted to 7.6 prior to use.

2.1.2.10 Weigerts Iron Heamatoxylin and van Gieson

Weigerts Iron Heamatoxylin:

Solution A	1% Heamatoxylin in absolute ethanol
Solution B	4 ml of 30% ferric chloride
	95 ml of Distilled water
	1 ml of concentrated HCL

Mix equal parts of A and B, stand for 30-60 min before use.

van Gieson:

Saturated aqueous picric acid	45 ml
1% Acid fuchsin	5 ml

2.2 Bronchoscopy and Bronchoalveolar Lavage

Fibreoptic bronchoscopy was performed in the bronchoscopy suite within the Department of Respiratory Medicine, Alfred Hospital. All subjects were sedated with intravenous midazalam (2-10 mg) (Roche, Paris, France) as clinically appropriate. Topical 2% lignocaine in 2 ml aliquots (maximum 14 ml) was used to anaesthetise the bronchial tree. After wedging the bronchoscope in a sub segment of the middle lobe or lingula, three 60-ml aliquots of phosphate-buffered saline warmed to 37^0 C were instilled via syringe. The fluid was immediately aspirated after each aliquot into a container at a negative pressure of approximately –80 mmHg. Ten ml of pooled bronchoalveolar lavage (BAL) fluid aspirate was retained for microbiological testing by the Alfred Hospital, Department of Pathology, including direct microscopy for the detection of CMV inclusion bodies, Pneumocytis carinii, fungi and mycobacteria. Culture for bacteria including legionella, mycobacteria, fungi and viruses were performed, and the presence of CMV and respiratory syncytial virus (RSV) genome were investigated by polymerase chain reaction amplification. The rest was immediately transported to the laboratory at 4 °C for processing and analysis.

2.3 Endobronchial and Transbronchial Biopsy

Following BAL six endobronchial biopsies (EBB), on average, were taken from lower lobe sub-carinae using alligator forceps (Olympus FB 15C, Tokyo, Japan). Between five and seven transbronchial biopsies (TBB) were also taken from the patients, but not from the controls, as part of the routine clinical follow up protocol for histological diagnosis of lung rejection in lung transplant recipients, which was done by the department of the Alfred Hospital, Department of Pathology. Table 1 summarizes the international grading scheme used for the grading of pulmonary allograft rejection (253). EBB specimens were snap frozen in liquid nitrogen-chilled isopentane slurry, embedded with ornithine carbamyl transferase (OCT), and stored at –80 °C until immunohistochemistry process.

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

A. Acute rejection
[with/without **(B)**]

Grade A0 – None
Grade A1 – Minimal
Grade A2 – Mild
Grade A3 – Moderate
Grade A4 – Severe

B. Airway inflammation – lymphocytic bronchitis/bronchiolitis

B0 – No airway inflammation
B1 – Minimal airway inflammation
B3 – Moderate airway inflammation
B4 – Severe airway inflammation
BX– Ungradeble because of sampling Problem, infection, tangential cutting, etc.

C. Chronic airway rejection – bronchiolitis obliterans
a. Active
b. Inactive

D. Chronic vascular rejection – accelerated graft vascular sclerosis

*Yousem SA. Et al. 1996. Revision of the 1990 working formulation for the classification of pulmonary allograft rejection: Lung Rejection Study Group. J Heart & Lung Transplantation. 15:1-15. (Ref. 28)

2.4 Tissue Processing

Some frozen EBB were post-processed into 100% ethanol fixative, embedded in paraffin as follows, and were used for collagen staining (Chapter III).

1. 100% Ethanol 30 min
2. 100% Ethanol 20 min
3. 100% Ethanol 20 min
4. 100% Ethanol 20 min
5. Xylene 40 min
6. Xylene 40 min
7. Xylene 40 min
8. Paraffin 40 min
9. Paraffin 40 min with vacuum
10. Embedded

Some EBB were post-processed into formalin fixtive, embedded in paraffin as follows, and were used for cell marker, TGF-β_1 and TNF-α stains (Chapter III).

1. 10% formalin fixation for 2 hours
2. 50% ethanol 20 min
3. 70% ethanol 20 min
4. 90% ethanol 20 min
5. 100% ethanol 20 min
6. 100% ethanol 20 min

7. 100% ethanol 20 min
8. Xylene 40 min
9. Xylene 40 min
10. Xylene 40 min
11. Paraffin 40 min
12. Paraffin 40 min with vacuum
13. Embedded in paraffin

2.5 BAL Process, BAL Cell Total and Differential Counting

Total BAL cell counts were determined on the unfiltered BAL fluid using a modified Neubauer haemocytometer. Cytocentrifuge preparations were made in duplicate using 200 µl of unfiltered BAL aspirate (Shandon Cytospin III, 850rpm, 10 minutes) and stained with "Diff-Quik". BAL cell differential counting was performed to count 500 cells on each of the slides with the results then averaged. BAL supernatants were obtained by filtering the pooled BAL fluid through a 200 µm nylon mesh, centrifuging at 1500 rpm for 10 min, and then aliquoting in 1.5 ml volumes, which were stored at –80°C for subsequent IL-8, TGF-β_1 and TNF-α assay. Some BAL cell pellets (not less than 2 million) were snap frozen and stored at –80°C for TGF-β_1 RT-PCR. Some cells (about 5 millions) were resuspended, and washed for alveolar macrophage culture experiments, which will be described later.

2.6 "Diff-Quik" Stain

1. Cytocentrifuge preparations of BAL cells were air dried
2. Fix in "Quick Dip Fixative" 4-8 dips
3. 4-6 dips in "Quick Dip I"
4. 10-12 dips in "Quick Dip II"
5. 15 dips wash in running tap water
6. Air dry and coverslip

2.7 Glass Slide Treatment

Glass slides were treated prior to immunohistochemical staining with 2% of aminopropyltriethoxysilane (AAS) solution to improve adhesion.

1. Load slides into racks
2. Dip the slides in the rack into 2% aminopropyltriethoxysilane solu n for 30 seconds
3. Rinse briefly in acetone
4. Rinse briefly in acetone
5. Rinse briefly in distilled water
6. Allow or dry at room temperature overnight. If slides are needed urgently they can be dried at 50°C.

2.8 Immunohistochemistry

The avidin-biotin-enzyme complex (ABC) method was applied in the study. This method utilizes the high affinity of avidin or streptavidin for biotin, and requires a biotinylated antibody as a link antibody. Horseradish peroxidase is commonly used as enzyme label. Avidin has four binding sites for biotin, but due to the molecular orientation of biotin-binding sites, fewer than four molecules of biotin will actually bind. Biotinylation is a mild process, whereby biotin is covalently attached to the antibody. The strong affinity of avidin for biotin and the mild biotinylation process make this method more sensitive than the direct and indirect methods.

Much of the early work with the ABC method focused on the identification of pituitary hormones and neoplastic tissue. Today, the ABC method has been used to the localization of numerous antigens in a variety of specimens (253-256). The sequence of reagent application is: primary antibody, biotinylated secondary antibody, preformed avidin-biotin enzyme complex (Fig. 1).

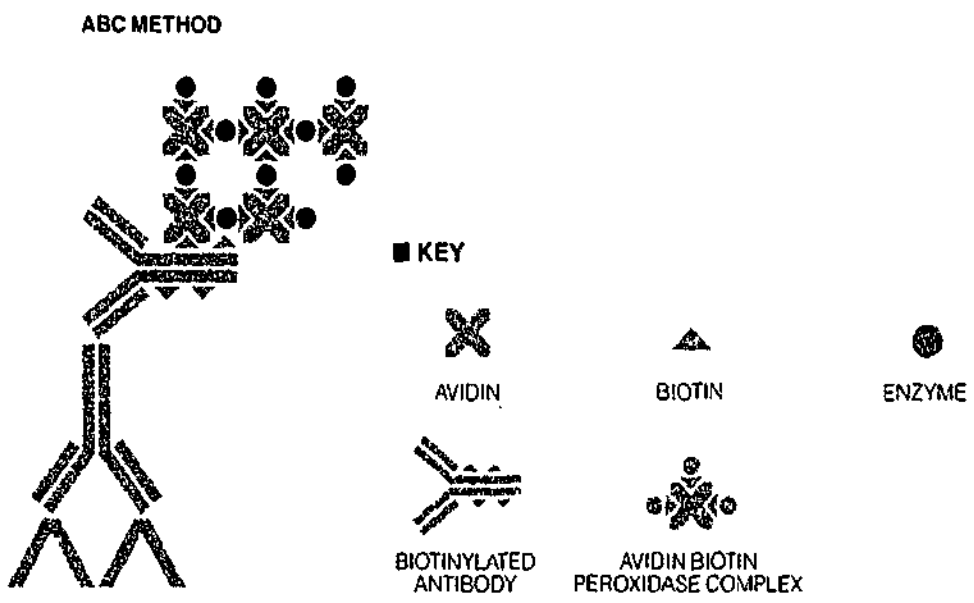


Fig. 1. Diagram of Avidin-biotin enzyme complex (ABC)

The general procedure of the ABC method is as follows:

1. Cut sections in 3 μm thick and onto 3-Aminopropyltrietheoxysilane (AAS) coated slides.
2. Deparaffinise the sections in xylene twice, 5 min in each,
3. Re-hydrate the sections through graded ethanol from 100%, 100% to 95% and 75%, 10 dips in each.
4. Gently rinse the slides with running tap water for 5 min.
5. Wash the slides in buffer (PBS or TBS) bath for 5 min.
6. Remove excess liquid from around the sections.
7. Apply 4-6 drops of normal (horse or rabbit) serum, diluted in 1:5-1:20. Incubate 45 min, to prevent nonspecific binding of immunoglobulins to tissue.
8. Tap off serum and wipe away excess. Do not rinse.
9. Apply 4-6 drops of primary antibody (mouse or rabbit anti human), diluted appropriately. Incubate for overnight at 4 °C in a moist chamber.
10. Repeat steps 5 and 6.
11. Apply 4-6 drops of biotinylated (horse anti mouse or rabbit anti goat) antibody, diluted appropriately. Incubate 45 min.
12. Repeat steps 5 and 6.
13. Block the "endogenous peroxidase activity" of the tissues:
    (1) Dehydrate the sections through graded ethanol from 75%, 95%, to 100% and 100%, 10 dips in each.
    (2) Incubate the sections in 0.12% methanolic hydrogen peroxidase for 10 min.
    (3) Rehydrate the section as step 3.
    (4) Repeat steps 5 and 6.

14. Apply 4-6 drops of avidin-biotin complex (mixed and diluted appropriately at least 30 min before use). Incubate 45 min.
15. Repeat steps 5 and 6.
16. Apply substrate-chromogen (Diaminobenzidine, DAB) solution and incubate until desired colour intensity has developed (in about 5-10 min).
17. Rinse the slides gently with running tap water for 5 min, counter-stain and coverslip.

2.9 Haematoxylin and Eosin (H and E) Stain

H-E stain was done for every biopsy specimen to assess the quality of the sections prior to immunohistochemical staining in this study. The procedures are as follows:

1. Cut sections in 3 μm thick and onto slides
2. Deparaffinise the sections in xylene twice, 5 min in each
3. Re-hydrate the sections through graded ethanol from 100%, 100% to 95% and 75%, 10 dips in each
4. Gently rinse the slides with running tap water for 5 min
5. Stain in haemotoxylin for 5 min
6. Rinse the slides with running tap water until water is clear
7. Differentiate with acid alcohol (1× dip)
8. Dip the slides 10 times in running tap water
9. Stain in eosin for 3 min
10. Dip the slides once in tap water

11. De-hydrate the sections through graded ethanol from 75%, to 95% and 100% (2x), 10 dips in each
12. Clear in xylene, coverslip

2.10 Masson's Trichrome Stain

Masson's trichrome stain is histochemical staining method for connective tissue used in this study.

Step 1-4 as above

5. Post fix the sections in prewarmed Bouins at 60 °C for 30 min
6. Rise in tap water
7. Stain with Weigerts haemotoxylin for 10 min
8. Wash in tap water
9. Differentiate with acid alcohol as required
10. Wash in tap water
11. Stain in 1% brilliant Crocein in water for 15 min
12. Rinse off stain with 1% phosphotungstic acid
13. Stain in 2% Light Green in 1% acetic acid for 10 min
14. Wash off with 1% acetic acid for 1 min
15. De-hydrate the sections through graded ethanol from 75%, 95% to 100% and 100%, 10 dips in each
16. Clear in xylene, coverslip

RESULTS:	Nuclei	black
	Collagen	green
	Cell cytoplasm (including muscle)	red

2.11 van Gieson Stain

van Gieson stain is an another histochemical staining method for connective tissue used in this study.

Step 1-4 as above.

5. Stain with Weigerts haemotoxylin for 10 min
6. Rinse in water
7. Differentiate with acid alcohol if required

   Note: some differentiation will occur with Van Gieson stain
8. Stain with Van Gieson for 10 min
9. Drain off excess stain
10. De-hydrate the sections through graded ethanol from 75%, 95% to 100% and 100%, 10 dips in each
11. Clear in xylene, coverslip

RESULTS:	Collagen	red
	Nuclei	black
	Cytoplasm and other tissue	yellow

2.12 Quantitation of Immunohistochemical and Histochemical Stainings

All the staining sections were quantified by using a computerized image analyzer (Video Pro 32, Leading Edge, Sydney, Australia) at a final magnification of ×400. To minimize subjective variation in interpretation of the staining, the staining for each of the markers from both transplant patients and controls was done in the same batch; the slides were coded before the assessment, and quantified "blindly" by one person (myself) without prior knowledge of clinical details of the subjects. Since the method of quantitation for individual staining markers were differed, the details are described in the individual studies of Chapter III.

2.13 Enzyme Linked Immunosorbent Assay

An Enzyme Linked Immunosorbent Assay (ELISA) was used for IL-8 and TGF-β_1 measurements in this study. ELISA employs the quantitative 'sandwich' enzyme immunoassay technique. A specific antibody for human IL-8 (Amersham, UK) or TGF-β_1 (Amersham, UK) had been coated on the microtitre plate provided with the kit. Samples were pipetted into the wells followed by incubation with biotinylated antibody reagent. If present, the IL-8 or TGF-β_1 was bound by the immobilized antibody and reacted with the biotinylated antibody. After washing away any unbound sample protein and biotinylated antibody, a streptavidin-horseradish peroxidase conjugate was added to the wells. Any IL-8 or TGF-β_1, which was bound by both the immobilized antibody and the biotinylated antibody during the first incubation, was bound by the streptavidin conjugate. Following a wash to remove

unbound conjugate, a substrate solution was added to the wells and color develop in proportion to the amount of IL-8 or TGF-β_1 bound in the initial step (Fig. 2).

In addition to the samples to be tested, a series of wells was prepared using known concentration of the standard (IL-8 or TGF-β_1). A curve, plotting the optical density versus the concentration of the standards, was prepared. By comparing the optical density of the samples to the standard curve, the concentration of the IL-8 or TGF-β_1 in the unknown samples were then determined.

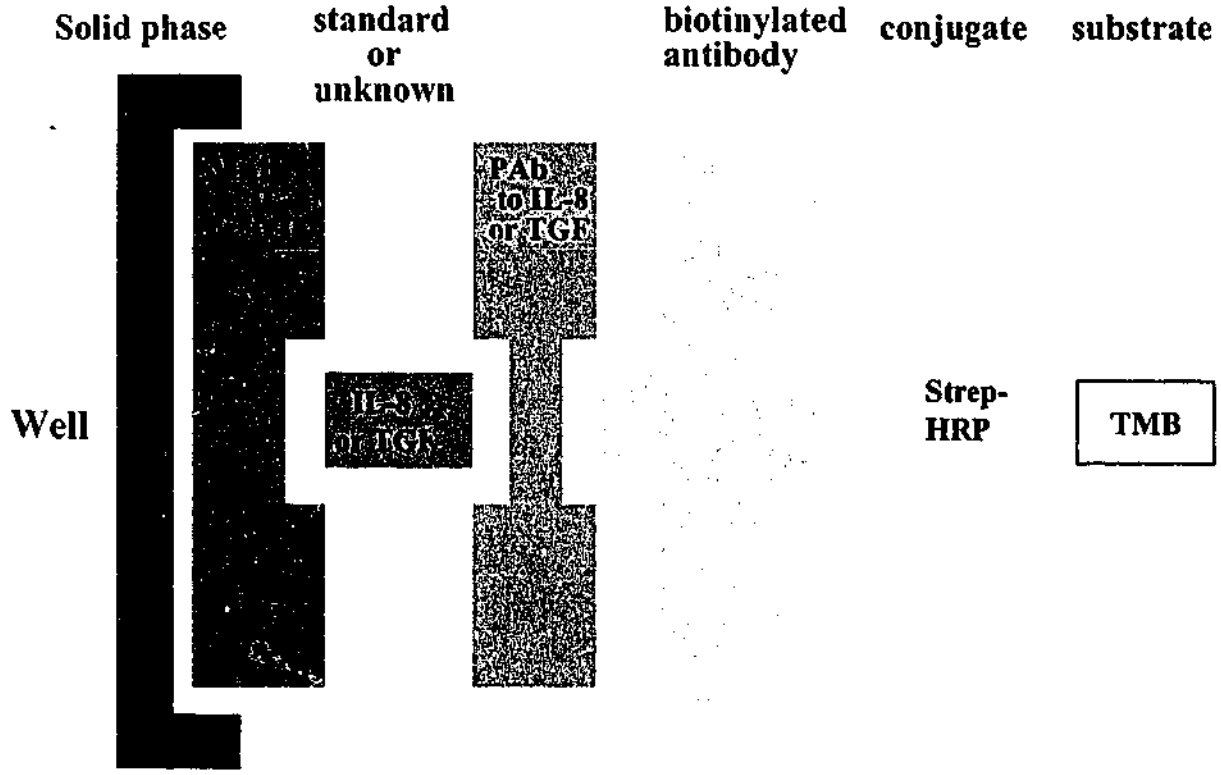


Fig. 2. Diagram of ELISA assay (**MAb:** Monoclonal Antibody; **PAb:** Polyclonal Antibody; **Strep-HRP:** Streptavidin Horseradish Peroxidase; **TMB:** 3, 3', 5, 5'-Tetramethylbenzidine.)

2.13.1 IL-8 ELISA Assay

The human IL-8 ELISA kits were purchased from Amersham UK. They had a detection range of 10 to 1000 pg/mL, and the sensitivity of <2pg/ml, which was defined as the concentration on the standard curve equivalent to 2.0 standard deviations above the zero standard. The procedure was as follows:

1. Prepare assay reagents and working standards as instructed by the manufacture
2. Add 50 µl of standard or sample per well, in duplicate. Cover with adhesive strip and incubate at room temperature (20-25 °C) for 60 min.
3. Aspirate or decant each well and wash, repeating the process twice for a total of three washes. Wash vigorously by filling each well with wash buffer using a squirt bottle. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining wash buffer by inverting the plate and blotting it against clean paper toweling.
4. Add 50 µl of the biotinylated antibody reagent to all wells. Cover with adhesive strip provided and incubate for 1 hour at room temperature (20-25 °C).
5. Repeat the aspiration/wash step as in step 3.
6. Add 100 µl of pre-diluted streptavidin-horseradish peroxidase conjugate. Cover with a new adhesive strip and incubate for 30 min at room temperature (20-25 °C).
7. Repeat the aspiration/wash step as in step 3.
8. Add 100 µl of TMB substrate solution into each well, incubate for 30 min at room temperature (20-25 °C). The plate should be developed in dark.
9. Add 100 µl of stop solution to each well.

10. Determine the optical density of each well within 30 min, using a spectrophotometer set to 450nm (Model 450, Bio-Rad, USA).
11. Average the duplicate readings for each standard and sample and subtract the zero standard optical density.
12. Plot the optical density for the standards versus the concentration of the standards, and draw the best curve (Fig. 3). Determine the concentration of IL-8 in the samples by interpolation from a standard curve, and express as pg/ml.

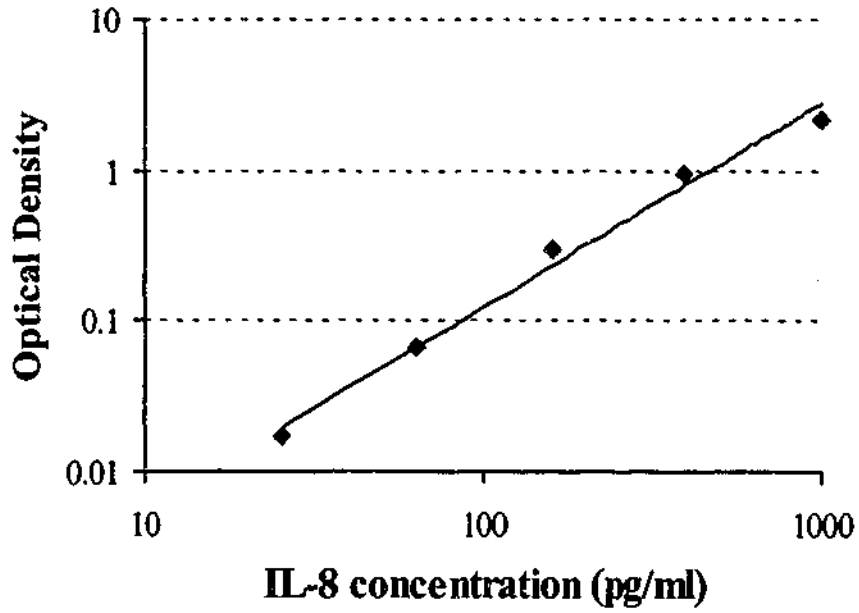


Fig. 3. Representative standard curve of IL-8 assays (data from Chapter III, 3.1).

2.13.2 TGF-β_1 ELISA Assay

The human TGF-β_1 ELISA kits were purchased from Amersham (Buckinghamshire, England, UK). They had a detection range of 15.6-1000pg/ml, and sensitivity of 4 pg/ml, which was defined as the concentration on the standard curve equivalent to 2.0 standard deviations above the zero standard.

TGF-β_1 is secreted from cells as an inactive latent complex form containing TGF-β and its propeptide, latency-associated protein (LAP), and in most cells, LAP is covalently linked to an additional protein, latent TGF-β binding protein (LTBP), forming the large latent complex (257). To bind to specific cell surface receptors, TGF-β_1 must be activated and released from this latent complex (257). *In vitro* activation can be accomplished by acid treatment.

Since the TGF-β_1 assays used in this study only detect the biologically active form of TGF-β_1, immediately prior to the assay, all samples need to be acidified. Briefly, 100µl of 1M hydrochloric acid was add to 500 µl of the specimens, mixed well and incubated for 10 minutes to achieve a pH of 1-2 during activation. After 10 minutes at room temperature, 100 µl of 1.2M NaOH/0.5M HEPES free acid was added to neutralize the acidified BAL supernatant, to achieve a pH of 7-8 after neutralization.

After acidification, 100 µl of TGF-β_1 standards (with know concentrations) and samples were incubated in microtitre wells precoated with mouse anti- TGF-β_1 antibody for 1 h at 37 °C followed by four time washes. Any TGF-β_1 present was bound to the wells, other components of the sample being removed by washing. TGF-β_1 was then detected using a polyclonal

antibody to TGF-β_1, and the biotinylated second antibody: streptavidin-peroxidase system (1 hour of incubation for each of them). HRP activity was determined by the addition of TMB substrate solution and incubated again for 30 min at room temperature. The reaction was stopped by addition of an acid solution (H_2SO_4, 0.19 M, 100 µl), and the resultant color were read at 450nm in a microtitre plate spectrophotometer (Model 450, Bio-Rad, USA). The concentration of TGF-β_1 in samples were determined by interpolation from a standard curve, corrected by dilution factor of 1.4 due to the acidification procedures. Sample TGF-β_1 levels were expressed as pg/ml.

2.14 TNF-α Chemiluminescent Immunoassay

This assay is a 6.5-hour solid phase chemiluminescent ELISA designed to measure TNF-α in cell culture supernatant, serum and plasma. It employs the quantitative 'sandwich' enzyme immunoassay technique as the ordinary ELISA described above. However, unlike the ordinary ELISA, following the last wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxidase substrate solution was added to the wells and light was produced in proportion to the amount of TNF-α bound in the initial step. A microplate luminometer was used to measure the intensity of the light emitted. The assay procedure was as follows:

1. Prepare all reagents and working standards as instructed by the manufacture
2. Add 50 µl of Assay Diluent QD-27 provided to each well.

3. Add 200 µl of standard or sample per well, in duplicate. Cover with adhesive strip provided. Incubate for 4 hours at room temperature on a horizontal orbital microplate shaker set at 500±50 rpm.
4. Aspirate or decant each well and wash, repeating the process once for a total of four washes. Wash vigorously by filling each well with wash buffer using a squirt bottle. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining wash buffer by inverting the plate and blotting it against clean paper toweling.
5. Add 200 µl of TNF-α conjugate to each well. Cover with new adhesive strip provided and incubate for 1 hour at room temperature on the shaker.
6. Repeat the aspiration/wash step as in step 4.
7. Add 200 µl of pre-prepared substrate solution to each well. Incubate for 20-40 min at room temperature on the benchtop (do not shake).
8. Determine the relative light unit (RLU) of each well using a luminometer (LumiCount™ Microplate Luminometer Packard Instrument Co. CT. USA) set with the following parameters: 1.0 min lag time; 1 second/well read time, summation mode; auto gain on.
9. Average the duplicate readings for each standard and sample and subtract the zero standard RLU.
10. Create a standard curve by plotting the RLU for the standards versus concentration of the standards, and determine the concentration of TNF-α in the samples by interpolation from a standard curve, and express as pg/ml.

2.15 Alveolar Macrophage Culture

Alveolar macrophages were obtained from BAL fluid aspirate. The BAL cell pellet was gently washed three times with phosphate buffered saline (PBS), and resuspended at a concentration of $1x10^6$ alveolar macrophages (determined by total cell count and cell differential count) in RPMI 1640 medium supplemented with L-glutamine (2mM), penicillin (200μ/ml) and streptomycine (200μg/ml). From this, 0.5×10^6 alveolar macrophages per well were seeded in 24-well tissue culture plates, and incubated at 37.0 °C in a 5% CO_2 humidified atmosphere for 1 hour, during which alveolar macrophages were separated from the rest of the BAL cells by adherence to the plastic culture plate. The nonadherent cells were removed by washing three times with the culture medium, and fresh supplemented RPMI 1640 medium was added. The cells were incubated for another 24 hours with and without the stimulus of Lipopolysaccharide (LPS) (1μg/ml) at 37.0 °C in a 5% CO_2 humidified atmosphere. The culture supernatants were harvested at the end of incubation, and were rendered cell-free by centrifugation, stored at –80 °C until assayed for TGF-β_1. The cultured alveolar macrophages were lysed by using Lysis Buffer RLT (Qiagen, Qiagen Pty Ltd Australia), and the stored at -80 °C until RNA extraction for PCR.

2.16 May-Grunwald-Giemsa Stain

May-Gruwald- Giemsa stain was used to assess the purity of alveolar macrophages that were separated from the rest of the BAL cells by adherence to the plastic culture plate in the study. After 1-hour adherence, one well of the alveolar macrophages from each specimen was gently harvested by rubber policeman, and resuspended in PBS. Cytocentrifuge preparations of the

purified alveolar macrophages were made as described above (Chaper II, 2.5), and stained with May-Grunwald-Giemsa as follows:

1. Air dried cytospin slides in neat May-Grunwald-solution for 3-5 min
2. Wash with MilliQ water (3 dips)
3. In Giemsa solution (1: 20 in MilliQ water, made freshly prior to use) for 15 min
4. Wash with tap water until clear
5. Air dry
6. Mount with Histopure BP and coverslip

2.17 Competitive Reverse Transcription PCR

2.17.1 Introduction

Competitive reverse transcription polymerase chain reaction (RT-PCR) is an assay based on competitive co-amplification of a specific target sequence together with known concentrations of a competitor template (or internal standard) in one reaction tube (258). To be effective, the competitor template has to share primer recognition sites with the specific template (i.e. specific native cDNA), both must be PCR-amplified with the same efficiency; and it must be possible to analyze the PCR-amplified products of specific template and competitor template separately, mainly on the basis of a small size difference.

To distinguish the two PCR products amplified in competitive PCR, competitor templates are designed to generate a PCR product of different size to the specific native PCR products, and

these products are separated by agarose or polyacrylamide gel electrophoresis, and then stained with a fluorescent dye. Since the ratio of competitor template to specific native template remains constant throughout the assay, quantitation can be then performed by comparing the PCR signals of the specific native template with PCR signals obtained with known concentrations of the competitor template (the internal standards), and the ratio is constant, the starting amount native RNA can be back calculated (Fig. 4).

2.17.2 RNA Extraction and Measurement

BAL cell and alveolar macrophage preparation has been described previously (Chapter II. [2.5 & 2.15} RNA extraction was performed with RNeasy Mini Kit (Qiagen, Australia) as follows:

1. Add Lysis Buffer RLT (700 µl/million cells) provided with the kit to the cells (2-Mercaptoethanol (β-ME) was added to Lysis Buffer RLT before use, 10 µl per ml Lysis Buffer RLT).
2. Homogenize the cells using syringe (2ml) and needle (20G): Pass the lysate through the needle attached to the sterile plastic syringe 10 times to achieve a homogenous lysate and to shear chromosonal DNA.
3. Add 1 volume of 70% ethanol to the homogenized lysate and mix well by pipetting. Do not centrifuge.
4. Apply up to 700 µl of sample, including any precipitate which may have formed, to an RNeasy mini spin column sitting in 2-ml collection tube (supplied) and centrifuge for 15

sec at 9500 × g. If the volume of the mixture exceeds 700 µl, successively load aliquots onto the RNeasy column and centrifuge as above.

5. Pipet 700 µl Buffer RW1 provided onto the RNeasy column and centrifuge for 15 sec at 9500 × g to wash.
6. Transfer RNeasy column into a new 2-ml collection tube (supplied). Pipet 500 µl Buffer RPE provided onto the RNeasy column and centrifuge for 15 sec at 9500 × g to wash. (Ethanol is added to Buffer PRE before use).
7. Pipet 500 µl Buffer RPE onto the RNeasy column. Centrifuge for 2 min at 17530 × g to dry the RNeasy membrane.
8. Transfer RNeasy column into a new 1.5-ml collection tube (supplied) and pipet 40 µl of Rneasy-free water directly onto the RNeasy membrane. Centrifuge for 1 min at 9500 × g to elute.

The extracted RNA was quantified by using capillary cuvette microsample spectrophotometry (Capillary Adaptor Cells, Helix, San Diego, CA, USA), and a Cary 1 spectrophotometer (Varian, Melbourne, Australia) with background correction at 320 nm. RNA purity was assessed by the absorbance (A) ratio $[(A_{260nm}-A_{320nm})/(A_{280nm}-A_{320nm})]$ measurements. Ratios of 1.9 to 2.0 indicate purified preparation of RNA. Samples with a ratio of less than 1.7 and/or yield less than 0.5 of total RNA were excluded from subsequent analysis.

2.17.3 Reverse Transcription and Competitive PCR

Complementary DNA (cDNA) was synthesized from up to 1 µg of RNA in a volume of 20 µl consisting of 1 × GeneAmp PCR buffer, 5 mMol/L $MgCL_2$, 1 mMol/L dexyribonucloside

triphosphate, 0.8 U of RNase inhibitor, 2 μMol/L Oligo $d(T)_{16}$, and 2 U of MuLV reverse transcriptase (all from Perkin-Elmer, USA) (Table 2.). The reaction was incubated for 1 min at 15 °C, and then the temperature was increased to 42 °C within 5 min followed by 1 hour at 42 °C, 5 min at 85 °C, and 1 min at 4 °C.

Table 2. Reverse Transcription Master Mix

	Volume (μl)	final concentration
GeneAmp PCR buffer (×10)	2	x1
25 mM $MgCl_2$	4	5 mM
10 mM dNTP mix	2	1 mM each
oligo (dT)	0.8	2 μMol/L
RNAsin	0.8	0.8 U/μl
MuLV reverse transcriptase	0.8	2 U/μl
RNA samples ± Nuclease-free water (or Nuclease-free water as RNA negative control)	9.4	</=1 μg
Final volume	20	

Following the reverse transcription reaction, competitive PCR was performed with 2 μl cDNA in a volume of 50 μl, consisting of 1× GeneAmp PCR buffer, 2 mMol/L $MgCl_2$, deoxyribonucleoside triphosphate, 2 U AmpliTaq Gold, primer (fluorescent labeled), and the competitor template (or internal standard). Thermal cycling was conducted in an MJ Research PTC-200 (Watertown, Mass, USA) with initial denaturation of 8 min at 94 °C, followed by 36 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, then concluding with 7 min at 72 °C and 1 min at 4 °C.

2.17.4 PCR Product Detection and Quantitation

The PCR products were resolved by electrophoresis in 3% agarose gels with Tris-acetate-EDTA buffer, pH 8.5 (TAE Buffer, 1 ×). The separated native and competitor products were then detected using a fluorescent scanner (FluorImager; Molecular Dynamics, Sunnyvale, CA, USA) (Fig. 4), and the PCR product band volumes were quantified with ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA). Native-competitor product ratios were calculated, and the levels of gene expression were reported as copies of mRNA per micrograms of total RNA.

Figure 4 summarizes the entire procedure and a representative TGF-β_1 competitive RT-PCR.

RNA
⇩
TGF-β_1 Competitor + cDNA
⇩
PCR
⇩
Electrophoresis
⇩
FluorImager scan
⇩
Quantitation

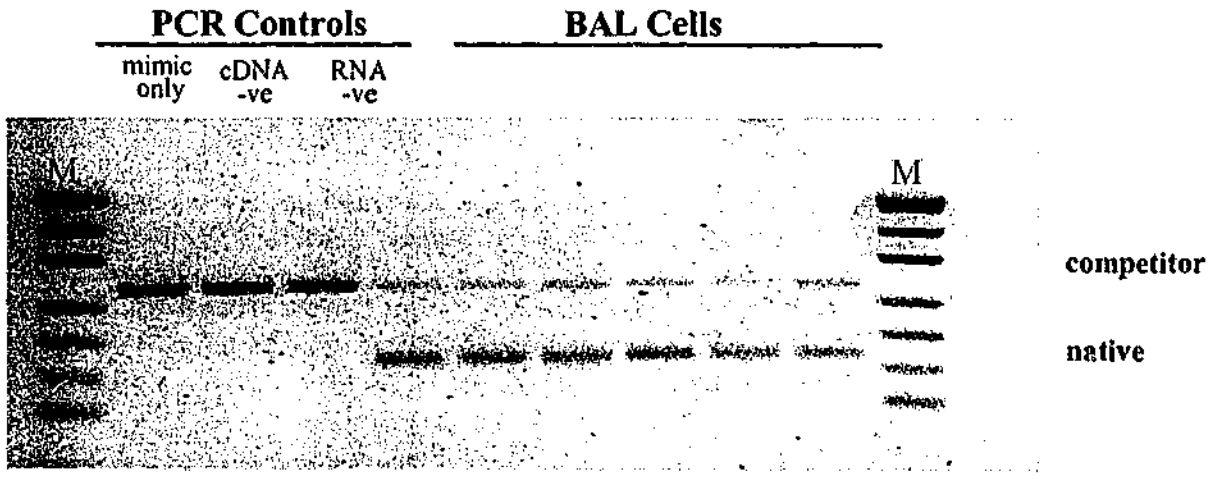


Fig. 4. Representative TGF-β_1 Competitive RT-PCR

2.18 Lung Function Tests

Lung function tests were performed in all the subjects immediately prior to bronchoscopy. A computerised rolling-seal spirometer (Sensor Medics 922, California, USA) was used to measure flow-volume loops. Each time, spirometer was pre-calibrated using a certified 3-liter syringe. $FEV_{1.0}$ and FVC were measured according to the American Thoracic Society standard (259), in which the best of three technically acceptable values were used and the values reported at body temperature and pressure saturated (BTPS). As part of routine post transplant follow up, regular spirometry was performed with the average of the best two $FEV_{1.0}$ measurements after surgery being used as the benchmark standard against which to assess later changes in lung function. In this study, the spirometric criteria for the diagnosis of BOS were based on the working formulation standardized by the International Heart Lung Transplantation Society in 1993 (Table 3.) (35), although an update has been proposed in 2001 (41), it had not yet published before the completion of the patient recruitment, and the data analysis of these studies. In addition, some relevant papers from these studies had been already published using the established 1993 working formulation.

Table 3. Staging System for Bronchiolitis Obliterans Syndrome*

Stage†	$\Delta FEV_{1.0}$, %‡	$FEV_{1.0}$, % of Baseline§
0	< -20%	>80%
1	-20% to -35%	65% to 80%
2	-35% to -50%	50% to 65%
3	>-50%	<50%

*Adapted from the working formulation for clinical staging (Ref. 35). The stages are based on fractional decrements in the FEV_1 from its baseline value, and each stage has a designator to indicate the absence or presence of BO.

† Subcategories a and b, without and with bronchiolitis obliterans, respectively.

‡ $\Delta FEV_{1.0}$ = [(current $FEV_{1.0}$ - baseline $FEV_{1.0}$)/baseline $FEV_{1.0}$)] x 100%.

§ Baseline $FEV_{1.0}$ is the average of the two best $FEV_{1.0}$s 3 to 6 wk apart.

2.19 Statistical Analysis

In the study, statistical analysis was performed by using the statistical package SPSS, version 10.0 (SPSS Inc. Chicago, Illinois, USA). Data were expressed as median and interquartile range unless otherwise specified. Student's unpaired t-test was used for comparisons of parametric variables between groups. The Mann-Whitney rank order test and Wilcoxon's signed rank test were used to examine the differences between and within groups respectively, when the data were not normally distributed. The Chi-square test was performed for comparisons if data were non-continuous. Correlation was evaluated by Spearman's rank method. P values of less than or equal to 0.05 were considered as statistically significant. In addition, "Best-fit" regression and the Bland-Altman method were employed in the individual studies, details are described in the individual sections of Chapter III.

CHAPTER III. STUDIES

3.1 Inflammatory Cellular Profiles in the Airways of Lung Allografts: Airway Neutrophilia in BOS

3.1.1 Introduction

The mechanisms by which chronic lung rejection lead to BOS remain poorly understood. Airway inflammation may have a pivotal role in the development of lung transplant BOS. There has been increasing evidence that persistent neutrophilic inflammation is associated with a number of chronic pulmonary fibrotic conditions, including Idiopathic Pulmonary Fibrosis (IPF), Asbestosis, and Adult Respiratory Distress Syndrome (ARDS) (144, 152, 260-262). Several previous studies have also demonstrated that BOS in lung transplant recipients (LTR) is associated with increased neutrophils and neutrophil activation in the lung allografts (130, 145-147, 263). The clinical relevance of these observations has recently been supported, but only in a small and retrospective series, by Henke and co-workers, who suggested that persistent BAL neutrophilia could predict mortality after lung transplantation (264).

However, most of previous studies on airway inflammation in lung allografts in humans have been primarily done using BAL samples, or on transbronchial biopsy (TBB) specimens (130, 145-147, 263), which provide only descriptive information on airway inflammation. There are little data directly from airway wall biopsies (129, 130). BAL findings may be particularly usefull for suggesting pulmonary infection after lung transplantation (263), but give no direct insight into the cellular infiltrate within the airway wall of the lung allograft. TBB, although regarded as the most specific specimens in detecting BO, has the disadvantage of great difficulty in consistently sampling bronchioles with up to 40% of samples not providing

adequate bronchiolar tissue (36, 37, 39, 40). Additionally, TBB does carry significant morbidity and mortality related to haemorrhage and pneumothorax (36, 37, 39, 40). Endobronchial biopsy (EBB), by directly sampling the airway mucosa, has been much used as a technique for studying the basic cellular, immunological, molecular abnormalities, cytokine profiles, and airway remodelling in asthma and COPD (265-267) in the last decade. The data obtained by biopsy of the bronchi have provided novel understandings in the pathogenesis of those airway diseases. By contrast, EBB has not been widely performed to study airway inflammation and structural changes in LTR (129, 130), which in where the work of this thesis is especially focused and novel.

We hypothesised that chronic neutrophilic inflammation is present in the airway wall compartment of lung allografts that have developed BOS. This study used EBB to investigate the cellular inflammation in the airway wall directly, and determine the relationship between the EBB cellular indices and clinical parameters in BOS patients.

3.1.2 Methods

3.1.2.1 Study Populations and Inclusion/Exclusion Criteria

Patients were recruited from the Lung Transplant Service of the Alfred Hospital. All were more than two months post lung transplantation. Inclusion criteria included: absence of any significant airway infection, (that is, afebrile, no new chest crackles or consolidation, clinically unchanged leucocyte counts and unchanged chest radiograph), and were without pathological evidence of acute allograft rejection on TBB for at least one month prior to the

study. The diagnosis of BOS was based on the criterion of at least a 20% decline in $FEV_{1.0}$ from their best post operative lung function in the absence of any other detectable complications that could cause a deterioration of lung function (35). In addition, there had to be absence of evidence of acute bronchitis or mucus on bronchoscopic assessment of the airways. Any microorganisms that were isolated on culture from the BAL fluid had to be considered microbiologically as "colonisers" and clinically as non disease producing and hence not requiring treatment. More specifically, gram stains for bacteria had to be negative in all cases and CMV cellular inclusions not detectable in any of the BAL or biopsy samples.

Normal volunteer subjects were healthy, non-smokers, free from known respiratory diseases, and asymptomatic with normal lung function.

Approval for this study was given by The Alfred Hospital Ethics Committee and each subject gave informed written consent.

3.1.2.2 Lung Function, BAL, EBB, , and Immunohistochemistry

All the subjects underwent lung function testing, BAL and EBB on the same day. The details of the procedures, including the subsequent BAL cell counting, cell differential counting, and EBB tissue process have been described previously in Chapter II. In our institution, surveillance bronchoscopy using BAL and TBB is carried out at 14 days, and then at 1, 2, 3, 6, 9, 12, 18 and 24 months post-transplant and annually after that. Additional BAL and TBB were performed whenever clinical parameters indicated deterioration in the patient's status. The normal controls underwent the procedure of BAL and EBB only on a single occasion.

Haemodynamic status and oxygen saturation levels were closely monitored throughout the bronchoncospic procedure.

Immunohistological staining for "panleukocytes", macrophages, and neutrophils were performed using monoclonal mouse anti-human antibodies against leukocyte common antigen (CD45), macrophage (CD68), and neutrophil elastase (all from Dako, Denmark) and amplified by using the avidin-biotin peroxidase complex (ABC) method described in Chapter II. Isotype control immunoglobulins (IgG1 Dako, Denmark) were used as negative controls. Serial consecutive 3 μm sections were cut from the post-processed formalin fixed tissue (three sections for one cell marker stain) and were deparaffinised in xylene, rehydrated through graded ethanol solutions, and washed with PBS (for CD45 and CD68 stains) or TBS (for neutrophil elastase stain). The quality of the sections obtained was assessed using standard haematoxylin and eosin staining. After pre-incubation with 20% normal horse serum for 20 min, the sections were stained with the primary antibodies to neutrophil elastase (diluted 1:600 in TBS), CD45 (diluted 1:500 in PBS) or CD68 (diluted 1:100 in PBS) at 4 °C in a moist chamber over night. The details of the procedure are described in Chapter II.

3.1.2.3 Quantitation of Immunohistochemical Staining

As stated in Chapter II, to minimise subjective variation in interpretation of the staining, the slides were coded before assessment. Positive staining for CD45 was most intense on the cell surface membrane, while for both CD68 and neutrophil elastase staining was exclusively intracytoplasmic. The total positive numbers of cells in the lamina propria of the airway wall in the EBB sections were counted by using a computerized image analyzer (Video Pro 32,

Leading Edge, Sydney, Australia) at a final magnification of ×400. Results were expressed as positive cells/mm^2 of lamina propria.

3.1.2.4 BAL IL-8 Assay

BAL IL-8 levels were measured in unconcentrated fluid with an ELISA by using a commercially available IL-8 ELISA kit (Amersham, U.K.) as described in Chapter II. The detection range was from 10 to 1000 pg/mL, with sensitivity of < 2pg/mL. The absorbency was measured with an ELISA reader (Model 450, Bio-Rad, USA) at 450 nm. The amount of IL-8 present in the samples was calculated by reference to the wells containing dilutions of the standard for IL-8.

3.1.2.5 Statistical Analysis

Results were expressed as medians and interquartile ranges unless otherwise specified. All comparisons between groups were made using the non-parametric Mann-Whitney rank order test. Correlation was evaluated by Spearman's rank method. A P value of less than or equal to 0.05 was considered as statistically significant.

3.1.3 Results

3.1.3.1 Patient demography and clinical characteristics

A total of twenty-seven LTR were recruited. Patient demography and clinical characteristics are shown in Table 1. 13 patients were clinically stable, 107-1930 days post-transplant, sustaining their best post operative lung function ($FEV_{1.0}$% best post-transplant: 99.3 ± 1.5%). 14 were diagnosed as having BOS and studied 77-2238 days post-transplant, with $FEV_{1.0}$% best post-transplant of 50.3 ± 15.7%. All patients were receiving a standard long-term maintenance regimen of immunosuppressive therapy comprising cyclosporine (to achieve a blood level of 200-350 μg/L by EMIT assay, Syva, California, USA), azathioprine (1-2 mg/kg/day) and prednisolone (0.15-0.25 mg/kg/day).

Twenty-one normal volunteers (5 females; mean ± SD age: 30±13 yr) were also recruited as controls. They were non-smokers, asymptomatic with normal lung function ($FEV_{1.0}$% predicted: 109.1 ± 11.8%).

3.1.3.2 Cellular Infiltration in Airway Walls

The distributions of panleukocytes, macrophages, and neutrophils in the normal airways and airways of lung allografts are showed by Figure 1. Panleukocytes and macrophages appeared to be evenly distributed throughout the entire bronchial lamina propria (Fig. 1 d-i), while neutrophils, particularly in the airways of lung allografts, were observed predominantly immediately beneath the epithelium (Fig. 1 a-c), and neutrophil infiltration of the epithelium

was often noted in LTR with BOS (Fig. 1j). Interestingly, in some specimens from LTR, neutrophil adhesion was also observed along the endothelium of airway vasculature (Fig. 1 k).

Compared with controls, there was a significantly increased number of neutrophils found in both the LTR without BOS and LTR with BOS (499 cells/mm^2, [330-606], and 419 cells/mm^2, [326-610] versus controls of 159 cells/mm^2, [108-360], p=0.03, and p=0.02 respectively) (Table 2, Fig. 2). However, there was no significant difference in panleukocytes numbers or the numbers of macrophages between LTR and normal controls (Table 2). Within LTR, the cell counts of panleukocytes, macrophages, and neutrophils did not significantly differ between the subjects without BOS and the subjects with BOS (Table 2).

3.1.3.3 Cellular Profiles in BAL

12 of the 14 BOS patients underwent adequate BAL (three 60-ml aliquots of PBS), but in 2 subjects, oxygen desatuation led to termination of the bronchoscopic procedure on clinical grounds before BAL. All 13 LTR without BOS and 21 normal controls underwent an adequate BAL procedure. However, BAL returns were significantly lower in BOS and stable LTR than in controls (59.5 ml, [43.5-75.8] and 85.0 ml, [59.0-101.0] vs controls of 120 ml, [108.0-124.0], p<0.0001 and p<0.001 respectively, Table 2).

The most striking feature of the BAL cellular findings was a significant elevation of neutrophils in the BOS group, both in the percentage (42.8%, [20.4-78]) and in the absolute counts (7.6 $\times 10^4$/ml, [2.2-25.7]) as compared with controls (1.4%, [1.2-2.9]; 0.2 $\times 10^4$/ml, [0.1-0.4], p<0.001) and the LTR without BOS (3.6%, [1.9-4.1]; 0.5 $\times 10^4$/ml, [0.3-0.8],

p<0.01), (Fig. 3, Table 2). The neutrophil counts in the LTR without BOS, though elevated only to a more modest extent, were also significantly different from the controls (p<0.05 for the percentage and absolute counts, Table 2). Reciprocally, there was a corresponding lower percentage of alveolar macrophages (p=0.002), but not absolute counts (p=0.09), in the patients with BOS compared to controls. The detail data are summarized in Table 2.

3.1.3.4 IL-8 Levels in BAL

The median BAL IL-8 level in normal controls was 25.6 (range 18.6-34.3) pg/mL. Compared with controls, IL-8 levels were markedly increased in BAL fluid from LTR with BOS (827 pg/mL, range 137-1056, p<0.0001), and to a lesser extent in BAL from LTR without BOS (32.9 pg/mL, range 26.5-65.7, p=0.03, Fig. 4). Between LTR groups the difference in BAL IL-8 levels was also highly significant (p=0.0002).

3.1.3.5 BAL bacterial culture and its association with BAL and airway neutrophil infiltrate

As previously stated, LTR were selected on the basis of absence of "clinical" infection. All BAL samples were negative on gram stain and did not have CMV inclusions in BAL cells. However, microbiological assessment by culture did isolate bacteria from BAL fluid on several occasions. Indeed, 4 of 13 LTR without BOS and 5 of 14 BOS LTR had positive bacterial cultures from BAL, but this inter-group difference was not statistically significant. The relationship between a positive BAL culture and neutrophil counts from both BAL and EBB are summarised in Table 3. From these data it is evident that in both the groups of LTR

with or without BOS, neutrophil percentages in BAL and neutrophil numbers in EBB were not significant different between positive and negative BAL culture.

3.1.3.6 Correlation Analysis

A significant correlation was found between BAL IL-8 levels and BAL neutrophil percentage (r=0.76, p=0.004, Fig. 5), as well as neutrophil absolute count (r=0.62, p=0.031) in BOS group, but not in LTR without BOS (r=0.11, p=0.72). Neither was there a significant correlation between BAL IL-8 and airway wall neutrophil density (r=-0.11, p=0.73 for LTR without BOS, and r=-0.03, p=0.94 for BOS group).

In both the LTR groups, the increased airway wall neutrophils correlated significantly with the total leukocyte numbers (CD45) (r=0.735, p=0.004 for BOS group; r=0.652, p=0.02 for LTR without BOS, respectively). Within the BOS group, both neutrophil density of airway walls and neutrophil percentage of BAL showed a negative correlation with $FEV_{1.0}$% best post-transplant (r=-0.533 p=0.03, and r=-0.713; p=0.009, respectively, Fig. 6a & b). There was a strong trend for a correlation between the airway wall and BAL neutrophilia (r=0.50), though not quite significant (p=0.059, Fig. 7) in BOS. There was no correlation between lung function parameters and airway wall macrophage and total leukocyte densities in the BOS group.

Table 1. Patient Demography and Clinical Characteristics

No	Sex	Age (yr)	Original disease*	LTx	Days Post LTx	TBB airway grading#	$FEV_{1.0}$% best–post LTx
LTR without BOS							
1	f	47	B	BSLTX	107	Bx	100
2	m	38	E	HLTX	1185	B0	100
3	m	22	CF	BSLTX	186	Bx	100
4	m	49	EM, VHD	HLTX	183	B0	100
5	m	40	B	BSLTX	189	B0	100
6	m	23	E	HLTX	1090	B0	99
7	m	38	CF	BSLTX	762	B0	100
8	f	35	E	HLTX	387	B0	96
9	f	45	PPH	HLTX	544	B2	100
10	f	35	PPH	BSLTX	287	Bx	96
11	f	37	E	HLTX	1108	B0	100
12	f	34	CF	BSLTX	788	B0	100
13	f	26	E	HLTX	1930	B0	100
		(36±8)					(99.3±11.8)
LTR with BOS							
1	m	24	CF	HLTX	1176	B0	51
2	m	23	CF	HLTX	1124	B0	64
3	m	26	CF	BSLTX	531	Bx	55
4	f	55	EM	SLTX	582	B1	78
5	f	31	PPH	BSLTX	396	B1	21
6	f	54	EM	SLTX	1186	B3	49
7	f	26	E	BSLTX	989	Bx	42
8	f	47	EM	SLTX	1518	B2	28
9	m	28	CF	BSLTX	929	Bx	60
10	m	61	EM	SLTX	2238	B1	57
11	m	50	IPF	SLTX	563	B0	53
12	m	31	CF	BSLTX	1500	B0	45
13	f	54	EM	SLTX	701	B1	57
14	f	55	EM	SLTX	77	B0	70
		(40±14)					(50.3±15.8)

*Original disease: B: bronchiectasis; CF: cystic fibrosis; E: Eisenmenger's syndrome; PPH: primary pulmonary hypertension; EM: emphysema; VHD: valvular heart disease.

LTx: lung transplantation; BSLTx: bilateral sequential lung transplant; HLTx: heart-lung transplant; SLTx: single lung transplant.

#Airway rejection defined as per ISHLT grading system (Ref. 28). Details are given in Chapter II, 2.3.

Table 2. Inflammatory Cellular profiles of Airway Walls and BAL Fluid

	LTR with BOS (n=14)	LTR without BOS (n=13)	Controls (n=21)
EBB			
Panleukocytes (cells/mm^2)	704 (425-1268)	651(462-756)	644 (489-919)
Macrophages (cells/mm^2)	683 (486-1129)	709 (619-846)	659 (549-911)
Neutrophils (cells/mm^2)	419 (326-610)*	499 (330-606)*	159 (108-360)
BAL			
BAL return (ml)	56.5 (43.5-75.8)**	85 (59-101)**	120 (108-124)
Total cell counts (×10^4/mL)	18 (8.5-36.5)	14 (11.5-34.5)	13 (10.5-15.0)
Macrophages (%)	43.4 (13.7-53.2)**	82.6 (56.8-89.5)	73.5 (67.6-80.1)
Macrophages (×10^4/mL)	6.3 (4.0-9.6)†	11.2 (9.3-11.3)	9.2 (7.6-11.3)
Lymphocytes (%)	6.8 (2.3-19.4)	9.3 (4.6-30.0)	20.8 (12.5-23.6)
Neutrophils (%)	42.8 (20.4-78.0)**†	3.6 (1.9-4.1)*	1.4 (1.2-2.9)
Neutrophils (×10^4/mL)	7.6 (2.2-25.7)**†	0.5 (0.3-0.8)*	0.2 (0.1-0.4)
Eosinophils (%)	0.4 (0.0-0.8)	0.0 (0.0-1.0)	0.3 (0.0-0.6)
Epithelial cells (%)	0.8 (0.0-4.2)	2.2 (0.6-4.7)	1.6 (0.3-4.2)
BAL IL-8 (pg/mL)	872 (137-1056)**†	32.9 (26.5-65.7)*	25.6 (18.6-34.3)

Data are shown as medians and interquartile ranges.
*P<0.05 vs controls; **p<0.001 vs controls; †p<0.01 vs LTR without BOS

Table 3. Comparison of BAL and Airway Neutrophils within LTR Based on BAL Bacteria Culture

	BAL Neutrophils (%)	EBB Neutrophils (cells/mm^2)
LTR with BOS		
BAL bacteria positive	30.8 (16.3-69.3)	543 (367-736)
BAL bacteria negative	37.1 (22.6-63.2)	335 (299-465)
LTR without BOS		
BAL bacteria positive	3.7 (1.6-4.9)	503 (497-509)
BAL bacteria negative	3.0 (1.9-4.3)	457 (286-639)

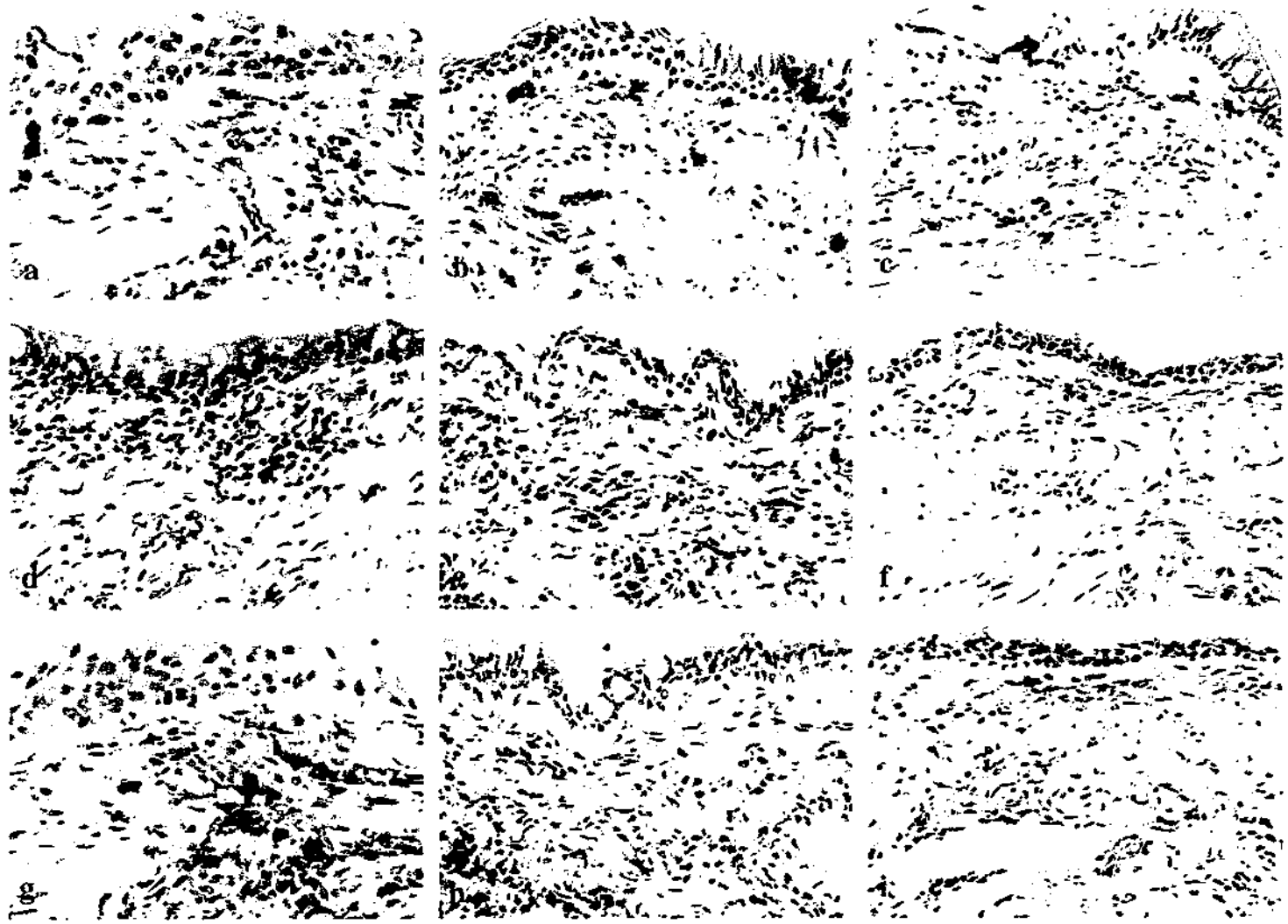


Fig. 1. Photomicrographs showing immuno-staining of bronchial biopsy specimens with monoclonal antibody to neutrophil elastase in (**a**) LTR with BOS, (**b**) LTR without BOS, and (**c**) controls; antibody to CD68 in (**d**) LTR with BOS, (**e**) LTR without BOS. and (**f**) controls; and antibody to CD45 in (**g**) LTR with BOS, (**h**) LTR without BOS, and (**i**) controls. (Each original magnification, ×400.)

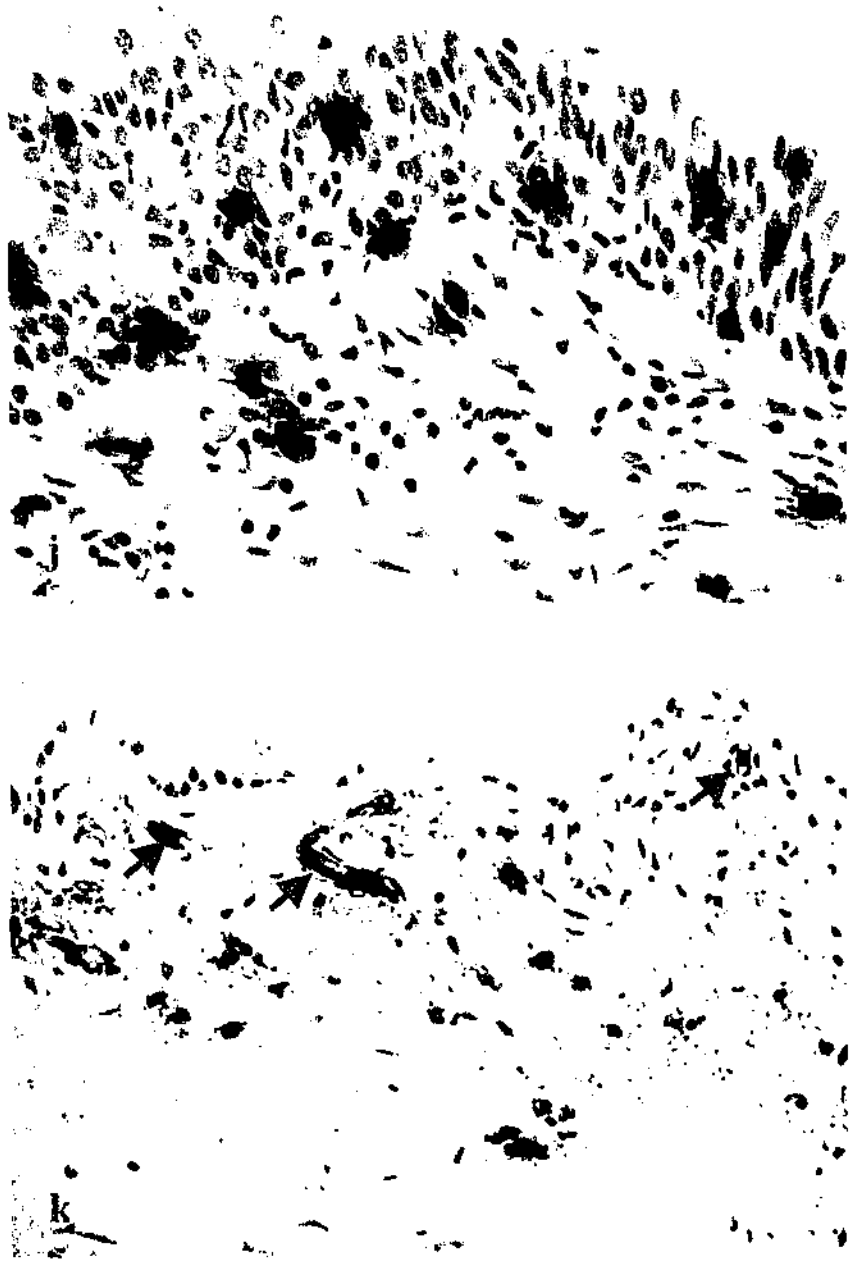


Fig. 1 j. & k. Photomicrographs of immuno-staining for neutrophils on bronchial biopsy specimens from LTR with BOS, showing: **(j)** neutrophil infiltration of the epithelium, and **(k)** neutrophil adhesion to the endothelium of airway vasculature. (Each original magnification, ×400.)

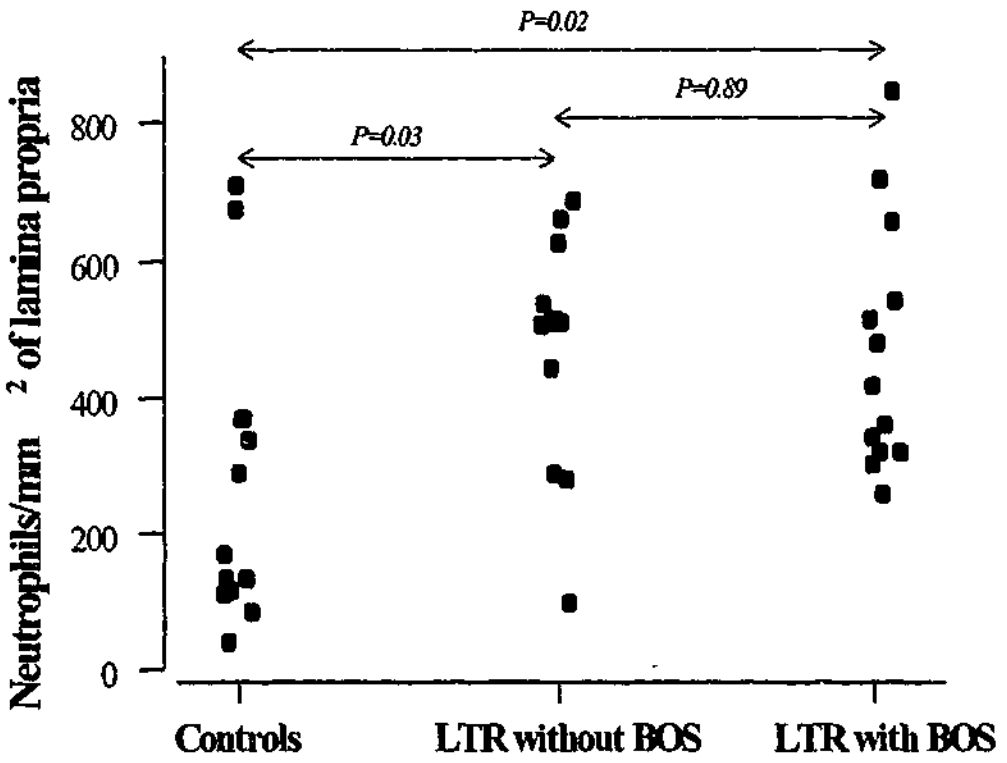


Fig. 2. Neutrophil elastase positive cells in the endobronchial biopsies from normal controls, lung transplant recipients without BOS, and lung transplant recipients with BOS.

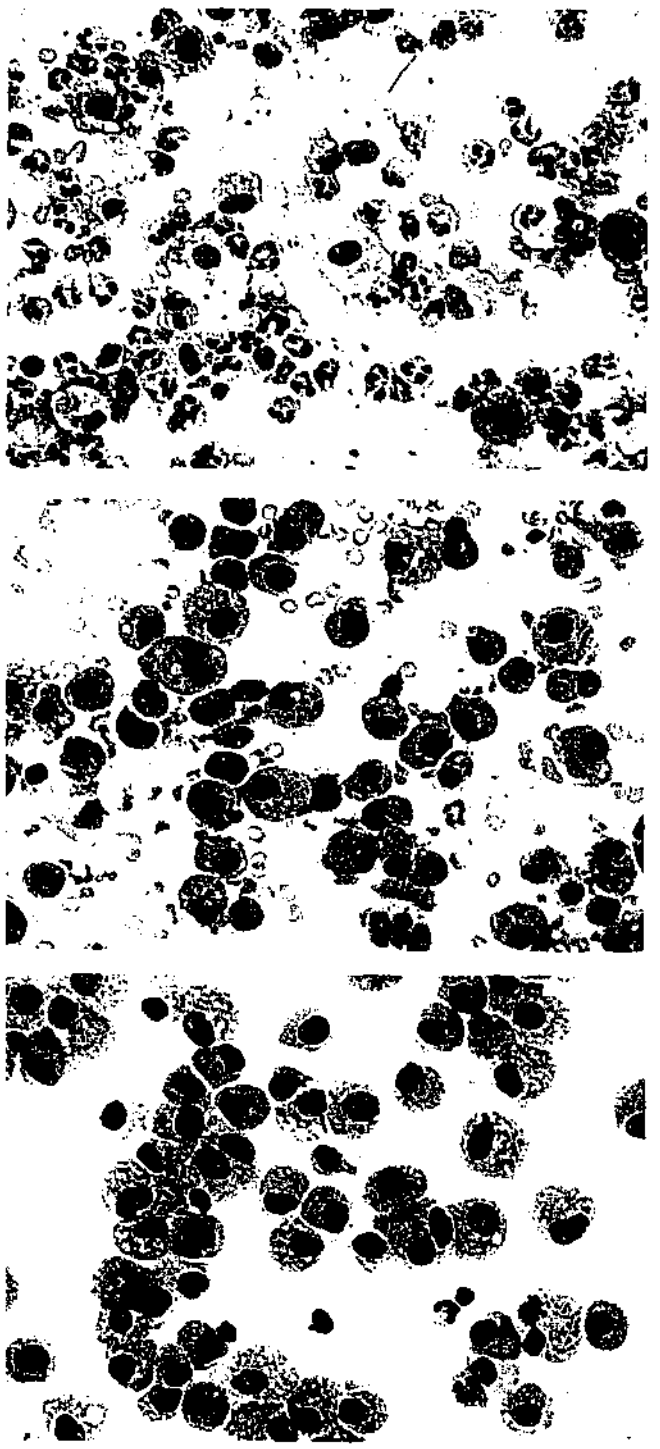


Fig. 3. Photomicrographs showing "Diff-Quik" staining for BAL cells from LTR with BOS (top), LTR without BOS (middle), and controls (bottom).

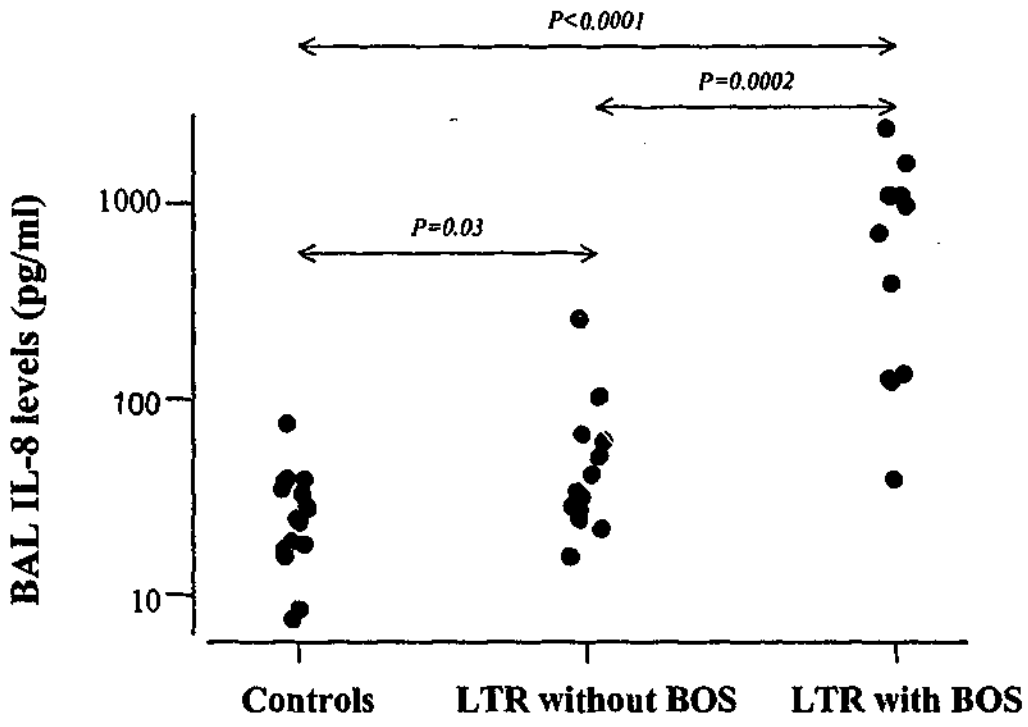


Fig. 4. IL-8 levels (pg/ml) in BAL from controls, lung transplant recipients without BOS, and lung transplant recipients with BOS.

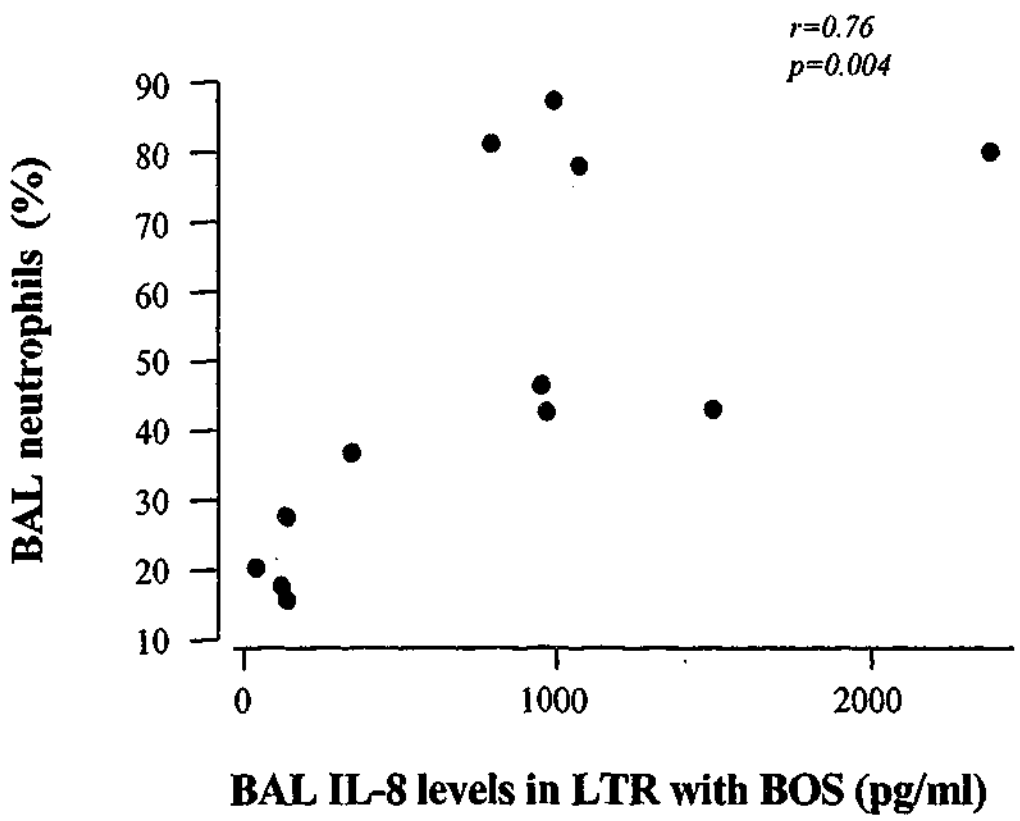


Fig. 5. Correlation between BAL IL-8 levels and BAL neutrophil percentage in the lung transplant recipients with BOS.

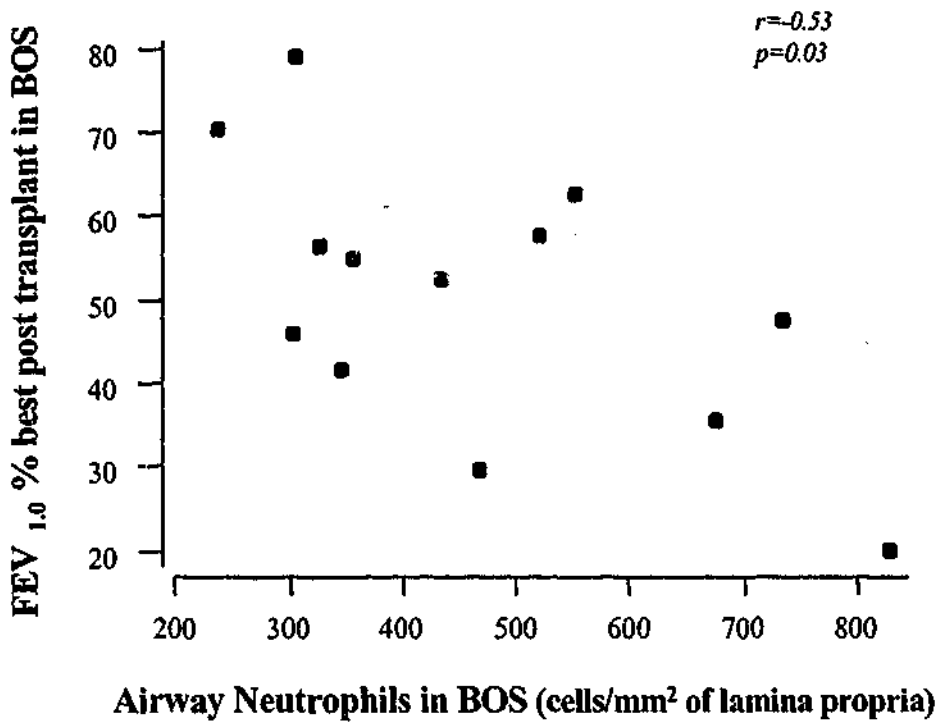


Fig. 6 a. Correlation between airway wall neutrophil density and lung function in the subjects with BOS.

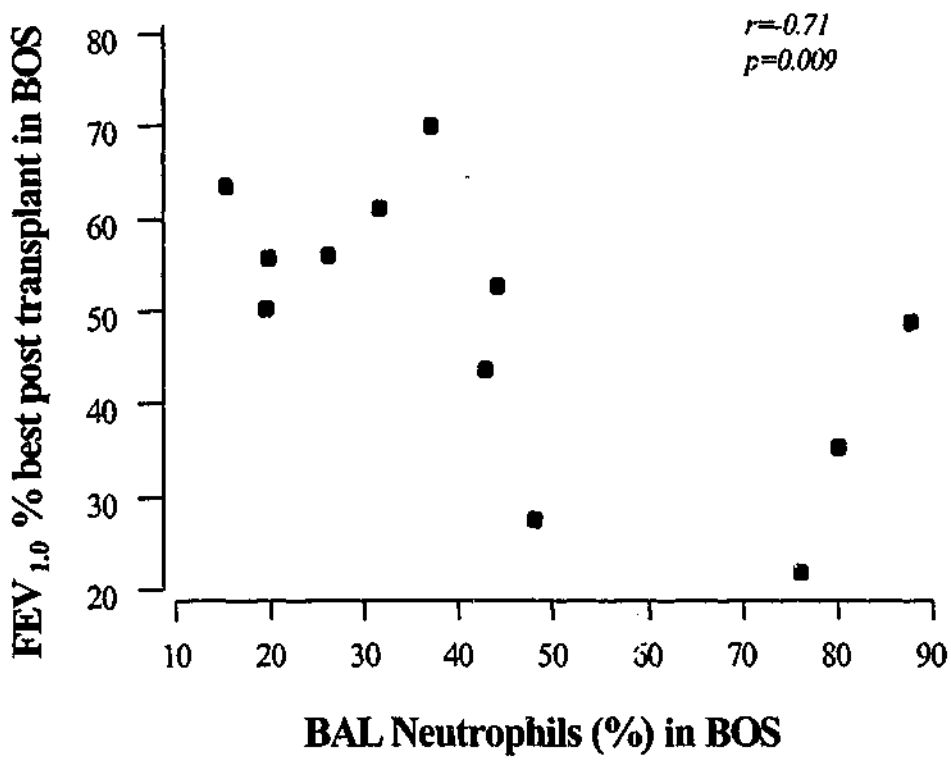


Fig. 6 b. Correlation between BAL neutrophil percentage and lung function in the subjects with BOS.

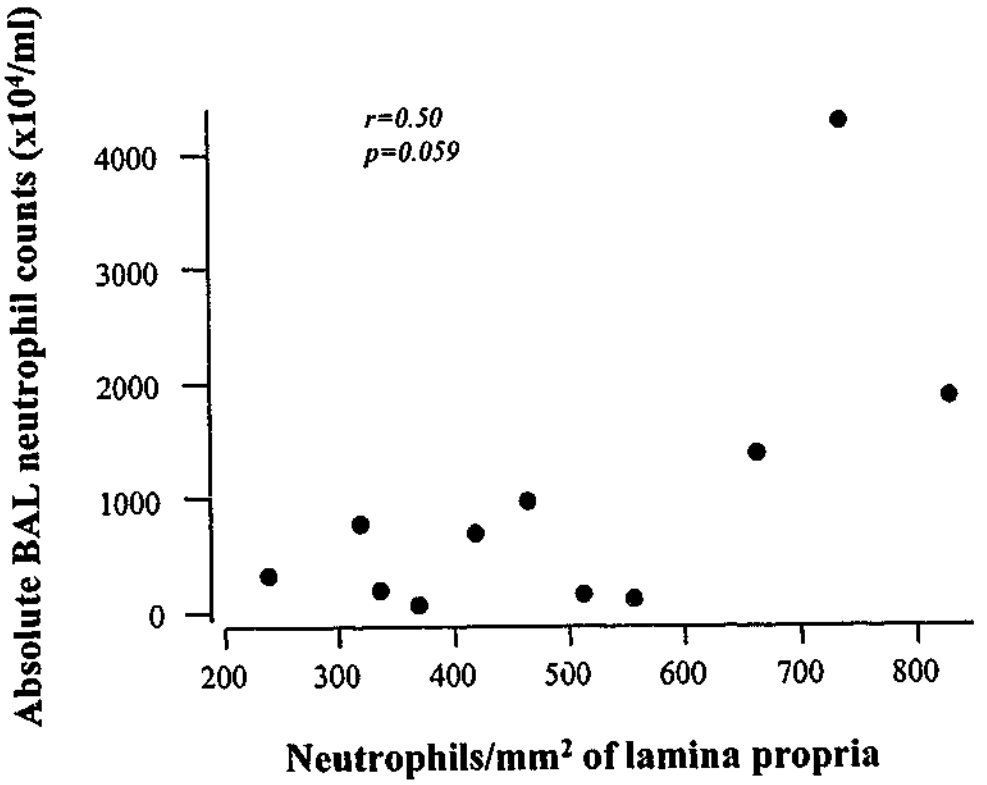


Fig. 7. Correlation between airway wall and BAL eutrophilia in the lung transplant receipients with BOS.

3.1.4 Discussion

This study used both BAL and bronchial biopsy technique to investigate cellular inflammation in the airways in LTR. For the first time, we have demonstrated a prominent airway wall neutrophilia in LTR both with and without BOS, which co-existed with the BAL neutrophilia, and correlated with the panleukocytes of the bronchial mucosa. However, no statistical difference was found in airway macrophage and panleukocytes density between LTR and normal controls. Although airway neutrophil density did not differ between the LTR who developed BOS, and those who did not, the percentage of BAL neutrophils in BOS was up to than ten fold higher than in the LTR without BOS, and it correlated significantly with the highly increased BAL IL-8 levels. This study has also revealed a significant reverse correlation between the decreased $FEV_{1.0}$% best post transplant and the increased airway wall neutrophil density, and BAL neutrophil percentage in BOS.

BOS post lung transplant has been considered as a form of chronic lung rejection. Analogous to the situation in acute rejection, research in BOS has focused on T-lymphocytes. However, no major differences in T-cell subpopulations have been found (130, 268), and in contrast, several studies have suggested that neutrophils may be the prominent cells associated with BOS (130, 145-147, 263). The current study provides further direct evidence of airway wall neutrophilia present in the lung allografts. However we did not observe an excessive neutrophil infiltrate in the airway wall lamina propria of BOS compared with their stable counterparts, which contrasts with the striking differences in BAL neutrophil numbers between the two LTR groups. A possible explanation for this discrepancy is that there may be a potent chemotactic gradient of IL-8 established from epithelium to vasculature within the

airways of BOS, so that the "excessive" airway neutrophils rapid migrate across the tissue to the lumen, which is then reflect in BAL constituents. Indeed, there was an almost significant correlation between BAL and airway wall neutrophils in BOS patients. To support this concept, a heavy neutrophil infiltration of the epithelium was often noted in the LTR with BOS as showed by immunohistochemical staining for neutrophils (Fig. 1j), although this was not separately quantified. This hypothesis is also supported by the observation of remarkably high levels of IL-8 in BAL from BOS as compared with their counterparts, although BAL IL-8 levels were also increased to a lesser degree in the LTR without BOS.

Neutrophils are rarely present in large number in normal airway and lung tissue (144). Traditionally, it is considered that neutrophil influx from the circulation to extravascular tissue is an important secondary event in host defense against microbial invasion. Therefore, before claiming a primary role for the neutrophil in the development of chronic lung rejection, it is important that confounding by occult airway infection is considered, although this is not easily achieved. In our study, what would normally be regarded as significant clinical airway infection had been excluded by subject recruitment criteria and specimen collection in the absence of overt infection. Thus, all BAL samples were negative on gram stain and did not have CMV inclusions, although microbiological organisms were still grown in some BAL samples. The presence of positive bacterial culture results, in the absence of a positive gram stain or clinical evidence of infection, argues that these bacteria were present in relatively low numbers and were considered "colonizers" rather than causing an active infective disease process of classic sort. In addition, organisms were not isolated more commonly in the BOS subjects (5 of 14) when compared to those without BOS (4 of 13), and within the BOS population, BAL neutrophil percentages and airway wall neutrophil numbers did not seem to

be particularly elevated in the presence of organisms. Two previous comparable studies showed the similar finding that when patients with BAL bacterial culture positive were excluded, the BAL neutrophils were still significantly elevated compared with controls (145, 146). In contrast, a recent study has suggested that it is BAL infection that resulted in a significant increase in BAL neutrophils in LTR (147). However, the studies by DiGiovine et al (145) (where LTR recipients were apparently excluded if any organisms were noted on BAL), and Riise et al (146) (where BAL bacterial load was used as a marker to exclude cases with infection), both found a similar BAL neutrophil percentage values to the present study. Neutrophilic infiltration of airways in smokers (269) and severe asthmatics (270) is also associated with airway wall remodelling and airflow obstruction mimicking the BOS, without any evidence of a current role for infection, at least as conventionally defined.

Airway inflammation leading to fibrosis and remodelling of the airways is the likely hallmark of BOS post lung transplant (145-147). Neutrophilic infiltration in the lungs, whatever the offending agent, has been shown to be associated with the lung injury and subsequent lung fibrosis in a diverse range of animal models (144, 152, 260-262, 271, 272). Some neutrophil products, such as proteases, matrix metalloproteinases, acid hydrolases, low-molecular-weight cationic proteins, and reactive oxygen metabolites, can potentially induce tissue injury at the site of neutrophil-dominated inflammation, and would lead to depletion of resident lung antioxidant defence, thereby facilitating the development of pulmonary fibrosis (144, 147-151). A most recent animal study demonstrated that bleomycin did not induce lung fibrosis in soluble E-selectin transgenic mice compared with the non-transgenic mice (271). In these transgenic mice there is excess secretion of soluble E-selectin into serum, which inhibits the E-selectin-mediated neutrophil extravasation to the lung by competitively binding to E-

selectin ligands on vascular endothelium (271). Another study of induced ARDS also showed that neutrophil depletion markedly decreased the extent of acute lung injury and subsequent development of irreversible scarring (272). In humans, a persistent neutrophil alveolitis has been observed to precede and play an important role in the pathogenesis of pulmonary fibrosis such as IPF (260) and ARDS (152). From our findings of airway and BAL neutrophilia presenting in both the LTR groups, and the reverse correlation between the airway/BAL neutrophilia and the $FEV_{1.0}$% best post-transplant in BOS, we would speculate that a persistent neutrophilic inflammation in lung allografts occurs prior to, and contributes to the development of BOS post transplantation. Similarly, DiGiovine, et al suggested that the development of BOS can be predicted by a twofold increase in BAL neutrophils and IL-8 levels prior to the later fall in lung function when compared with a long term stable non-BOS group (145), although their data was retrospective.

Neutrophilic infiltration in the lung allografts may be associated with increasing disease severity in BOS, as both airway and BAL neutrophilia correlated negatively with the decline of lung function in BOS found in this study. These findings are in agreement with results previously reported in smokers with severe COPD, where airway neutrophils were not only increased, but also correlated with the degree of airflow limitation (266, 273). There is also a higher number of airway neutrophils in patients with severe asthma compared with mild asthma (270), and in asthma and chronic bronchitis during exacerbation (269). In further support of the association between neutrophilia and severity of disease, several studies have reported a positive correlation between BAL neutrophilia or neutrophil products (neutrophil elastase) with the clinical severity of IPF and ARDS (144, 152). Taken together, these studies

suggest a pathogenic role for neutrophils in the severity and secondary damage in fibrotic pulmonary diseases.

In the recruitment of neutrophils to the lung, chemoattractants serve as a homing mechanism to precisely target neutrophils to the sites of inflammation. IL-8 is one of the most potent neutrophil chemoattractants (122). It is primarily produced by monocytes/macrophages, but also by other cells, including human mesothelial cells, bronchial epithelium, pulmonary fibroblasts, and endothelial cells (122). IL-8 concentrations in BAL fluid is markedly increased in several situation of neutrophil-mediated lung injury and lung fibrosis, such as IPF (260), ARDS (152), and CF (275). In addition, administration of a neutralizing antibody to IL-8 can prevent neutrophil-mediated tissue injury, and neutrophil infiltration in an animal models of pulmonary fibrosis (122). These results, alongside the present study, the studies of DiGiovine et al (145) and Riise et al (146) raise the possibility that IL-8 could be a potential novel target for therapeutic intervention in neutrophil-mediated fibrotic pulmonary disorders in general, but particular in lung transplant BOS.

Macrophages are constitutively present in the normal lung. This study did not find a significant difference in airway macrophage density between normal controls and LTR, although a previous study from our group showed an increase in airway wall macrophages in clinical stable LTR, but not in BOS, as compared with normal controls (129, 130). This can probably be explained by a different sample size in the two studies. There were 19 LTR without BOS in the previous study, while EBB specimens were obtained from only 13 LTR for this study. It is also possible, on the other hand, that airway macrophages contribute to bronchial obliteration, or an airway fibrotic process in the lung allografts mainly by releasing macrophage-derived pro-

inflammatory cytokines and growth factors such TNF-α, TGF-β, and PDGF. However, a prospective longitudinal study is needed to clarify the role for macrophages in the development of lung transplant BOS.

The panleukocyte density (CD45) in the bronchial mucosa in LTR was not statistically significantly increased as compared with controls, although it correlated significantly with the increased numbers of airway neutrophils, in the both LTR groups. This may suggest that the sensitivity of the two antibodies investigated (anti-CD45 and anti-neutrophil elastase antibodies), which stain for neutrophils, is perhaps different. Indeed, according to the manufacturer, anti-CD45 labels polymorphs usually weakly, although it reacts with all the nucleated cells of haemotopoietic origin, while anti-neutrophil elastase specifically and strongly labels neutrophil elastase-containing cells, namely predominantly the neutrophils. This technical limitation could lead to an under-estimation of the panleukocytes with anti-CD45 antibody, especially when the increased neutrophils become a major component of airway cell numbers, as demonstrated in the airway walls of LTR by this study.

In conclusion, this study has further characterised the features of airway inflammation occurring in the lung allografts by using airway biopsies. In stable LTR and those with BOS, there were not only an increased BAL neutrophilia and elevated IL-8 levels, but also a prominent airway wall neutrophilia. It implies a role for recipient neutrophils in the development of BOS post lung transplantation. A further study is needed to define the contribution of subclinical infection as a co-factor for BOS, for example, potentially by stimulating IL-8 production in the airways. Endobronchial biopsy specimens continue to provide useful insights into airway immunopathology beyond that, which can be

demonstrated with BAL and TBB alone, as it done clinically. If longitudinal studies can confirm a link between airway neutrophils and/ or increased IL-8 levels and the development of airway damage/remodelling in BOS, then this could create novel potential targets for future therapeutic interventions.

3.2 Bronchial Vascular Changes in the Airways of Lung Allografts: Contribution to Airflow Limitation in BOS

3.2.1 Introduction

The lung has two distinct blood supply systems: the pulmonary circulation and bronchial circulation. The pulmonary circulation is a low-pressure vascular system that has primarily a gas-exchange function. The bronchial circulation is a high-pressure system that provides oxygenated blood and nutrition to the walls of the entire bronchial tree, the large pulmonary blood vessels, some hilar structure, including lymph nodes, the lung parenchyma, and the visceral pleura. Between these two systems, anastomoses at the precapillary, capillary and postcapillary levels have been demonstrated throughout the entire human airway (276).

The physiological function of these anastomoses between the pulmonary and bronchial circulation is assumed to be particularly important post-lung transplantation. In the human lung transplant, although clinically applicable methods for direct restoration of bronchial circulation is available (277), with current surgical techniques the bronchial arterial supply to the tracheobronchial tree is generally not reconstructed at the time of transplantation, which makes lung allografts unique among solid organ transplantation. The survival of donor airway post-lung transplantation is probably totally dependent on the pulmonary circulation for its nutrition via these anastomoses between pulmonary circulation and bronchial circulation. This fundamental change in the airway blood supply post-lung transplantation stimulated our interest in investigating the airway vascularity in the lung allograft, and its potential for contributing to airflow dysfunction.

Since it is a significant fraction of the airway wall, the airway microvasculature has been of considerable interest as to its contribution to increased airway wall thickness, lumenal narrowing and hyperresponsiveness in asthma (197, 198, 278-281), but no information on airway vascularity is available in human lung allografts. In this study, given its potential for contributing to airway wall thickening, the vascularity of the proximal airways in bronchial biopsies from transplant recipients with and without BOS was investigated, and the association of airway vascularity with airflow obstruction post-lung transplantation was addressed.

3.2.2 Methods

3.2.2.1 Study Population

The inclusion criteria for patients were described in the previous section (Chapter III, 3.1). Twenty-two lung transplant recipients (LTR) were studied. 15 were clinically stable, 107-762 days post-transplant, sustaining their best post operative lung function ($FEV_{1.0}$ % best post-transplant: 99.4 ± 1.4%). 9 were diagnosed as having BOS and studied 489-1181 days post-transplant. BOS diagnosis was based on at least a 20% decline in $FEV_{1.0}$ from their best post operative lung function ($FEV_{1.0}$% best post-transplant: 50.07 ± 17.6%) in the absence of any other complications that could cause deterioration of graft function (35). All patients were receiving a standard long-term maintenance regimen of immunosuppressive therapy comprising cyclosporine (to achieve a blood level of 200-350 µg/L by EMIT assay, Syva, California, USA), azathioprine (1-2 mg/kg/day) and prednisolone (0.15-0.25 mg/kg/day). Individual patient details are recorded in Table 1.

Fourteen normal non-smoking volunteer subjects (10 males with mean age of 30 ± 12 yr; and 4 females with mean age of 22 ± 1.3 yr) were recruited as controls. They had no history of airway symptoms, had normal physiology and airway responsiveness to inhaled methacholine challenge.

3.2.2.2 Endobronchial Biopsies and Lung Function Test

Endobronchial biopsies from LTR were taken at routine bronchoscopy surveillance performed under intravenous sedation with midazalam (Roche, France), as decribed in Chapter II. Six endobronchial biopsies were taken from lower lobe sub-carinae using alligator forceps (Olympus, FB 15C, Japan), and were snap frozen in a liquid N_2-chilled isopentane slurry, embedded with ornithine carbamyl transferase (OCT), then stored at -80°C until immunohistochemical procedure.

Lung function tests were performed in all the patients immediately prior to bronchoscopy with a computerised rolling-seal spirometer (Sensor Medics 922, California, U.S.A.). The details were described in Chapter II.

3.2.2.3 Immunohistochemistry

The airway vessels were identified by typical staining of type IV collagen in the true basement supporting the endothelium (Fig. 1) Immunohistological staining for collagen type IV was performed by a standard three layer immunoperoxidase method (ABC method, described in Chapter II) with monoclonal antibody for collagen type IV (DAKO, Denmark).

Isotype IgG1 (DAKO, Denmark) was used as a negative control. Human nasal polyp sections were used as positive control in each staining run. Duplicate 7 μm sections were cut on a Cryocut 1800 (Reichert-Jung, Heidelberg, Germany) cryostat, onto poly-L-lysine coated slides. Sections were fixed in paraformaldehyde-lysine-periodate and were incubated with 10% normal rabbit serum for 15 min to prevent nonspecific binding of immunoglobulins to tissue. Then the sections were covered with antibody to human collagen type IV diluted in 1% normal rabbit serum/PBS (1:3200) at 4 C° in a moist chamber overnight. In the subsequent steps of the staining (30 min each), labeling was done with biotinylated rabbit antimouse antibody (1:400 in PBS) (DAKO, Denmark) followed by horseradish-peroxidase conjugated streptavidin (1:600 in PBS) (DAKO, Denmark). Metal enhanced diaminobenzidine (Pierce, Illinois, USA) was used as substrate. Sections were then counterstained with haematoxylin and mounted in synthetic mounting medium.

3.2.2.4 Quantitation of Immunohistochemical Staining

By using a computerised image analyser (Video Pro 32, Leading Edge, Sydney, Australia), the total vessel number in the subepithelial lamina propria seen in each biopsy section was counted. The total area of vessels, area of the subepithelial lamina propria and percentage area occupied by vessels were assessed at a final magnification of ×400, excluding smooth muscle and glands from the area assessed. For each subject, the total number of vessels was divided by the total area of lamina propria to be expressed as number of vessels /mm^2 of the subepithelial lamina propria; and the total vessel area was also divided by total area of lamina propria to determine the percent vascularity; the average vessel size was estimated by dividing the total vessel area by the total number of vessels.

To minimize subjective variation in interpretation of the staining, the slides were coded, then assessed by a blinded observer (myself), and randomly checked for quality control by a second observer. The coefficient of variation (CV) of between observers for the number of vessels/mm^2 was 14%, and for the percent vascularity was 4%.

3.2.2.5 Statistical Analysis

Parametric variables (lung function data) were assessed using Student's unpaired t-test. Vessel data were expressed as medians and interquartile ranges, and analysed using the non-parametric Mann-Whitney U test. Differences were considered significant with $P < 0.05$. "Best-fit" regressions were used to examine the relationship of airway vessel data to $FEV_{1.0}$ and $FEV_{1.0}$% best post-transplant in the LTR with BOS, where airway vessel data were the independent variable and lung function data were the dependent variable in these analyses.

3.2.3 Results

3.2.3.1 Airway Vascularity in LTR

The average total area of lamina propria examined per patient was very similar for the three groups (Table 2). However, the percent vascularity was greater in LTR both with BOS (median 15.0%; range 12.9-18.9%) and without BOS (median 13.3%; range 12.3-16.3%) compared with normal controls (median 11.5%; 7.4-13.3%), $P<0.05$. There was no significant difference in the percent vascularity between LTR with and without BOS (Fig. 2).

No significant difference was found in the vessel number/mm^2 of lamina propria, nor in average vessel size between the LTR groups and normal controls (Fig. 3, 4). Interestingly, however, in subgroup analysis, the percent vascularity and average vessel size were increased in the LTR with bilateral sequential or single lung transplantation compared with LTR who had heart-lung transplantation and controls (Table 3).

3.2.3.2 Association Between Airway vascularity and Airway Obstruction in BOS

In spite of the lack of specificity of vascularity changes in the BOS group, we were able to fit a simple exponential model for the relationship of airway vessel size to $FEV_{1.0}$ % best post-transplant ($R^2 = 0.49$; $P = 0.036$) in LTR with BOS (Fig. 5). This fitted model demonstrated an increase in airway vessel size with decreasing $FEV_{1.0}$% best post-transplant, indicated by the negative values of the exponential coefficient (-0.0014). No significant correlation was found between the percent vascularity, vessel number/mm^2 of lamina propria and lung function parameters.

Table 1. Clinical Details of the Lung Transplant Recipients Studied

No	Sex	Age (yr)	Original disease*	LTx	Days Post LTx	$FEV_{1.0}$(L)	$FEV_{1.0}$% best post-LTx
LTR without BOS							
1	m	47	B	BSLTx	107	2.07	100
2	m	40	B	BSLTx	189	2.29	100
3	f	31	CF	BSLTx	58	2.16	100
4	m	38	E	HLTx	1185	3.46	100
5	m	22	CF	BSLTx	186	3.72	100
6	m	42	E	HLTx	1090	4.33	99
7	m	38	CF	BSLTx	762	1.98	100
8	f	35	E	HLTx	387	3.48	96
9	f	43	PPH	HLTx	369	2.52	100
10	m	49	EM/VHD	HLTx	183	3.04	100
11	f	35	PPH	BSLTx	287	3.52	96
12	f	37	E	HLTx	464	3.51	100
13	f	23	CF	BSLTx	102	3.36	100
14	f	23	E	HLTx	1144	2.48	100
15	f	34	CF	BSLTx	788	1.84	100
						(2.9±0.8)	**(99±1.4)**
LTR with BOS							
1	m	24	CF	HLTx	1176	2.60	51
2	m	23	CF	HLTx	1124	1.88	64
3	f	25	PPH	HLTx	729	2.04	58
4	f	55	E	SLTx	582	1.00	78
5	f	31	PPH	BSLTx	396	0.52	21
6	f	54	E	SLTx	1186	0.72	49
7	f	26	E	BSLTX	989	1.16	42
8	f	47	EM	SLTX	1518	0.56	28
9	m	26	CF	BSLTx	252	2.76	59
						(1.5±0.9)*	**(50±17.8)***

Original disease: B: bronchiectasis; CF: cystic fibrosis; E: Eisenmenger's syndrome; PPH: primary pulmonary hypertension; EM: emphysema. VHD:

LTx: lung transplantation; BSLTx: bilateral sequential lung transplant;
HLTx: heart-lung transplant; SLTx: single lung transplant:

*P<0.05 LTR with BOS vs LTR with BOS.

Table 2. **Vascularity assessed in endobronchial biopsies from Lung transplant recipients (LTR) and controls**

Subjects	Total Area (μm^2)	Percent Vascularity	Vessels per mm^2	Vessel Size (μm^2)
Control				
1	290196	13.0	312	465.6
2	56436	8.7	284	314.61
3	133018	7.6	323	576.1
4	89125	12.6	303	449.5
5	62297	5.1	257	198.2
6	58860	15.2	340	447.8
7	69440	12.2	418	291.1
8	71686	4.3	153	281.1
9	53571	12.3	451	298.9
10	166250	9.3	337	271.6
11	57266	14.8	349	408.5
12	116667	14.1	404	375.1
13	119791	6.9	334	234.5
14	140775	10.8	355	303
median	**80405**	**11.5**	**356**	**308.8**
interqutile range	**(58462-134957)**	**(7.4-13.3)**	**(298-367)**	**(278.8-448.2)**
LTR w/out BOS				
1	42337	10.0	331	465.7
2	82185	13.3	480	294.3
3	86539	16.3	310	512.2
4	70866	13.3	480	283.6
5	89569	13.1	335	389.0
6	138650	14.5	383	386.5
7	133474	29.0	450	625.7
8	54979	5.9	346	159.8
9	149351	13.0	315	411.0
10	84546	12.4	173	808.4
11	120619	17.1	389	518.8
12	64543	12.2	302	391.1
13	46694	13.3	300	439.4
14	122886	12.3	439	288.4
15	118083	17.3	143	1203.1
median	**86539**	**13.3**	**335**	**411.0**
Interqutile range	**(64543-122886)**	**(12.3-16.31)**	**(302-439)**	**(294.3-518.7)**
***p Value**	**0.98**	**0.028**	**0.71**	**0.12**
LTR with BOS				
1	69282	11.8	325	263.5
2	51090	10.3	392	268.3
3	68563	20.1	536	389.6
4	166585	18.7	355	457.4
5	283369	13.8	152	927.9
6	44303	19.2	386	456.3
7	81470	15.0	379	371.1
8	82001	15.1	244	634.6
9	86551	14.4	268	656.9
median	**81470**	**15.0**	**355**	**456.3**
Interqutile range	**(59827-126568)**	**(12.8-18.9)**	**(256-389)**	**(319.7-645.8)**
***p Value**	**0.63**	**0.011**	**0.83**	**0.14**

*p: vs control

Fig. 1. Photomocrographs of type IV collagen staining in endobronchial biopsies indicating vessels below the true basement membrane in LTR with BOS (top), LTR without BOS (middle), and normal controls (bottom). (Each original magnificatuion, × 400).

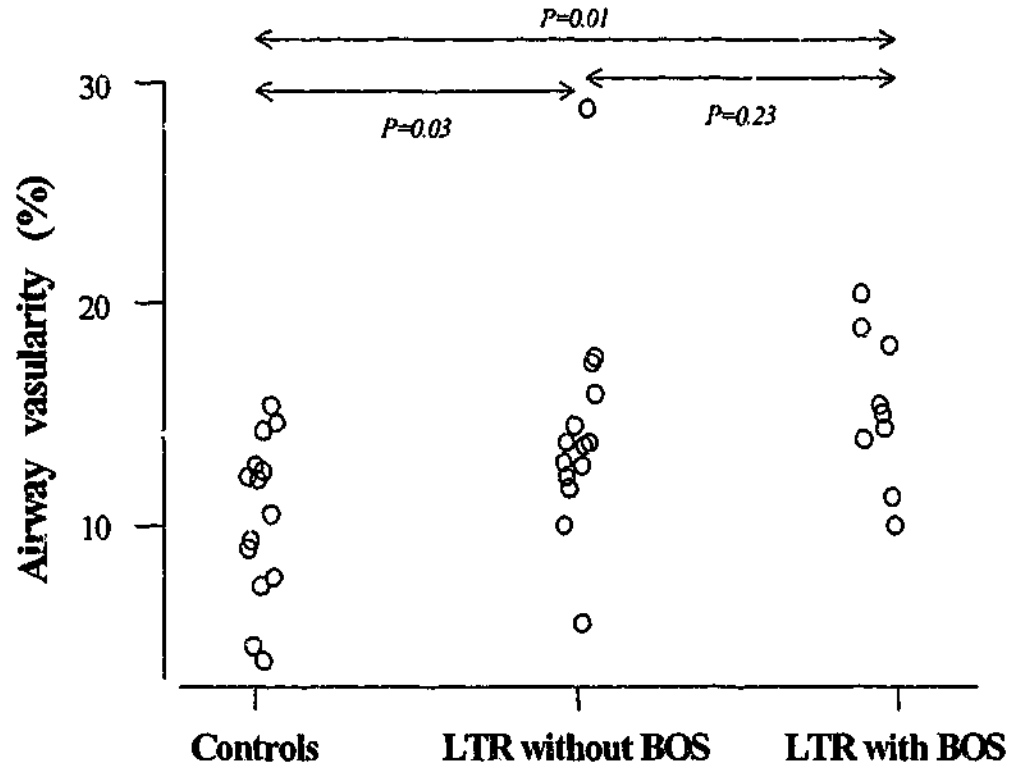


Fig. 2. Comparison of airway vascularity (%) between lung transplant recipients and normal controls.

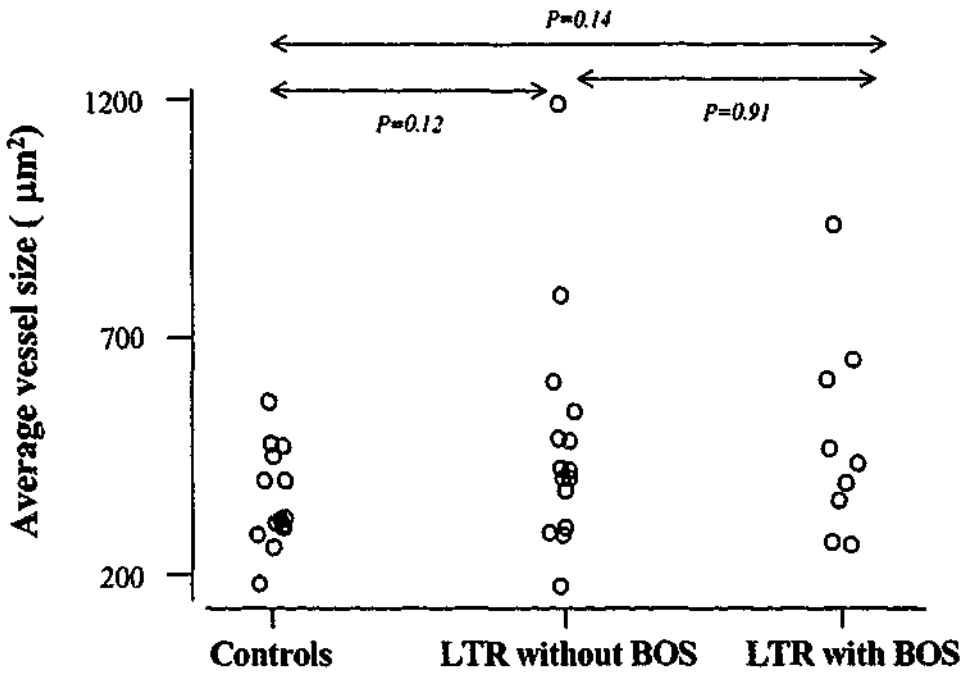


Fig. 3. Comparison of average vessels size (μm^2) of lamina propria in endobronchial biopsies from lung transplant recipients and normal controls.

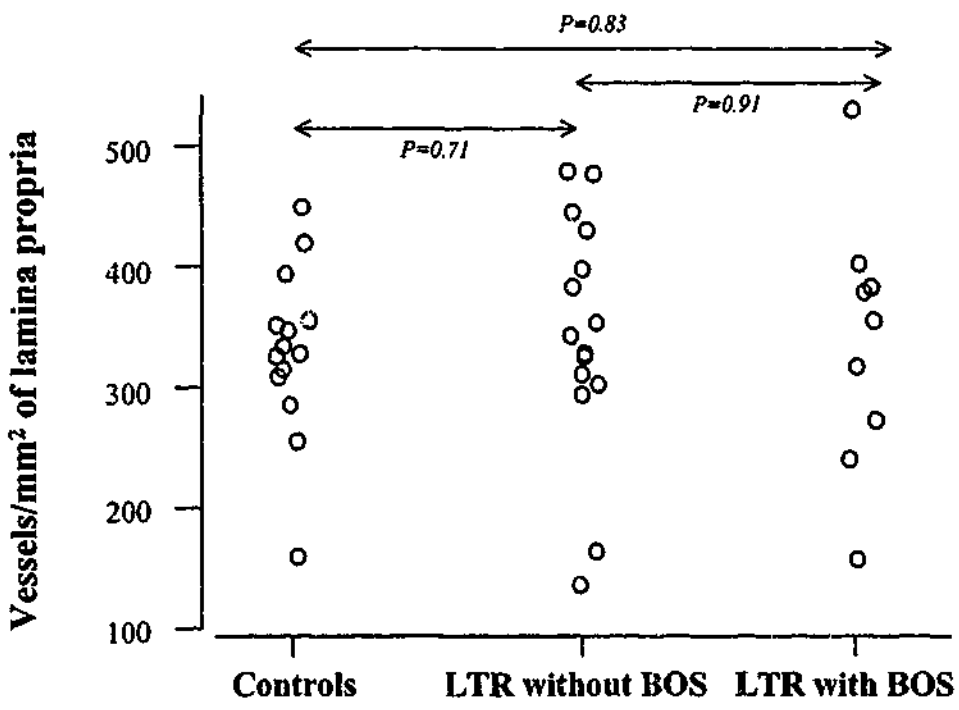


Fig. 4. Comparison of vessel numbers per mm^2 of lamina propria in endobronchial biosies from lung transplant recipients and normal controls

Table 3. **Comparison of Airway Vascularity by Different Types of Lung Transplant**

	N	Percent Vascularity	Vessels per mm^2	Vessel Size (μm^2)
HLTx	10	12.4 (11.4-13.6)	364.1 (312-450)	337.5 (267.1-396.1)
BSLTx & SLTx	11+3	15.1 (11.4-17.6)*†	333 (262-387)	489.0 (426.8-640.2)*†
Controls	14	11.5 (7.4-13.3)	336 (298-367)	308.8 (278.8-448.2)

*P < 0.05 vs controls;
†P < 0.05 vs HLTx.

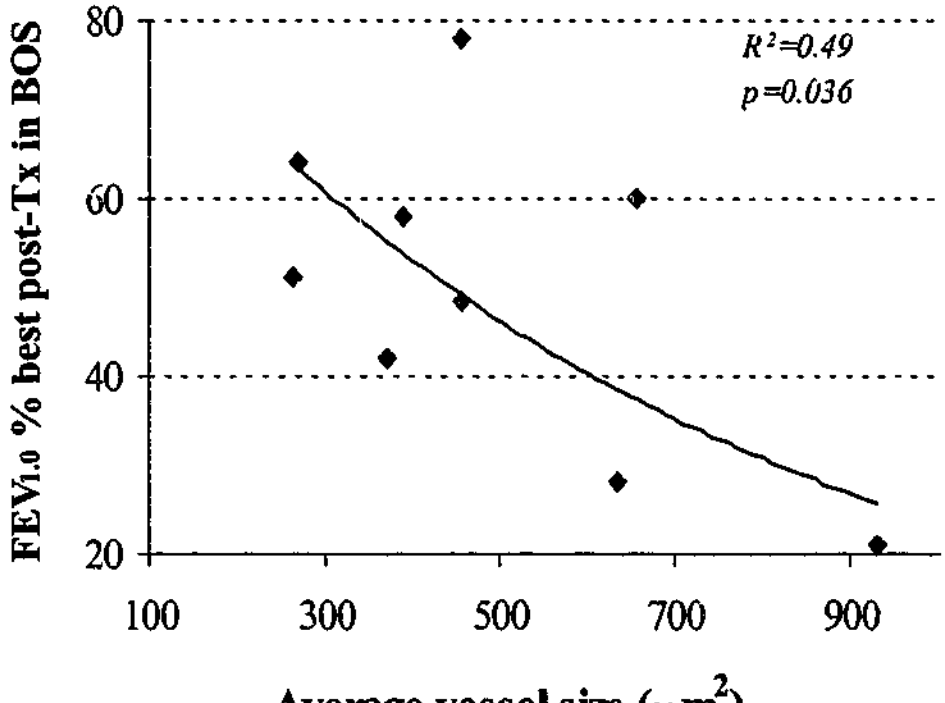


Fig. 5. Relationship of airway vessel size to the $FEV_{1.0}$% best post-transplant (Tx) in LTR with BOS.

3.2.4 Discussion

This study has demonstrated, for the first time, that overall the vascularity of the subepithelial lamina propria in the segmental airways of lung allografts is increased compared with controls. The recipients with bilateral sequential or single lung transplant showed a higher airway vascularity and larger airway vessel size than those with a heart-lung transplant and controls. As the airway vessel size increased, the $FEV_{1.0}$% best post-transplant decreased exponentially in the LTR with BOS. An explanation of these findings may be important in understanding the pathophysiology of post-transplant airway complications.

Although the gross anatomy of the tracheobronchial circulation has been described in detail (276), because of methodological difficulties, there are only a few descriptions of the detailed organisation and structure of the subepithelial microvasculature (282). In humans bronchial artery anatomy is highly varied, but the bronchial arteries generally arise directly either from the aorta or the intercostal arteries and are present along the entire length of the bronchial tree as far distally as the terminal bronchioles. They form two bronchial vascular plexuses within the airway walls: the peribronchial plexus, located in the adventitial space between the muscle layer and the surrounding lung parenchyma, and the lamina propria vascular plexus, located beneath the epithelial layer.

In this study, we investigated the vasculature of the subepithelial plexus in the segmental level of airways as sampled by bronchial biopsies, using the method previously described (198). We found a similar airway vascularity in terms of percent of lamina propria in normals to that

previously reported (198), but a lower vessel number/mm^2 of the lamina propria compared with that study (198). This discrepancy can probably be explained by different tissue fixation methods applied in the studies. Compared with snap frozen tissue used in this study, ethanol fixed tissue used previously may be more dehydrated, so that the number of vessels counted per square mm of lamina propria may therefore be higher, but the percentage of the mucosa occupied by the vasculature should be less affected by the different tissue fixatives. Importantly, this study essentially confirmed the previous findings (198) that the subepithelial microvascular bed constitutes about 10% of the lamina propria tissue volume in the segmental level of normal airways.

The observation of an increase in airway vascularity of lung allografts but without significant increase in vessel number in LTR both with and without BOS suggests that vascular engorgement or structural vessel dilatation is a component of airway wall remodelling occurring post-lung transplantation. The bronchial vasculature is innervated mainly by adrenergic and peptidergic nerves and normally dilates in response to hypoxia and hypercarbia (283-285). Under normal circumstances, blood flow to the trachea is almost entirely from the systemic circulation (284), and 97% of the blood supply to the intraparenchymal airways down to 1 mm in diameter is also from the bronchial circulation (286), while the pulmonary circulation helps to perfuse the bronchial vasculature of the smaller airways and, to a diminishing extent, the larger airway up to the lower trachea (284, 286.). In contrast, under the circumstance of lung transplantation, since the bronchial artery to the donor airway is disrupted in the harvest procedure, and the bronchial circulation is generally not revascularized at the time of transplantation, the blood supply to the donor airway is likely to be almost entirely

dependent on a low-pressure, mixed-venous retrograde blood flow from the pulmonary artery. We postulate that in lung allografts the relative oxygen desaturated and hypercarbic pulmonary arterial blood supply to the donor airways from the pulmonary circulation could contributes, at least partially, to the bronchial vascular engorgement or dilatation observed. The denervation may also affect vascular tone and permeability (287). In addition, the rich subepithelial microvascular networks may be functionally related to the high metabolic rate of the epithelium, which is very active in secretory processes and ciliary beating. After lung transplantation, the nutritional and metabolic oxygen demands of airway tissue may be higher than can be supplied by the pulmonary arterial flow, and vascularity changes could potentially be induced by an acidic local pH and further hypercarbia.

Although the long-term functional capacity of airways deprived of their bronchial arterial supply is unknown, post-operative bronchial ischaemia appears to be the most important factor influencing the process of airway healing (28). Therefore, some groups have performed direct bronchial artery revascularization (BAR) for a number of lung transplant recipients, and investigated the effects of BAR on BOS/BO post transplant recently (96-100). Good BAR may postpone the onset of BOS/BO (99). In one series, the total incidence of BOS at three years was 33% (100), which seems lower than the reported rate of BO/BOS without BAR (11-13). Direct airway damage due to ischaemia and lack of perfusion may, therefore, be a factor in BOS development.

Interestingly, in view of our findings of differences in airway wall vasculature in the different procedural subgroups, clinical evidence has shown that human heart-lung transplantation has

been associated with fewer airway necrosis problems in comparison to double lung transplantation, probably because of a better-sustained systemic circulation (288). A collateral flow from coronary artery to bronchial arteries has been demonstrated radiographically following heart -lung transplantation, and preservation of bronchial artery flow by means of these connections is thought to be responsible for the generally more satisfactory airway healing with the combined heart-lung transplant (288). Our evidence of more normal airway vascularity and a smaller vessel size in the recipients who had a heart-lung transplant compared to those with bilateral sequential or single lung transplant, might imply that the changes seen in the other groups are indeed due to relative inadequacy of the bronchial wall blood supply when provided solely by retrograde flow from the pulmonary artery collateral system.

Hyperaemia is also one of the cardinal manifestations of airway inflammation, caused by the release of many immunological and inflammatory mediators from the airway epithelium and migratory leukocytes (289). Such hyperaemia may lead to oedema and swelling of the mucosa and an increased exudation onto the luminal surface. In the transplanted lung, the airways are subjected to a wide range of insults induced by ischaemia-reperfusion, the humoral immune and cell-mediated immune responses, rejection, and potentially extrinsic infections. All are associated with release of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 (290, 291). In the previous section (Chapter II, 3.1) I have shown that despite standard triple immunosuppression, in the LTR both with and without BOS, there is ongoing inflammation present in the airways. The changes in vascularity seen could therefore be secondary to this inflammatory process. It is possible that the bronchial vascular hyperaemia assists in both inflammatory and healing processes by facilitating the influx of inflammatory cells into the

airway wall but also rapidly clearing locally released chemical mediators. However, the difference between heart lung and isolated lung transplants groups is less easy to rationalize on an inflammatory basis.

Physiologically, the bronchial vascular hyperaemia could obstruct peripheral bronchi by increasing the thickness of the airway wall. As long ago as 1960, von Hayek hypothesed that in order for the airways in the lung to contract, some space outside the airways must 'enlarge (292). He believed that the blood vessels in the adventitia of the airway wall could serve this function, and demonstrated a histological section of a severely contracted airway with numerous and extremely large adventitial vessels (292, 293). It has also been suggested that the volume of tissue internal to the muscle layer may determine both the responsiveness of the airway to contractile stimuli and also the degree of airway narrowing (294). In chronic asthma, there is increasing evidence suggesting that an increase in the size and number of bronchial vessels appears to correlate with severity of asthma. Patients with fatal asthma showed an increase in the number and size of their bronchial vessels (209). In this study, a considerably larger fraction of the bronchial wall occupied by blood vessels have been demonstrated in LTR both with and without BOS compared with normal airways. Furthermore, in the BOS group there was a relationship between vessel size and loss of airway calibre. Such findings are reminiscent of the previously observed effects of intravenous administration of 2 L of warm normal saline, that produced profound decrements in $FEV_{1.0}$ in 8 asthmatic subjects and moderate reductions in 8 normals (281). Brown et al (280) also showed in dogs that, as the loading volume of normal saline or homologous blood increased, the airway luminal area decreased, and the airway wall thickness increased. Change in airway carlibes is functionally

important, because airway resistance is inversely proportional to the fourth power of airway radius. Taken together, these data suggest that bronchial vascular hyperaemia, engorgement or structural vessel dilatation could be one of the factors contributing to the airflow limitation seen in BOS.

In summary, bronchial vascular changes in transplanted lung are probably an important adaptive process and may function to maintain the viability of allograft tissues and structure. However, such changes are not without their potential adverse effects and may contribute to airway narrowing and to chronic graft dysfunction in this aspect. A longitudinal study to gain better understanding of the natural history of airway remodelling in the allograft and its physiological sequela are necessary. These may provide information, which will allow development of a means for improvement in long-term allograft survival.

3.3 Collagen Deposition in the Airways of Lung Allografts: The Role of TGF-β_1 and TNF-α

3.3.1 Introduction

Fibrosis is a pathological process in which normal tissue is replaced by excessive and deranged deposition of extracellular matrix (ECM) proteins with accumulation of mesenchymal cells. The sequence of events leading to organ fibrosis usually involves an injury causing an inflammatory reaction followed by an excessive repair process, which eventually results in functional impairment. Lung transplant BOS manifests itself, histologically, as obliteration of the bronchiolar lumen by either polyps of organizing connective tissue, or mucosal fibrosis, which is present in both large and small airways of lung allografts (29, 78). Although neither its cellular nor molecular pathogenesis has been fully understood, lung transplant BOS is believe to be the consequence of airway tissue repair and remodelling, in which ECM deposition is an important part, in response to graft injury, whatever the offending agent.

Collagens of various types constitute 60-70% of protein found in the ECM of normal human lung (295). They form a three-dimensional network with other ECM proteins such as elastin, proteoglycans, fibronectin, and laminin to maintain both structural and functional integrity of the lung. The collagen family consists of at least 13 genetically distinct types, each with unique physical and chemical characteristics (295). Collagen type I, III and V are the fibril-forming collagens in human lung, and contain long stretches of triple helix structure (295). A dynamic equilibrium between synthetic and degradative mechanisms controls the metabolism of

collagen. Inappropriate change in this balance may lead to increased deposition of collagen, resulting in pathological fibrosis (295).

Various cytokines have been implicated in the pathogenesis of respiratory tract fibrosis, including transforming growth factor beta (TGF-β_1), tumor necrosis factor alpha (TNF-α), platelet-derived growth factor (PDGF), and the interleukins (IL-1, IL-8) (296). Of those investigated so far, the TGF-β family has the most potent stimulatory effect on ECM deposition (124, 297). TGF-β is the prototype of a superfamily of growth factors and cytokines that is subdivided on the basis of their structural similarities (124, 297). The name is derived from the observation that TGF-β stimulates normal cells to grow in soft agar as though they had been virally transformed (297). In mammals, TGF-β has three isoforms, TGF-β_1, TGF-β_2 and TGF-β_3, whose biologic properties are nearly identical (124). TGF-β_1 is the best studied isorform of these three (178, 298-303). It stimulates fibroblast procollagen gene expression and protein synthesis and inhibits collagen breakdown (124). TNF-α is generally considered to be a pro-inflammatory cytokine (142), but evidence also suggests a role for TNF-α in the pathogenesis of pulmonary fibrosis (159, 161, 304). I*n vitro,* it can promote fibroblast replication and collagen synthesis, and *in vivo,* pulmonary TNF-α gene expression rises after administration of bleomycin in a pulmonary fibrosis model in mice (305).

In the previous sections of this chapter (Chapter III, 3.1 & 3.2), I have demonstrated features of airway inflammation and bronchial vasculature changes in lung allografts. In this study, I have investigated scar collagen (collagen type I, III and V) deposition in the airway walls of lung allografts, and evaluated the potential role of TGF-β and TNF-α in the pathogenic process.

3.3.2 Methods

3.3.2.1 Study Populations and Exclusion Criteria

Patients were recruited from the Lung Transplant Service of the Alfred Hospital. The recruiting criteria for patients were described in the previous section (Chapter III, 3.1). The diagnosis of BOS was based on the criterion of at least a 20% decline in $FEV_{1.0}$ from their best post operative lung function in the absence of any other detectable complication that could cause a deterioration of lung function (35). All patients were receiving a standard maintenance regimen of immunosuppressive therapy comprising cyclosporine (to achieve a blood level of 200-350 μg/L by EMIT assay, Syva, California, USA), azauioprine (1-2 mg/kg/day) and prednisolone (0.15-0.25 mg/kg/day).

Control subjects were normal healthy volunteers, who were non-smokers, free from known respiratory diseases, and asymptomatic with normal lung function.

Approval for this study was given by The Alfred Hospital Ethics Committee, and each subject gave written informed consent.

3.3.2.2 Lung Function, BAL, and Endobronchial Biopsy

Lung function testing was done in all the subjects on the same day and immediately prior to bronchoscopy. BAL and EBB were obtained from the subjects through a bronchoscopy

procedure under intravenous sedation with midazolam (Roche, Paris, France) as described in Chapter II. BAL supernatants were stored at -80°C until TGF-β_1 and TNF-α assays were performed. EBB was embedded in OCT, snap frozen in liquid nitrogen-chilled isopentane slurry, and stored at -80°C until immunohistochemical analysis.

3.3.2.3 Tissue processing and Immunohistochemistry

Since formalin fixation and paraffin embedded tissue is recommended for TGF-β_1 and TNF-α staining by the manufacturers of the monoclonal antibodies used, and ethanol fixation and paraffin embedded tissue were shown to work well for collagen subtype staining in preliminary experiments, the frozen EBB tissues were post-processed into 100% ethanol for 30 min and into 10% formalin for 2 hours respectively, then embedded in paraffin using an automated tissue processor (Histokinette, Shandon, Pittsburgh, PA, USA). The details of tissue process procedures are described in Chapter II.

Immunohistological staining for collagen subtype I, III and V was performed on 3μm sections with highly purified collagen subtype specific antibodies (Chapter II, 2.1) and by using an avidin-biotin peroxidase complex (ABC) method described in Chapter II. The working dilution of the antibodies was pre-determined by staining normal skin and nasal polyp tissue with serial dilutions of the antibodies. The optimal dilution was 1:50 for anti-type I and V collagen, and neat for anti-type III collagen. Isotype control immunoglobulins (IgG1 and IgG2a; DAKO, Denmark) were used as negative controls, and human skin tissue was used as positive control in each of the assays. Since the primary antibodies to type I and V collagens were goat anti-

human, and to type III collagen was mouse anti-human, biotinylated rabbit anti-goat IgG, or rabbit anti-mouse IgG were employed as the second antibodies in the staining for collagen type I, V and III respectively. In addition, preliminary optimisation runs had indicated that prior to the staining process all the tissue sections need to be treated with 0.025% protease XIV (Sigma, USA) at 37°C for 3 min in order to "unmask" the antigens of the collagen subtypes.

As recommended by the manufacturers, formalin fixed EBB specimens were used for the TGF-β_1 and TNF-α stains in this study. Immunohistological staining for TGF-β_1 and TNF-α was performed on 3 μm sections with a monoclonal mouse anti–human antibodies against human TGF-β_1 (1:4000 in PBS) and TNF-α (1:50 in PBS) and amplified by the methods outlined in Chapter II. No pre-treatment to "unmask" antigen were needed for either TGF-β_1 or TNF-α stains. The details of the antibodies are described in Chapter II.

At least 2-4 consecutive sections from each specimen were stained with each of the antibodies against the collagen subtypes, TGF-β_1 and TNF-α.

3.3.2.4 Histochemical Stains

Serial sections of each specimen were stained with van Gieson and Masson's trichrome to compare the detection for collagen with the immunohistochemical stains, and also stained with Haematoxylin and Eosin (H and E) to check the quality of the tissue blocks prior to immunohistochemical stain (Chapter II).

3.3.2.5 Quantitation of Immunohistochemical Staining

Collagen deposition was measured in the total area of the lamina propria seen in the EBB sections. The lamina propria was defined as the area between sub-epithelial and the smooth muscle layers and/or mucous glands of the airway walls. The absolute area stained for collagen in the lamina propria and the corresponding area of lamina propria were measured by using a computerized colour image analysis system (Video Pro 32, Leading Edge, Sydney, Australia) at a final magnification of ×400. Collagen deposition in the lamina propria of the airways was calculated as the total collagen area/total corresponding lamina propria area, and expressed as a percentage.

As described in previous publications (178, 298-303, 306), positive staining for TGF-β_1 was located in the ECM of bronchial lamina propria and homogeneously in the bronchial epithelial cells. The intensity of the staining in the ECM was graded as follows: - (negative), + (weak but detectable staining), ++ (moderate staining), and +++ (marked staining), and in the epithelium as: – (negative) and + (positive) under light microscope (126, 176, 178, 298, 299, 301, 302).

Positive staining for TNF-α was found within the cytoplasm of inflammatory cells, endothelial cells in bronchial lamina propria, and bronchial epithelial cells. The total number of cells and vessels positively stained for TNF-α in the lamina propria of the airway wall in the EBB sections were counted by using the computerized image analyzer (Video Pro 32, Leading Edge, Sydney, Australia) at a final magnification of ×400. Results were expressed as positive cells/mm^2 of lamina propria, and positive vessels/mm^2 of lamina propria respectively. In

addition, the absolute area of epithelium stained for TNF-α was measures and expressed as a percentage of the corresponding total epithelial area (178), rather than counting positively stained cells, in order to avoid the influence of the pseudostratified epithelial structure, hyperplasia or metaplasia on epithelial cell counts.

3.3.2.6 TGF-β_1 and TNF-α Assays

The concentration of TGF-β_1 in the unconcentrated BAL fluid was measured using a commercially available sandwich ELISA kit (Amersham UK) according to the manufacture's instructions. Since the TGF-β_1 assays used in this study only detect the biologically active form of TGF-β_1, immediately prior to the assay, all samples need to be "activated" by transit acidification using 1 M HCl as described in Chapter II. The detection range of the assay was 15.6-1000 pg/ml with sensitivity of 4 pg/ml, defined as the concentrations on standard curve equivalent to 2.0 standard deviation above the zero standards. This assay is reported by the manufacture to show no detectable crossreactivity with TGF-β_2 or TGF-β_3, nor with a range of other growth factors and cytokines.

TNF-α in the unconcentrated BAL fluid was measured using a chemiluminescent ELISA with commercially available kits (R&D, USA) according to the manufacture's instructions (Chapter II). The detection range of this assay was 0.7-700 pg/ml with sensitivity of 0.5 pg/ml.

All the measurements for both the TGF-β_1 and TNF-α assays were performed in duplicated and all in a single batch.

3.3.2.7 Statistical Analysis

Results were expressed as medians and interquartile ranges unless otherwise specified. Parametric variables (lung function data) were assessed using Student's unpaired t-test. All other comparisons between groups were made using the non-parametric Mann-Whitney rank order test, when the measurements were not normally distributed, and Chi-square test was performed for comparisons if the data were non-continuous. The Bland and Altman method (307, 308) was employed to analyse the agreement between two staining methods for a collagen deposit in tissue sections, i.e. comparison of histochemical and immunohistochemical stains. Correlation was evaluated by Spearman's rank method. A P value of less than or equal to 0.05 was considered as statistically significant.

3.3.3 Results

3.3.3.1 Patient demography and clinical characteristics

Thirty-two LTR were recruited. Patient demography and clinical characteristics are shown in Table 1. 15 were clinically stable at a median of 369 (183-1090) days post-transplant, and essentially maintaining their best post operative lung function ($FEV_{1.0}$ % best post-transplant: 98.8 ± 2.6%). 17 were diagnosed as having BOS and studied 729 (102-1343) days post-transplant, with $FEV_{1.0}$% best post-transplant of 52.7 ± 13.8%.

The 15 normal volunteers (3 females; age: 33±13 yr) recruited for bronchoscopy as controls had an $FEV_{1.0}$% predicted of 107 ± 12.9%.

3.3.3.2 Deposition of Collagen Types I, III and V in the Bronchial Lamina Propria

As demonstrated in Figure 1a-c, specific staining for collagen subtypes revealed that collagen type I, III and V were present and co-distributed throughout the lamina propria. The non-specific van Gieson and Masson's trichrom stains also revealed subepthelial collagenous matrix in the same areas of the lamina propria (Fig. 1d-e).

Table 2 summarises the quantitation of staining for type I, III and V collagen deposition in the lamina propria of the airways from the both LTR groups. Similar staining for collagen type I, III and V were found in the lamina propria of the airways in the LTR without BOS (Fig. 1 a-c, and Table 2), and the ratio of collagen type III to type I was 1.0. By contrast, in the BOS group, type III collagen deposition was more predominant than that of type I collagen (p=0.002, Table 2) with the ratio of collagen type III to I being 1.5. Between group comparisons showed that in BOS (Table 2), type III collagen deposition was significantly increased compared with non-BOS LTR group (p=0.048), and so was the ratio of collagen type III to I (1.5 vs 1.0, p=0.004, Fig. 2). The amount of type I collagen was significant lower in the BOS group (p= 0.004) compared with non-BOS group, while type V deposition was similar in the two groups (p = 0.31, Table 2).

3.3.3.3 Histochemical versus Immunohistochemical Stains for Collagen

Comparison of histochemical with immunohistochemical stains for collagen was performed in the total of 15 LTR subjects (8 LTR without BOS and 7 LTR with BOS), and data are shown in Table 2. Both the van Gieson and Masson's trichrome stains failed to detect the increased amounts of collagen deposition in the lamina propria of the airways in BOS, in contrast to the immunohistochemical stains with antibodies specific to collagen subtypes. Analysis (307, 308) showed poor 95% limits of agreement (i.e. mean difference of the two staining methods ± 1.96 SD) between the histochemical and immunohistochemical stains for collagen (Table 3, Fig. 3). For example, compared with collagen type I stain (Table 3), the van Gieson stain detected between 12.5% more and 21.3% less collagen deposition than the immunohistochemical stain, while between 21.8% more and 21.3% less with Masson trichrome stain (Fig. 3).

3.3.3.4 Expression of TGF-β_1 in Bronchial Biopsy and BAL Fluid

Immunostaining for TGF-β_1 was performed in a total of 33 EBB specimens (7 from controls, 13 from LTR without BOS, and 13 from LTR with BOS). TGF-β_1 immunoreactivity was found to be localized in bronchial epithelium and in the ECM of the lamina propria (Fig. 4). No labeling was observed when anti-TGF-β_1 antibody was omitted from the immunohistochemical protocol, or when non-immuno isotype IgG replaced the primary antibody.

Bronchial Epithelium. The positive staining was localized in both ciliated and basal epithelial cells with homogeneous intensity within each sample (Fig. 4). The presence of TGF-β_1 was

scored in the intact areas of the bronchial epithelium. Chi-square testing showed no significant difference in the presence of epithelial TGF-β_1 among the three groups (x^2=1.93, p=0.38). A summary of the results of anti-TGF-β_1 staining is given in Table 4.

Lamina Propria. In contrast to the epithelial staining, the overall intensity of the staining varied markedly and strikingly among the three groups (Fig. 4, Table 4). The ECM of the lamina propria in controls and LTR without BOS were brightly positive, while those in the LTR with BOS were consistently negative or at best displayed a weak signal (Fig. 4, $p<0.05$ vs both controls and LTR with BOS). Positive staining for TGF-β_1 in BOS was also observed in some inflammatory cells and bronchial vessels of the lamina propria in the EBBs with a weak or negative matrix stain, but were not quantifiable above the matrix staining in other groups, because the intensity of matrix staining did not allow differentiation of cell or vessel staining.

BAL Fluid. BAL data for fluid volume recovery, total cell counts and cell differential counts are shown in Table 5. The BAL fluid from the LTR, both with BOS (66 pg/ml, [42-112], p=0.0028) and without BOS (56 pg/ml, [40-61], p=0.0023) showed significantly increased TGF-β_1 levels compared with normal controls (21 pg/ml, [10-29]). In the BOS group, a non-significant trend toward higher TGF-β_1 levels was observed when compared with the LTR without BOS (Fig. 5, p=0.27), with a possible type II error as a results of the large variation of the TGF-β_1 levels in the LTR without BOS group. In addition, consistent with the data in the previous section, there was a higher percentage of neutrophils (with a corresponding lower percentage of alveolar macrophages), and also lower return of BAL fluid in the BOS group

compared to the normal controls and LTR without BOS ($p<0.05$ for all the comparisons; Table 5).

3.3.3.5 Expression of TNF-α in Bronchial Biopsy and BAL Fluid

Unlike the pattern of positive staining for TGF-β_1, ECM of the lamina propria did not stain for TNF-α. TNF-α staining was predominantly localised to the bronchial epithelium, inflammatory cells and endothelial cells in the lamina propria of the airways (Fig. 4). In addition, no significant differences were found among the three groups in the percentage of epithelium, the number of cells/mm^2 of lamina propria, or the number of bronchial vessels/mm^2 of lamina propria, which expressed TNF-α (Table 6).

Table 5 also summarizes the comparisons of TNF-α levels in the BAL fluid from the normal controls, and LTR with and without BOS. No statistical difference was found in the BAL TNF-α levels among the three groups ($p>0.05$ for all the comparisons, Table 5).

3.3.3.6 Correlation Analyses

The $FEV_{1.0}$ % best post-transplant, (but not absolute $FEV_{1.0}$ or $FEF_{25-75,}$) correlated inversely with the increased ratio of type III to type I collagen ($r=-0.60$, $p=0.039$, Fig. 6a) and TGF-β_1 BAL levels ($r=-0.57$, $p=0.034$, Fig. 6b). However, BAL TGF-β_1 levels did not correlate directly with collagen staining, although there was a weak trend for correlation between BAL TGF-β_1 and the ratio of type III to type I collagen in the BOS group (Fig. 6c, $r=0.44$, $p=0.2$). There was

no correlation between BAL TNF-α levels with collagen deposition, or with lung function measurements.

Table 1. Subject Demographics and Clinical Characteristics

	LTR w/out BOS (n=15)	LTR with BOS (n=17)	Controls (n=15)
Gender (f/m)	7/8	9/8	3/12
Mean age (yr) (mean ± SD)	36±8	41±15	33±13
$FEV_{1.0}$% predicted	--	--	107.1±12.9
Days post LTx	369 (183-1090)	729 (450-1343)	--
$FEV_{1.0}$ (L) (mean ± SD)	2.9±0.8	1.3±0.7	--
$FEV_{1.0}$ % best LTx[§] (mean ± SD)	98.8±2.6	52.7±13.8*	--
$FEF_{25-75\%}$ (L) (mean±SD)	4.2±1.4	0.6±0.5*	--
Original Diseases:			
Cystic fibrosis	n=5	n=5	--
COPD (or Emphysema)	n=0	n=7	--
Eisenmenger's syndrome	n=4	n=1	--
Primary pulmonary hypertension	n=2	n=2	--
Interstitial lung disease	n=0	n=1	--
Beonchiectasis	n=2	n=0	--
α_1-AT def. Emphysema	n=1	n=1	--
Emphysema &Valvular heart disease	n=1	n=1	--
[§]LTx:			
SLTx	n=0	n=9	--
BSLTx	n=7	n=5	--
HLTx	n=8	n=3	--

[§]LTx: lung transplantation; SLTx: single lung transplant; BSLTx: bilateral sequential lung transplant; HLTx: heart-lung transplant.
*$p<0.05$ compared with LTR without BOS;
#α_1-AT def. Emphysema: α_1-antitrypsin deficiency emphysema.

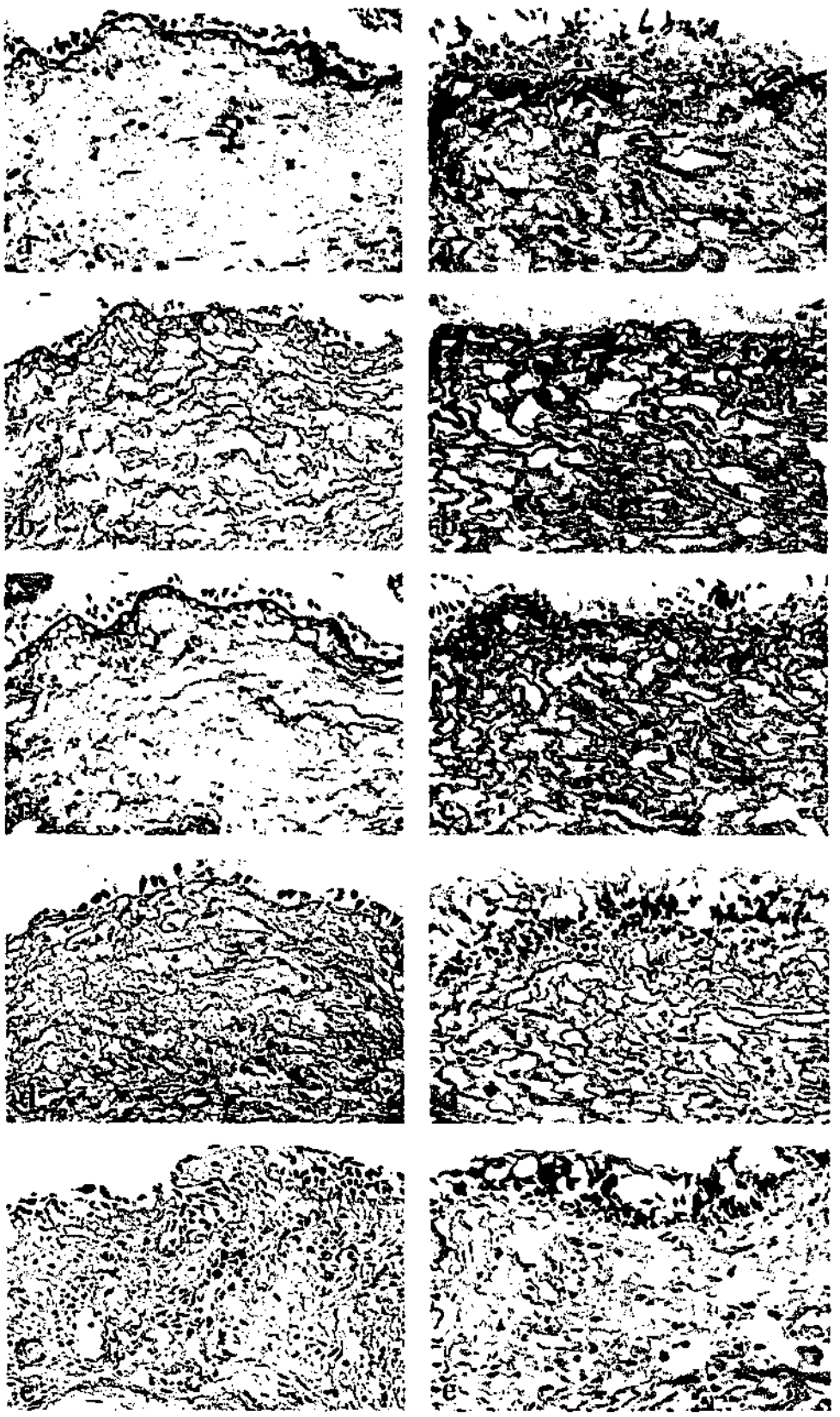


Fig. 1. Photomicrographs of immunohistochemical and histochemical stains for collagen in serial sections of EBB from a LTR with BOS (left) and a LTR without BOS (right): a) type I collagen; b) type III collagen; c) type V collagen; d) van Gieson stain; e) Masson's trichrom stain. (Each original magnification ×400)

Table 2. Comparison of Collagen Deposition between LTR with and without BOS

	LTR w/out BOS (n=10)	LTR with BOS (n=12)	*P value*
Collagen deposition, %			
Type I	49.5 (43.1-56.4)	37.4 (30.5-41.6)	*0.004*
Type III	48.3 (46.7-50.1)	54.5 (45.0-61.2)	*0.048*
Type V	49.8 (44.6-53.9)	46.9 (41.1-51.4)	*0.31*
Ratio of III/I	1.0 (0.8-1.1)	1.5 (1.2-1.8)	*0.0004*
van Gieson stain	42.4 (32-49)	34.3 (30-43)	*0.028*
Masson's trichrom stain	43.3 (39-52)	44.8 (37-48)	*0.845*

Data are shown as medians and interquartile ranges;
Collagen deposition is expressed as % of lamina propria;
Van Gieson and Masson's trichrom stains were only done in 8 LTR w/out BOS and 7 LTR with BOS.

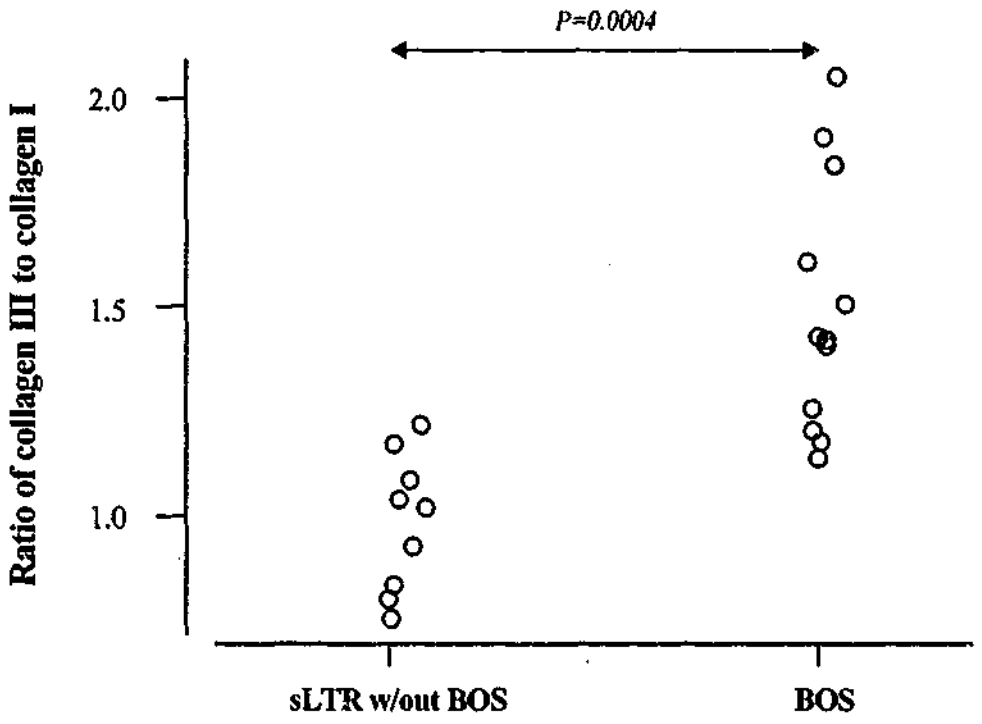


Fig. 2. The ratio of collagen type III to type I in the bronchial mucosa from LTR with and without BOS

Table 3. Agreement of Histochemical with Immunohistochemial Stains for Collagen

	*95% limit of agreement (%)	95% CI (%)
van Gieson-type I	12.5- -21.3	0.31- -10.6
van Gieson-type III	7.1- -35.5	8.3- -20.1
van Gieson-type V	3.8- -26.8	-7.3- -15.8
Masson's trichrom-type I	21.8- -21.3	6.4- -6.0
Masson's trichrom-type III	7.3- -26.4	-3.4- -15.8
Masson's trichrom-type V	7.8- -20.3	-2.2- -10.3

*95% limit of agreement (%): mean difference of the two staining methods ± 1.96 SD.

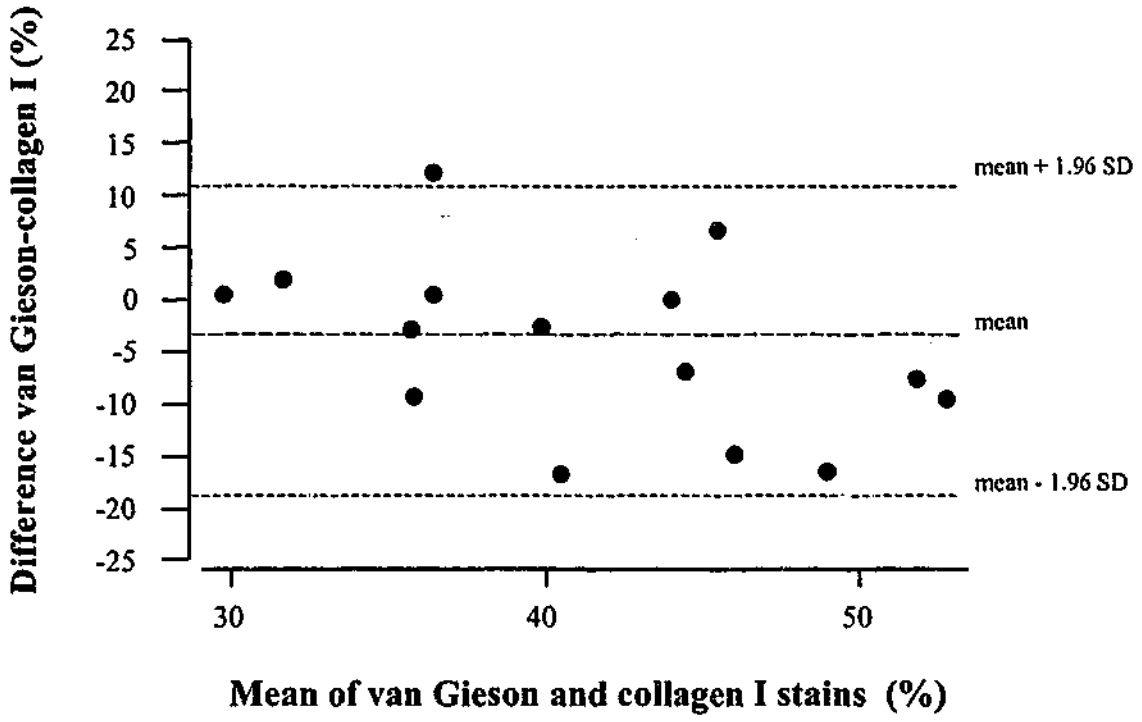


Fig. 3. Representative graphics showing the 95% limits of agreement between van Gieson and collage type I stain (top) and between Masson's trichrome and collagen I stains (bottom).

Table 4. Localization and Intensity of Immunoreactive TGF-β_1 in Bronchial Biopsies from Controls and LTR

	Bronchial epithelium*	ECM of lamina propria§
Controls:		
1	+	+++
2	NA	+++
3	-	+++
4	-	++
5	+	++
6	+	+++
7	+	++
8	NA	++
LTR without BOS:		
1	+	+++
2	+	++
3	-	+
4	-	+++
5	+	+++
6	-	++
7	+	++
8	-	+++
9	+	++
10	-	++
11	+	+++
12	+	++
13	+	+++
LTR with BOS:		
1	-	++
2	-	+
3	-	+
4	-	+
5	-	-
6	-	++
7	-	++
8	-	+
9	+	++
10	+	+++
11	NA	+++
12	+	+
13	+	+++

*Staining in the bronchial epithelium was scored as positive (+) and negative (-); NA: no intact epithlium available. § Staining in the ECM of lamina propria was scored as intensity: - = no staining; + = weak but detecble staining; ++ = moderate staining; +++ = marked staining.

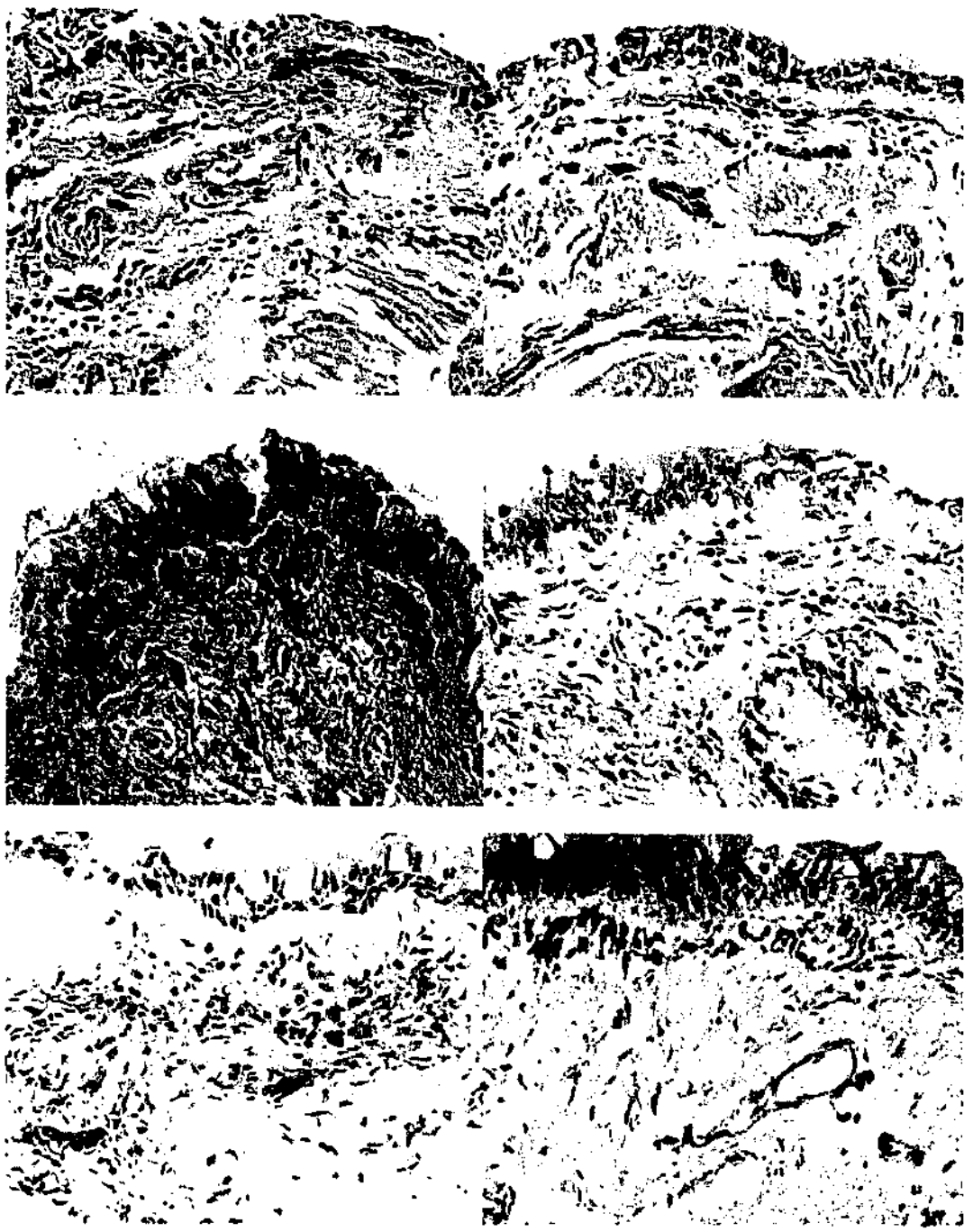


Fig. 4. Immunostainings for TGF-β_1 (left) and for TNF-α (right) in sections of bronchial biopsies from control (top); LTR without BOS (middle); LTR with BOS (bottom). (Each original magnification ×400)

Table 5. Comparison of BAL Cellular Profile and BAL TGF-β, TNF-α Levels Among LTR and Control Groups

	LTR with BOS (n=14)	LTR without BOS (n=12)	Controls (n=13)
BAL TGF-β_1 (pg/ml)	66.0 (42.0-112.1)**	56.1 (40.4-61.3)**	22.4 (10.4-29.0)
BAL TNF-α (pg/ml)	0.9 (0.1-8.1)	0.3 (0.27-0.79)	0.5 (0.3-0.7)
BAL return (ml)	59 (45-75)*	85 (59-100)*	111 (108-120)
Total cell counts (x10^4/ml)	13.4 (8.8-33.5)	15.0 (12.0-31.0)	12.8 (10.0-14.9)
Macrophages (%)	52.4 (37.2-55.3)	82.6 (52.6-88.9)	72.5 (66.4-76.8)
Lymphocytes (%)	11.4 (4.8-18.5)	9.3 (4.8-30.8)	19.8 (15.8-30.0)
Neutrophils (%)	34.0 (19.1-44.71)**†	3.6 (1.8-4.2)*	1.40 (1.15-2.94)
Eosinophils (%)	0.5 (0.0-0.9)	0.0 (0.0-0.6)	0.3 (0.0-0.58)
Epithelial cells (%)	1.5 (0.0-3.8)	2.2 (0.4-4.4)	1.2 (0.3-5.2)

Data are shown in medians and interquartile ranges.
*P<0.05 vs controls; **p<0.01 vs controls; †p<0.01 vs LTR without BOS

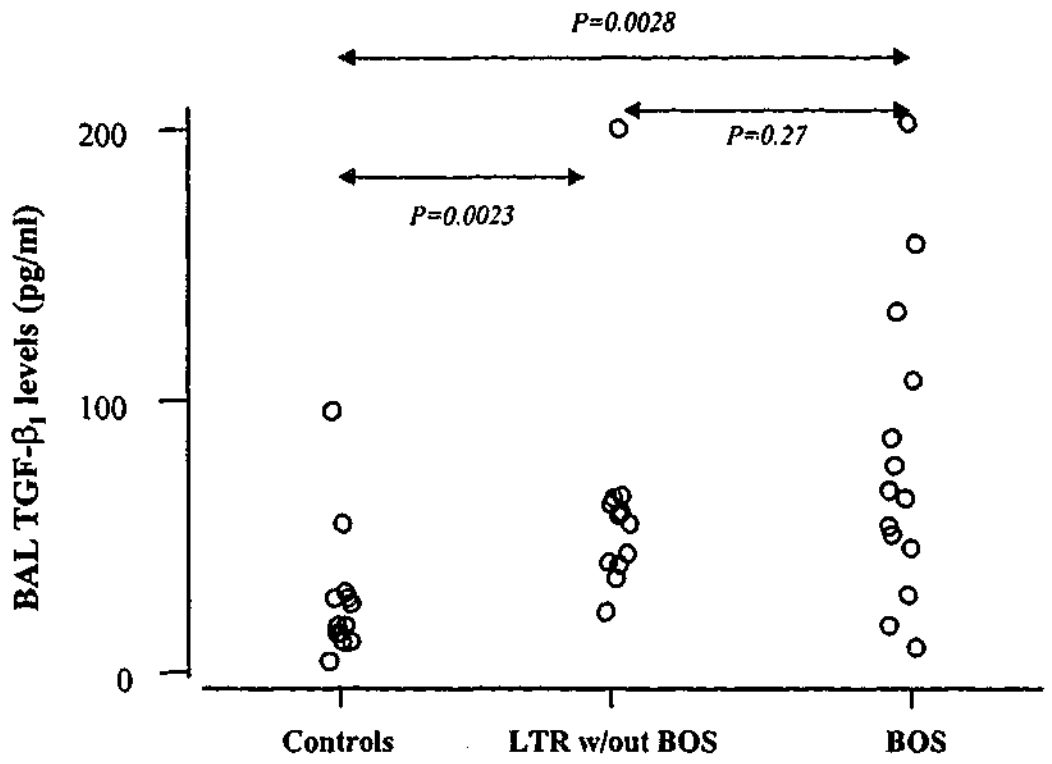


Fig. 5. Comparison of BAL TGF-β_1 levels between healthy controls and LRT with and with out BOS groups (pg/ml)

Table 6. TNF-α Expression in the Bronchial Biopsies from LTR and Controls

	LTR with BOS (n=12)	LTR w/out BOS (n=11)	Controls (n=12)
Bronchial epithelium (%)	16.2 (11.1-19.4)	17.7(10.3-24.7)	13.2 (7.8-17.8)
Bronchial vessels/mm^2 of lamina propria	100 (72-122)	126 (48-150)	145 (96-232)
Cells/mm^2 of lamina propria	160 (110-280)	238 (120-297)	330 (190-510)

Data are shown in median and interquartile range.

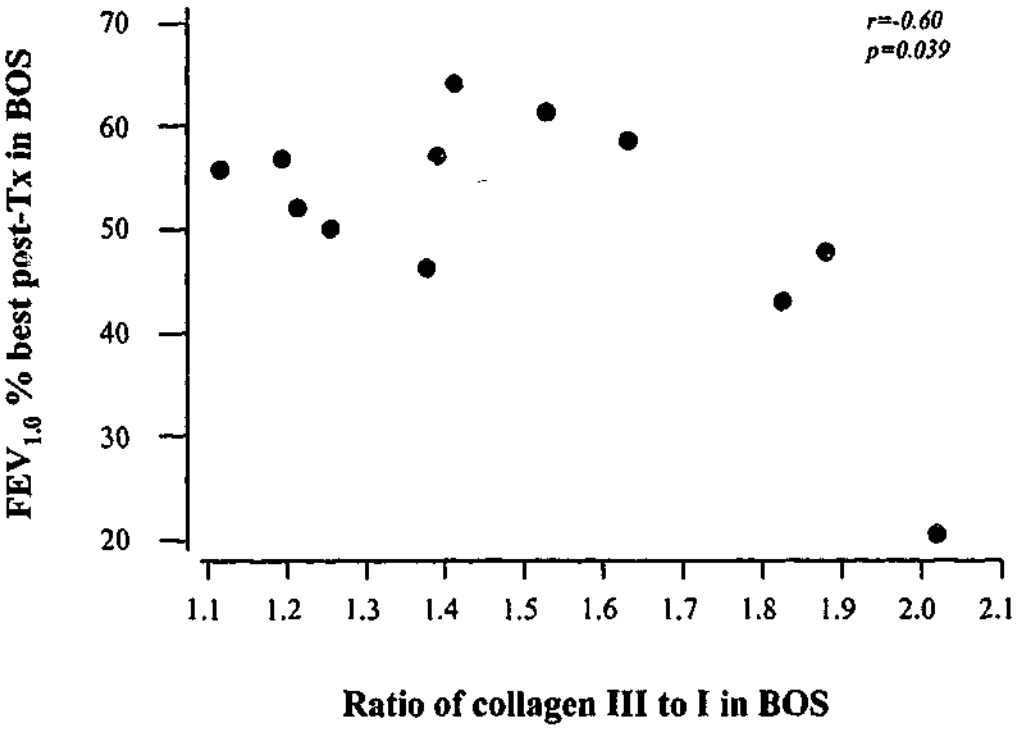


Fig. 6a. Correlation between the ratio of collage type III to type I and $FEV_{1.0}$% best post transplant (Tx) in LTR with BOS.

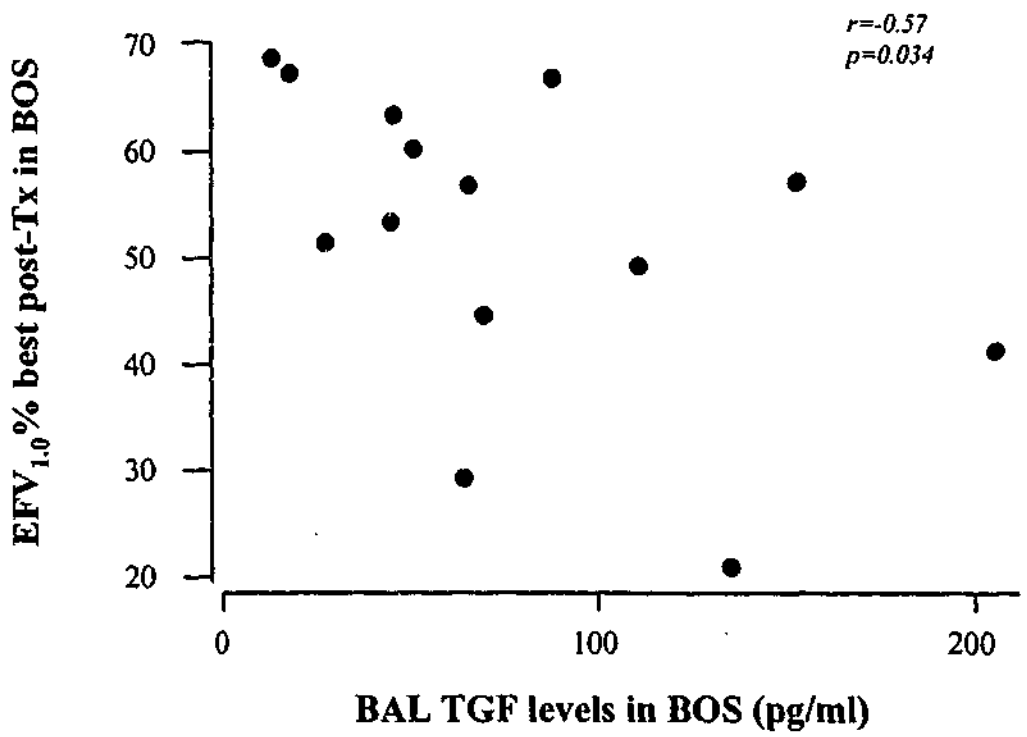


Fig. 6b. Correlation between BAL TGF-β_1 levels (pg/ml) and $FEV_{1.0}$% best post transplant (Tx) in LTR with BOS

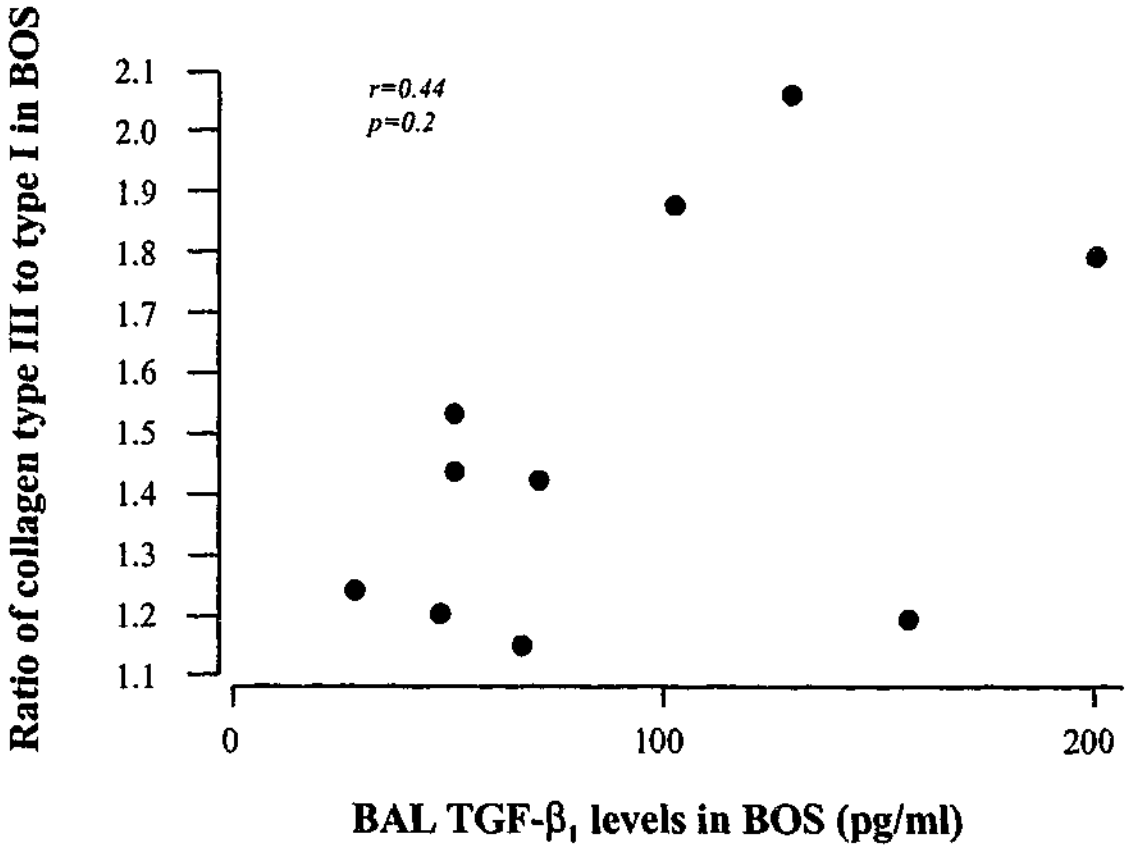


Fig. 6c. Correlation between BAL TGF-$_1$ levels (pg/ml) and the ratio of collagen type III to type I in LTR with BOS.

3.3.4 Discussion

This study compared the deposition of the three fibrillar collagen types (I, III and V) in biopsies of segmental level airways in lung allografts with and without BOS, and compared the distribution and expression of TGF-β_1 and TNF-α in the airway biopsies and BAL fluid from LTR and normal control subjects. In the LTR with BOS, there was a significant increase in type III collagen deposition and also in the ratio of type III to type I in the lamina propria of the airways, which correlated inversely with $FEV_{1.0}$ % best post-transplant. In addition, BAL TGF-β_1 levels were markedly elevated in the LTR both with and without BOS, and they correlated with $FEV_{1.0}$ % best post-transplant negatively in the BOS subjects. However, the difference in BAL TGF-β_1 levels was not significant between the two transplant groups. In contrast, the expression of TGF-β_1 in bronchial epithelium of the airways was similar in all the three groups, but it was significantly weaker in the lamina propria of the airways from BOS subjects when compared with both controls and LTR without BOS. TNF-α levels in BAL fluid and TNF-α expression in airway biopsies were not significantly different among the studied groups. No correlation was found in BOS between collagen deposition and the expression of TGF-β_1 or TNF-α in airway walls, nor in BAL fluid.

The clinical correlate of chronic airway rejection in the lung allograft is thought to be BOS, manifest pathologically as extensive scarring of the large and small airways, i.e. BO (29). It is generally accepted that open lung biopsy is the gold standard for histopathological diagnosis of BO, with sensitivity approaching 100%. However, sequential open lung biopsy

in transplant recipients is clearly impractical (29, 75). Transbronchial lung biopsy is likely to be the most specific way of defining chronic rejection changes in the small airways, but bronchioles are difficult to assess quantitatively, and the sensitivity of making a diagnosis in this way is not clear (75). We have demonstrated excessive scar collagen deposition, presumably secondary to chronic airway inflammation in the airways of BOS subjects; our data suggest that endobronchial biopsies may be helpful in monitoring airway remodelling occurring as a part of chronic lung rejection.

Several subtypes of collagen proteins have been found in the normal human lung. In the healthy adult, approximately 90% of lung collagens consist of the fibrillar types I and III in a ratio of 2:1 (295, 309, 310). They are concordantly distributed in the interstitium, airways, and blood vessels (295). It would seem that collagen types I and III play critical roles in maintaining the structural and functional integrity of the pulmonary system. Type V collagen has been found in small amounts in basement membranes and in association with type I collagen in the interstitium (295).

Previous studies of collagen in fibrotic disorders have suggested that the deposition of collagen type III is a phenomenon observed in the early stage of fibrosis, whereas collagen type I is the predominant collagen deposited at a later pathological stage (295). In skin, for example, increased type III collagen has been demonstrated in early scar tissue in both animal and humans as compared with increased type I collagen in older scare (311, 312). In the lung, immunohistochemical techniques have indicated that areas of "early" fibrosis in the expanded interstitium contain more type III collagen than the normal lung, whereas in areas

of dense or established "late" fibrosis, type I collagen predominates (306, 313, 314). These findings are in concordance with biochemical studies (310), and have been confirmed by serial studies of immunohistochemical staining of collagen types in several fibrotic lung and non-respiratory diseases such as IPF, sarcoidosis, ARDS, asthma, liver and renal fibrosis (195, 295, 306, 313-317). In this context, the current finding that the proportion of collagen type III was increased in airway biopsy samples from LTR with BOS might be a potential signal for early stage progression to terminal chronic lung allograft dysfunction.

The fact that there was a relationship between the ratio of collagen III and I to airway function gives this hypothesis biological plausibility. The physiological implication of changes in collagen ratio is increasingly recognized. Shifts in the ratio of collagen types have been reported in IPF (310, 316), ARDS (309), cirrhotic liver (316), and atherosclerotic blood vessels (318). In this study, the ratio of collagen type III to type I in the airways in BOS LTR was significantly increased with an increase in the amount of type III and decrease in the amount of type I compared to LTR without BOS. It suggests that there is newly synthesised collagen type III in the airways of lung allograft in LTR with BOS in our study. Human lung fibroblasts have been shown to be the major producer of both type I and type III collagen. In normal adult lung, there is a balance between synthesis and degradation of collagens to maintain the amount of collagen type I and III constant (295, 306, 309, 310). Presumably, in a fibrotic process, there is a dynamic shift in collagen types in response to inflammation and other stimuli, with a distinct population of fibroblasts proliferating and initially switching to predominant synthesise of type III collagen to form early scar collagen deposition (295, 306, 310). Later there may be a switch to type I synthesis to form the mature scar (295, 306, 310).

There have been few studies of collagen V performed in human airways. One study showed that the thickened ECM associated with the epithelial basement membrane in asthmatic airways stained strongly for collagen V (195). Experimental studies have suggested that type V collagen may regulate the assembly and hence the size of type I collagen fibrils (319, 320). A recent study suggested a role for collagen type V in preventing development of allograft rejection in *vitro*, as collagen type V may have a MHC-like sequence and MHC-derived peptides have been used to induce tolerance in allografts other than lung (321). However, we did not find a significant change in collagen V deposition in LTR with BOS compared to their stable counterparts.

Compared with immunohistochemical staining for collagen, the histochemical methods of van Gieson and Masson's trichrome failed to detect the increased amount of collagen deposition in the lamina propria of the airways in BOS, which is supported by previous studies of collagen types in human fibrotic lung diseases (313, 322). It appears that immunohistochemical methods can be a more useful and sensitive approach for detecting small increase in lung and airway collagens, possibly at stages when they are not detectable with the non-specific histochemical stains for connective tissue, and can be also an important application for defining active or early pathological fibrosis.

TGF-β family has been shown to be the most potent stimulator of collagen production (172, 323, 324). It maximises the amount of collagen produced through stimulating procollagen gene transcription (325-328), increasing mRNA stability (325, 328), decreasing intracellular

degradation of procollagen (329) and limiting extracellular degradation of collagen (330-332). However, in the current study, we were not able to demonstrate a direct association between our collagen measurements and the assessments of TGF-β_1 expression in either airway biopsy or BAL specimens from BOS subjects. But this cannot rule out a role for TGF-β_1 in the development of transplant BOS, as the increased BAL TGF-β_1 levels in the BOS patients did correlate significantly with the their lung function ($FEV_{1.0}$% best post-transplant), the main physiological consequences of airway fibrosis in LTR who developed BOS. This observation of a negative correlation between BAL TGF-β_1 levels and lung function was subsequently confirmed in LTR by an another recent study (333) reported subsequent to this work. In addition, a lack of correlation between airway collagen deposition and TGF-β_1 expression has been reported also in patients with asthma (179, 322). This is perhaps not surprising, as it would be the cumulative effect of growth factors, individually and together, which is important, and this influence may not be well represented in a single time point measurement of a single growth factor. In addition, individual growth factors may affect ECM metabolism not only directly by stimulating synthesis, but also indirectly by affecting cell proliferation, such as fibroblast proliferation. Such indirect relationship may be difficult to detect with the limitation of the methods used.

TGF-β_1 was not only increased in BAL fluid from BOS, but also from LTR without BOS, which is supported by the recent study that compared BAL TGF-β_1 concentrations in 28 clinical stable LTR with normal healthy controls (334). This could mean that all LTR are at risk to a varying degree of development of airway fibrosis, and may suggest that an increase in BAL TGF-β_1 could serve an early predictive and prognostic marker of airway fibrosis. By

using competitive reverse transcription polymerase chain reaction (RT-PCR), Charpin et al (335) and Bergmann et all (336) demonstrated more direct evidence that patients with chronic lung rejection had marked peaks of TGF-β_1 mRNA expression by BAL cells, and these peaks preceded the diagnosis of chronic rejection by several months. Magnan, et al have also reported similar findings from their study that suggested an increased TGF-β_1 secretion by alveolar macrophages from LTR preceded the functional diagnosis of BOS (141). Therefore, monitoring TGF-β_1 levels in BAL fluid could be of practical value in early detection of lung transplant BOS in LTR, but this need to be tested in prospective studies.

In the present study, we also demonstrated that immunoreactive TGF-β_1 is present in bronchial biopsy specimens from both LTR and healthy controls. In both cases, the major sites of localization were extracellular matrix (ECM) and bronchial epithelial cells. The pattern of staining appeared to be specific, as no immuno-labeling was detected in control sections in which the primary antibody was either omitted or replaced by an irrelevant mouse IgG protein. However, in contrast to the findings of BAL TGF-β_1, the overall intensity of matrix-associated TGF-β_1 was weaker in the bronchial biopsies from BOS than from LTR without BOS and health controls, while the presence of epithelial TGF-β_1 was indistinguishable among the groups. TGF-β_1 is one of several cytokines that bind avidly to a variety of ECM components such as elastin, fibronectin, laminin, basement membrane proteins, proteoglycans, and decorin (124, 337, 338). This association of TGF-β_1 with ECM proteins suggests a critical role for ECM in modulating the activity of TGF-β_1. It may also explain the immunohistochemical localization of TGF-β_1 to ECM in human airway walls

demonstrated by our study as well as by others (178, 298-303, 306), and the apparent decrease in binding in BOS may be very relevant.

Of particular relevance in the context of ECM protein interaction is the binding of TGF-β_1 to decorin, because this interaction has been shown to neutralise the biological activity of TGF-β_1 both *in vitro* and *in vivo* (337-340). Decorin, a small dermatan sulphate proteoglycan, is an abundant component of ECM (3), and has been found to be co-localized with TGF-β_1 at subepithlial connective tissue sites in normal and asthmatic airways (337). Therefore, it allows speculation that ECM provides an extracellular site in which a pool of TGF-β_1 can be sequestered and may be released in active form in response to appropriate stimuli (337, 338). In the context of transplant BOS, one stimulus could be graft injury induced by either immunological or non-immunological attack, which would release and activate the bound TGF-β_1 from the ECM of the airway walls. Although I was not able to show a significant increase in BAL TGF-β_1 levels in BOS group compared with the non-BOS LTR in the study, this could possibly be due to type II error as a results of the large variation of the TGF-β_1 levels in the LTR without BOS group (Fig. 5). In addition, or alternatively, the scarcity of matrix-associated TGF-β_1 seen in BOS could be also due to the increase amount of type III collagen in the ECM of the airways, as decorin has been particularly associated with type I collagen in tissues (295). Co-distribution of TGF-β_1 staining with procollagen type I have been also shown in human IPF and in a silica-induced model of pulmonary injury (303, 341).

Two previous studies contrast with our results (126, 342). They found a greater expression of TGF-β_1 in transbronchial biopsy specimens from BO patients in comparison to patients

without BO (126, 342). More likely, the difference in the nature of samples or in subject population studied accounts for this discrepancy. Thus in these reports, they studied transbronchial lung biopsies obtained from subjects diagnosed pathologically, i.e. with BO rather than the clinical diagnosis of BOS (126, 342), and were likely to be more acutely unwell, and indeed probably had acute lung rejection. In contrast, our specimens were endobronchial biopsies from patients with a physiological diagnosis of BOS, and in a subacute or probably chronic illness phase. Moreover, in both the previous studies, the antibody used was polyclonal (126, 342), while we used a monoclonal antibody that recognises the TGF-β_1 isoform more specifically.

Bronchial epithelial cells appear to be a major source of TGF-β_1 (298, 343), although most cellular constituents of the airway wall have the potential to contribute to expression of this cytokine in the airways, including endothelial cells, macrophages, lymphocytes, fibroblasts and smooth muscle (165, 168, 171, 299, 344, 345). However, in the literature, immunohistochemistry studies on TGF-β_1 expression by airway epithelium have yielded conflicting data, at least in asthma (178, 299-301, 337). In some studies TGF-β_1 expression was found to be increased in asthmatic airways (178), but not in others (299, 300, 337), while another studies showed even negative or only faint positive staining for TGF-β_1 (301). Anthony et al have reported an increased concentration of TGF-β_1 in BAL fluid of asthmatics (179), but failed to find evidence of a difference in TGF-β_1 expression in airway biopsies between asthmatic and control subjects (337), which is consistent with our differential findings on TGF-β_1 expression in the airway epithelium versus BAL fluid among LTR and control groups. The significance of our observation is unknown and will be difficult to

elucidate at least in human, as TGF-β is synthesised, stored and secreted in a latent form and must be activated in the airway microenvironment for its biological activities in inflammatory and remodelling process after secretion (124).

Although TNF-α has been implicated in the pathogenesis of pulmonary fibrosis (305, 346, 347), I failed to find a difference in TNF-α expression in either the BAL fluid or airway biopsy specimens from normal control versus LTR groups. However, these results are supportive of other recent findings by Elssner et al (333), who also reported an increase in TGF-β_1 concentrations, but not in TNF-α, in BAL fluid from BOS patients, and by Magnan et al (141, 163), who observed that in contrast to acute rejection, CMV and bacterial pneumonia, the production of TNF-α and IL-6 by alveolar macrophages was not significant elevated, whereas TGF-β was, in BOS. It is thought that there are two phases in the inflammatory reactions of tissues: a phase of tissue injury followed by a phase of repair. Distinct cytokines probably orchestrate each of these phases. The pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 participate in the first phase, whereas potentially anti-inflammatory and pro-fibrotic cytokines and growth factors, including TGF-β_1 and PDGF participate later to organise tissue repair or remodelling to limit the acute inflammatory response (141).

This study, although, was not designed to investigate the fibrogenic effect of CsA on lung allografts, accumulating evidence from recent studies (111, 112, 348) lead me to speculate that the use of CsA in LTR may account for a number of my findings, such as the increased airway wall collagen III deposition in BOS and expression of TGF-β_1 in both BAL fluid and

airway biopsies from the studied LTR. For example, Bicknell et al showed that the expression of mRNA for collagen III in renal biopsies was significantly higher in patients receiving CsA therapy than in those taking tacrolimus (112). Mohamed et al reported a significant greater expression of active TGF-β_1 in renal biopsy specimens from patients receiving CsA compared with tacrolimus (111), and a *in vitro* study strongly suggested a direct fibrogenic effect of CsA on human tubulointerstitial cells (348). It appears that despite its important role as a potent immunosuppressant, CsA up-regulates TGF-β_1 production and promotes collagen deposition, at least, in human renal allografts (111, 112, 348), but little is currently known in human lung transplant context. Thus, further studies are needed to clarify the potential fibrogenic role for CsA in human lung allografts. Such an association between CsA and fibrosis, if it exists, would have significant effect on long-term graft outcome. The dual action (immunosyppressive and fibrogenic) of CsA might explain why this drug, although revolutionary in organ transplantation, might not be optimal for long-term graft survival.

In conclusion, this study revealed, for the first time, pathological evidence of excessive collagen deposition in the airways of LTR who have developed BOS, and suggests that these structural changes may be a consequence of fibrogenic growth factor (TGF-β_1) release associated with ongoing airway inflammation after lung transplantation. Monitoring TGF-β_1 levels in BAL fluid routinely during the follow-up of lung transplant recipients could be of practical value in predicting the likely development of airway fibrosis occurring in the lung allografts, but this needs further longitudinal work for confirmation. Bronchial biopsy, by directly sampling airway wall mucosa, has provided much valuable and novel data to help

better understand the airway remodelling that occurs in chronic lung rejection, and its physiological implications.

3.4 Expression of TGF-β_1 mRNA and TGF-β_1 Protein in Alveolar Macrophages from Lung Allografts

3.4.1 Introduction

Pulmonary or lung macrophages consist of several subpopulations, such as alveolar macrophages, airway macrophages and interstitial macrophages, as defined by their anatomical locations in the lung (349). Alveolar macrophages (AM) reside on the alveolar epithelial surface (350). Airway macrophages are found on and in the epithelial lining of conducting airways and in bronchus-associated lymphoid tissue (351-354). Interstitial macrophages are located in perivascular, peribronchiolar, and visceral pleural sites, as well as in the interstitium of the alveolar region (349). By conventional bronchoalveolar lavage (BAL), more than 90% of the macrophages obtained are AM, whereas the airway macrophages, that are present on the intraluminal surface of conducting airways, account for 5-8% of the them (355).

Pulmonary macrophages have come to be recognised as critical cytokine secretory cells involved in orchestrating local inflammatory and immune response of the lungs. In accord with their pluripotent role, the macrophage secretory products can be proinflammatory (IL-1, IFN-γ, TNF-α), anti-inflammatory (IL-10), mitogenic for mesenchymal cells (PDGF, FGF, IGF-1), or directly fibrogenic (TGF-β) (356). Thus, macrophages are armed to play a critical role in every aspect of the pathogenesis of pulmonary fibrosis and at each stage of airway disease (356). In both human fibrotic lung disease and animal models, there is increasing evidence of macrophage activation, and increased production of profibrotic cytokines (296).

In the previous section (Chapter III, 3.3), I have given data demonstrating markedly elevated BAL TGF-β_1 levels in LTR, both with and without BOS, and an association between the increased BAL TGF-β_1 levels and the decline of lung function in BOS patients. This was also supported by another previous study from our group (334). It could mean that all LTR are at risk to a varying degree of development of airway fibrosis following transplantation, and may suggest that an increase in BAL TGF-β_1 could serve as an early predictive and prognostic marker of airway fibrosis. Although TGF-β_1 can be generated by a number of different cell types (124), AM are one of the potential sources of TGF-β. In this study, we sought to determine whether AM from LTR were likely to be responsible for elevated BAL TGF-β_1 levels.

3.4.2 Methods

3.4.2.1 Study Population and Exclusion Criteria

Fourteen clinically stable LTR were recruited from the Lung Transplant Service of the Alfred Hospital between 1996 and 1998. The recruiting criteria for patients were described in the previous section (Chapter III, 3.1). All patients were receiving a standard maintenance regimen of immunosuppressive therapy comprising cyclosporine (to achieve a blood level of 200-350 μg/L by EMIT assay, Syva, California, USA), azathioprine (1-2 mg/kg/day) and prednisolone (0.15-0.25 mg/kg/day).

We also recruited 14 control subjects, who were normal healthy volunteers, non-smokers, and free from known respiratory diseases. They were all asymptomatic with normal lung function.

Approval for this study was given by The Alfred Hospital Ethics Committee, and each subject gave written informed consent.

3.4.2.2 Bronchoalveolar Lavage

BAL was performed in all the subjects through a bronchoscopy procedure under intravenous sedation with midazolam (Roche, Paris, France) as described in Chapter II. BAL supernatants were stored at -80°C until TGF-β_1 assays. BAL cell counts and cell differential counts were determined, viability was assesses by exclusion of trypan blue stain (Chapter II). Some BAL cells pelleted (not less than 2 million/specimen) were snap frozen and stored at –80°C until TGF-β_1 PCR. Other cells (about 5 millions/specimen) were resuspended, and washed for the following AM culture experiments.

3.4.2.3 Alveolar Macrophage Culture

AM were obtained and separated from BAL cells as previously described in Chapter II. Of the 14 LTR and 14 controls, AM were obtained from 9 patients and 10 controls, where there were enough BAL cells for doing the experiments. Briefly, on average 5 million BAL cells with viability of >85% were retained from each BAL specimens for the cell culture. After

three washes with PBS, the BAL cells was resuspended at a concentration of $1x10^6$ AM/ml (determined by total cell count and cell differential count) in RPMI 1640 medium supplemented with L-glutamine (2 mM), penicillin (200 μ/mL) and streptomycin (200 μg/ml). Then, the cells were allowed to adhere onto plastic tissue culture plates (0.5×10^6 AM per well, 24 well plate) at 37.0 °C in a 5% CO_2 humidified atmosphere for 1 hour to separate the AM from the rest (357). After adherence, purity of the cells was assessed by May-Grunwald-Giemsa stain, more than 95% were macrophages.

The purified AM were incubated for 24 hours in the presence or absence of LPS (1μg/ml) at 37.0 °C in a 5% CO_2 humidified atmosphere. The culture supernatants were then recovered and stored at –80 °C until assayed for TGF-β_1. The cultured AM were lysed by using Lysis Buffer RLT (Qiagen, Qiagen Pty Ltd Australia), and the lysed AM were stored at –80 °C until RNA extraction for RT-PCR.

3.4.2.4 Measurement of TGF-β_1 Protein in BAL and AM Culture Supernatants

Concentrations of TGF-β_1 in the unconcentrated BAL fluid and AM culture supernatants were measured using a commercially available sandwich ELISA kit according to the manufacture's instructions. Samples were "activated" before assay by transit acidification using 1 M HCl; the details were descried in Chapter II. The detection range of the assay was 15.6-1000pg/ml. This assay is reported by the manufacture to show no detectable crossreactivity with TGF-β_2 or TGF-β_3, nor with a range of other growth factors and cytokines. All the measurements were performed in duplicated.

3.4.2.5 RNA Extraction and Measurement

Total RNA was extracted from BAL cells and the cultured AM by employing RNeasy Mini Kits (Qiagen, Qiagen Pty Ltd Australia). Cells were homogenised in 700 µl of lysis buffer RLT by aspiration 10 times through 21-gauge syringe needle to shear chromosomal DNA. RNA was eluted from the spin column with 40 µl of RNase-free water provided (Qiagen, Qiagen Pty Ltd Australia). Aliquots of 5 µl of RNA were quantitated by capillary spectrophotometry by using an Absorbance Capillary Adaptor Cell (Helix, San Diego, CA, US), background correction at 320 nm and a Cary 1 spectrophotometer (Varian, Melbourne, Australia). Samples with $(A_{260}-A_{320})/(A_{280}-A_{320})$ ratio less than 1.7 and/or yields less than 0.5 µg of total RNA were excluded from subsequent analysis.

3.4.2.6 Reverse Transcription

cDNA was synthesised from up to 1 µg of RNA in a volume of 20 µL consisting of 2 µl of GeneAmp PCR buffer (10 fold concentrated), 2 mMol/L $MgCl_2$, 1 mMol/L deoxyribonucleoside triphosphate, 0.8U of Rnase inhibitor, 2 µMol/L Oligod$(T)_{16}$, and 2U of MuLV reverse transcriptase (all from Perki-Elmer, USA). Reactions were incubated for 1 minute at 15°C, and then the temperature was increased to 42°C over 9 minutes followed by 1 hour at 42°C., 5 minutes at 85°C, and 1 minute at 4°C.

3.4.2.7 Competitive PCR

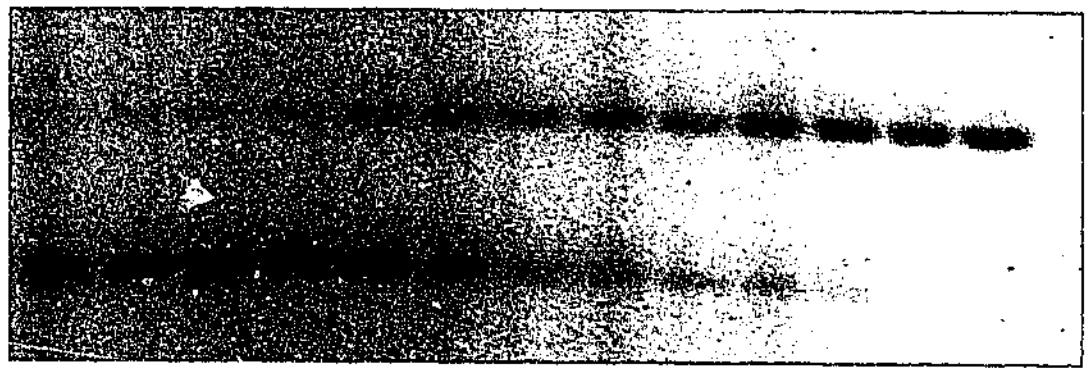
Competitor Template and Primers: A DNA competitor template for TGF-β_1 was obtained from Clontech commercially, with a PCR product size of 270 bp (Palo Alto, CA, USA). It has been successfully tested in a competitive PCR experiment using CLONTECH's Transforming Growth Factor-β_1 Amplimer Set and the positive control PCR product that accompanies these primers by the manufacturer. The primers (upstream GCCCTGGACACCAACTATTG, and downstream AGGCTCCAAATGTAGGG) were synthesised by GeneWorks, and redesigned using Oligo 5.0 Primer Analysis Software (National Biosciences, Plymounth, MN, USA) to improve their specificity and annealing profiles. The upstream primer was labeled with fluorescein during synthesis by the manufacturer to facilitate PCR product detection by fluorescent scanning of agarose gels in our FluorImager.

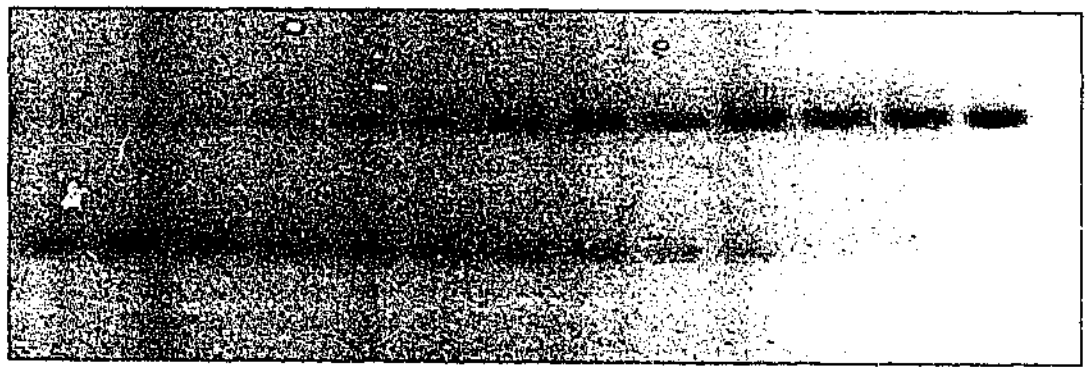
Validation of Competitive PCR Assay: As described in Chapter II, competitive PCR is based upon the co-amplification with gene-specific primers of an "experimental" cDNA produced from the native RNA and an artificial internal standard (the competitor) of different size, where the input ratio of native to competitor remain unchanged throughout the PCR amplification and the subsequent detection process, or the output ratio should be equal to the input ratio.

To test this, a "changing ratio" experiment was performed, in which the concentration of native cDNA synthesised from one of the cultured AM samples was used and kept constant in the PCR, while the amount of DNA competitor for TGF-β_1 was varied in 2 fold steps of

increase from 10 to 20680 copies/reaction. The "changing ratio" test was prepared in triplicate reactions (50 μl/reaction) and amplified for 37 cycles, which were determined as optimal in preliminary experiments. The PCR products were resolved by electrophoresis in 3% agarose gels (Fig. 1). The PCR product band volumes were quantified with ImageQuaNT software (Molecular Dynamics, USA), and expressed as output ratio of native to competitor.

Although the concentration of input template cDNA was constant in the PCR reaction for the input/output experiments, the concentration of input native copies of target gene was unknown. Thus, relative input ratios were calculated by obtaining an estimation of the input native copies from the average results of the triple PCR at the output ratio closest to 1.0 (358), and then, plotted against output ratio (Fig. 2). It was found that in the assay the competition between DNA competitor and native cDNA for TGF-β_1 was not ideal in terms of balanced rates of amplification, but did follow a linear regression of [Log (output ratio)= 0.49Log (input ratio) + 1.48×10^{-6}] (Fig. 3) (358). Therefore, this equation was used as a relative standard curve for TGF-β_1 competitive PCR in this study in calcu. ng TGF-β_1 mRNA copy numbers in patient samples (358).





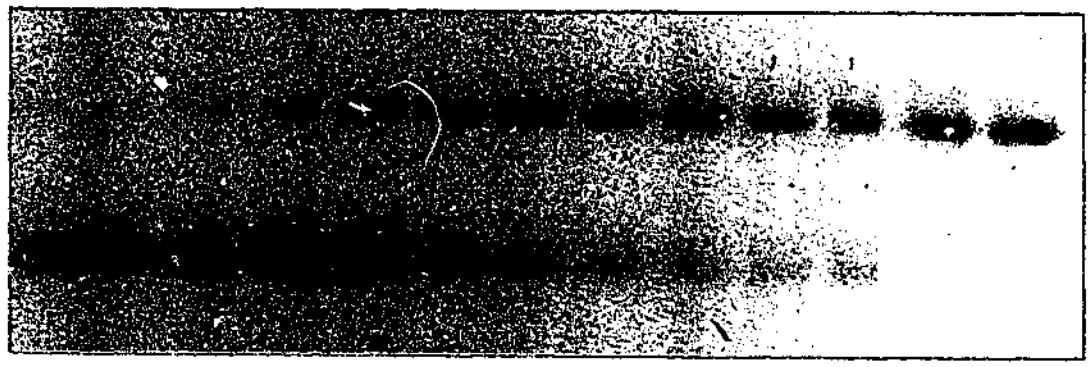


Fig. 1. Agarose gels of the triplicate change ratio experiments of TGF-β_1 competitive PCR. TGF-β_1 competitor was titrated in triplicate 2-fold steps against a constant aliquot of cDNA from cultured AM

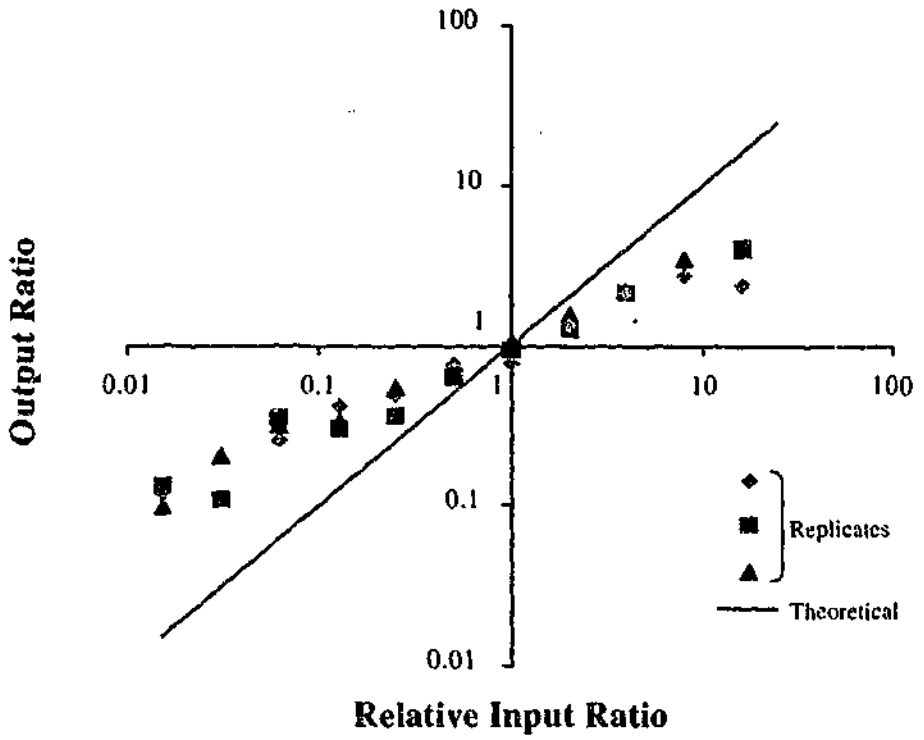


Fig. 2. Plot of output ratios from the three replicate change ratio of TGF-β PCR against the relative input ratios. The average results of the three replicates close to 1 were used to calculate the relative input ratio. The data do not conform to a theoretical line of slope 1 required for direct calculation of native native cDNA from the output ratio (Analysis done by Dr. E. Glare [Ref 358]).

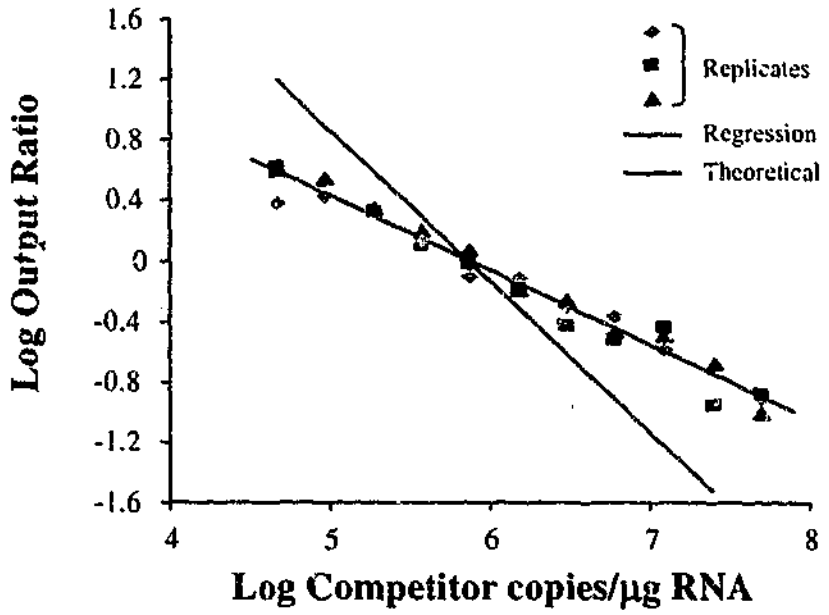


Fig. 3. Plot of native to competitor output ratios against the number of molecules competitor added. The data fit the regression well but do not conform to theoretical line of slope-1 required for quantitative competition. (Analysis done by Dr. E. Glare [Ref 358]).

TGF-β_1 Competitive PCR of Sample RNA: For competitive PCR, 20 µl of cDNA synthesised from BAL cells or cultured AM was made up to a 50 µl reaction, consisting of 2 U AmpliTaq Gold, 5 µl of GeneAmp PCR buffer, 2.5 mMol/L $MgCl_2$, deoxyribonucleoside triphosphate (all from Perkin-Elmer), 0.2 µMol/L upper and lower primers and appropriate amount of competitors. The temperature profile commenced with initial denaturation of 8 min at 94 °C, followed by 37 cycles of 94 °C for 1 min, annealing at 60 °C for 1 min, and 72 °C for 1 min, concluding with 7 min at 72 °C and 1 min at 4 °C.

The PCR products were resolved by electrophoresis in 3% agarose gels and Tris-acetate-EDTA buffer, and quantified with ImageQuaNT software (Molecular Dynamics, USA), as described in Chapter II. Native-competitor product ratio were calculated on the relative standard curve as outlined above, and the levels of gene expression were reported as copies of mRNA/µg RNA/10^6 AM in BAL cells, and as copies of mRNA/µg RNA in cultured AM.

3.4.2.8 Statistical Analysis

Results were expressed as medians and interquartile ranges unless otherwise specified. Differences between groups were examined with the Mann-Whitney rank order test, and within group were examined with the Wilcoxon's signed rank test when the measurements were not normally distributed. Correlation was evaluated by Spearman's rank method. P values of less than or equal to 0.05 were considered as statistically significant.

3.4.3 Results

3.4.3.1 Patient demography and clinical characteristics

Patient demography and clinical characteristics are shown in Table 1. All the LTR were clinically stable, 190 (range 240-616) days post-transplant with $FEV_{1.0}$% best post-transplant: 99 ± 2.2%.

The normal volunteers were 8 females, and 6 males with an average age of 36±12 yr, and $FEV_{1.0}$% predicted of 106 ± 11%.

3.4.3.2 TGF-β_1 protein levels in BAL fluid from LTR

Basic BAL fluid data including volume recovered, cell count, cell viability, and cell differentials are provided in Table 2. The BAL fluid from the LTR showed significantly increased TGF-β_1 levels (51.0 pg/ml, [40.9-71.4]) compared with normal controls (28.2 pg/ml, [14.3-36.6], p=0.0013, Fig. 1).

BAL volume returns in LTR were significant lower than in normal controls (p=0.0001), but the total cell counts ($\times 10^3$/ml of BAL fluid) were significantly elevated in LTR (p=0.002). However, no statistical difference was found in the absolute total cell counts recovered in total BAL return between the two groups (p=0.23, Table 2). As described in the previous sections, there were higher percentage (p=0.007) and absolute counts of neutrophils

(p=0.005), with a corresponding lower percentage of alveolar macrophages (p=0.01) in the LTR group compared with the normal controls, whereas the absolute numbers of macrophages were in the same range in both groups (p=0.85, Table 2).

3.4.3.3 TGF-β_1 mRNA expression by BAL cells from LTR

TGF-β_1 mRNA was detected in all specimens, with PCR controls being negative as shown by the representative competitive PCR of TGF-β_1 from the studied BAL specimens (Fig. 2). Given the differences in BAL cell populations between LTR and normal controls, and because the AM is one of major cellular sources of TGF-β_1, TGF-β_1 mRNA levels in BAL cells were "normalised" as copies of mRNA /μg RNA/10^6 AM in this study.

Despite the significantly difference in BAL TGF-β_1 protein levels between LTR and normal controls (Fig. 1), TGF-β_1 mRNA levels in LTR (2.03×10^5 copies mRNA/10^6 AM, [1.61-6.67×10^5]) did not quite differ statistically from normal controls (7.85×10^5 copies mRNA/10^6 AM, [3.38-15.47×10^5], p=0.078, Fig. 3).

3.4.3.4 TGF-β_1 protein production by AM from LTR

Neither the spontaneous nor the LPS-induced TGF-β_1 production by AM from LTR was significantly different from that in normal controls, p=0.21 and 0.15 respectively (data are shown in Table 3, Fig. 4a & b). LPS (1 μg/ml) did not significantly modulate the release of

TGF-β_1 by AM from either the LTR (LPS+: 57.5 ng/ml, [39.8-90.8] vs LPS-: 71.2 ng/ml, [0.4-83.6], p=0.26) or normal controls (LPS+: 34.0 ng/ml, [12.9-72.2] vs LPS-: 25.0 ng/ml, [6.0-41.7], p=0.14), (Table 3, Fig. 5a & b).

3.4.3.5 TGF-β_1 mRNA expression by AM from LTR

Figure 6 shows a representative competitive PCR of TGF-β_1 from the cultured AM specimens. Consistent with TGF-β_1 protein production by AM from the two groups, TGF-β_1 mRNA expression by AM either LPS unstimulated or LPS stimulated was not significantly different between LTR and normal controls (Table 3, Fig. 7a & b, p=0.65 and 1.0 respectively). LPS stimulation did not up-regulate the expression of TGF-β_1 mRNA in either group, p=0.68 and 0.59 respectively (data are shown in Table 3, Fig. 8a & b).

3.4.3.6 Correlation analyses

Table 4 summarises the results of correlation analyses between BAL TGF-β_1 protein levels and mRNA expression with BAL macrophage counts, and between TGF-β_1 protein production and mRNA expression by AM in both normal controls and LTR.

In neither group did BAL TGF-β_1 protein levels or mRNA copy numbers correlate significantly with BAL macrophage counts, nor did BAL TGF-β_1 protein levels and mRNA copy numbers correlate significantly (Table 4).

No correlation was found between TGF-β_1 produced by cultured AM in the presence or absence LPS with the corresponding mRNA levels in either group (Table 4).

In addition, 9 of the LTR studied have been followed subsequently for over two years, and 7 of those subjects developed BOS at an average of 1002±445 days post transplant. Their BAL TGF-β_1 levels, time to the development of BOS, and lung function changes are shown in Table 5. It seemed that high BAL TGF-β_1 levels give short BOS free-time, or BAL TGF-$\beta_1$increased earlier than their lung function deterioration in the studied LTR. However, TGF-β_1 protein produced by AM *in vitro* did not seem to be associated with the development BOS, nor did the expression of TGF-β_1 mRNA by BAL cells or by AM. Due to relative limitation of sample size, no statistical analysis was done with the data.

Table 1. Patient Demographics and Clinical Characteristics

	Controls (n=14)	**LTR** (n=14)
Mean age (yr) (mean ± SD)	36±12	42±14
Gender (f/m)	8/6	5/9
$FEV_{1.0}$ (L) (mean ± SD)	3.7±0.6	2.4±0.8
$FEV_{1.0}$% predicted	106.9±10.9	--
$FEV_{1.0}$ % best post LTx[§] (mean ± SD)	--	99.3±2.1
Days post-LTx*	--	190 (94-616)
Original Diseases		
Cystic fibrosis	--	n=3
COPD (or Emphysema)	--	n=1
Eisenmenger's syndrome	--	n=2
Primary pulmonary hypertension	--	n=2
Bronchiectasis	--	n=3
IPF	--	n=1
Extrinsic allergic alveolitis	--	n=1
Emphysema/VHD[§]	--	n=1
LTx		
SLTx	--	n=4
BSLTx	--	n=8
HLTx	--	n=2

*LTx: lung transplantation; SLTx: single lung transplant; BSLTx: bilateral sequential lung transplant; HLTx: heart-lung transplant.

Table 2. **BAL Cell Counts and Cell Populations***

	Controls (n=14)	**LTR** (n=14)	***P value***
BAL return (ml)	114.5 (106-128)	85.5 (78-98)	***<0.001***
Total cell counts ($\times 10^3$ cells/ml)	137.0 (118.3-180.0)	269.5 (162.0-381.2)	***0.002***
Viability (%)	89.1 (84.5-90.3)	84.5 (81.5-89.7)	*0.31*
Mac ($\times 10^6$)	13 (10.5-20)	16.4 (7.7-23.9)	*0.87*
Mac (%)	86 (79.3-92.2)	75 (52.3-83.2)	***0.01***
Lym (%)	11.7 (6.4-15)	15.3 (4.4-29.9)	*0.38*
Neu (%)	1.4 (0.8-2.4)	4.5 (2.2-8.4)	***0.01***
Eos (%)	0.4 (0.1-0.5)	0.0 (0.0-0.5)	*0.15*
Epc (%)	0.5 (0.2-1.7)	0.6 (0.0-2.0)	*0.83*

* Data are shown in median and interquartile range.
Abbreviations: MAC: Macrophage, LMY: Lymphocyte, NEU: Neutrophil, EOS, Eosinophil, EPC: Epithelial Cell.

Table 3. **TGF-β_1 Protein and mRNA Expression by Alveolar Macrophages in Lung Transplant Recipients***

	Controls	LTR	*P value*
TGF-β_1 protein levels:			
BAL fluid (pg/ml)	28.2 (14.3 - 36.6) (n=14)	51.0 (40.9 - 71.4) (n=14)	***0.001***
AM culture supernatants (pg/ml)			
-LPS	25.0 (6.0 - 41.7)	71.2 (0.4 - 83.6)	*0.19*
+LPS (1μg/ml)	34.0 (12.9 - 72.2) (n=10)	57.5 (39.8 - 90.8) (n=9)	*0.142*
TGF-β_1 mRNA expression:			
BAL cells (copies/μg RNA/10^6AM)	7.9×10^5 (3.4×10^5 - 15.5×10^5) (n=14)	2.02×10^5 (1.6×10^5 - 6.8×10^5) (n=14)	*0.08*
Cultured AM (copies/μg RNA)			
-LPS	12.6×10^5 (1.8×10^5 - 38.6×10^5)	17×10^5 (4.1×10^5 - 54×10^5)	*0.62*
+LPS (1μg/ml)	12.82×10^5 (5.6×10^5 - 19×10^5) (n=10)	11.2×10^5 (0.6×10^4 - 30.8×10^5) (n=9)	*0.97*

*Data are shown in median and interquartile range.

Table 4. **Correlation Analyses Between TGF-β_1 mRNA and Protein Expression with BAL Macrophages Counts**

	TGF-β_1 mRNA in BAL cells (copies/μg RNA/10^6 Mac)	**BAL TGF** (pg/ml)	**TGF-β_1 mRNA in cultured AM** (copies/μg RNA)	
			-LPS	+LPS
	r(p)	*r(p)*	*r(p)*	*r(p)*
LTR				
(n = 14)				
BAL TGF-β_1 (pg/ml)	*-0.19 (0.54)*	--	--	--
BAL Macrophages (%)	*-0.38 (0.18)*	*-0.37 (0.22)*	--	--
BAL Macrophages ($\times 10^6$)	*-0. 50 (0.07)*	*-0.01 (0.99)*	--	--
(n = 9)				
AM TGF-β_1 (pg/ml, -LPS)	--	--	*0.54 (0.14)*	--
AM TGF-β_1 (pg/ml, +LPS)	--	--	--	*0.02 (0.97)*
Controls				
(n = 14)				
BAL TGF-β_1 (pg/ml)	*0.01 (0.96)*	--	--	--
BAL Macrophages (%)	*-0.10 (0.73)*	*-0.15 (0.61)*	--	--
BAL Macrophages ($\times 10^6$)	*-0.06 (0.84)*	*-0.15 (0.61)*	--	--
(n = 10)				
AM TGF-β_1 (pg/ml, -LPS)	--	--	*-0.48 (0.16)*	--
AM TGF-β_1 (pg/ml, +LPS)	--	--	--	*-0.07 (0.86)*

Table 5. **Changes of Lung Function in Nine of the LTR studied over Two-Year Period**

Patient	BAL TGF-β_1 (pg/ml)	Development to BOS	Days post-Tx to BOS	$FEV_{1.0}$% best post-LTx at last assessement	Δ $FEV_{1\text{-}0}$%post-LTx
MV	114.1	yes	952	57	-43.0
PW	74.5	yes	378	67	-33.0
AW	74.8	yes	1113	80	-15.7
CM	68.3	yes	1078	75	-21.0
GW	68.3	yes	455	70	-33.0
JT	46.3	yes	1358	68	-32.0
SH	19.6	yes	1680	73	-24.0
CA	40.8	no	--	--	--
WE	39.4	no	--	--	--

LTx: Lung transplantation

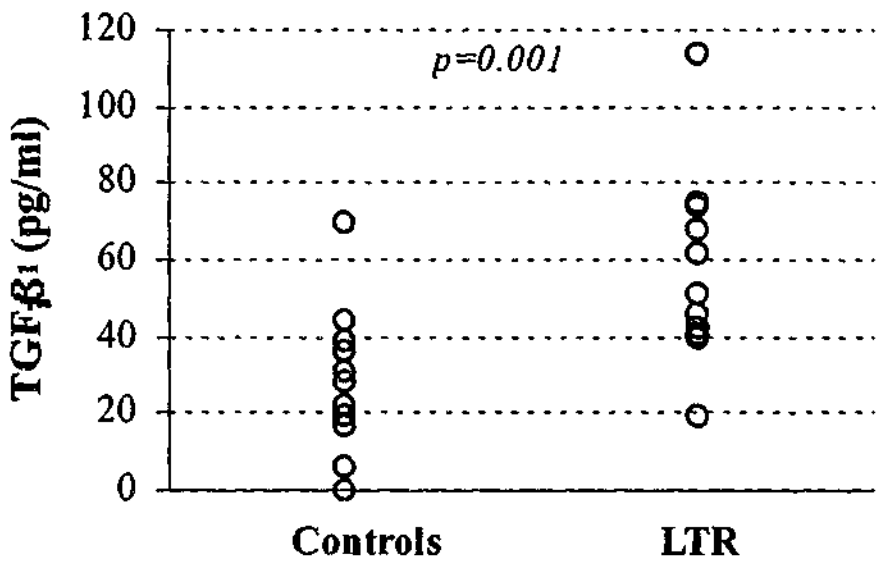


Fig. 1. Comparison of TGF-β_1 protein levels in BAL fluid from normal controls and lung transplant recipients (LTR).

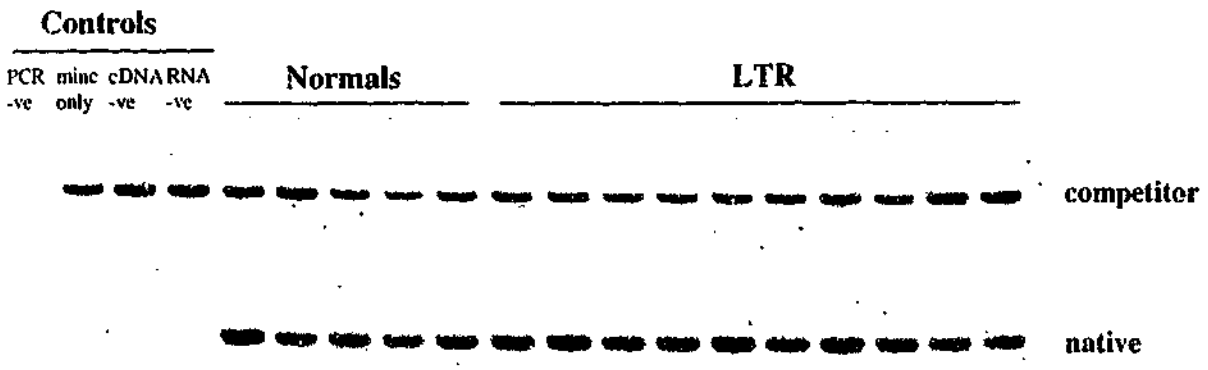


Fig. 2. Representative competitive PCR of TGF-β_1 mRNA from BAL cells obtained from normal subjects (n=5) and lung transplant recipients (LTR, n=10).

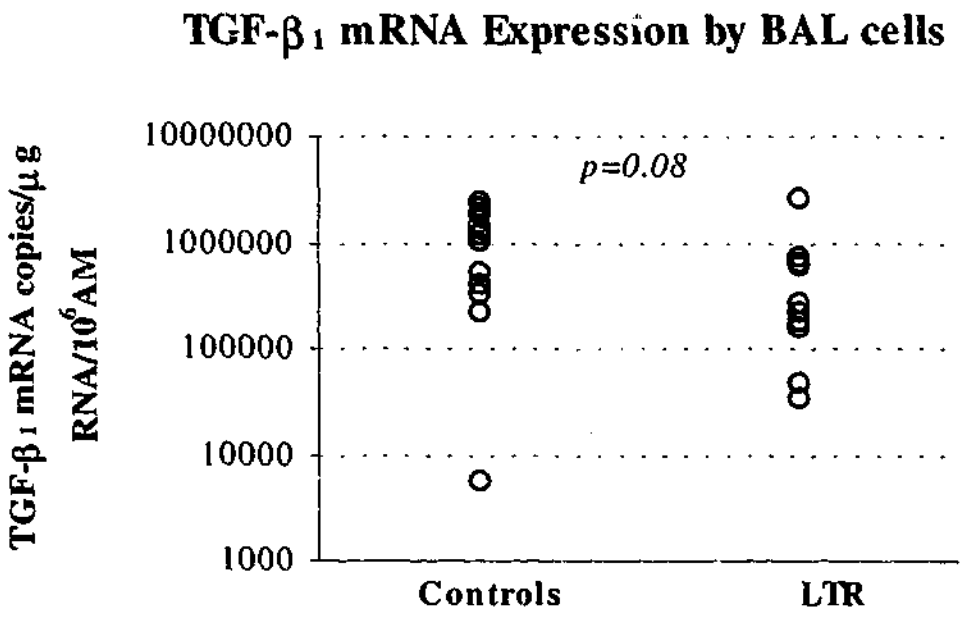


Fig. 3. Comparison of TGF-β_1 mRNA levels in BAL cells from normal controls and lung transplant recipients (LTR).

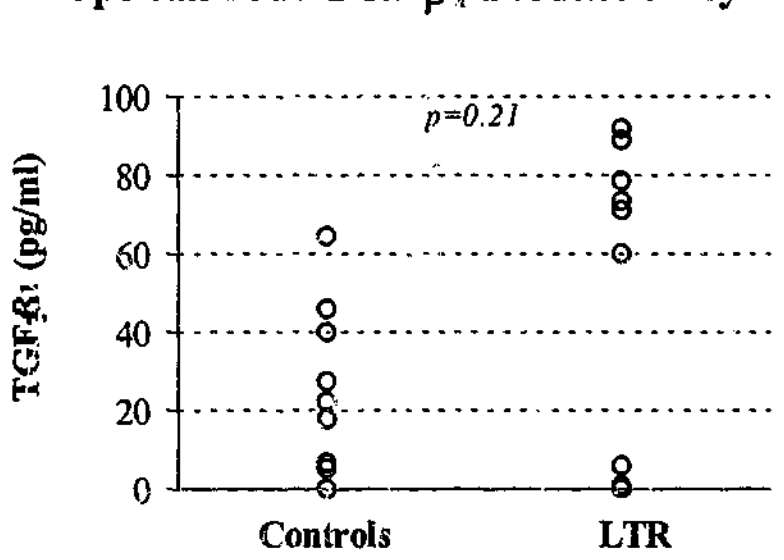


Fig. 4 a. Comparison of spontaneous production of TGF-β by alveolar macrophages (AM) between normal controls and lung transplant recipients (LTR) *in vitro*.

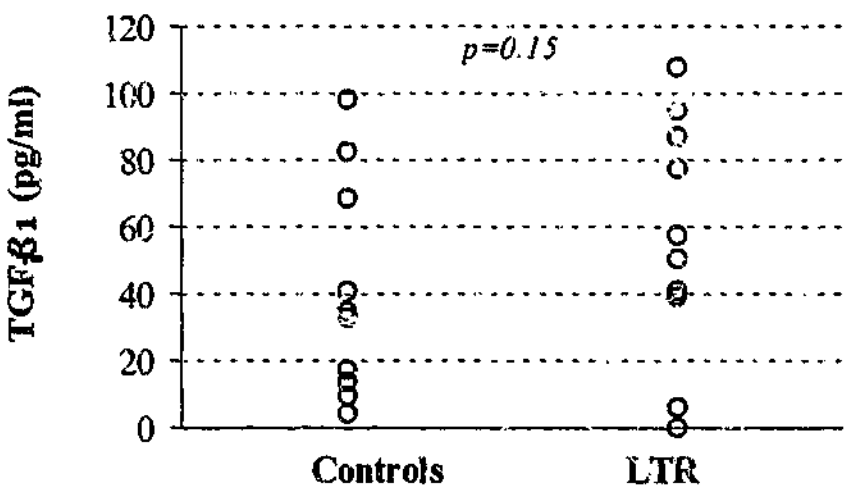


Fig. 4 b. Comparison of LPS-induced TGF-β_1 production by alveolar macrophages (AM) between normal controls and lung transplant recipients (LTR) *in vitro*.

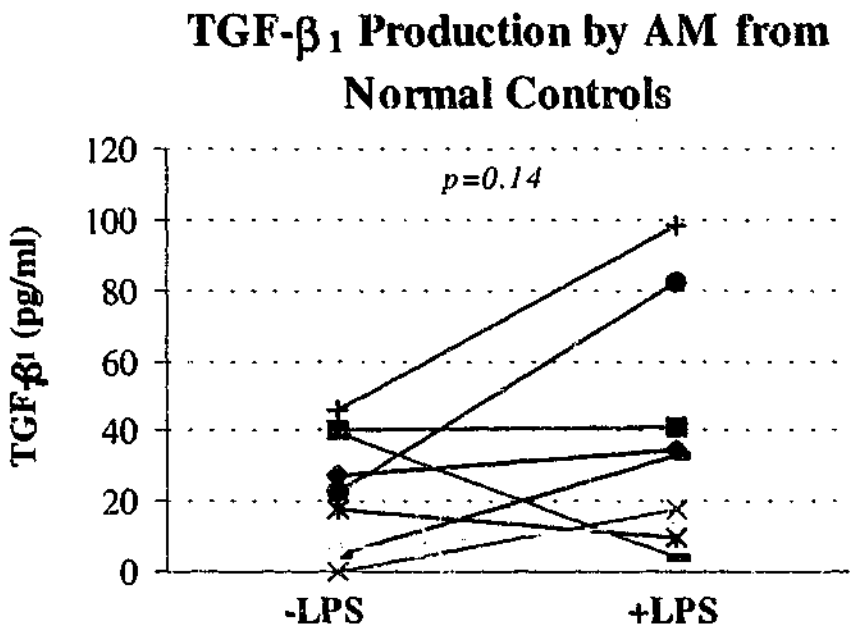


Fig. 5 a. Comparison of spontaneous and LPS-induced TGF-β_1 production by alveolar macrophages (AM) from normal controls *in vitro.*

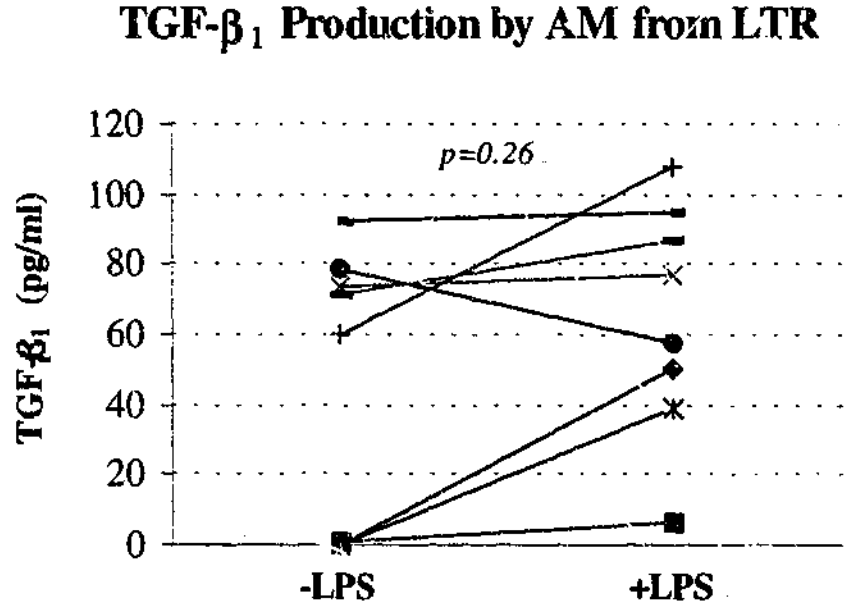


Fig. 5 b. Comparison of spontaneous and LPS-induced TGF-β_1 production by alveolar macrophages (AM) from lung transplant recipients (LTR) *in vitro.*

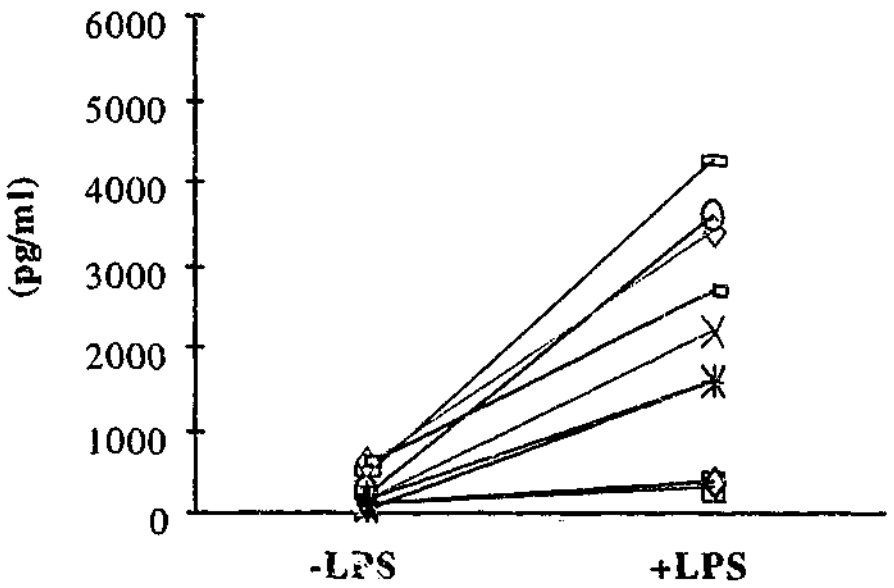


Fig. 5 c. Control experiment showing response of AM to LPS stimulus (1μg/ml). TNF-α release by AM from the same normal subjects was significant up-regulated by LPS *in vitro*

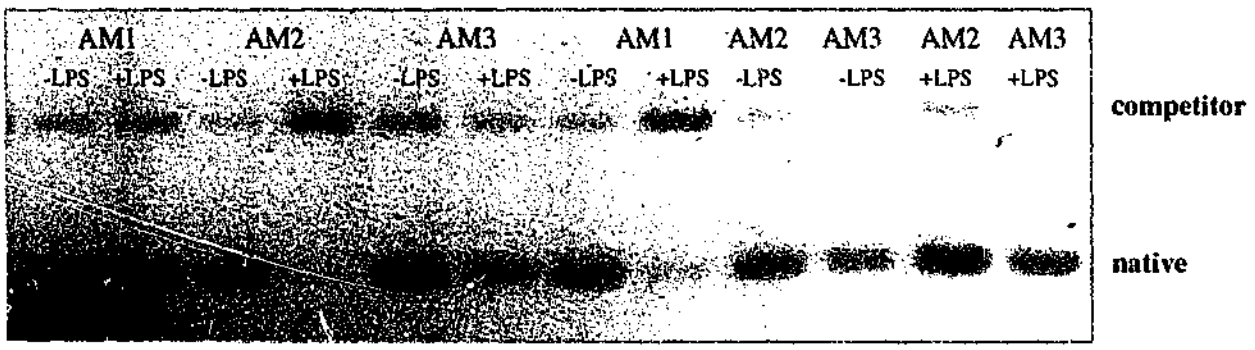


Fig. 6. Representative competitive PCR of TGF-β_1 mRNA from cultured alveolar macrophages from normal controls and lung transplant recipients with and without LPS stimulus.

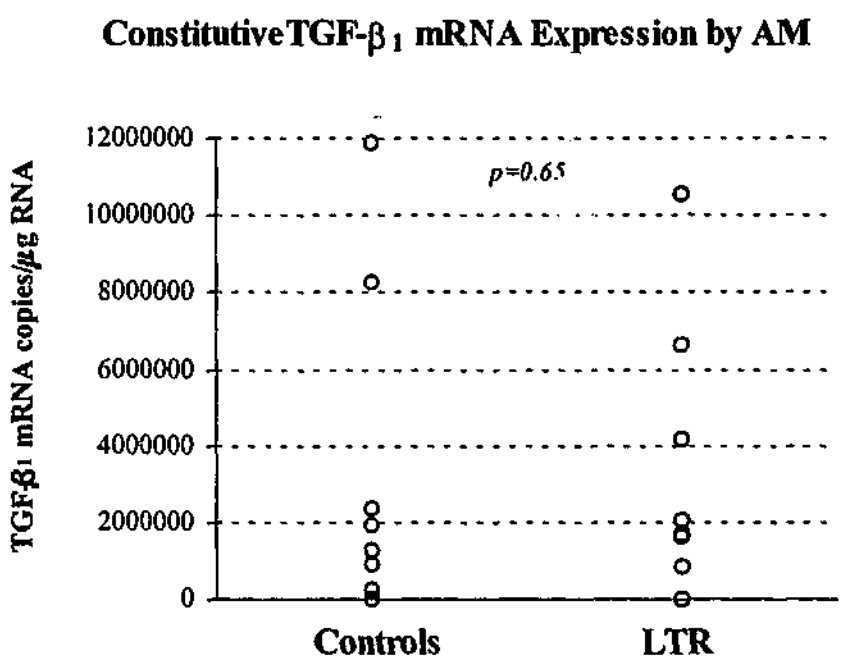


Fig. 7 a. Comparison of constitutive expression of TGF-β_1 mRNA in alveolar macrophages from nromal controls and lung transplant recipients (LTR).

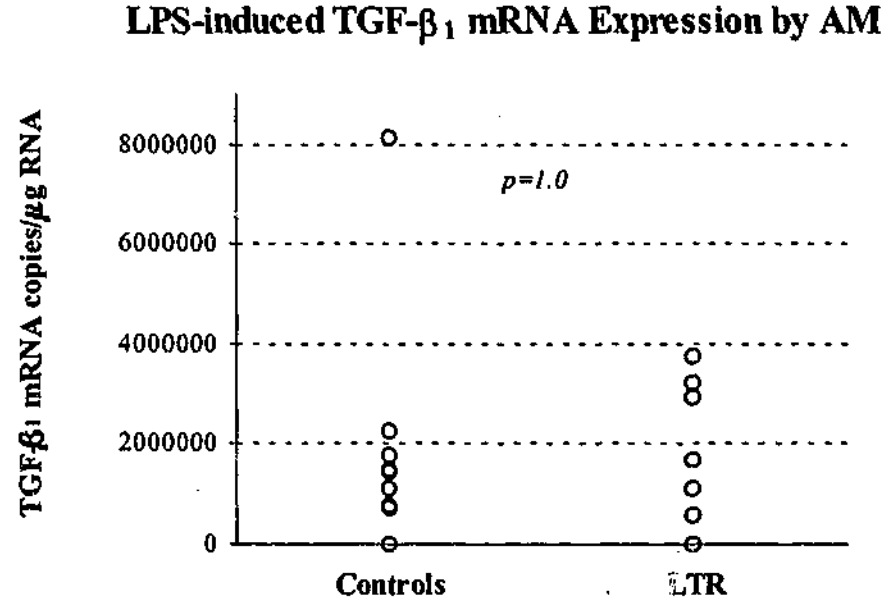


Fig. 7 b. Comparison of LPS-induced expression of TGF-β_1 mRNA in alveolar macrophages from normal controls and lung transplant recipients (LTR).

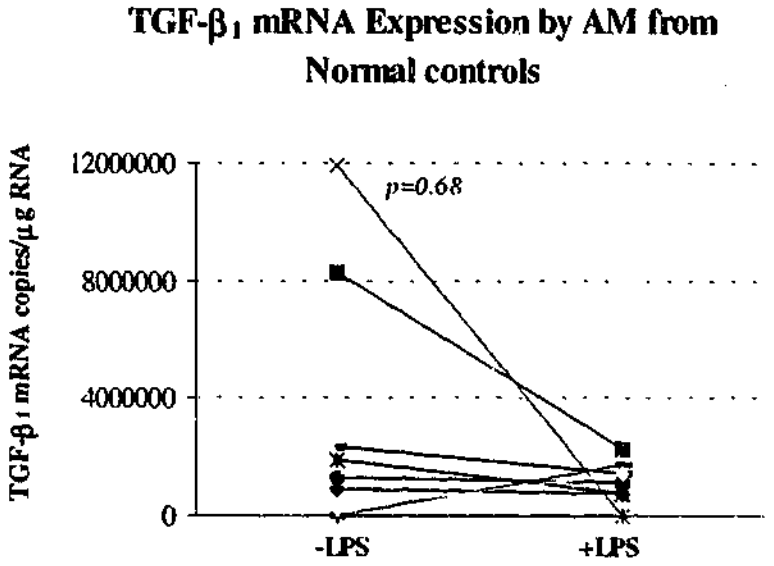


Fig. 8 a. Comparison of spontaneous and LPS-induced TGF-β_1 mRNA expression by alveolar macrophages (AM) from normal controls *in vitro.*

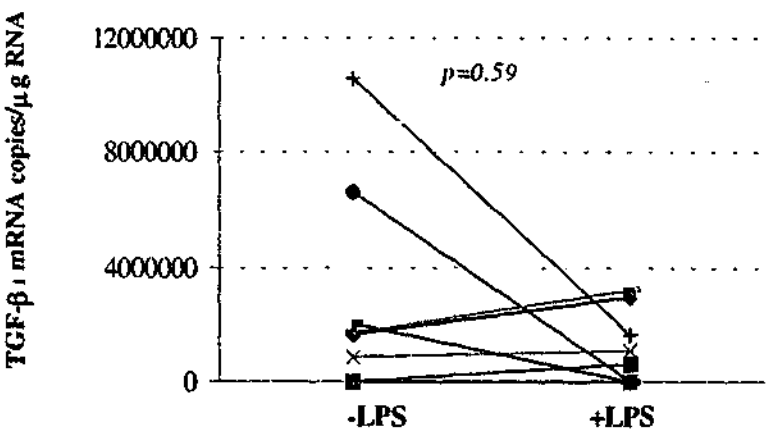


Fig. 8 b. Comparison of spontaneous and LPS-induced TGF-β_1 mRNA expression by alveolar macrophages (AM) from lung transplant recipients (LTR) *in vitro.*

3.4.4 Discussion

In this study, I have shown that TGF-β_1 mRNA expression could be detected in BAL cells, and in cultured AM isolated from BAL specimens as reported in previous publications (335, 336, 359). Compared with normal controls, BAL TGF-β_1 protein levels were significant elevated in LTR. However, the expression of TGF-β_1 mRNA from BAL cells did not significantly differ from controls, and no correlation was found between TGF-β_1 protein levels and mRNA expression in either normal or transplant group. In addition, I was unable to find any significant difference between normal controls and LTR in either the spontaneous or LPS-induced production of TGF-β_1 protein by AM isolated from the BAL cells *in vitro*. Nor was there a significant difference in TGF-β_1 mRNA expression by AM between the two groups.

The subjects included in this study were not the same individuals reported in the previous section (Chapter III, 3.3). Therefore, this study confirmed the previous findings of increased BAL TGF-β_1 protein levels in LTR, but failed to find evidence of up-regulation of TGF-β_1 mRNA in BAL cells from the LTR. These data may reflect a consequence of translational and post-translational control of TGF-β, which may permit increases in TGF-β protein secretion without alteration in mRNA levels.

On the other hand, however, BAL cytokine levels, including growth factors, may have been derived not only from inflammatory cells but also from epithelial or other structural cells lining the sampled airways (360). TGF-β_1 can be synthesised and secreted by a number of inflammatory cells, including monocytes/macrophages, neutrophils, eosinophils, and also by

structural cells such as endothelial cells, epithelial cells, fibroblasts and airway smooth muscle cells (124). Although alveolar macrophages have been suggested as an important source of TGF-β_1 in interstitial lung diseases (IPF) (297, 359), asthma, and bronchitis (180), several lines of evidence have also demonstrated that bronchial epithelium may be the major source of TGF-β_1 in both normal (298) and fibrotic lungs (124). Whatever its source, in the context of lung transplant recipients, my data would suggest that AM are likely not the major source of TGF-β_1 elevation in BAL. In support of this speculation, TGF-β mRNA expression did not appear to be up-regulated either in alveolar macrophages harvested by BAL from the same LTR subjects. Although in the previous section (Chapter III, 3.3) I reported the apparent paradox that TGF-β_1 immunoactivity in bronchial epithelium of the airway biopsies tended to be weaker in LTR, particularly in BOS patients, than in controls, this may indicate an increased release or secretion of TGF-β_1 from the epithelium of lung allografts.

Given the potential role of the bronchial epithelium in airway inflammation and airway fibrosis in lung allografts, Elssner and colleagues investigated the expression of TGF-β_1 mRNA in both BAL cells and bronchial epithelium, as well as BAL TGF-β_1 protein levels in lung transplant patients with and without BOS (333). However, there were no normal controls included in their study. Even so, compared with non-BOS LTR, BAL fluid from BOS patients showed significantly increased levels of TGF-β_1 protein, while the expression of TGF-β_1 mRNA by BAL cells again was not significant different between the two groups (333). However, TGF-β_1 mRNA levels in bronchial epithelial cells from the BOS patients did show a trend toward higher expression, though this did not reach statistical significance (333).

Although neither the molecular nor the cellular pathogenesis of lung transplant BOS has been fully elucidated, the most widely held hypothesis is that both alloantigen-dependent and -independent mechanisms could be relevant. Bronchial epithelium of lung allografts could potentially be the pivotal cells that initiate alloantigen-dependent inflammatory insults, as they express donor class II MHC antigen (79, 116). In addition, they are abundant and constantly the targets for non-immunological attacks following the transplant, such as ischaemia, viral and bacterial infections, which, in turn, could amplify alloantigen independent insults. Therefore, further studies are needed to compare bronchial epithelium from both normal health controls and lung transplant subjects to clarify the contribution of the bronchial epithelium of lung allografts in the production of TGF-β_1.

In contradiction to my findings, one study has suggested that the AM content and secretion of TGF-β_1 is increased in patients with BOS compared with clinically stable LTR (141). Another two studies found a trend toward higher expression of TGF-β_1 mRNA in BAL cells from patients with BOS (336) or chronic lung rejection (335). Although no normal healthy controls were studied in any of these studies (141, 335, 336), the contribution of AM to growth factor production in BAL fluid from lung transplant patients still cannot be completely ruled out. My data, though, would be very much against such role of AM, is important negative information. However, there may be present a possibility of type II statistical error due to the relative limitation of power in the *in vitro* experiment part of the study.

In addition, the effects of immunosuppressive drugs, such as CsA and corticosteroids, on TGF-β_1 expression need to be taken account to explain the paradoxical findings of increased TGF-β_1 protein levels in BAL fluid *in vivo*, but not in AM culture supernatants *in vitro* in our

studied LTR. The effects of gluco-corticosteroids (GCs) on TGF-β *in vitro* are not uniform (361-369), as TGF-β has bidirectional actions, namely inducing or inhibiting cell replication depending on the cell type, the presence of other growth factors, and the state of activation of the cells before exposure to TGF-β (124). Some lines of evidence suggest that GCs up-regulated TGF-β_1 mRNA expression in unstimulated and mitogen-activated T cells (361), fibroblasts (364), osteoblasts (365), and other cells (362, 363) in a concentration and time dependent matter. Other reports provided opposing evidence that GCs did not up-regulate TGF-β_1 mRNA expression (366, 367) or protein secretion (369), but rather suppressed its expression (368). However, those finding regarding GCs effect on TGF-β_1 expression were largely from *in vitro* studies. It remains to be determined what the effects of GCs are on TGF-β_1 expression *in vivo*. By contrast, CsA, despite its importance as a cornerstone of immunosuppressive therapy in human organ transplantation, has been shown recently to enhance the production of TGF-β_1 protein, as well as the expression of its receptor and mRNA both *in vivo* and *in vitro* (109-114). Therefore, the increased TGF-β_1 protein levels in BAL fluid from LTR *in vivo*, whatever its source, could, at least partly, be attributable to the receiving of CsA therapy.

Several studies including those cited above (141, 333, 335, 336) have suggested that post human lung transplant, the increase in TGF-β_1 production by AM, or high and early peaks of TGF-β_1 mRNA expression in BAL cells or AM preceded the functional diagnosis of BO or BOS. More importantly, the early occurrence of such peaks (≤6 months post transplant) not only preceded BOS by several months but was associated with a relentless evolution of chronic lung rejection, leading to the death of some of the patients (335). These findings were retrospective, but argue for a causal role for TGF-β_1 in the development of lung transplant

BOS. To evaluate the predictive value of TGF-β_1 in early detection of BOS post transplantation, nine LTR in this study were followed up prospectively for over two years. Among them, 7 subjects eventually developed BOS between 378 to 1680 days post transplant. Interestingly 6 out of 7 were among those with higher levels of BAL TGF-β_1 compared with the rest. In the previous section (Chapter III, 3.3), I also reported an association between the increased BAL TGF-β_1 levels and lung function ($FEV_{1.0}$% best post-transplant) in BOS patients. Taken together, these studies give further evidence in support of the hypothesis that TGF-β contributes to the development of BOS in lung transplant subjects, and at least the expression of TGF-β protein in BAL specimens could potentially serve as an early predictive and prognostic marker of airway fibrosis occurring in BOS.

Monocytes and macrophages are exquisitely sensitive to low levels of LPS (357, 359). I was surprised that in this study, moderate concentration of LPS (1 μg/ml) had little effect on TGF-β_1 production by AM, nor on TGF-β_1 mRNA expression in AM from either the normal healthy controls or LTR. This is unlikely to be due to an inadequate stimulus by 1 μg/ml of LPS to AM, as with the same concentration, TNF-α release by the AM was significantly up-regulated in the same subjects (Fig. 5c), as was also demonstrated in my previous study (357). In support of my results, Shaw and colleagues also failed to shown an increase in TGF-β mRNA in AM from normal individuals or patients with interstitial lung disease after incubation in the presence of LPS at any concentration between 0.1ng/ml to 10μg/ml (359). Put together, the evidence would suggest that LPS is unlikely to be the major stimulus to TGF gene activation in AM.

In summary, this study has confirmed the constitutive presence of TGF-β_1 mRNA in alveolar macrophages shown by others (335, 336, 359), but failed to find conclusive evidence of up-regulation of TGF-β_1 release and mRNA levels in the alveolar macrophages from clinically stable LTR. The paradoxical findings of increased TGF-β_1 protein levels in BAL fluid *in vivo*, but not in AM culture supernatants *in vitro,* nor in the TGF-β_1 mRNA levels of the cultured AM or BAL cells from LTR, may suggest that AM are unlikely to be the major cells causing the increased BAL TGF-β_1 levels in the LTR studied. Further studies will need to look at other cell origins such as the bronchial epithelium.

CHAPTER IV CONCLUSIONS

Although lung transplant recipients are complex group of patients to study with many variations in underlying age, diseases, drug therapy, and infections, they are linked by the realisation that BOS translates as function loss of small airway, which will ultimately lead to graft failure in the majority.

Despite these difficulties, these cross-sectional airway biopsy studies have further characterised the features of airway inflammation and remodelling occurring in human lung allografts. Compared with normal healthy controls, in stable LTR and those with BOS, there was not only a BAL neutrophila as reported previously, but also found were a prominent airway wall neutrophilia, and a significant increase in airway mucosal vascularity. Compared with LTR without BOS, I have demonstrated evidence of pathologically excessive scar collagen (collagen type III) deposition in the airways of LTR who had developed BOS.

These pathological features are likely to lead to airway wall thickening and stiffness, and may have a profound effect on airway function, i.e. contributing to the development of BOS. Thus, significant reverse relationships were also found between lung function parameters and airway neutrophilia, increased airway vasculature, and collagen deposition in BOS patients.

Expression of TGF-β_1 in BAL fluid, but not in airway biopsies, was significantly increased in both LTR with and without BOS, which may suggest that all LTR are at risk to a varying degree of development of airway fibrosis. Monitoring BAL TGF-β_1 levels routinely during the follow-up of LTR could be of practical value in predicting the likely development of airway fibrosis in lung allografts. This needs further longitudinal study for confirmation.

Although were BAL TGF-β_1 levels increased significantly in LTR *in vivo*, I was unable to find evidence of up-regulation of TGF-β_1 production and mRNA levels in the alveolar macrophages from the same group of patients. These paradoxical findings may imply that alveolar macrophages are unlikely to be the major cell origin of the increased BAL TGF-β_1 levels in the LTR studied, or it could also be merely a type II error due to the relatively limited power in the *in vitro* experiment. In addition, the effect of immunosuppressive drugs, such as CsA and Corticosteroids, on TGF-β_1 production *in vivo* could have confounded the findings.

In accordance with previous studies, markedly increased BAL IL-8 levels and a significant correlation between BAL IL-8 and BAL neutrophilia were also found in patients with BOS in the study.

These cross-sectional observations need to be followed up in long term. If longitudinal studies can confirm a link between airway neutrophils and/or increased IL-8 levels and the development of airway damage/remodelling in BOS, then this could create novel potential target for further therapeutic interventions. However, the role of infection in the development of lung transplant BOS remains to be clarified.

Bronchial biopsy, by directly sampling airway wall mucosa, has provided much valuable and novel data to help better understand the airway remodelling that occurs in chronic lung rejection, and its physiological implications.

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Responses to Examiners' Comments

I shall take the opportunity to thank the Examiners for their comments on my PhD thesis. The following represent point-by-point responses, amendments and correction of typographical errors, to the comments from A/Professor A Glanville:

Title: Airway Inflammation and Remodelling post Human Lung Transplantation

- **Specific Questions and Comments:**

1. **Page 20. The allegation that improved results are directly related to refinements in tissue typing cannot be supported by evidence.**

This comment is well taken and this statement have been retracted from the thesis. Data in the literature have been conflicting on HLA mismatching, with most series showing no significant associations between HLA mismatching and BOS development in lung transplant recipients. Therefore, it is uncommon for any lung transplant centre to have more than a few HLA-matched recipients. The new sentence (page 20 paragraph 2 lines 5-8) now reads *"The outcomes have been progressively improved secondary to greater understanding of patient physiology, better surgical and peri-operative techniques, and more effective immunosuppressive modalities with coincident appreciation of their risk".*

2. **Page 22-23. There appears to be some lack of understanding that BOS is the physiological description of loss of graft function after transplant. More care needs to be used to be precise in usage of BOS and OB.**

The attempt on page 22-23 was to try and summarise the definitions of lung transplant BO and BOS from literature.

"Bronchiolitis Obliterans" (BO) (without organisation) in the non-transplant setting, refers to a rare clinical syndrome characterised by progressive airflow obstruction (19), which is classically associated with toxic gas exposure, viral infection, or systemic connective-tissue diseases (20-23). The most common histological findings are constrictive bronchiolitis with bronchiole inflammation, fibrosis, scarring, and stenosis but without the intraluminal plugs or polyps that are found in bronchiolitis obliterans organising pneumonia (BOOP).

Lung transplant BO is a major cause of allograft dysfunction. By definition, it manifests itself **histologically** as obliteration of the bronchiolar lumen by fibrous scaring tissue. This fibrous scaring can be eccentric with formation of a fibrous plaque in the wall of the airway; concentric with the interposition of a "donut" of collagen tissue; or granulation tissue may completely obliterate the lumen of the airway, reducing the air passage to stenotic cords of scar tissue (41).

Because BO is difficult to document histologically, due to transbronchial biopsy specimens being often insufficiently sensitive for diagnosis (41), in 1993 a committee sponsored by the International Society for Heart and Lung Transplantation (ISHLT) proposed a "clinical" description of BO, termed Bronchiolitis Obliterans Syndrome (BOS), and defined by **pulmonary function changes** rather than histology. The Spirometric criteria for the diagnosis and stages of BOS have been standardized by the International Society for Heart and Lung Transplantation in 1993 (35), and updated in 2001 (Table 1) (41). Although this system does not require histologic diagnosis, it does recognise it as a likely fundamental underlying pathological process.

Table 1. Staging System for Bronchiolitis Obliterans Syndrome*

BOS Stage[徐]	$FEV_{1.0}$, % of Baseline[§]	FEF_{25-75}, % of Baseline
BOS 0	>90%	>75%
BOS Potential	81-90%	≤75%
BOS 1	66-80%	--
BOS 2	51-65%	--
BOS 3	≤50%	--

* Estenne M, et al. 2001. J. Heart Lung Transplant (in press). (Ref. 41).

§ Baseline $FEV_{1.0}$ is the average of the two highest $FEV_{1.0}$ obtained over > 4 weeks.

徐Subcategories "a" and "b", without and with bronchiolitis obliterans, respectively.

Thus, the diagnosis of BOS in lung transplant recipients does not necessary require histological confirmation. In contrast, the term of BO should be used only when histology demonstrates dense fibrous scar tissue affecting the small airways (41).

In the studies presented in this thesis, patients were diagnosed clinically as BOS rather than histologically as BO, and there was no consistent histological confirmation. Underlying BO was assumed but not proven.

3. The use of the St Louis Data Base from 1996 is questioned as these data are now old and certainly incomplete compared with the recent ISHLT 2001 data.

I agree that compared with the recent ISHLT 2001 data, the St Louis Data Base from 1996 can be incomplete. The main reason to cite the St Louis Data Base in my literature review was that the Si Louis data summarised the **Causes of Death after Lung Transplantation,** which was the topic of that part of literature. In addition, the writing up of the thesis took about 10 to 12 months intermittently, by the time of writing for the literature review part, the ISHLT registry report 2001 was not yet published.

After the examiner's comments, the **ISHLT registry report 2002** has been published just recently. It provided the most updated data on **Causes of Death after Lung Transplantation.** Therefore, the Table 3 (on page 26) now reads:

Table 3. Causes of Death of recipients of adult lung transplants performed between 1987 to 2001

	0-30 days (n=962)	31 d to 1 yr (n=1230)	>1 to 3 yr (n=953)	>3 to 5 yr (n=479)	5+yr (n=301)
CAD	1.2%	0.8%	1.2%	1.5%	1.3%
Cardiac	9.3%	3.6%	2.1%	3.1%	2.3%
Malignancy, other	0.0%	2.0%	4.1%	7.9%	9.0%
Lymphoma	0.1%	3.3%	2.4%	1.7%	4.0%
CMV	0.5%	4.1%	1.7%	0.6%	0.7%
Acute rejection	5.9%	1.8%	2.1%	0.8%	0.7%
Infection, non-CMV	24.6%	39.3%	24.9%	18.8%	16.3%
Bronchiolitis	0.7%	6.1%	30.0%	33.0%	34.2%
Primary graft failure	16.4%	6.1%	5.8%	4.0%	4.7%
Graft failure, other	14.7%	11.4%	10.2%	13.8%	8.3%
Technical	8.8%	3.1%	0.9%	0.2%	0.3%
Other	17.7%	18.5%	14.7%	14.6%	18.3%

*Data from ISHLT registry report 2002 (Ref. 55).

4. Page 96-97. The references 36, 37, 39, and 40 are used to support the assumption that the sensitivity of transbronchial biopsy (TBB) to diagnose OB is the same as the sensitivity of TBB to enable grading of bronchial tissue……"

We are aware of the examiner's group's opinions that TBB can sample small airways in 97% of individuals. However, data have been variable from centre to centre, and a previous publication of our group showed that bronchiolar material was present in only 78% of surveillance TBB procedures performed in the Alfred (Ward C, et all ERJ 10:2876-2880, 1997). Perhaps it would be appropriate to state that histological confirmation of BO is difficult, because TBB specimens "often" are not sufficiently sensitive for diagnosis (41), and a negative TBB does not exclude BO (1).

Therefore, these new sentences, found on page 96 (paragraph 2, line 6) now reads *"TBB, although regarded as the most specific specimens in detecting BO, are often not sufficiently sensitive for diagnosis (36, 37, 39, 40, 41)."* To support this statement, the most recent revision of the ISHLT working classification of lung rejection (41) is also cited.

5. Page 98. The definition of what constitutes a colonising organism is a vexed one……

We agree with that. BAL neutrophilia in BOS has been reported by a number of studies (145, 146, 147) prior to the current one. This study attempted to confirm those previous findings and investigate airway wall neutrophil

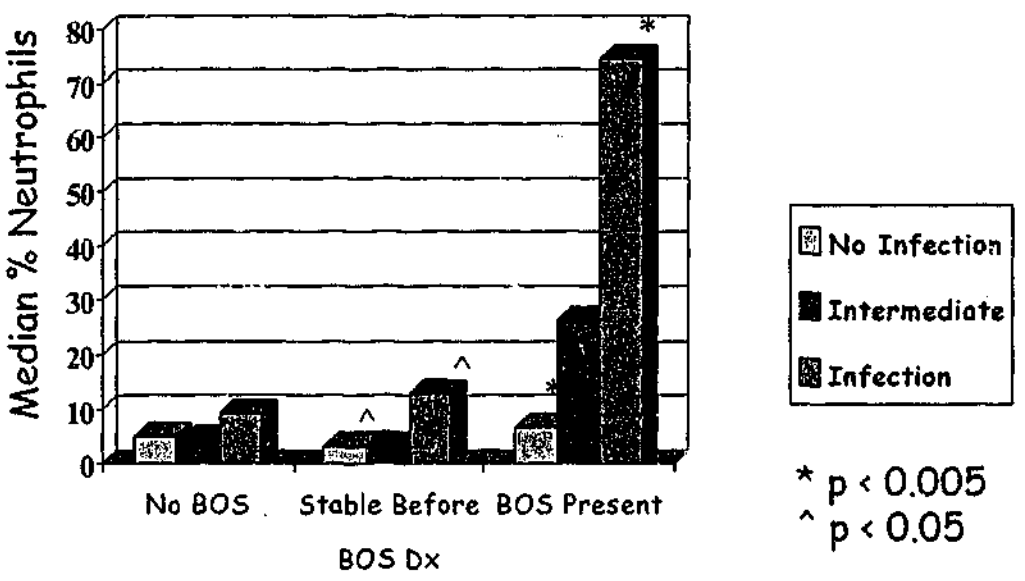
infiltrate in BOS for the first time. However, traditionally, it is considered that neutrophil influx from the circulation to extravascular tissue is an important secondary event in host defence against microbial invasion-the "Innate" immune system. Therefore, before claiming a primary role for the neutrophil in the development of chronic lung rejection, it is important that confounding by occult airway infection is considered although we agree that this is not easily achieved.

In our studies, patients were recruited only if (1) they were clinically free of infection, for example, afebrile, no new chest signs with unchanged leukocyte count, unchanged chest radiography; (2) there was absence of evidence of acute bronchitis or mucus on bronchoscopic assessment of the airways, (3) BAL samples were negative on gram stain; (4) CMV cellular inclusions were not detectable in any of the BAL or biopsy samples, (5) any micro-organisms that were isolated on culture from the BAL fluid had to be considered microbiologically as "colonisers", e.g. cytomegalovirus (CMV), or commensals. This would have been the definition of the micro Lab. BAL micro-organisms culture positive samples were only found in 5 of the 14 BOS patients and 4 of the 13 patients without BOS in my study. Numbers were not sufficient for a sub-analyse of these patients in terms of microbial load and pathogenicity of the organisms. The presence of positive bacterial culture results, in the absence of a positive gram stain or clinical evidence of infection, would argue that these bacteria were present in relatively low numbers and were considered "colonisers" rather than causing an active infective disease process of a classic sort.

In addition, two previous comparable studies, one from DiGiovine et al (145) (where LTR recipients were apparently excluded if any organisms were noted on BAL), and another one from Riise et al (146) (where BAL bacterial load was used as a marker to exclude cases with infection), found a similar BAL neutrophil percentage values to the present study.

However, in one of our ongoing studies, a sub-analysis of BAL neutrophilia was performed in lung transplant patients, who were "by clinical definition" classified as **No Infection**, (i.e. no secretions on bronchoscopy; negative gram stain and culture; negative viral, fungal and AFB cultures); regarded as **Intermediate** (i.e. no secretions on bronchoscopy/asymptomatic patient; but positive bacterial, viral or fungal staining or culture); and who were defined as having airway **Infection** (i.e. secretions on bronchoscopy/symptomatic patient; positive bacterial, viral or fungal staining &/or culture; treatment initiated). The results are shown by Figure 1. These suggested that BAL neutrophil% increase in BOS is associated with the common situation of infection and microbial colonization (H Whitford, et all. J Heart & Lung Transplant. 20:260, 2001).

Fig. 1. Comparisons of BAL Neutrophil % in lung transplant recipients with and without infections.



6. **Page 105. It is noted that 7 of the 14 patients with BOS were recipients of single lung transplants whereas none of the 13 without BOS were recipients of single lung .....**

This is very interesting. The difference in the number of **single lung transplants** between the groups with and without BOS studied is well pointed out. But there is little information available from the literature suggesting a significant difference in the incidence of BOS between single lung and double lung transplant recipients.

However, whether a 20% fall in $FEV_{1.0}$ means the same for a single lung transplant (SLTx), a bilateral sequential lung transplant (BSLTx) or a heart-lung transplant (HLTx) is very unclear. As an extreme example a 20% fall in $FEV_{1.0}$ in SLTx recipients for pulmonary vascular disease may actually represent a 40% fall in graft function. However in a population this is likely to be only small confounding factor. BOS was fairly equally distributed amongst SLTx and BLTx recipients.

Moreover, none of the unrelated native lung diseases in the single lung transplant patients recruited into the study were associated with infections, and patients were excluded from the study, if there were evidence of infections clinically or microbiologically as re-addressed on point 5.

7. Reference 21 and 81 are duplicated.

The citation of reference 81 is omitted from the following text:

1) Page 29, Table 4, line 4 reads "Infection (particularly CMV)------1, 2, 21, 31, 34, 75, **77-80, 82-87**"

2) Page 34, paragraph 1, lines 1-4 now reads "Certain infectious agents have been implicated in the development of BO in the nontransplanted population, particularly viruses, including respiratory syncytial virus, adenovirus, rhinovirus, coronavirus, influenza, and parainfluenza, measles, mumps, and cytomegalovirus (CMV) (21, **80, 82**)".

8. Reference 233 gives year 1996 but the text states that it refers to 1990. Only one of these can be correct.

Year 1996 is correct. Page 52, paragraph 2, lines 5-7 reads now "In **1996**, a transbronchial biopsy study demonstrated airway basement membrane thickening in BO patients (233), suggesting that subepithelial collagen deposition occurs in the process of BO/BOS development".

9. Page 212, paragraph 1. Does simply not make sense and should either omitted it or re-written.

The re-written paragraph 1 found on page 212 reads *"Lung transplant recipients are a complex group of patients to study, with many unique variations in their clinical course influenced by a complex interaction of factors such as age, original underlying disease, episodes of acute rejection and infection, and the requirement for maintenance immunosuppressive medications. However, the loss of function in the lung allografts through the process of BOS remains the single commonest problem limiting long-term survival in all lung transplant recipients."*

10. Page 207, Line 5. The reference 79 refers to a theoretical hypothesis rather than data on MHC II epithelial expression.....

I would thank the examiner for this comment on the citation of two references (reference 79 and 166) in the thesis, which are not completely appropriate for supporting my statement on the expression of MHC II by human airway epithelium.

Therefore, on page 223, reference 116 is changed from the previous one: "Romaniuk, A., J. Prop, A. H. Petersen, C. R. Wildevuur, and P. Nieuwenhuis. 1987. Expression of class II major histocompatibility complex antigens by bronchial epithelium in rat lung allografts. Transplantation. 44: 209-214" to the current one: ***"Daar, A. S., S. V. Fuggle, J. W. Fabre A. Ting and P. J. Morris. 1984. The detailed distribution of MHC Class II antigens in normal human organs. Transplantation. 38:293-298."***

Page 207, lines 3-5 now reads *"Bronchial epithelium of lung allografts could potentially be the pivotal cells that initiate alloantigen-dependent inflammatory insults, as they express donor class II MHC antigen (116)".* The previously cited reference 79 is omitted form this statement.

• General Comments on English Usage:

All the incorrect usage in the language, and typographical errors listed by the examiner are appreciated, and have been corrected in the thesis as below. I appreciate the examiner's patience and constructive criticisms, although English is not my first language.

1) Page 5, paragraph 1, line 3, page 8, paragraph 2, line 1, page 47, paragraph 2, line 3, and page 58, paragraph 1, line 2, "subepithelial....." reads "**sub epithelia**....."

2) Page 17, line 20, and page 93, paragraph, line10, "International Society of Heart and Lung Transplantation" reads "International Society **for** Heart and Lung Transplantation".
3) Page 20, paragraph 2, line 7, "preoperative....." reads "**peri**-operative....."
4) Page 22, paragraph 2, line 2, "Burke and colleague....." reads "Burke and **colleagues**....."
5) Page 25, paragraph 2, line 1, "antimetabolite....." reads "**anti-metabolite**....."
6) Page 28, paragraph 1, line 4 reads, "Bronchiolitis obliterans (BO) is the pathologic hallmark of the airway component (27, 28, 31)" where "....likely to be....." is omitted.
7) Page 40, paragraph 1, line 4 reads, "In contrast, bronchial epithelial cells are abundant, and **are** constantly exposed to extrinsic infectious agents,....."
8) Page 50, paragraph 1, line 10 reads, "Collagen deposition within the airway wall is thought to result in the irreversible stiffness of the airways in asthma (193-195), which could, in **turn**, lead to poorer response to bronchodilator therapy, ..."
9) Page 56, figure legend reads, "**Fig. 3.** The effect of inner wall thickening. In this example, the normal airways have an inner wall area of 20%. When 30% airway **smooth** muscle (ASM) shortening **occupies**, the airway resistance (R) increases from an arbitrary **volume** of 1 to 7.6. An increase in wall area to 40% produces a small increase in resistance to 1.8 × baseline, but with the thicker wall, 30% ASM **shortening** increases resistance to 80 × baseline. (Data from Kuwano A, et al. 1993. Am Rev Respir Dis 148: 1220-1225. [Ref. 209])."
10) Page 69, line 13 & 14 reads, "....**Haematoxylin**....."
11) Page 70, line 5 reads, "All subjects were sedated with intravenous **midazolam** (2-10 mg) (Roche, Paris, France) as clinically appropriate.
12) Page 140, paragraph 2, line 14 reads, "Furthermore, in the BOS group there was a relationship between vessel size and loss of airway **calibres**."
13) Page 168, paragraph 3, lines 3-5 reads, "In skin, for example, increased type III collagen has been demonstrated in early scar tissue in both animal and humans as compared with increased type I collagen in older **scar** (311, 312)."
14) Page 176, paragraph 1, lines 6-9 reads "It appears that despite its important role in **immunosuppression**, CsA up-regulates TGF-β_1 production and promotes collagen deposition, at least, in human renal allografts (111, 112, 348), but little is currently known in human lung transplant context."
15) Page 214, Ref 9 reads, "Yousem, S. A., J. A. Dauber, R. Keenan, I. L. Paradis, A. Zeevi, and B. P. Griffith. 1991. **Does** histological acute rejection in lung allografts predict the development of bronchiolitis obliterans? Transplantation. 52: 306-309."
16) Page 218, Ref 52 reads, "European Mycophenolate Motetil Cooperative Study Group. 1995. Placebo-controlled study of mycophenolate mofetil combined with cyclosporin and **corticosteroids** for the prevention of acute rejection. Lancet. 345: 1321-1325."
17) Page 219, Ref 67 reads, "Paul, L. C. 1994. Functional and histological **characteristics** of chronic renal allograft rejection. Clin. Transplant. 8:319-323.
18) Page 219, Ref 70 reads, "Sanfilippo, F. 1994. The role and methods of **histocompatibility** testing in transplantation. In: P. C. Kolbeck, R. S. Markin, and B. M. McManus, Editors. Transplant Pathology. ASCP Press. 19-44.
19) Page 222, Ref 99 reads, "Norgaard, M. A., C. B. Andersen and G. Pettersson. 1998. **Does** bronchial artery revascularization influence results concerning bronchiolitis obliterans syndrome and/or obliterative bronchiolitis after lung transplantation? Eur. J. Cardiothorac. Surg. 14: 311-318."
20) Page 222, Ref 103 reads, "Hess, M. L., A. Hastillo, J. A. Thomopson, D. J. Sansonetti, S. Szentpetery, G. Barnhart, and R. R. Lower. 1987. Lipid mediators in organ transplantation: **does** cyclosporine accelerate coronary atherosclerosis? Transplant. Proc. 19: 71-73.
21) Page 223, Ref 133 reads, "Liu, Y. J. 2001. Dendritic cell **subsets** and lineages, and their functions in innate and adaptive immunity. Cell. 106: 259-262."
22) Page 236, Ref 277 reads, Svendsen, U., H. Arendrup, M. NØrgaard, T. Olsen, J. Thiis, S. Mortensen, and G. Pettersson. 1995. Double lung transplantation with bronchial artery revascularization using mammary artery. **Transplantation** Proceedings. 27: 3485.
23) Page 237, Ref 287 reads, "Higenbottam, T., M. Jackson, T. Rashdi, S. Stewart, C. Coutts and J. Wallwork. 1989. Lung rejection and bronchial hyperresponsiveness to methacholine and distilled water in **heart-lung transplant** recipients. Am. Rev. Respir. Dis. 140: 52-57."
24) Page 240, Ref 321 reads, "Mares, D. C., K. M. Heidler, G. N. Smith Jr, O.W. Cummings, E. R. Harris, B. Foresman, and D. S. Wilkes. 2000. Type V collagen modulates **alloantigen** induced pathology and immunology in the lung. Am. J. Respir. Cell Mol. Biol. 23:62-70."

Responses to Examiner' Comments

I shall take the opportunity to thank the Examiners for their comments on my PhD thesis. The following represent point-by-point responses to the comments from A/Professor KD McNeil.

Title: Airway Inflammation and Remodelling post Human Lung Transplantation

1. Whether these studies compare and contrast Bronchiolitis Obliterans Syndrome (BOS) to Asthma?

The aim of these studies was to investigate airway inflammation and aspects of airway remodelling, that occur in the airway walls of lung allografts by using the techniques of endobronchial biopsy (EBB), cell biology, immunohistochemistry, and molecular biology. Although no attempt was made to directly compare or contrast lung transplant BOS airways to asthmatic airways, studies from our group did suggest that the features of airway inflammation and remodelling in lung transplant recipients (LTR), who develop BOS are markedly different from that reported in asthma. The comparison and contrast are in shown in Table 1.

Table 1. Comparing asthma, COPD & BOS

Feature	Asthma	COPD	BOS
Steroid responsive	+++	+	-
Angiogenesis	++	?	+
BHR	+++	+	++
Role of infection	?	+	++
Fibrosis	+	++	+++
Inflammation	CD4/Th2	CD8	CD8
	Eosinophils	Neutrophil	Neutrophil
	Mast cells	Macrophages	Macrophages?

2. In terms of your understanding of the concept of BOS, can you tell me whether or not you consider all LTR with post transplant BO to have BOS?

"Bronchiolitis Obliterans" (BO), in the lung transplant setting, refers to pathological changes of chronic lung rejection that occurs in small airways of lung allografts. By definition, it manifests itself **histologically** as obliteration of the bronchiolar lumen by fibrous scaring tissue. This fibrous scaring can be eccentric with formation of a fibrous plaque in the wall of the airway; concentric with the interposition of a "donut" of collagen tissue; or granulation tissue may completely obliterate the lumen of the airway, reducing the air passage to stenotic cords of scar tissue (41). Therefore, the term of BO should be used only when histology demonstrates dense fibrous scar tissue affecting the small airways (41),

Because BO is difficult to document histologically, due to transbronchial biopsy specimens often being not sufficient sensitive for diagnosis (41), in 1993 a committee sponsored by the International Society for Heart and Lung Transplantation (ISHLT) proposed a clinical description of BO, termed **Bronchiolitis Obliterans Syndrome (BOS),** to be defined by **pulmonary function** changes rather than histology (35). The Spirometric criteria for the diagnosis and stages of BOS have been standardized by the International Society for Heart and Lung Transplantation in 1993 (35), and recently updated in 2001 (41). Although this system does not require histologic diagnosis, it does recognise it being the underlying process.

3. Do you consider all chronic pulmonary allograft dysfunction, where a definitive aetiology cannot be assigned, to represent BOS?

Essentially "yes", by definition that is widely accepted. Chronic allograft rejection in the lung is a clinical-pathologic **syndrome** of graft dysfunction that is characterised histologically by BO and physiologically by airflow limitation (35). However, BO can be also caused by nonimmunologic conditions, and airflow limitation can be related to factors other than BO. Thus, the term BOS has been applied to pulmonary allograft dysfunction with obstructive physiology; histological proof of BO is not necessary, but **other causes of airflow obstruction must be excluded**, such as acute rejection, infection, or airway complications.

4. **[illegible] selection of candidates for the clinical studies.....where do you draw the line in terms of time [illegible] transplant to enable a valid comparison between patients with and without BOS? It is generally [illegible]dered that the patients within three months of transplant are not at risk of developing BOS.....**

I acknowledge this comment very much. Indeed there was just one patient recruited as stable LTR without BOS at 58 days post transplant, while the criteria for BOS established in 1993 by ISHLT defined that patients must be **3 months or more** after transplantation to be considered evaluable under the working formulation (35). At two months post transplant and well the patient clearly did not have chronic rejection. The 3 months cut off was put in place to stop the inclusion of patients prior to this time who weren't well and a non-BOS diagnosis was more likely. Clinical follow up of the patient did not suggest a development of BOS in the next 3 years. Statistically, it would not alter the results presented in the study, if this patient was excluded from the analysis.

Many factors have been reported as risk factors for BOS. In the recent published update of the diagnostic criteria for BOS by ISHLT (41), those factors have been summarised as probable, potential, and hypothetic risk factors (Table 2). Although patients must be **3 months or more** after transplantation to be considered evaluable under the diagnostic criteria for BOS (35), those risk factors do exist throughout the whole course of post transplant.

Table 2. Risk Factors for BOS

Probable risk factors
Acute rejection
Lymphocytic bronchitis/bronchiolitis
CNV pneumonitis
Medication non-compliance
Potential risk factors
CMV infection (without pneumonitis)
Organising pneumonia
Bacterial/fungal/non-CMV viral infection
Older donor age
Longer graft ischemic time
Donor antigen-specific reactivity
Hypothetic risk factors
Underlying disease
HLA-mismatching
Genotype of recipient
Gastroesophageal reflux with aspiration

5. **In the studies on inflammatory cell content in BAL, ... Could you please state for instance, whether or not a certain or initial percentage of BAL recovery was not included in the cellular analysis to exclude airway contamination etc?**

All the procedures of bronchoscopy and bronchial alveolar lavage (BAL) in the studies were performed under European Guidelines for Standardization of BAL (Haslam PL, et al ERJ 14:245-8; 1999). After wedging the bronchoscope in a sub segment of the middle lobe or lingula, three 60-ml aliquots of phosphate-buffered saline warmed to 37 ^{0}C were instilled via syringe. The fluid was immediately aspirated after each aliquot into a container at a negative pressure of approximately –80 mmHg. It is generally considered that the **initial percentage of BAL recovery (approximately 20%)** represent "bronchial lavage", which contains more neutrophils than in "alveolar lavage" (Haslam PL, et al. ERJ 14:245-8; 1999). Our Lab has never separated the first aliquots of BAL return, and this is not recommended in the current guidelines (Haslam PL, et al. ERJ 14: 245-8; 1999).

In the studies, the **initial percentage of BAL recovery** was not excluded from the cellular analysis, as it might reflect, to some extent, the inflammatory cellular content of the bronchial wall compartment, that we were particularly interested in. For example, by conventional bronchoalveolar lavage, although more than 90% of the macrophages obtained are "alveolar" macrophages, the airway macrophages, that are present on the intraluminal surface of conducting airways, account for 5-8% of the macrophage numbers (355). In addition two comparable studies (145, 146) cited by my study did not suggest excluding the initial part of BAL recovery from the analysis.

I entirely agree with the examiner that there is maybe ongoing inflammation in the native airways of lung transplant recipients with cystic fibrosis (CF), which may contaminate the BAL cellular profiles of lung allografts during the passage of the bronchoscope. However, there was similar proportion of CF patients in each group of the patients studied, therefore, the data analysis of the studies were statistically valid.

6. **..... apart from the standard bacterial culture and studies for identifiable respiratory viruses, where any other so called 'atypical' organisms excluded as potential cause of infection.....(eg Mycoplasma, Chlamydia) etc.**

Although both **Mycoplasma** and **Chlamydia** are potentially significant pathogens in immunocompromised hosts, particularly organ transplant recipients (Andersen P. Thorax 53:302-307; 1998, Gass R, et al, Clinical Infection Dis. 22:567-568; 1996), microbiological tests for Mycoplasma and Chlamydia were only performed for lung transplant patients in the Alfred when specifically clinically suspected, due to the difficulty in culture of these organisms and the poor specificity of respective blood tests. To our knowledge, none of the studied patients had infections with these organisms, and these organisms are generally rarely recognised as pathogens in lung transplants. Notwithstanding, an ongoing study in our centre is currently investigating the prevalence of Mycoplasma and Chlamydia infections post lung transplantation, and its role in the development of BOS.

7. **The link between neutrophils and BOS..... If this is a consistent theme, why then does chronic lung allograft dysfunction take the form of airway pathology rather than interstitial diseases as occurs in IPF and ARDS?**

In the recruitment of neutrophils to the lung, chemoattractants serve as a homing mechanism to precisely target neutrophils to the **sites of inflammation**. For example, IL-8 is one of the most potent neutrophil chemoattractants (122). It is primarily produced by monocytes/macrophages, but also by other cells, including bronchial epithelium, pulmonary epithelial cells, pulmonary fibroblasts, and endothelial cells (122). Presumably, whatever the offending agent, there may be a potent chemotactic gradient of IL-8 established from airway epithelium to vasculature **within the airway walls of BOS**, while in **interstitial lung diseases**, a significant chemotactic gradient of IL-8 established between pulmonary epithelium and alveolar vasculature, which culminate in the recruitment of neutrophils into airway walls in BOS, or into the parenchyma of the lung in IPF and ARDS respectively. This is specifically described by DiGiovine et al (145) that IL-8 is localised to peri-bronchial areas shown by immunohistochemistry on transbronchial biopsy specimens from lung transplant patients. However, regardless of the inflammation sites, neutrophilic infiltration into pulmonary tissues has been shown to be associated with the lung injury and subsequent lung fibrosis in a diverse range of animal models and human lung diseases (144, 152, 260-262, 271, 272) and in human lung allografts (145-147).

8. **Clearly, the situation is not the same in asthma and lung transplantation..... so what led you to choose the asthma model of mucosal congestion/hyperaemia as a comparator?**

As previously, we are not directly comparing these situations, but most data relating chronic airway inflammation with subsequently airway damage comes from asthma research.

"Airway remodelling" was quite a new concept few years ago, and refers to the structural changes seen in the airways of airway diseases, such as Asthma and Chronic Obstructive Pulmonary Disease (COPD) (191-193). **Changes in airway vascularity** is one the aspects of airway remodelling. There may be two types of vascular remodelling, sprouting angiogenesis and microvascular enlargement. The changes in the number or calibre of airway mucosal blood vessels are likely to be functionally important, because even modest increases in airway wall thickness can potentially amplify the narrowing of the airway lumen produced by a fixed amount of bronchial muscle contraction (211).

Since it is a significant fraction of the airway wall, the airway microvasculature has been of considerable interest as to its contribution to increased airway wall thickness, lumenal narrowing and hyperresponsiveness in asthma (197, 198, 278-281). The most available observations in asthmatic airways from literature have provided valuable information on the impacts of changes in airway microvasculature to airway physiology. Thus, those studies were frequently referred by the current study as references to the potential for these structure changes to be important, but not as a direct comparator. Actually, as described in point 1, there are some similarities between BOS and asthma-notably the presence of BHR.

In the human lung transplant, although clinically applicable methods for direct restoration of bronchial circulation are available (277), with current surgical techniques the bronchial arterial supply to the tracheobronchial tree is generally not reconstructed at the time of transplantation, which makes lung allografts unique among solid organ transplantation. The survival of donor airway post-lung transplantation is probably totally dependent on the pulmonary circulation for its nutrition via anastomoses between pulmonary circulation and bronchial circulation. This fundamental change in the airway blood supply post-lung transplantation was one factor, which stimulated our interest in investigating the airway vascularity in the lung allograft, and its potential for contributing to airflow dysfunction.

Although our current data suggest that bronchial vascular hyperaemia, engorgement or structural vessel dilatation could be one of the factors contributing to the airflow limitation seen in BOS, further studies are needed to investigate the changes in airway vascularity in lung allografts longitudinally.

9. **"..... The incidence of BOS has been shown in many studies to be the same in heart-lung transplantation and bilateral lung transplantation. Given then, the fact that you have shown that airway vascularity differ between these two groups, your conclusion regarding the finding of this section and the implication of these findings need to be rethought.**

Indeed, there are no significant difference in the incidence of BOS between heart-lung and bilateral lung recipients as shown in many studies. The findings of this study suggested a more normal airway vascularity in the recipients who had a heart-lung transplant compared to those with bilateral sequential or single lung transplant. This was in accordance with clinical and radiographic evidence that human heart-lung transplantation has been associated with fewer airway necrosis problems in comparison to double lung transplantation, probably because of a better-sustained systemic circulation (288).

However, the mechanism of BOS development in lung transplant setting is not yet fully understood. It is more likely a process involving multiple factors, including airway vascularity changes in the lung allograft. Therefore, I concluded this part of study with a certain caution in the thesis: "*In summary, bronchial vascular changes in transplanted lung are probably an important adaptive process and may function to maintain the viability of allograft tissues and structure. However, such changes are not without their potential adverse effects and may contribute to airway narrowing and to chronic graft dysfunction. A longitudinal study to gain better understanding of the natural history of airway remodelling in the allograft and its physiological sequelae are necessary.*"

10. **"..... Work done by authors cited in your bibliography has demonstrated that most CF patients surviving to transplant (at least in their cohort) can be genotyped as "high" TGF-β_1 producer. Is it thus valid to compare TGF-β_1 production in lung transplant patients to normal healthy controls? Should this not be done by using potential lung transplant recipients with disease group similar those of your lung transplant population as the "controls"?....."**

This comment is very reasonable. As noted by the examiner, about 1/3 of the patients in the study were CF patients before lung transplantation. If their underlying disease leading to systemic production of high TGF-β_1, this could be reflected in BAL levels, particularly when the lung capillary permeability is increased. In the study, the systemic levels of TGF-β_1 were not investigated, however, a sub-analysis has been performed to compare the BAL TGF-β_1 levels between CF and non-CF LTR (Table 3). Although the BAL TGF-β_1 levels from CF LTR did not seem to be significantly different from the non-CF LTR, it would be worthwhile to measure its systemic levels in future studies. In general, the use of normal healthy subjects as controls in these studies was used to enable us to appreciate whether or how abnormal were the lung transplant patients studied.

Table 3. Comparison of BAL TGF-β_1 levels between CF and non-CF lung transplant recipients (LTR)

	BAL TGF-β_1 (pg/ml)	P value
CF LTR	48.1 (30.8-84.5)	
non-CF LTR	61.12 (37.2-82.5)	**0.064**