Immunosuppression and Obliterative Airway Disease in Experimental and Clinical Lung Transplantation: The Role of Transforming Growth Factor-beta (TGF- β) and basic Fibroblast Growth Factor (bFGF)

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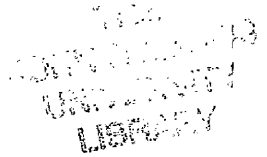

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Abstract

Obliterative bronchiolitis (OB) is the most common cause of morbidity and mortality following clinical lung transplantation affecting around 50% of patients at 4 years. It is characterised by small-airway inflammation and occlusion by fibrous tissue. The aetiology of OB is still unclear. Various factors have been implicated such as lack of bronchial circulation or lymphatic drainage, infections and allograft rejection. Chronic rejection has been identified as the most likely cause of OB in several clinical reports and animal studies. More recently, different growth factors have been implicated in the pathogenesis of OB particularly the ubiquitous Transforming Growth Factor-beta (TGF-β). The aims of the thesis were to establish a reproducible animal model for OB and to study the effect of different immunosuppressive agents on OB, identifying the role of TGF-β in the pathogenesis of OB in the animal model. The final part of the thesis is to assess the influence of the new immunosuppressive agent Mycophenolate Mofetil (MMF) on TGF-β, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) expression in lung transplant patients and its relation to acute rejection and OB in a randomised clinical trial.

A heterotopic tracheal transplant rat model with omentopexy was set up at Manchester University, Immunology Department. The experimental work was divided into 3 parts (or chapters). In the first part (chapter 2), a reproducible rat model for isografts and allografts was designed and established. It consisted of 30 PVG-RT1^u recipient rats. These rats received tracheal implants in their greater omentum from donors across defined genetic barriers. Nine tracheal segments were isografts (PVG-RT1^u donors). The recipient animals were sacrificed at 7, 14, and 28 days. Six tracheal segments were harvested from 3 AO donor rats that share MHC antigens but are diverse in the minor antigens compared with the recipient. The recipients animals were sacrificed at 14 and 28 days. Six tracheal segments were harvested from 3 PVG/c donors rats that share minor histocompatible antigens but are MHC incompatible. The recipients rats were sacrificed at 14 and 28 days. Finally 6 tracheal segments were harvested from 3 donor Lewis rats that are diverse at both minor and MHC antigens. The recipient rats were sacrificed at 14 and 28 days. Retrieved tracheal segments were sectioned, stained with haematoxylin and eosin (H & E) stain and assessed by two independent

observers, one of whom was a Consultant Histopathologist. The epithelial integrity, obliterative airway disease (OAD), submucosal fibrosis (SMF), and mononuclear cell (MNC) infiltrate were graded.

The next study (chapter 3) established the influence of a range of immunosuppressive agents on OAD, SMF, MNC infiltrate and epithelial regeneration. Work reported in chapter 4 was designed to assess the role of TGF-β in the pathogenesis of OAD and to test the hypothesis that TGF-β is over-expressed in OAD. In this chapter, immunohistochemical staining of tracheal biopsies was carried out on all biopsies. Chapters 3 and 4 are part of the same animal experiment. It consisted of forty two recipient PVG-RT1^u rats transplanted with tracheal segments from 21 male PVG Lewis rat donors. The recipients were divided into 7 groups consisting of 6 animals in each. The recipient groups were: controls, Mycophenolate Mofetil (MMF), Cyclosporine (CyA), Tacrolimus (Tac, FK), combination of MMF and CyA, MMF and FK groups. Finally 6 animals were treated with Vitamin E. The animals were sacrificed at 14 days and 28 days. Tracheal segments were assessed for epithelial integrity, OAD, submucosal fibrosis and MNC infiltrate. In addition, all tracheal sections underwent immunohistochemical staining for TGF-β1 (chapter 4).

Work in the fifth chapter of the thesis was to establish the potential beneficial effect of MMF on OAD/OB and acute rejection in the clinical setting and whether it is moderated, directly or indirectly, through suppression of growth factors particularly TGF-β. To achieve this aim, we studied all biopsies from 17 lung transplant patients who formed part of an "International randomised study of MMF versus Azathioprine in lung transplantation" in patients receiving Cyclosporine based immunosuppression. Biopsies from these patients were assessed for acute rejection and OB as well as stained for three growth factors, TGF-β, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF).

In chapter 2, results from the animal model confirmed normal epithelial regeneration, minimal mononuclear cell infiltrate and absence of OB and fibrosis in isografts at 14 and 28 days. Animals receiving grafts from AO or PVG/C donors showed moderate/severe OAD as well as abnormal epithelial regeneration, significant fibrosis and moderate MNC infiltrate.

In chapter 3, no treatment (control) groups developed complete epithelial loss, severe

OAD (complete obliteration) and extensive MNC infiltration. Vitamin E groups developed almost complete epithelial loss, moderate-severe OAD, moderate MNC infiltrate and moderate submucosal fibrosis (SMF). Immunosuppressive treatment with MMF alone had moderate OAD, MNC infiltrate, SMF and epithelial regeneration. CyA, Tac and their combination with MMF seemed to give good epithelial regeneration with normal or near normal epithelium, no OAD, mild/moderate MNC infiltration, and mild SMF.

In chapter 4, TGF immunohistochemical staining showed that TGF-β1 was severely over-expressed in controls, moderate in Vitamin E, mild/moderate over-expression in CyA animals groups. TGF-β was only mild in Tac, MMF, MMF+CyA, MMF+Tac groups.

It is clear from this study that prevention of acute rejection is a very important component in the control of OAD. Hence adequate immunosuppression must be used to prevent the development of OAD in allografts. On the other hand, the relationship of TGF-β1 over-expression with OAD, fibrosis and epithelial regeneration is rather complex. In the CyA group, there was minimal OAD and well developed epithelial lining despite mild/moderate TGF-β1 staining. By contrast, in controls high TGF-β1 over-expression was associated with complete epithelial loss and severe OAD.

In chapter 5, the clinical study further supported the findings from the previous chapter. The results suggested that MMF reduced the incidence of acute rejection and probably that of OB. The MMF group showed no significant reduction in the intensity and distribution of TGF-β1 and EGF expression in lung biopsies when compared with azathioprine treated patients. However, there was a significant reduction in bFGF expression. This reduction was particularly in the distribution of staining of bFGF in lung biopsies.

Based on data from the clinical and the animal studies, it appears that acute rejection is a significant risk for OB or OAD, and TGF-β overexpression is important in the pathogenesis of OB. MMF when combined with CyA (or Tac) has an additive or, may be, a synergistic effect that seems to result in superior and more balanced immunosuppression, with better control of acute rejection. For the first time, inhibition of growth factor over-expression was observed with MMF therapy. Inhibition of TGF-β overexpression was seen in the rat tracheal biopsies and in the

expression of bFGF in the lung biopsies. It is still not clear yet whether this is a direct or an indirect effect. This effect on growth factors may, at least in part, play an important role in the prevention of OB, particularly in cyclosporine treated patients.

Declaration:

This thesis is based on results from animal work performed at the University of Manchester, Department of immunology over a period of 6 months started in March 1997. The laboratory work was carried out predominantly in the transplant laboratory at Wythenshawe hospital. The work was done during a clinical post as Associate surgical specialist in cardiac surgery for 3 years and as a consultant cardiothoracic surgeon in the last 2 years.

No portion of this work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or another institute of learning.

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Papers arising from this work:

1. Tracheal allograft transplantation in rats, the role of immunosuppressive agents on development of obliterative airway disease **NA.Yonan,** P Bishop, A El-Gamel, IV Hutchinson. **Transplantation Proceedings: 1998; 30(5); 2207.**

2. Tracheal Allograft Transplantation: The impact of different immunosuppressive regimes on Transforming Growth Factor Beta expression and development of Obliterative Airway Disease. **NA.Yonan**, Gillian M. Entwistle, P. Bishop, IV Hutchinson
Submitted for publication to Transplant Immunology in August 2002

Chapter I:

Introduction

1.i Lung transplantation:

I.i.a: History and development:

The first successful human lung transplant with long term survival was achieved by Reitz and associates in 1981 (1). Since then, lung transplantation has been successfully used world-wide. The advances in the surgical techniques particularly in single and bilateral sequential lung transplantation, improvement in recipient and donor selection and management, advances in immunosuppressive therapies with development of new drugs, and improvements in diagnosis and treatment of infection with more potent antibiotics, anti-viral and anti-fungal therapies have improved results dramatically. The operative mortality for lung transplants is around 10 percent. The 1, 3, and 5 year actuarial survivals for all transplants are 71%, 60%, and 46% , respectively (2) (figure 1).

The eighteenth official registry report of the International Society for heart and lung transplantation (ISHLT) have shown that a total of 15475 lung transplants were performed world wide (2). There were 7201 single lungs, 5419 bilateral lung transplants and 2855 heart and lung transplants (figures 2 and 3). Despite the rapid expansion of lung transplantation in less than 2 decades, problems remain, most notably chronic lung rejection which manifests itself as small airway obstruction which is well recognised and has been named obliterative bronchiolitis (OB) or Bronchiolitis Obliterans Syndrome (BOS) (3).

I.i.b. Clinical lung transplantation:

The first successful clinical lung transplant was carried out by Hardy and associates in 1963 (4). Despite the short lived success of this pioneering procedure as the patient died on the 18th post operative day, it demonstrated the surgical feasibility of single lung transplantation (SLT). Also it generated interest in pulmonary transplantation world-wide.

Despite the initial euphoria and wide spread interest in lung transplantation, the number of procedures in the next 20 years were very small. A total of sixty procedures were carried out in that period. The main reason was lack of long term survival in those early patients (5, 6). In 1983, the first long term single lung transplant

survival was achieved by Cooper and associates in Toronto (7, 8). By then, the first human lung transplant long term survivor was already achieved by Bruce Reitz in March 1981 when he carried out a successful heart and lung transplant on a 45 year old patient (9, 10).

The success of heart /lung transplantation(HLT) was attributed to the good healing of the tracheal anastomosis due to cononary-tracheal collaterals, contrary to the poor bronchial healing of SLT. Hence the indication for HLT expanded from being exclusively used in patients with pulmonary vascular disease, particularly those with pulmonary hypertension secondary to congentital heart disease (Eisenmenger syndrome) to include intrinsic lung disease like emphysema (11).

Double lung transplantation (DLT) as well as bilateral sequential lung transplantation (BSLT) was introduced to clinical practice by the Toronto group in the late Eighties (12, 13). BSLT superseded DLT because bronchial healing at the anastomotic site was becoming more successful following the introduction of omental wrap and telescoping anastomotic techniques. These advances reduced serious bronchial complications drastically. Tracheal healing in DLT on the other hand, remained a major problem despite improved surgical techniques and this procedure was ultimately abandoned. Currently, SLT and BSLT have become the established modalities for the management of end stage lung disease (14).

I.i.c: Physiology of the transplanted lung:

1. The early post-transplant lung function:

Ischaemic and reperfusion injury are the main causes of early post transplant graft dysfunction. This is manifested clinically by hypoxaemia, pulmonary oedema, raised mean pulmonary artery pressure and raised airways pressure. Radiologically, it manifests itself as pulmonary oedema, pulmonary infiltrate, hilar flare and pleural effusion (15). Reperfusion injury is worse in patients with pre-existing pulmonary hypertension (16).

Functional changes following lung transplantation are partly caused by impaired

surfactant function during re-implantation as a result of high levels of inhibiting serum proteins and low level of phosphatidyl choline (17). This results in atelectasis and pulmonary oedema (18).

Infection is a major problem post transplantation. This can be related to factors such as the transmission of donor pathogens during transplantation. Recipient factors may include a constant source of contamination like chronic infection in the nasal sinuses or upper airways (Cystic fibrosis) or infective foci in the contralateral lung. It is important to remember that impaired bronchial mucociliary clearance by the transplanted lung is a major predisposing factor to increased infection incidence (19). It is also worth noting that the lung is the only transplanted organ that is in continuity with the atmospheric environment.

The cough reflex is absent following lung and heart lung transplantation due to vagal denervation (20). The lymphatic drainage is also severed during transplantation with lymphatic leak into the pleura. It is estimated that lymph drainage of the lungs is around 15% of total thoracic duct lymph (21). This may cause prolonged pleural drainage following transplantation.

2. Ventilation and perfusion:

In single lung transplantation, the blood flows to the lung with lower pulmonary vascular resistance (PVR). Hence, the blood flows preferentially to the transplanted lung as it has a lower PVR. It has been demonstrated that over 95% of blood flow is into the transplanted lung in patients with pulmonary hypertension who undergo SLT (22). This will result in a significant decrease in the mean pulmonary artery pressure and subsequent reduction in right ventricular afterload that eventually leads to a decrease in right ventricular dilatation and tricuspid incompetence (23).

With regard to ventilation, there is less preference of ventilation to the transplanted lung compared to native lung. This may result in some ventilation/perfusion mismatch. Symptomatic severe V/Q mismatch may develop if the bronchus of the transplanted lung is occluded by retained secretions.

In SLT for emphysema, hyperinflation of the native lung may occur. This is due to poor compliance of the transplanted lung compared to native lung particularly in the early post-operative period (24).

I.ii: Immunology of the transplanted lung:

The immune system plays a vital role in the survival of individuals. It has the ability to recognise "self" from "non self" and is able to destroy "non self" through a variety of mechanisms. In order to have a successful transplant (without immunosuppression), the organ has to have an identical genetic code to that of the host. The host immune system will then recognise it as "self" and hence does not mount an immune response. This is the scenario in transplantation between identical twins (isograft).
Transplantation between unrelated individuals (allografts) will result in immune cells recognising that these cells are "non self" and begin the process of destruction of that organ. This process is summarised as "rejection" which ultimately ends with the destruction of the graft unless the immune system is circumvented or suppressed (25) (figure 4). The initial work on transplant immunology was carried out by Sir Peter Medawar and colleagues who in a series of experiments identified the "laws of transplantation" and showed elegantly that rejection is a systemic process governed by lymphocytes (26, 27).
The initial studies on mouse skin grafts led to the identification of the Major Histocompatability Complex (MHC) antigens. MHC binds the processed antigens and presents them to T lymphocytes (28) (figure 5). T cells recognise antigens as peptides, derived from processed intracellular proteins that are bound to the cell surface and presented at the cell surface by the MHC glycoproteins (29, 30).
Antigen presentation is central to the process of immune recognition that triggers the activation of helper and cytotoxic T lymphocytes (31, 32). The helper T cells have a regulatory function. They produce mediators called cytokines or lymphokines. These agents deliver messages to the effector cells helping to orchestrate the immunological response. Helper cells are also essential for the differentiation of B lymphocytes into antibody producing cells such as macrophages.
Cytotoxic T lymphocytes, on the other hand, are effector cells that can directly kill specific cells that are infected by a virus or other pathogen. This process is triggered

by the recognition of "non self" on the target cell surface (figure 6).

It is recognised that both helper and cytotoxic T cells use the same mechanism of antigen recognition via similar receptors (33). The reason behind differential activation of helper and cytotoxic T cells resides in the properties of the antigen presenting MHC molecules (34). It is generally accepted that many cytotoxic T cells recognise antigens as presented by class I MHC molecules while most of helper T cells are restricted to class II MHC molecules (34). Class I restricted T cells subsets are CD8 positive while class II restricted T cells subsets are CD4 cells. These subsets have distinctly different surface protein markers. They recognise specific sites of corresponding MHC molecules (35). Although class I and Class II have many structural and genetic similarity, there are marked differences in tissue distribution, regulation of surface expression and peptide binding dynamics (36).

I.ii.a. Transplant rejection:

The activation of the naive and memory recipient T cells is the main mechanism of acute rejection (figure 4). The initial phase is sensitisation of the immune system by passenger leukocytes in the graft being recognised by the recipient CD4+ T lymphocytes. The effector phase of rejection starts when activated CD4 cells (helper cells) are stimulated to produce lymphokines/cytokines (figure 7). This initiates a cascade of responses which involves activation of antibodies as well as activated lymphocytes and macrophages, resulting in cellular infiltration into the allograft, inflammatory process leading to tissue injury and ultimately to graft destruction (37).

Allograft rejection is a process that involves activation of antigen presenting cells, T helper cells, release of pro-inflammatory cytokines or interleukins: IL-1, 2, 6, interferon-gamma, and tumour necrosis factor-alpha (figure 7). Alternatively, other cytokines may be released that suppress graft rejection such as IL-4, 5, 10, and transforming growth factor beta (TGF-β) (38).

In summary, cytokines play an important role in the co-ordination and regulation of transplant rejection. The process of rejection is a systemic inflammatory process, involving pro-inflammatory cytokine stimulation. Donor epithelial cells may actively participate in the host response by producing chemokines and other inflammatory mediators.

I.ii.b. The human major histocompatability complex:

The major MHC antigens known in humans as human leukocyte antigens (HLA) are glycoproteins encoded by the major histocompatability complex located on the short arm of chromosome 6 (39).

MHC molecules are of two isotypes: Class I which include HLA-A, HLA-B, HLA-C and class II which includes HLA-DR, HLA-DP, HLA-DQ (figure 8). The class III region lies between class I and II regions and contains genes related to the complement system particularly C2 and C4 and the tumour necrosis factors. Class I and II molecules are cell surface antigens. Class I antigens are expressed on the surface of almost all nucleated cells in the body while Class II antigens are expressed on the surface of antigen presenting cells (APC). These include B-lymphocytes, macrophages and dendritic cells (33, 40). MHC class I and class II loci are highly polymorphic and as allogenic cells can express different MHC molecules, this enhances the ability of T cells to recognise foreign MHC antigen derived from the donor organ (41).

I.ii.c. The activation of T cells :

T cell activation occurs by 2 distinct pathways (42) (figure 9). The first is the direct pathway, in which T cells recognise donor MHC antigen molecules, class II peptide, expressed on the surface of donor APCs. The second is the indirect pathway in which peptides derived from the catabolism of donor molecules, including allogenic MHC molecule, are presented to the T helper cells by the MHC molecules on host APCs. The direct pathway may be responsible for the immune responses in acute rejection while the indirect pathway may have a major role in chronic rejection as donor APCs in the graft will be replaced by host APCs gradually.

In the direct pathway, T cells can recognise donor APCs in the graft or in the recipient spleen (43). While in the indirect pathway, donor antigen recognition may occur in the graft or in the recipient lymphoid tissue (44).

Activation of T helper cells by either mechanism will result in the release of different cytokines that can stimulate macrophages and B-lymphocytes. Activated B-lymphocytes produce alloantibodies while activated macrophages and helper cells will

contribute to graft rejection by the delayed type hypersensitivity response. In addition, cytokines can act as growth and activation factors for cytotoxic T lymphocytes resulting in the destruction of the graft by lysis of donor cells (45).

I.ii.d. Chronic rejection:

Chronic rejection in lung transplantation is manifested as small airway disease resulting in inflammation and later fibrosis or obstruction, a typical pathological feature of obliterative bronchiolitis (OB). It has also been described in lung transplantation as bronchiolitis obliterans syndrome (BOS). The incidence of OB following lung transplantation is common and estimated to be around 40-50% in long term survivors (46, 47). Initially, It was thought that OB was restricted to HLT and would not occur in isolated lung transplants. However, a few years later, this complication was reported in SLT and BSLT (48-50). The pathological picture in the active phase of OB demonstrates injury of the bronchiolar epithelium with submucosal lymphocytic infiltrate. The subsequent development of granulation tissue occurs with acute and chronic inflammatory cells, eventually leading to formation of scar tissue. In severe cases this may eventually result in total occlusion of the entire lumen of the bronchiole (51, 52) (figure 10).

Immunologically, acute lung injury due to acute lung rejection is manifested by increased infiltration with recipient MHC class II cells in the perivascular, peribronchial, interstitium and alveolar spaces. Furthermore, expression of class II antigens is induced on donor bronchial epithelium and vascular endothelium. This is found as early as two days post-transplant. The intensity of class II antigen expression on the bronchi and pulmonary vasculature of the graft increases with the progressive deterioration of rejection (53-58). The severity of rejection correlated well with biochemical markers in the bronchioalveolar lavage (BAL) fluid obtained at the time of bronchoscopy. These markers include tumour necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), interleukin 2 (IL-2) and soluble IL-2 receptors (sIL 2R) (59-61). The abnormal expression of class-II antigens on the vascular endothelium and bronchial epithelium, as well as abnormal increase in cytokines levels of the BAL fluid taken from the rejecting lung, can be suppressed by cyclosporin A (CyA) treatment (58, 61, 62). Once the bronchial epithelium and

vascular endothelium become positive for MHC class-II antigens, they are likely to be targets for immune injury resulting in low grade rejection which may ultimately result in the development of BOS and small vessel vasculopathy (63).

The presence of OB following lung transplantation is a clinical diagnosis based on lung function test. As it is essentially a disease of the small airways, changes in forced expiratory volume in first second (FEV_1) is the most commonly used marker. Forced expiratory flow (FEF) is also a useful marker. As OB progresses, the patient becomes more breathless and their FEV_1 and FEF 25%-75% decline to 50-75% of normal value for that patient (64).

Histologically, OB can be diagnosed by transbronchial biopsy (TBB). Usually numerous biopsies are required from each lobe to establish the histological diagnosis. The sensitivity of transbronchial biopsy for obliterative bronchiolitis is poor with a high incidence of false negatives (46).

In addition to OB, chronic lung rejection can occur as chronic vascular rejection. Here, there is fibromyxoid thickening of the subendothelial zones of arteries and veins. This thickening is due to proliferation of smooth muscle cells, fibroblasts and myofibroblasts. The presence of accelerated graft atherosclerosis is difficult to identify on TBBs but can readily be seen on post-mortem histology sections from the pulmonary graft (65).

I.iii: The operative procedure and post-operative period.

I.iii.a. Patient selection:

Patients with end-stage lung disease who have the best opportunity for long-term survival with a capacity for full rehabilitation are considered for lung transplantation (66). Careful consideration of the type of transplant procedure, deemed suitable for different individuals with a specific disease entity, has been elucidated (67-70).

I.iii.b. Indications for different transplant procedures:

Heart and lung transplantation: This is the procedure of choice for patients with pulmonary hypertension and severe right ventricular dysfunction due to uncorrectable congenital heart disease (Eisenmenger Syndrome) (71), primary pulmonary

hypertension with ventricular dysfunction and severe concurrent cardiac and pulmonary disease (71).

Single lung transplantation is the procedure of choice for patients with pulmonary fibrosis, older patients with emphysema, patients with sarcoidosis or asbestosis with end stage pulmonary disease and for selected patients with primary (PPH) and secondary pulmonary hypertension with good left ventricular function and easily correctable congenital defects (13, 72-75). It also seems to be the most commonly used procedure for a re-transplant in patients who have previously had HLT or BSLT (76-78).

Bilateral lung transplantation (BSLT): The primary indication for BSLT is a septic lung condition. Cystic fibrosis is the most frequently encountered condition in this category (14). Emphysema (in younger patients), bronchiectasis and PPH are less common indications.

I.iii.c. Post-operative management:

Ventilatory management post transplant is based on the principles of prevention of re-perfusion injury, positive end expiratory pressure (PEEP) and lowest adequate inspired oxygen. To reduce lung trauma, ventilatory pressure should be the minimal necessary to provide normal oxygenation.

Fluid management is of paramount importance. The transplanted lung has a tendency to trap excess fluids hence the fluid management is based on adequate diueresis and blood or colloid infusion. Patients are extubated as early as possible if they have a clear chest X-ray and normal oxygenation (79).

I.iii.d. Infection and rejection:

Despite aggressive immunosuppression, acute rejection is a common and troublesome problem following lung transplantation. When symptomatic, it presents with breathlessness and hypoxaemia. Acute lung rejection can be a very rapidly progressive process and can lead to a marked deterioration in the patient's clinical condition within a few hours (80, 81). Apart from breathlessness, patients may have low grade fever, leukocytosis, perihilar or interstitial infiltrate on the chest X-ray (58, 63). This clinical picture is typically seen in the first 3 weeks post transplant. Furthermore,

a similar clinical picture can be seen in acute pulmonary infection that is common in the early post-transplant period because of augmented immunosuppression and the likelihood of donor transmitted infection (82-84). Taking the clinical picture alone into consideration, it is usually difficult to differentiate between infection and acute rejection. Furthermore, both processes can occur concurrently. Therefore special investigations are required which include, in addition to haematological and biochemical profiles and plain chest radiograph, CT scans and fibro-optic bronchoscopy, bronchioalveolar lavage (BAL) and transbronchial biopsy (TBB). The latter is the most useful tool for the histological diagnosis of rejection (85) and assessment of its severity using the International Society for Heart and Lung Transplantation grading system (86). TBB is also an invaluable tool that can differentiate infection from acute rejection (87, 88). BAL has not been a useful diagnostic tool for the diagnosis of rejection, but it is extremely important in the diagnosis of opportunistic infections which are fairly common in immunosuppressed patients.

Transbronchial lung biopsy is the gold standard for the diagnosis of acute rejection in lung transplantation. The typical histological appearance is that of a perivascular lymphocytic infiltrate (figure 11). TBB was reported to have a specificity of 100% and a sensitivity of 84% (85). Multiple biopsies are required from each lobe and on average 16 biopsies are taken per session in order to avoid false negative results (85). TBB and BAL are carried out routinely following transplantation and according to a specific protocol, specific for individual units. TBB is an invasive procedure that carries a small risk of complications including pneumothoax and bronchial haemorrhage. Careful attention to a clotting disorder prior to biopsy reduces the incidence of this complication (89).

I.iii.e: Chronic rejection and obliterative bronchiolitis (OB):

Obliterative Bronchiolitis (OB) is the most common late pulmonary complication among lung and heart-lung transplant recipients (3, 47, 90, 91). It remains the most common cause of morbidity and mortality following clinical lung transplantation affecting around 50% of patients at 4 years (92). The aetiology of OB is still unclear. Lack of bronchial circulation or lymphatic drainage (93), infections (94) and rejection (95) are possible causes. Chronic rejection, however, has been

identified as the most likely cause of OB in several clinical reports (96, 97) and animal studies (98-101).

Recently, growth factors (102-104) are thought to play a central role in the pathogenesis of OB particularly Transforming Growth Factor Beta (TGF-β). TGF-β is a potent cytokine that promotes fibrosis by enhancing the synthesis of extracellular matrix components (104, 105). The repair process following lung allograft injury replaces lung parenchyma with fibrous tissue, leading to pulmonary dysfunction. TGF-β has a major role in co-ordinating tissue repair, hence its over-expression may result in exaggerated or aberrant tissue repair processes which may ultimately result in tissue fibrosis (104).

The clinical presentation of chronic rejection is primarily a form of obliterative bronchiolitis with a fall in the forced expiratory volume in the first second of expiration (FEV_1) which proceed dyspnea, the clinical presentation commonly seen in these patients. Although the pathological hallmark of chronic rejection is actually OB, sometimes the terms are used interchangeably. This is misleading as it may be that most chronic lung rejection is presented as OB, not ALL OB cases are of immunological aetiology, as other factors such as aspiration or chronic infection may result in OB (106-108). Because of the heterogeneity in the causation of OB, a consensus conference sanctioned by the ISHLT gave a new terminology to chronic allograft dysfunction as bronchiolitis obliterans syndrome (BOS) (109, 110). This obviously will focus on the clinical observations like sudden or gradual progressive reduction in FEV_1 rather than based purely on histology.

Treatment strategies for BOS are of limited success and based on augmentation of immunosuppression using a variety of agents. Corticosteroids are generally used as a first line. More recently other modalities such as total lymphoid irradiation (TLI) (111), cytolytic therapy (112), MMF (113, 114) and tacrolimus (115-117) have been used. In end stage OB retransplantation is the only option available (76, 77, 118).

I.iii.f. Infection post lung transplantation:

Infection in the lung transplant patients is fairly common and is the major cause of morbidity and mortality (64). The lungs are the only transplanted organs with direct

communication with the atmosphere hence infections from the surrounding hospital environment, as well as infection from upper airways and nasal sinuses is common, particularly in cystic fibrosis patients. Moreover, contamination can be transmitted by donor lungs. The commonest infectious organisms are bacteria (64) followed by viruses, most notably the cytomegalovirus (CMV) (82, 84, 119-122). Protozoa like pneumocystis pneumonia (PCP) is less common since most units use septrin prophylaxis. Toxoplasma, another protozoa, can also be transmitted by donor tissue. In patients with mismatch to toxoplasma, i.e serologically are donor positive and recipient negative, prophylaxis is generally given using pyrimethamin for 3 months to prevent active infection. Finally fungi are encountered more commonly in lung transplantation particularly Candida Albicans. Less commonly seen but much more difficult to treat is Aspergillus infection which seems more common with more aggressive immunosuppressive regimes.

I.iii.g. Immunosuppression:

Most clinical lung transplant programs use the standard triple immunosuppression therapy of cyclosporine, azathioprine and prednisolone (123-126). Cyclosporine, a calcineurin inhibitor, is the mainstay immunosuppressive agent that acts primarily by inhibiting IL-2 synthesis and expression of IL-2R on T helper cells (figure 12). It is given in doses ranging from 5-10 mg/kg/day in 2 divided doses and its main side effect is nephrotoxicity. Azathioprine is given in doses ranging between 1-2mg/kg/day in one single daily dose. It acts by inhibiting the division of rapidly multiplying cells like lymphocytes through the inhibition of purine synthesis. It is primarily a bone marrow depressant **(REF).** Corticosteroids, have wide ranging immunosuppressive properties, given at doses of 1gm IV at surgery followed by reducing oral dose, starting at 1mg/kg/day to be reduced to 0.2mg/kg/day at 3 weeks post operatively. Newer immunosuppressive agents have been introduced to lung transplantation primarily tacrolimus which, like cyclosporine, is a calcinurin inhibitor (figure 12). It has had some success as a primary as well as a rescue agent in lung transplantation (116, 117, 127). The other new agent is mycophenolate mofetile (MMF), which acts by inhibiting nucleic acid synthesis, hence inhibiting T and B lymphocyte proliferation. It is primarily used to replace azathioprine in CyA or tacrolimus based

immunosuppression regimes (113, 128, 129). It has been shown to improve one year survival in lung transplant patients in a randomised trial against azathioprine (130).

I.iv: Lung fibrosis and Obliterative Bronchiolitis:

I.iv.a. Lung fibrosis:

Fibrosis is the final pathological process that occurs in most allografts including, heart, kidneys, liver and lungs. The process is complex and has three overlapping stages. The first is the inflammatory stage which follows tissue injury such as rejection, infection or ischaemia. The second is the healing stage, the formation of granulation tissue. The final stage is the re-modelling stage, the formation and remodelling of extracellular matrix (131).

The fibrotic process as described is similar to normal wound healing where parenchymal cells infiltrate the wounded tissue, leading to extracellular matrix deposition. This results in the growth of normal granulation tissue and ultimately scar formation (132).

Lung fibrosis can be caused by certain disease processes, the aetiology of which is sometimes known (e.g. asbestosis), or it can be of unknown cause (133). The vascular endothelial cells are the first cells to be affected, then the type I pneumocytes with basement membrane are involved. This tissue injury can be accompanied by local inflammation. The inflammation begins as infiltration with polymorphonuclear leukocytes or neutrophils (PMN) which lasts a few hours to a few days. These cells are replaced by a mononuclear cell infiltrate (monocytes and macrophages or MNC) which lasts few a days to a few weeks. The inflammatory process is essential to the healing process initially by amplifying and disrupting the normal architecture caused by the local injury and by the accumulation of mesenchymal cells in the local milieu (132). The inflammatory process of the vascular endothelium causes alteration in capillary permeability leading to a rapid leakage of inflammatory cells, platelets and serum proteins into the interstitial and alveolar spaces (134).

Cytokines can be produced and released from inflammatory cells and platelets as well as mesenchymal cells. These cytokines play a major role in the fibrotic process by

recruiting more inflammatory and mesenchymal cells and by promoting proliferation and migration of smooth muscle cells to the alveoli. Recruitment of mesenchymal cells leads to accumulation of the extracellular matrix. This leads to progressive deposition of collagen, elastin, fibronectin and proteoglycans resulting in excessive formation of granulation tissue that leads to tissue fibrosis (132).
In the lung transplant setting, the graft is exposed to multiple injuries caused initially by ischaemia and re-perfusion injury, acute rejection and infection. This injury, as mentioned above, will result in injury to vascular endothelium and pulmonary epithelium which initiates the cascade, ultimately leading to graft fibrosis (135).

I.iv.b. Cytokines and growth factors:

Cytokines are soluble polypeptides molecules which are secreted by activated T-helper lymphocytes (CD4), macrophages and other cells and tissues (figure 13). Cytokines act by binding to high affinity receptors on the surface of target cells and by inducing biochemical signals within those cells that have profoundly been affected by their behaviour. The term cytokine, was used initially to differentiate a group of immunomodulatory proteins, also called immunotransmitters, from other growth factors that modulate the proliferation and bioactivities of non-immune cells. However, this clear-cut distinction cannot be maintained and may not be meaningful altogether (136). Some cytokines are produced by a rather limited number of different cell types while others are produced by almost the entire spectrum of known cell types. The initial concept "one producer cell, one cytokine, one target cell" has been falsified for almost every cytokine investigated more closely (136, 137). Recently the term cytokine is used as a generic name for a diverse group of soluble proteins and peptides, which act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment (138, 139). The biological activities of cytokines are similar to those of hormones produced in specialised glandular tissues. Some cytokines behave like classical hormones as they act at a systemic level.
In general cytokines act on a wider spectrum of target cells than hormones. The

important characteristic that distinguishes cytokines from hormones is that cytokines are not produced by cells that are organised in specialised glands, i. e. there is not a single organ source for these mediators (138, 139).

Cytokines can be divided into different subgroups, these are: Growth factors, lymphokines and interleukins, colony stimulating factors (CSFs), transforming growth factors (TGFs), tumour necrosis factors (TNFs), interferons (IFNs) and chemokines. Cytokines have different functions, such as the control of cell proliferation and differentiation during embryonic development and in later life, regulation of the immune response to foreign antigens and invading organisms, defence against viral infection and other intracelluar pathogens. They play an essential role in cellular regeneration and wound healing, regulating the development of cellular and humoural immunity and mediating the inflammatory response. Alteration in the production or action of cytokines can be responsible for different diseases. Advances of technology and a better understanding of the role of cytokines in humans may help in using them as therapeutic agents against some inflammatory diseases and even some forms of cancers as well as better control of acute and chronic allograft rejection (140).

I.iv.c. T-helper CD4+ lymphocytes:

T-helper lymphocytes can be divided into two functional subsets, T-helper 1 (TH1) and T-helper 2 (TH2) categorised by the profile of cytokines they produce (141). TH1 cells produce IL-2, IFN-γ, TNF-α, IL-12. TH1 cytokines tend to promote cell mediated responses by inducing cytotoxic T cells and the delayed type hypersensitivity reaction that is mediated by macrophages. On the other hand, TH2 lymphocytes produce IL-4, IL-5, IL-10, IL-13. TH2 cytokines are responsible for B-cell activation and antibody mediated immune responses. A third pattern of cytokine production is attributed to TH0 cells that are the precursor cells that can fully differentiate to TH1 and TH2 (figure 13). The differentiation of the naive Th0 cells can be affected by the environment and *in-vitro* studies show that the early presence of IL-4 induces differentiation towards the TH2 subset while the presence of IL-12 promotes the differentiation towards TH1 cells (142). It is interesting to note that IL-4 and IL-10 have the ability to inhibit the expression of Th-1 cytokines whereas IFN-γ and IL-12 can inhibit the expression of Th-2 cytokines.

Cytokines can play an important role in the development of lung allograft fibrosis. This is either by recruitment of mesenchymal cells and increasing intracellular matrix production, or by contributing to the inflammatory reaction. Different cytokines play different roles. IFN-γ and TNF-α could be described as inflammatory cytokines while, transforming growth factor-β (TGF-β) and platelet derived growth factor-β (PDGF-β) can enhance the synthesis of the extracellular matrix components.
Human IFN-γ is a 20 kilo Dalton (kDa) cytokine that plays a central role in the cellular immune response (143). It can be produced by a number of T-lymphocytes that include T helper cells, cytotoxic cells and natural killer (NK) cells (144, 145). IFN-γ has been shown to increase the expression of MHC class I and class II molecules on the surface of different cells such as macrophages, epithelial and endothelial cells which may be vital in the initiation of an inflammatory response (146). It can also augment the infiltration of inflammatory cells into injured tissue by enhancing the re-organisation of endothelial cell basement membrane (147).
In addition to its pro-inflammatory action, IFN-γ can modulate fibroblast collagen matrix deposition by increasing the expression of the cell surface receptor for collagen (148). IFN-γ can also increase the expression of the cell surface marker (CD40) on the surface of mesenchymal cells including fibroblasts. Fibroblasts can interact with T-cells through this surface marker leading to the activation of both cell types. This process is important for normal wound healing process but, if left unchecked, can be pathogenic and may result in exuberant tissue fibrosis (149).

I.iv.d. Tumour necrosis factor-alpha:

This is a pro-inflammatory cytokine composed of a 26 kDa peptide. It is mainly produced by monocytes and macrophages. TNF-α has the ability to activate endothelial cells and macrophages (150). It can also induce the expression of MHC class I molecules on endothelial cells but it does not seem to stimulate the release of class II antigens. The activation of endothelial cells can also result in expression of adhesions molecules like ELAM-1, VCAM-1 and ICAM. It also enhances the expression of IL-8 and other chemokines (151, 152). This expression results in the binding of granulocytes and monocytes to the vascular endothelium leading to enhanced migration of leukocytes to the site of inflammation which enhances the

development of the inflammatory state. TNF-α may play a major role in immuno-regulation by enhancing the proliferation of T-lymphocytes (153).

I.iv.e. Platelet-Derived Growth Factor:

Platelet-Derived Growth Factor (PDGF) is a polypeptide growth factor that was first identified in the serum. It is secreted from the α-granules of platelets during the process of clotting. It can also be secreted from the smooth muscle cells, endothelial cells, and macrophages (154). Functional PDGF is secreted as a dimer with two chains, A and B chain (PDGF-AA, PDGF-AB, PDGF-BB) and it can be bound by two distinct receptors, α and β (155). PDGF is a powerful mitogen for connective tissue cells including dermal fibroblasts, arterial smooth muscle cells, epithelial and endothelial cells (156-158). In addition to its mitogenic properties, PDGF is a chemotactic agent for fibroblasts and smooth muscle cells (159). In addition, PDGF can stimulate formation of type V collagen (160) and stimulate collagenase activity 3 fold (161). In summary, PDGF is mitogenic and chemotactic for fibroblasts smooth muscle cells, also has the ability to stimulate and degradate matrix proteins. PDGF can be involved in the wound healing process as well as in pathological processes like fibrosis, inflammatory arthritis, atherosclerosis and tumour growth (162).

I.iii.f. Basic fibroblast growth factor (bFGF):

Fibroblast growth factors are polypeptide growth factors that include acidic FGF (aFGF or FGF-1) and basic FGF (bFGF or FGF-2) molecules. Its main function is an inducer of growth and a chemoattractant for the migration of smooth muscle cells (SMC) (163). Both aFGF and bFGF are prototypic members of a family of structurally related molecules (164). They act as potent mitogens for mesodermal derived cells *in vitro* and potent hormonal inducers of angiogenesis *in vivo* (165, 166). The biological activity of FGF involves productive cell surface interaction with high affinity specific receptors such as FGFR-1, FGFR-2, FGFR-3 and FGFR-4 (167, 168).

Prototypic FGF proteins were identified initially in neural tissue (169). They were later identified, isolated and characterized in the bovine kidney (170).

Apart from their role as inducers of neurogenic repair, they has been associated with

glomerular as well as vascular lesions in chronic renal transplant rejection (171). They have also been associated with angiogenesis, growth enhancement and chemoattraction of smooth muscle cells, fibroblast as well as promoting angiogenesis in area of intimal hyperplasia associated with cardiac and renal allograft vasculopathy (172).

In lung transplantation, high levels of bFGF have been detected in the bronchioalveolar lavage of patients with OB compared to non OB patients. This suggests that bFGF may play a significant role in the pathogenesis of OB. Cytokines such as bFGF can mediate lung allograft injury by being directly toxic to the tissues. It may enhance the rejection processes by stimulating the proliferation of T lymphocytes and other immunocytes. In chronic lung rejection, where bronchiolar as well as vascular rejection occurs, there is fibro-intimal hyperplasia and scar formation. The participation by proliferative growth factors is extremely likely. Previous studies have suggested a significant role for bFGF in the pathogenesis of OB (52). This is probably due to its mitotic activity for fibroblasts, vascular endothelial cells, myoblasts and smooth muscle cells (173). bFGF can also modulate pulmonary mesenchymal and fibroblast cell migration , replication and granulation tissue deposition (52). It is very likely that bFGF may also act directly or indirectly as an angiogenic promoting factor (174).

I.iii.g. Epidermal growth factor:

Epidermal growth factor (EGF) is a polypeptide cytokine with fibrogenic property (175). It is a growth-promoting peptide (176) which has a potent chemoattractant property for vascular endothelial cell line (RHEC) in human as well as rats (177). Many studies indicated that EGF-like factors may function *in vivo* to control, in part, the angiogenic events that occur during tissue repair (177). Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-alpha), strongly decreased IL-18 mRNA expression in HaCaT keratinocytes (178). Other studies suggested that EGF and TGF-alpha play an important role in the implantation process by directly stimulating trophoblast development (179). EGF may also play a role in the maintenance and repair of airway epithelial cells in adult humans, and whether EGF receptor (EGFR) is expressed in these cells (180).

The role of EGF has been investigated in the kidney (175, 176, 181, 182). It

has been suggested that it may have a possible role in chronic renal damage. EGF is also involved in tubular regenerative responses to injury. On the other hand, EGF does not appear to contribute to the development of injury in chronic renal rejection. It may instead exert a protective effect on tubular structures.

EGF is a growth-promoting peptide that is synthesized in the distal tubules of the kidney and excreted in urine. It may play a role in repair after renal tissue damage, as well as in compensatory growth of the remaining kidney after uninephrectomy. Compensatory increase of around 30% in urinary EGF excretion from the remaining kidney occurs after uninephrectomy in healthy humans. Whether EGF plays a role in the adaptive processes in the remaining kidney or whether changes in EGF excretion are merely of a secondary nature is still uncertain (176).

The role of EGF in tubular regeneration after ischaemia has been investigated to identify its role in post-ischaemic repair. There was a reduction of EGF immuno-staining and disappearance of EGF receptors during the mitogenic response (182).

In kidney transplantation, the urinary excretion of epidermal growth factor (EGF) was studied in 15 patients. Urine creatinine increased prior to the increase in urine EGF. Patients who received cyclosporine immunosuppressive treatment excreted little or no EGF during the first month, while patients who received prednisone and azathioprine excreted EGF as early as four days post- transplantation (181).

EGF has been found in different parts of the GI tract. It has been implicated in the control of gastrointestinal tract growth, maturation, and protection (183).

In the heart, EGF acts as a potent chemoattractant for human as well as rat heart vascular endothelial cell lines. Human EGF and TGF-alpha have been shown to be active as chemo-attractants for these cells. This indicates that EGF-like factors may function in vivo to control, in part, the angiogenic events that occur during tissue repair (177). In a model of rat allograft aortic transplantation between major and minor histoincompatible rat strains, levels of epidermal growth factor, insulin-like growth factor-1 (IGF-1), and PDGF in grafts from angiopeptin-treated recipients were 35% to 75% of levels in grafts from nontreated recipients. SMC in the media and intima was reduced by 30% to 90% and intimal thickening by approximately 50%. This suggests that growth factors like EGF play a role in the vascular response and interference in the growth factor response may be one way of reducing or preventing

vascular injury (184).

In heart transplant patients, Hosenpud and colleagues suggested that growth factors including EGF may upregulate cell mediated activation of vascular smooth muscle cells resulting in stimulation of smooth muscle cell proliferation and migration to intimal sites (185).

In the lung, the role of EGF in the maintenance and repair of airway epithelial cells in adult humans was investigated using immunohistochemical staining. EGF receptor (EGFR) expression in these cells was also investigated. Lung biopsies from thirteen adult patients with lung carcinoma were examined for the expression of EGFR in peripheral lung tissue and bronchial tissue. The study concluded that EGFR is not expressed, at least at an immunohistochemically detectable level, by airway epithelial cells of adult humans, not only in the quiescent state but also under conditions in which epithelial cells are stimulated to replicate. Thus, it appears unlikely that the EGF/EGFR system plays an important role in either maintenance or repair of airway epithelial cells in adult humans (180).

In patients with obliterative bronchiolitis, anti-HLA antibodies development has been shown to play an important role in its pathogenesis. However, the nature of non-HLA antibodies developed after lung transplantation and their role in OB pathogenesis have not been determined. Antibody-positive samples were tested for induction of proliferation and growth factor production in two selected airway epithelial cell (AEC) lines. Results from this study indicates that anti-AEC antibodies may play a role in the immunopathogenesis of OB in the absence of anti-HLA antibodies (186).

I.v. Transforming Growth Factor-Beta:

I.v.a. Introduction:

Transforming growth factor-β is a multifunctional polypeptide growth factor (figure 14) which was identified in the late 1970's and early 1980's (187). It is a cytokine with potent chemoattractant properties for fibroblasts. In addition, it is a regulator of their growth potential. It also upregulates transcription and translation of matrix components to promote the healing of inflamed tissues. Recently, TGF-β has been shown to accelerate the healing process of incisional wounds and to cause extensive mononuclear cell infiltration as well as accumulation of fibroblasts with enhanced

collagen deposition (188). Because TGF-β has a specific enhancing effect on wound healing, it has been suggested that it also contributes to certain fibro-proliferative diseases, such as kidney sclerosis in which high levels of TGF-ß were reported (189).

Many cells can synthesise TGF-β and all of them have specific receptors for this peptide. It has been accepted that TGF-β acts by both autocrine and paracrine mechanisms to regulate the behaviour of almost all cells in the body (190). TGF-β is present in five different isoforms TGF-β 1,2,3,4 and 5. All isoforms share similar biological activities in certain established cell lines (191).

The normal healing process is characterised by an ordered accumulation of acute inflammatory cells (neutrophils) and platelets, followed by macrophages and lymphocytes and later on fibroblasts at the injury site (192). It is interesting that subcutaneous injection or even topical application of TGF-ß produces a similar histological pattern to normal healing with inflammatory cell recruitment, fibroblast accumulation and microvascular growth (132, 188, 193).

It is well known that the pathological process of chronic lung rejection (OB) following lung transplantation is similar to the effect of TGF-β on lung healing after lung injury. This is characterised by an inflammatory cell infiltrate, fibroblasts and collagen deposition which ultimately result in pulmonary fibrosis. OB, which affects around 40% of long term lung transplant patients, is characterised by excess fibrous tissue mainly in the small air-ways resulting in airway obstruction and graft failure. It is logical to assume that TGF-β may play a key role in this process (194-198).

I.v.b. Structure:

The structure of TGF-β1 is similar to other isoforms (figure 15). It is synthesised in a latent form (199) as a 25kDa di-sulphide linked dimer and has 390 amino acids, while the active form of TGF-β is a cleavage product of two identical chains of 112 amino acids, which correspond to the mature bioactive form. Each chain is synthesised as a C-terminal domain of a 390 amino acid precursor that has also N-terminal secretory signal polypeptides and a long precursor segment (200). The C-terminal region is cleaved from the remaining precursor segment following a series of four basic amino acids (201). There are three N-linked glycosylation sites in the precursor and nine conserved cysteines in the 112 amino acids chain, eight of which

form intramolecular disulphide bonds, and the last cysteine residue from each chain forms an intramolecular disulphide bridge (200). TGF-β1 is stored in platelets as a latent complex which also contains a 125-190 kDa glycoprotein, the function of which is unknown (202). The amino acid sequence of human TGF-β1 is around 71% homologous to that of TGF-β2 and 77% homologous to TGF-β3, while TGF-β2 is approximately 80% homologous to TGF-β3 (203).

The active forms are very highly conserved over evolutionary time. The general genomic structures, including the regulatory regions, of the TGF-β genes are conserved too, although each isoform differs somewhat in its transcriptional control.

I.v.c. Sources of TGF-β:

TGF-β1 is synthesised in a latent form. Many sources have been identified but one of the main sources in humans is platelets. It is released from the α-granules and is also released by lymphocytes, monocytes, macrophages, endothelial cells, fibroblasts, bone matrix, kidney, placenta and some tumour cell lines (204).

As mentioned earlier, TGF-β1 is activated by cleavage of the C-terminal from the precursor form. The activation, *in vitro*, can be triggered either by change in ionic strength, acidification or by the action of certain proteolytic enzymes such as plasmin or cathepsin-D (205). *In vivo*, the mechanism of activation of latent TGF-β1 is not known but is very likely to be mediated by plasmin or cathepsins (206). The activation may take place at cell surfaces where the precursors bind to mannose-6 phosphate receptors (207). The active TGF-β1 binds to various extracellular matrix components such as biglycan, thrombospondin, decorin, fibronectin and other collagens. The binding may cause sequestration of the active form (208). TGF-β1 can rapidly be cleared following activation. This process is induced by binding of the active form to α2-macroglobulin (209).

I.v.d. Biological function of TGF-β1 :

As described earlier, TGF-β is produced by many cell types, including lymphocytes, monocytes, macrophages and other inflammatory cells (210). It has a wide range of functions that mainly involve the control of the proliferation and differentiation of many cell types (190, 211, 212). These effects are related to cell type and their differentiation, growth conditions and presence of other growth factors (213). More recent studies suggest important roles in different situations like immune cells,

connective tissue cells, epithelial cells, wound healing, extracellular matrix and others (213).

I.v.e. Effect on cell proliferation:

TGF-β1 is a strong inhibitor of cellular proliferation. This is achieved by limitation of the supply of mitogen and by the action of negative growth factors (214). This growth inhibitory function can affect a variety of cells that include bronchial epithelial cells, T and B lymphocytes, embryo fibroblasts, keratinocytes, and haemopoietic cells (215-219). TGF-β1 can cause inhibition of cell proliferation by opposition to specific mitogens such as EGF in keratinocytes, lymphocytes, IL-2, IL-1 and IL-3, GM-CSF and CSF-1 (220). TGF-β1 can inhibit proliferation by inhibiting cell cycle progression. This inhibition is caused by stopping the cells from entering the S phase of the cell cycle and the cells are arrested at the G1 phase (221).

Despite this, TGF-β1 is generally considered an inhibitor of cell proliferation, it can sometimes stimulate cell proliferation, for example, of NRK-49F rat fibroblasts cultured in semi-solid medium (187).

I.v.f. Effect of Immune cells:

TGF-β1 is a regulatory agent for immune cells. It can suppress the proliferation of T and B lymphocytes and at the same time it can enhance monocyte function (217). The inhibitory function of TGF-β1 on immune cells makes it a powerful immunosuppressive factor. TGF-β1 also suppresses the proliferation of thrombocytes (222) and natural killer cells (NK) (223). It can also inhibit both lymphokine-activated killer cells (LAK) and allospecific cytotoxic T lymphocytes (224). In addition, TGF-β1 can inhibit the production of Interleukins IL-1, IL-2, IL-3 (225). It also been shown to suppress the effects of IFN-γ on MHC class II antigens (226).

It has also been shown that TGF-β1 can inhibit the secretion of immunoglobulin G (IgG) and immunoglobulin M (IgM) by activated B-lymphocytes (218) but at the same time, it can it can upregulate the secretion of immunoglobulin A (IgA) by splenic lymphocytes. This upregulation can be inhibited if treatment with TGF-β1 is continued (227).

TGF-β gene "knockout" mice (genetically unable to make TGF-β) always die at 3-4 weeks of age from a diffuse inflammatory syndrome involving massive mononuclear cell infiltration of the lungs and other vital organs (228, 229). It appears that

under some circumstances TGF-β is a regulator of inflammation and therefore may be of use in the treatment of autoimmune disease, transplant rejection or ischaemic damage after myocardial infarction.

In addition to its effect as an immunosuppressive agent, it contributes to the inflammatory process, being a powerful chemotactic agent for monocytes and fibroblasts (230, 231). At the inflammatory site, TGF-β1 activates monocytes which in turn causes the release of several growth factors such as fibroblast growth factor (FGF), IL-1, TNF-α and TGF-β1 itself (232, 233). As soon as monocytes are differentiated into macrophages, TGF-β1 can inhibit cytotoxicity by suppressing the production of hydrogen peroxide (H_2O_2) (234). The above observations reflect the major role of TGF-β1 in coordinating the response to injury and its contribution at the site of repair and inflammation.

I.v.g. The role of TGF-β1 in fibrosis:

TGF-β1 plays a central role in the processes of tissue repair and fibrosis. It initially acts as a chemotactic factor for acute and chronic inflammatory cells like neutrophils, lymphocytes, monocytes, and fibroblasts at the site of injury. Then, it promotes the deposition of extracellular matrix components. The accumulation of matrix in the tissues will lead to the pathological picture of fibrosis which is the hallmark of certain diseases (235).

I.v.h. Controlling effect of TGF-β1 on the extracellular matrix:

TGF-β1 promotes the accumulation of extracellular matrix proteins and cell responses to those proteins following tissue injury. It has three effects (figure 16): It activates gene transcription and increases synthesis and secretion of tissue matrix proteins. It decreases the synthesis of proteolytic enzymes and stimulates protease inhibitors. It also augments the transcription and translation of cell receptors that bind to those extracellular matrix proteins (198). The effect of TGF-β1 on tissue matrix has been shown in different fibroblastic cell lines and with different animal models. It has been shown to increase the synthesis of type I, III, IV, and V collagen and fibronectin (236-238). It is thought that the mechanism responsible for this effect could be the accumulation of mRNA for type I, III, IV, V collagens and fibronectin (237).

The other important effect of TGF-β1 is its regulation of gene expression which control the degradation of the synthesised matrix protein. This can occur in two ways:

TGF-β1 inhibition of biosynthesis and the secretion of proteases such as thiol protease, serine protease, and metalloproteinases by altering the mRNA level of the affected gene. The second is by increasing the expression of the gene encoding protease inhibitors such as plasminogen activator inhibitor (PAI) and tissue inhibitors of metalloproteinases (TIMPS) (191).

Another effect of TGF-β1 is to increase the interaction of cells with extracellular matrix (239). This is done by increasing the cell membrane receptors for cell adhesions. These receptors named integrins consist of 2 subunits α and β (240) and the expression of these subunits may be regulated by TGF-β1 (241). Through this regulatory process, TGF-β1 promotes and enhances adhesion to fibronectin and collagen in different cell types (239).

I.v.i. TGF-β1 receptors:

This cytokine binds with specific receptors to a wide variety of cell types (242). A number of proteins have a high affinity to TGF-β1 which include cellular matrix proteins and cell surface receptors (243). The most widely distributed receptors with high affinity to TGF-β on the cell surface are type I, II, III receptors. The cloned type II receptor has affinity for TGF-β1 and TGF-β3 (244). The TGF-β type II receptor is a member of the transaminase family, serine / threonine kinase, which include many type II activin receptors. It forms a heteromeric complex with the type I receptor which has proved to be essential for the signal transmission of TGF-β receptors (245). TGF-β receptor type I is again a transmembrane serine/threonine kinase that shares conserved sequences in the extracellular as well as intracellular domain with type II receptors (245). TGF-β receptors type I and II are components of the TGF-β receptor signalling complex. They are involved in two other distinct signalling pathways triggered by TGF-β. These are: growth inhibition and gene regulation (246). The TGF-β type III receptor is a transmembrane proteoglycan with a highly conserved cytoplasmic domain that lacks an apparent signalling motif (247). The cell surface expression of type III receptors modulates the binding of TGF-β to the type II receptor (248).

I.vi. Aims and Objectives:

Obliterative Bronchiolitis is the most common late pulmonary complication among lung and heart-lung transplant recipients. Although chronic rejection is considered to be the most likely cause of OB, the exact aetiology and pathogenesis of OB remain uncertain. TGF-β is a potent, profibrotic cytokine that promotes fibrosis by enhancing the synthesis of extracellular matrix components (104). In this work, we hypothesised that following lung injury, excessive expression of TGF-β and other growth factors in the allograft lungs may result in exaggerated or exuberant tissue repair. This may result in the lung parenchyma being replaced by fibrotic tissue leading to OB. We also suggest that periodic lung injury caused by recurrent acute rejection is a major predisposing factor to the development of OB.

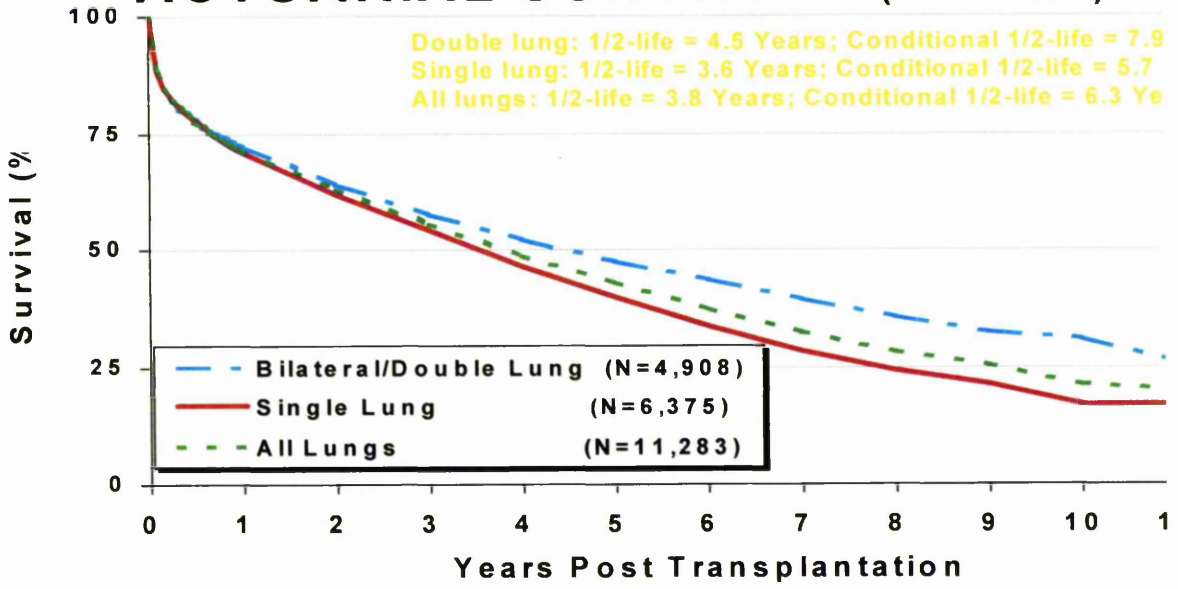
Based on the above hypothesis: The study was designed with the following objectives:

1. To set up a reproducible small animal obliterative airway disease (OAD) model to test the above hypothesis.
2. To establish that OAD in this model can be triggered by the rejection process.
3. To assess the effectiveness of different, newer, clinically available immunosuppression regimes on the prevention of OAD.
4. To assess whether TGF-β is over-expressed in OAD by performing detailed immunohistochemical analysis of tracheal biopsies.
5. To correlate TGF-β tissue expression with each immunosuppressive treatment.
6. Finally, to compare the effect of two immunosuppressive regimes on the expression of TGF-β as well as bFGF and EGF in a randomised clinical trial and correlate it with rejection and OB in lung biopsies.

The demonstration of a relationship between TGF-β over-expression and OAD, and the effect of modern immunosuppressive agents on OAD and TGF-β expression will assist in developing a more effective immunosuppressive strategy to avoid or delay OB in long term lung allograft recipients.

Chapter I: Figures (1-16)

Figure 1: LUNG TRANSPLANTATION ACTUARIAL SURVIVAL (1983-2000)



(ISHLT web site)

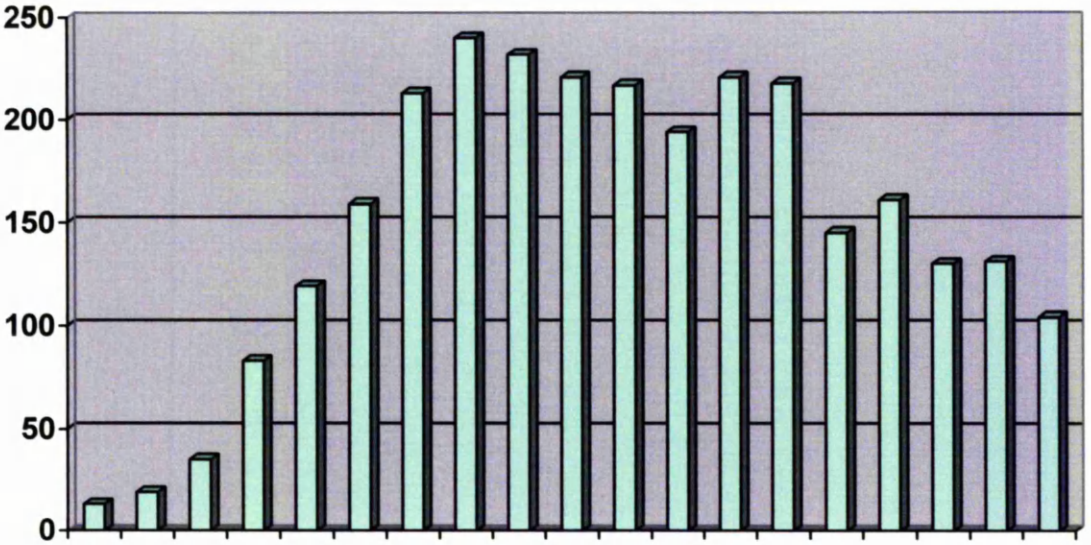


Figure 2: No. of heart and lung transplants per year from 1982 to 2000
(ISHLT web site)

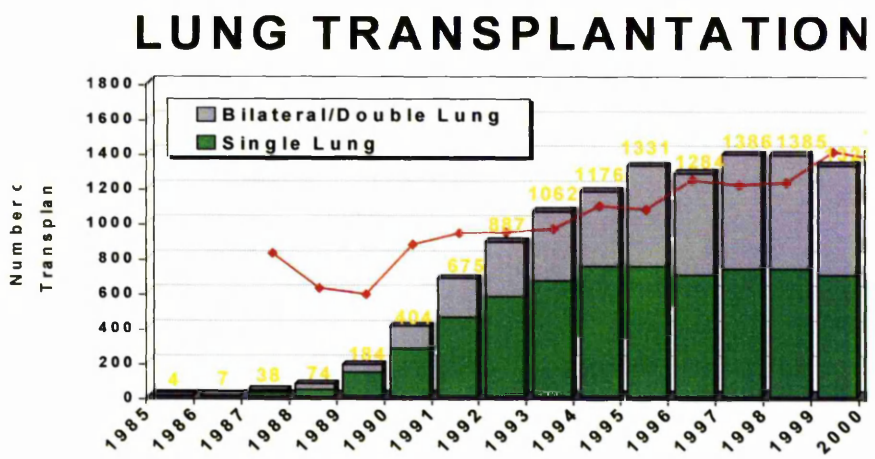


Figure 3: No. of lung transplants per year from 1985-2000
(ISHLT web site)

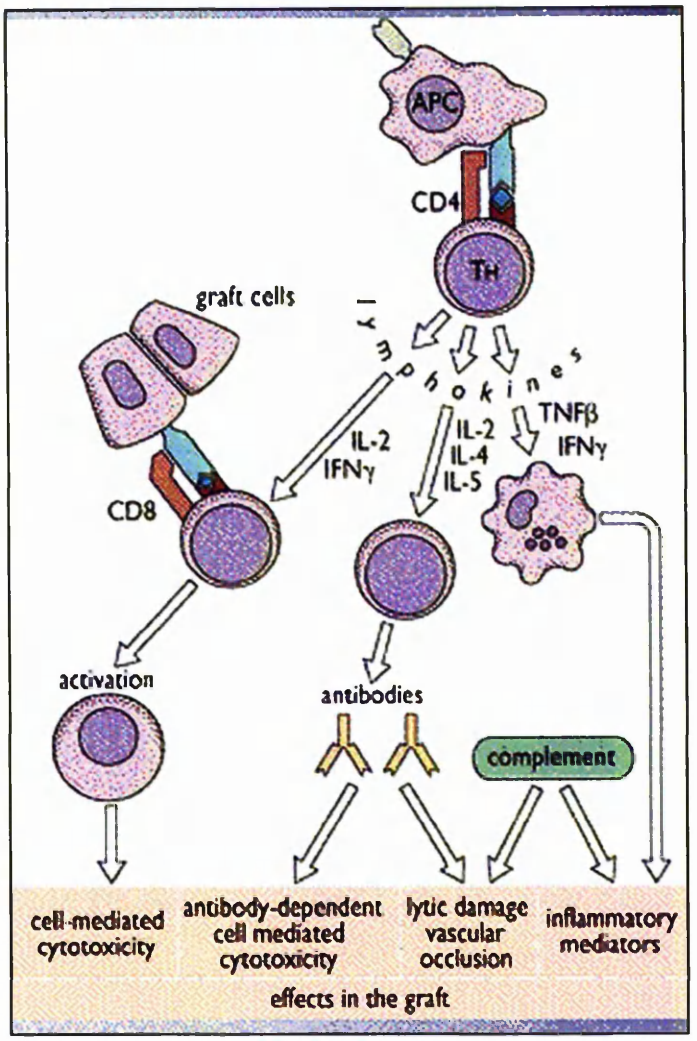


Figure 4

Mechanisms of rejection in transplantation

(Roitt Immunology, third edition)

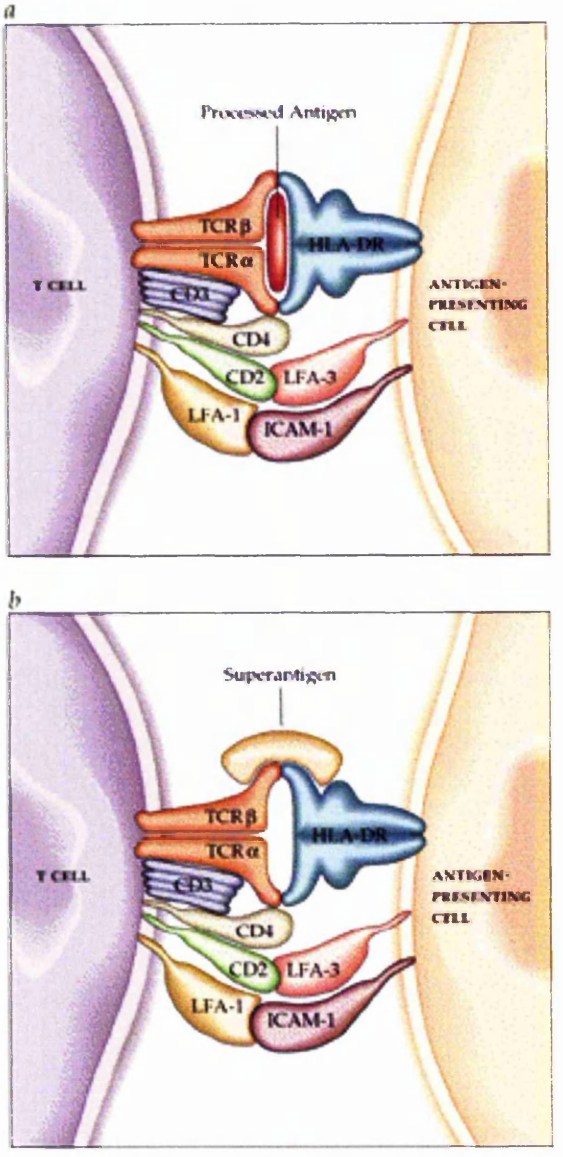


Figure 5

MHC and antigen presentation

(Scientific American Web site)

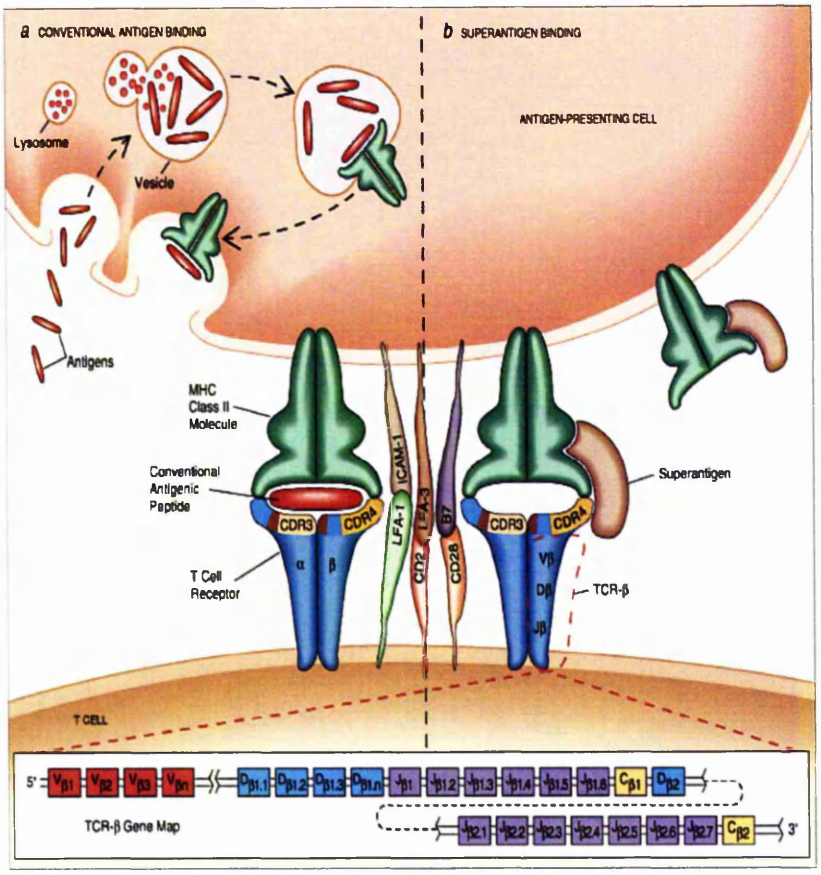


Figure 6

MHC recognizing foreign antigens

(Scientific American Web site)

CLASSIFICATION OF CYTOKINES POTENTIALLY RELEVANT TO TRANSPLANTATION

Group	Important examples
haematopoietins	IL-2, IL-3,, IL-5, IL-12, IL-13, GM-CSF
interferon family	IFN-γ, IFN-α/β, IL-10
TNF family	TNF-α, TNF-β/lymphotoxin
TGF-β	TGF-β1, β2, β3
chemokines	α (or C-X-C) chemokines e.g. IL-8 β (or C-C) chemokines e.g. MIP, RANTES
others	IL-1

Events in transplant immunology and candidate cytokine mediators to support that role:

T cell clonal expansion	IL-2
macrophage and endothelial cell activation	IFN-γ, TNF-α
B cell growth and differentiation	IL-4, IL-10
MHC and adhesion molecule induction in endothelial/epithelial cells	IFN-γ, TNF-α
chemotaxis of leucocytes in graft	chemokines (e.g. RANTES)
capillary leak	TNF-α
cytotoxic effects on targets	TNF-α
fibroblast growth, collagen formation	TGF-β
immunosuppressive/anti-inflammatory at times	IL-4, IL-10, IFN-γ, TGF-β

Figure 7

Cytokines relevant to transplantation
(adapted from "Handbook of transplant Immunology 1995")

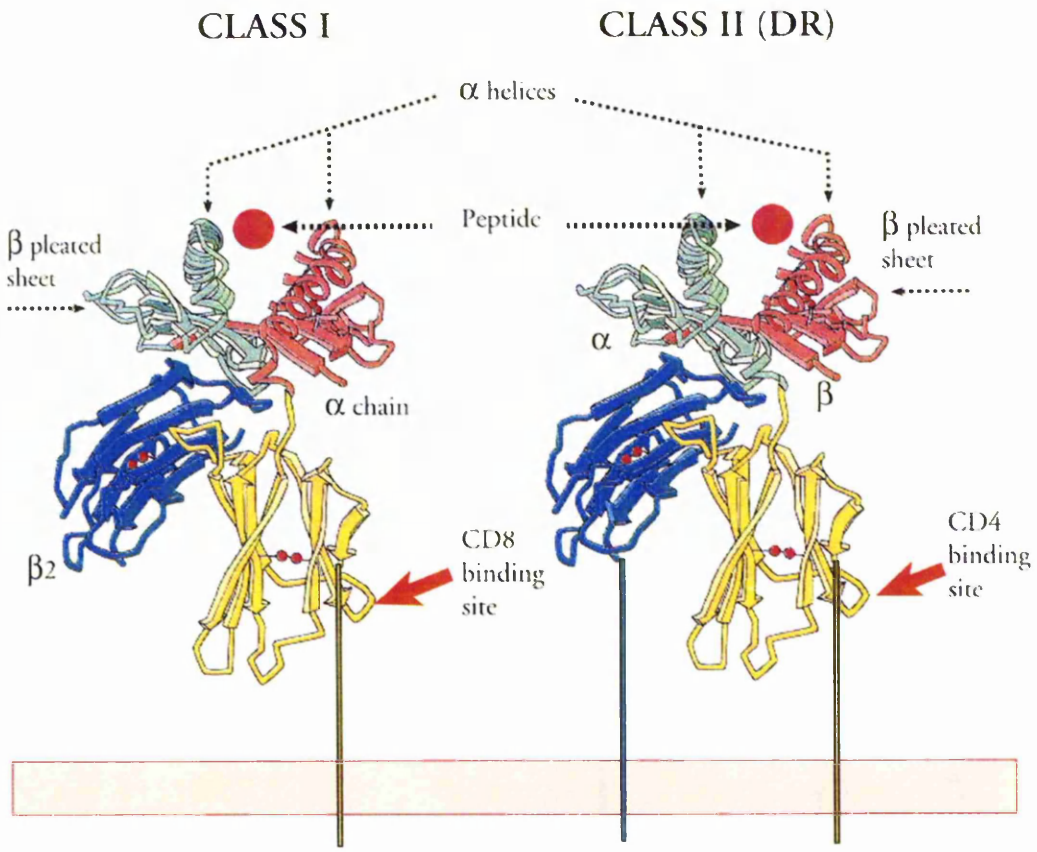


Figure 8
MHC antigens class I and II

(adapted from "Handbook of transplant Immunology 1995")

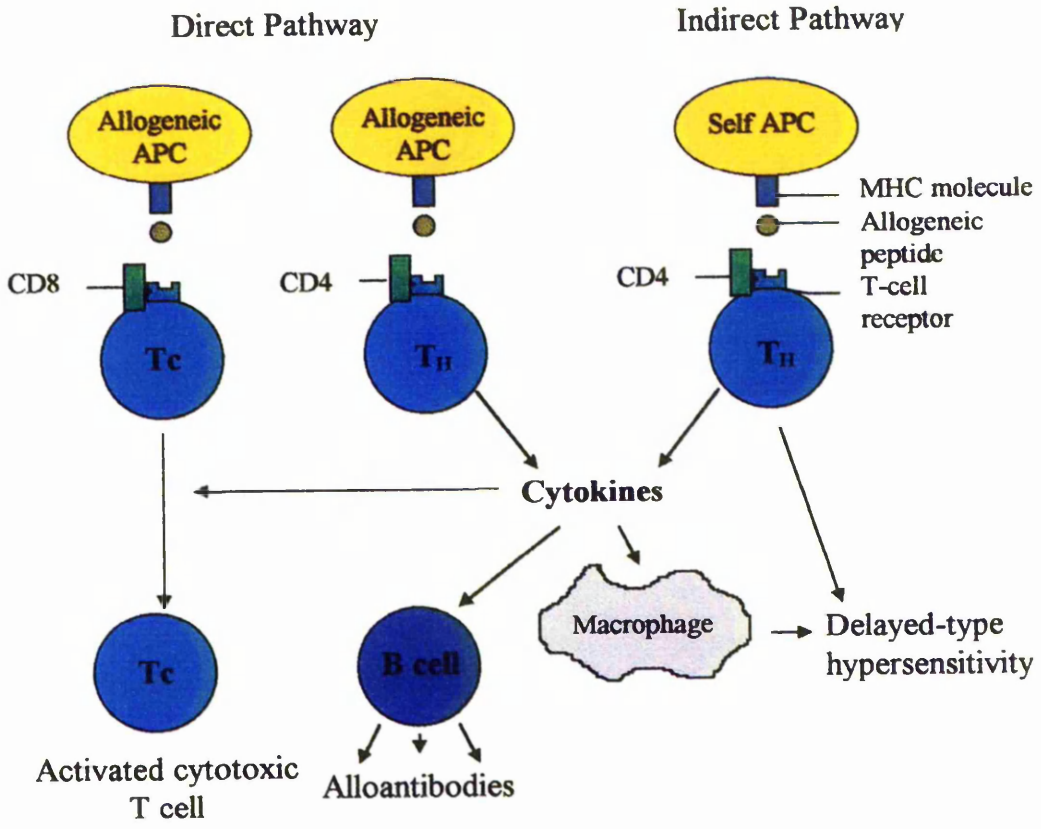


Figure 9

Pathways of recognition of allogeneic MHC and mechanisms of Graft rejection.
(Adopted from Sayegh 1998)

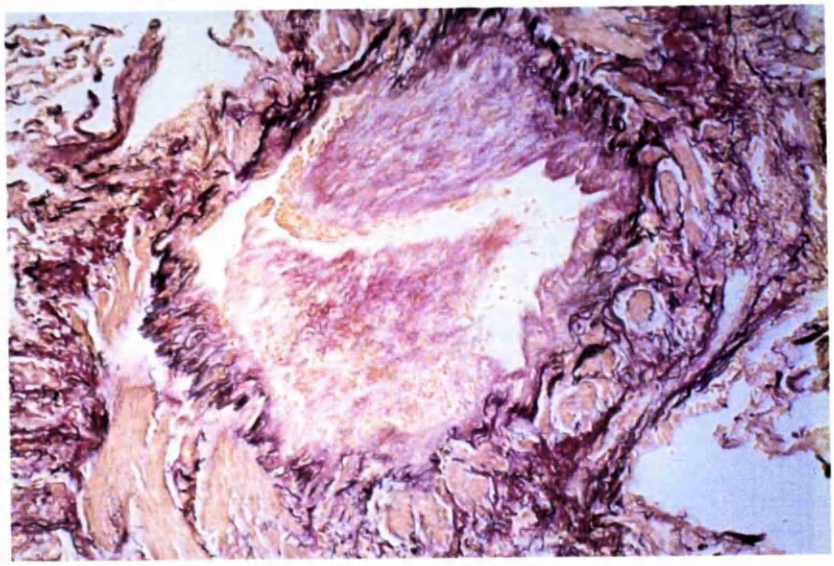


Figure 10
Obliterative bronchiolitis, histological features

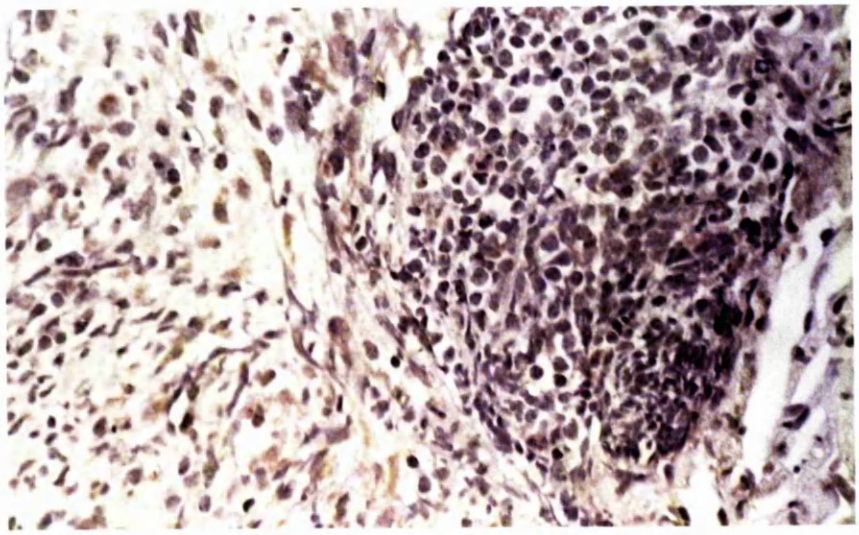
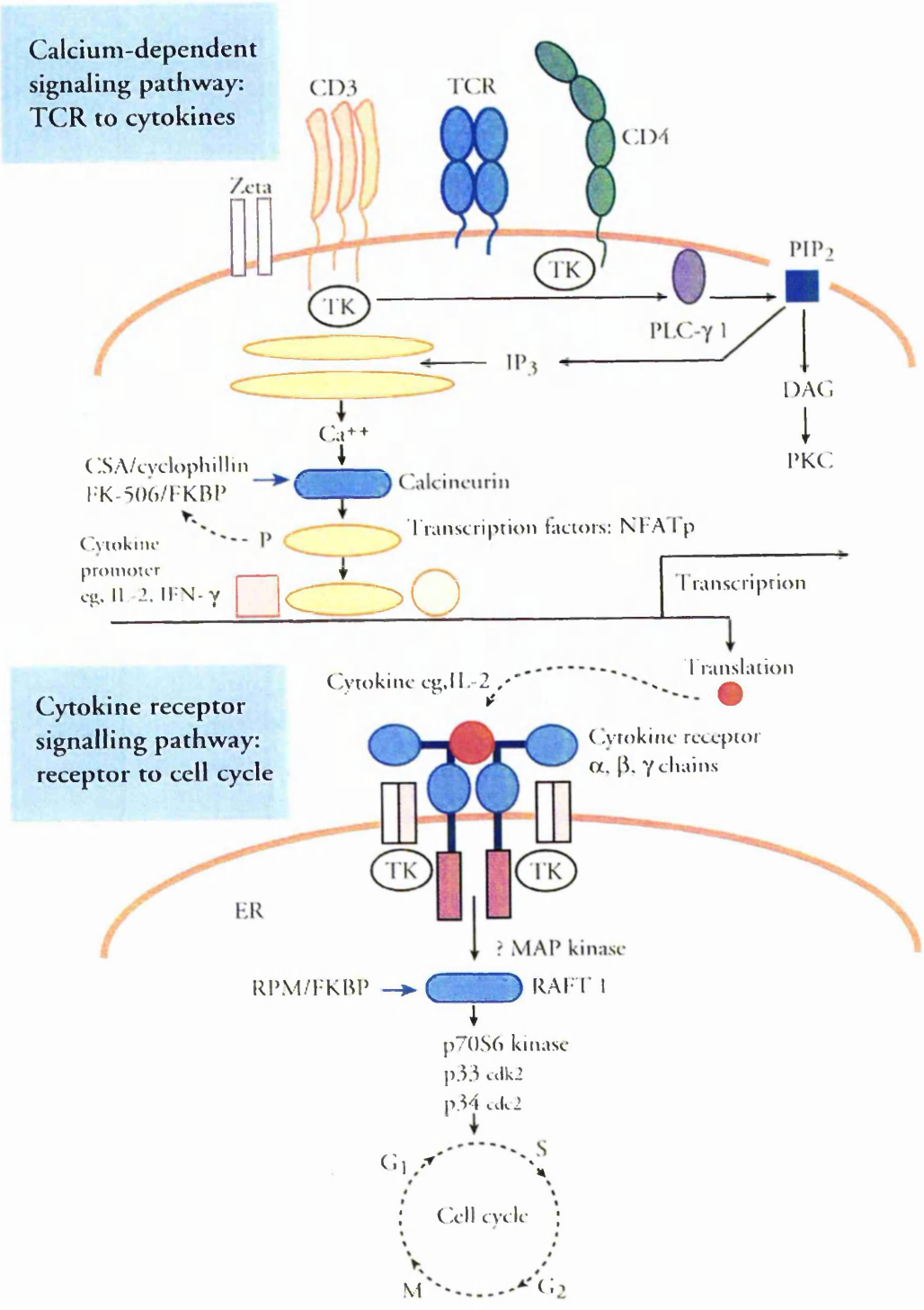


Figure 11
Acute pulmonary rejection



Reproduced through the courtesy of Dr. Philip F. Halloran, Departments of Medicine and Immunology, University of Alberta, Edmonton, Alberta, Canada.

Figure 12
The molecular mechanisms for the immunosuppression effect of cyclosporine, tacrolimus and rapamycin
(adapted from "Handbook of transplant Immunology 1995")

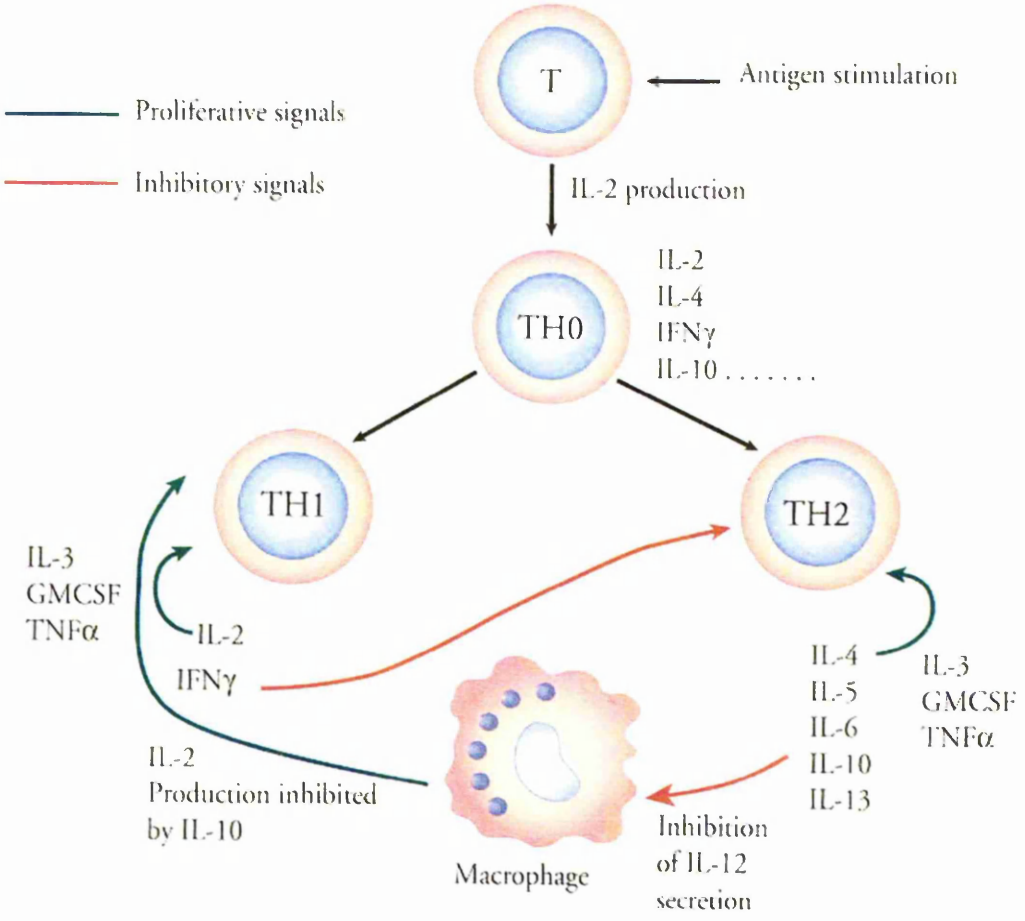


Figure 13
Differentiation of T cells into TH1 and TH2 with different profile of cytokine production

(adapted from "Handbook of transplant Immunology 1995")

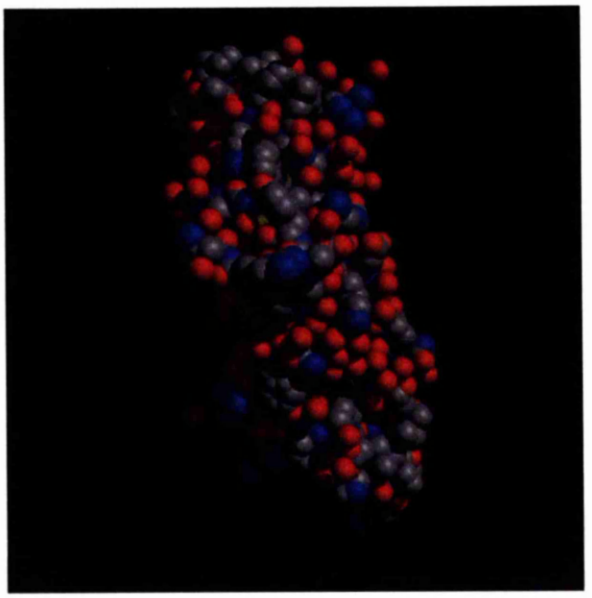


Figure 14

TGF-β molecules (Cytokine web site)

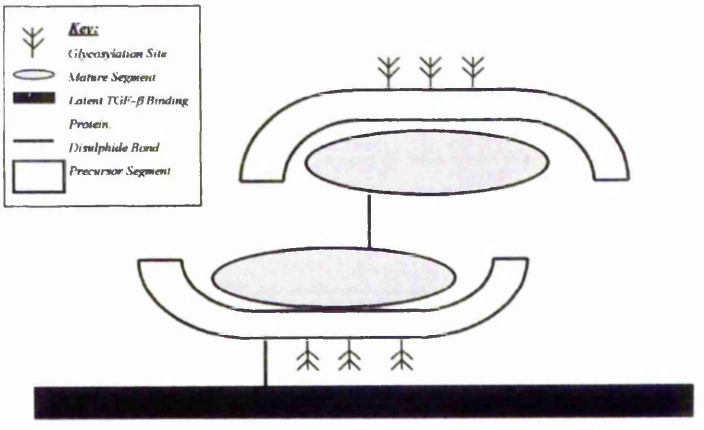


Figure 15

Structure of the secreted TGF-β1 latent complex
(Adapted from Gitelman and Derynck 1995)

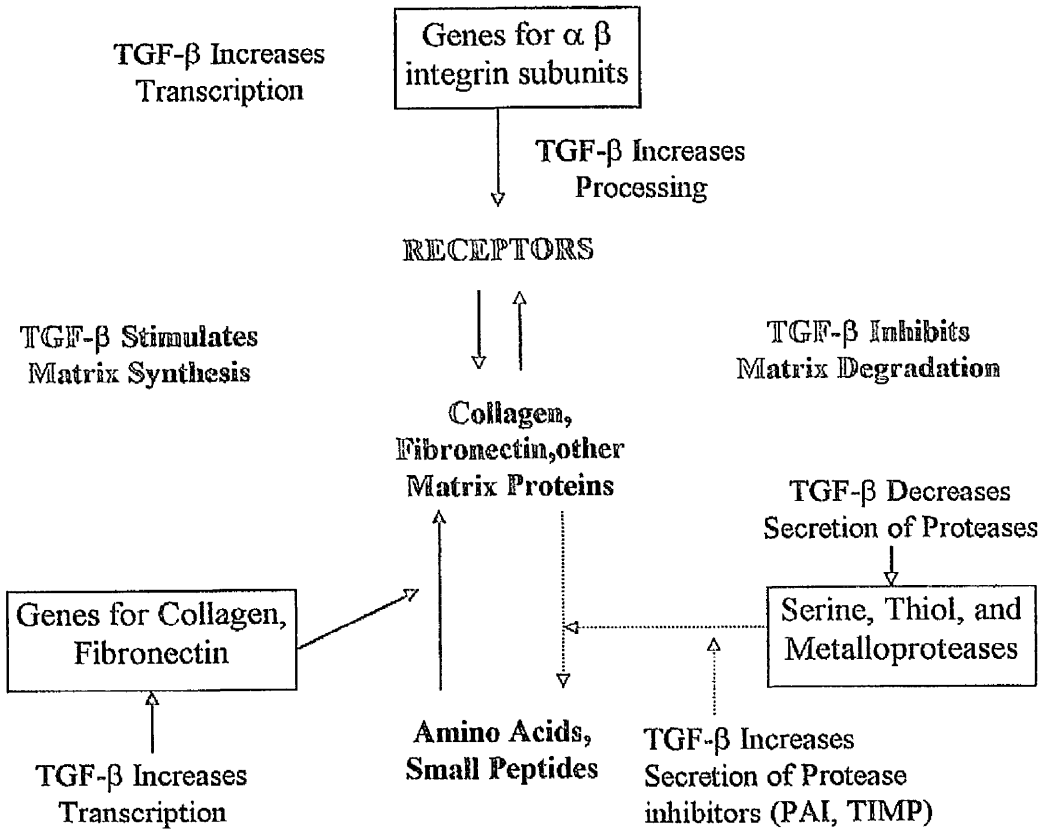


Figure 16

The three stages of the fibrotic process
(adopted from Rajendra 1991)

CHAPTER II

Establishment of the rat heterotopic tracheal allograft model for obliterative airway disease (OAD)

II.i: Introduction:

Obliterative bronchitis is the most common late complication among lung and heart lung transplant recipients (47). It is the commonest cause of morbidity and late death (91). Clinically, it manifests itself as worsening dyspnea with progressive airflow obstruction. The pathology of OB is that of fibro-proliferative disorder that seams to be restricted to the respiratory and terminal bronchioles. There is mucosal and submucosal fibrous thickening and scarring that in severe cases occludes the lumen of the entire bronchiole, the hallmark of OB (95). The aetiology of OB is still unclear. Various factors have been suggested as possible causes but recurrent acute rejection and chronic rejection seems to be the most likely causes (249, 250). In the swine lung transplant OB model (101), a study was designed to assess the effect of acute rejection on the development of OB. This was achieved by tapering the immunosuppression at 3 months post transplant over one month and discontinued for 2 months. TBB and BAL were obtained regularly. TBB showed severe peribronchiolar lymphocytic infiltration and BAL showed an increase in CD8 cells. Histology of the lung after sacrifice (6 months) showed progressive fibrous inflammatory occlusion of the bronchus. This clearly suggests that OB can be caused by inadequate immunosuppression (101).

A new concept in the pathogenesis of OB was suggested in a recent clinical study by El-Gamel and associates. In that study, OB was associated with an increased expression of TGF-β in lung biopsies (102). TGF-β is a major cytokine/growth factor which seems to orchestrate the process of healing through its effects on recruitment of inflammatory cells and fibroblasts to area of injury as well as to stimulate the synthesis of extracellular matrix (105). TGF-β over-expression has been associated with exuberant tissue repair resulting in fibrosis. Studies of human lung diseases as well as animal research suggest that the initiating factors for OB are a combination of pulmonary injury and the recruitment of inflammatory cells, mainly macrophages. TGF-β has been found in the injured lung. It is also produced by inflammatory cells removed from the lung. In an animal model of pulmonary fibrosis, TGF-β production is increased prior to collagen synthesis and is mainly produced by tissue macrophages. In idiopathic pulmonary fibrosis, extensive TGF-β deposition can

be detected by immunohistochemical staining, mainly in epithelial cells in areas of lung regeneration and remodelling. This would strongly suggest that the pathogenesis of the progressive lung fibrosis may be an aberrant repair process (104). In order to test our hypothesis and confirm the association between OB, lung rejection and TGF-β in standardised laboratory conditions, we designed and tested a reproducible heterotopic rat obliterative airway disease model at the Immunology research laboratory, Manchester University.

II.ii: The Animal Model:

The pathogenesis of OB is still poorly understood and there is no effective therapy yet. Clinical investigations are hampered by the limited amount of biological material obtained from patients and the small number of patients in each centre. The clinical set-up is complex with large number of variables. Hence, the development of a reproducible animal model for OB is highly desirable. These studies allow the sequential study of the fibroinflammatory processes in the airways, the assessment of possible aetiological factors and the testing of promising new therapies.

A number of animal models have been designed to reproduce obliterative bronchiolitis. Most of these models are based either on orthotopic lung transplantation or heterotopic tracheal implantation. The orthotopic lung transplant model has been successful in large animal models like pigs and dogs (101, 251). In small animals, Jochum Prop and Charles Wildevuur from the University Hospital, Groningen pioneered and popularized the technically difficult model of rat orthotopic left lung transplantion (95, 250, 252). This model was very promising as it reproduced many characteristic changes of OB. However, it suffered from 2 major limitations; firstly it required specialized microsurgical equipment and techniques, limiting its applicability. Secondly, the coexistence of severe vascular and paranchymal injury complicate the study of airway pathology which is the main lesion in OB.

The second type of model is based on the heterotopic implantation of the donor tracheal/ bronchial tissue into the subcutaneous tissue (98) or more commonly into the greater omentum of recipient animals. This seems to be successful in rat and mouse models (99, 253) as well as in larger animals such as canine models (254). This type of model offers the advantages of simplicity, control of MHC

disparity, and lower cost. It replicates the histopathological features of OB independently of other pulmonary lesions. Furthermore, the evaluation of tracheal viability is independent of the pulmonary to bronchial retrograde circulation. Because of these factors, we used the heterotopic tracheal rat model with omentopexy in this study. It is a model of rapidly progressing obliterative airway disease (OAD) with histological features characteristic of OB in lung allograft recipients.

II.iii: The aims of the study:

1. Design a suitable heterotopic tracheal transplant rat model in which a tracheal segment was taken from a donor rat and implanted into the greater omentum of a recipient rat.
2. Assessment of tracheal isografts to confirm normal epithelial regeneration and the presence or absence of obliterative airway disease (OAD). Histological assessment of tracheal allografts to assess the severity and magnitude of OAD at different MHC disparities (between donor and recipients). To define the appropriate time intervals between implantation and explantation. Also to confirm the reproducibility of the model.
3. Assess the most suitable donor and recipient rat strains for the next phase of the study based on the most severe immunological and OAD responses.
4. The choice of appropriate rat strains for the study will be based on their suitability, MHC characteristics as well as availability. For all recipients, the PVG $RT1^u$ strain was used.

For donor, the following strains were used:

Isografts: PVG $RT1^u$

Allografts 1: AO which have the same MHC antigens, but differ in minor antigens

Allografts 2: PVG/c which have the same minor histocompabability antigens, but differ in MHC antigens i.e. class I and II antigens.

Allografts 3: PVG Lewis rats. This stain is diverse at both major and minor histocompatability antigens.

II.iv: Material and methods:

The heterotopic tracheal transplant rat model with omentopexy was used. All procedures were carried out using inbred rats obtained from the biological services unit (BSU), Manchester University, Manchester UK. Recipient procedures were carried out on 27 male (200-300g) inbred PVG RT1^u. Complete tracheal segments were harvested from a total of 14 donor rats, divided into two segments and were implanted into the greater omentum of 27 PVG RT1^u recipient rats. Nine segments of donor tracheas were harvested from five (each trachea was divided into two segments) PVG RT1^u animals (isografts) for use in nine recipient animals. Six tracheal segments were harvested from three AO male and were implanted in 6 PVG RT1^u recipients. Six tracheal segments were harvested from 3 PVG/c male rats and were implanted into six PVG RT1^u recipient rats. Finally, in order to have maximum immunological desparity, six tracheal segments were harvested from three 250-300gms donor Lewis rats and were implanted into six PVG RT1^u recipient rats.

The experimental animals were divided into 4 groups as follows:

Group 1: Nine isografts were divided into 3 subgroups depending on the timing of the sacrifice procedure:

Subgroup a: Three animals sacrificed at 7 days.

Subgroup b: Three animals sacrificed at 14 days

Subgroup c: Three animals sacrificed at 28 days.

Group 2: Six AO allografts sharing MHC antigen only.

Divided into two groups:

Subgroup d: Three animals sacrificed at 14 days.

Subgroup e: Three animals sacrificed at 28 days.

Group 3: Six PVG/c allografts sharing minor antigens only (diverse in MHC). Divided into two groups:

Subgroup f: Three animals sacrificed at 14 days

Subgroup g: Three animals sacrificed at 28 days.

Group 4: Six Lewis allografts across both MHC and minor antigens. Divided

into two groups:

Subgroup h: Three animals sacrificed at 14 days.

Subgroup i: Three animals sacrificed at 28 days.

No active treatment was given to any animal group.

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, the "Guide for the Care and Use of Laboratory Animals" and the "Guide to the Care and Use of Experimental Animals" formulated by the Home office. The experimental protocol was approved by Manchester University, Department of Immunology. Project and personal licences were granted by the Home Office.

II.iv.a: Donor Procedure (figure 17)

Donor rats were anaesthetised using inhaled anaesthetic ether in an induction chamber. The chest and upper abdominal wall were prepared by shaving the area and cleaning of the skin using 95% ethanol. In a surgically clean environment, the neck and the chest were opened using a midline incision with division of the sternum. The trachea was exposed to its bifurcation using combined sharp and blunt dissection. The whole length of the trachea was excised and flushed with 5-10ml isotonic saline solution. The trachea was then divided into two 1-1.5cm segments and stored in cold (+4°C) normal saline ready for implantation into two recipients.

II.iv.b: Recipient Procedure (Figure 18)

PVG.RT1^u rats were anaesthetised in an induction chamber using ether. This was followed by continuous ether anaesthesia with spontaneous ventilation. The upper abdomen was prepared for surgery by shaving and cleaning of the skin with 95% ethanol. An upper midline abdominal incision (1-2 cms) was carried out, the greater omentum was identified, the tracheal segment was wrapped in greater omentum and then secured with two 8-0 Prolene (Ethicon UK Ltd)® sutures. The peritoneum and abdominal musculature were closed in one layer using a 4-0 Surgisilk® (Sutures Ltd,

Vauxhall Industrial Estate, Ruabon, Clwyd U.K.) continuous suture, and the skin was closed with a continuous 4/0 silk suture.

II.iv.c: *Retrieval of transplanted tracheas:*

After 7, 14 or 28 days, recipient rats were anaesthetised in an induction chamber with anaesthetic ether, followed by continuous ether anaesthesia with spontaneous ventilation. Using a midline abdominal incision, the tracheal segment and surrounding omentum were excised. The recovered tracheas were divided into 2 equal sections, one fixed in 5% acetic acid/95% methanol, and the other stored at -80°C.

II.v: Histological techniques:

II.v.a. Tissue Processing:

Tracheal segments retrieved from the recipient rat omentum at the sacrifice operation were processed at the Immunology Laboratory, Manchester University to produce stained microscopic slides for pathological diagnosis. The rest of the specimens were coded and stored for future experiments.

II.v.b. Specimen Accessioning

Tracheal specimens retrieved at the sacrificed operation were divided initially into 2 segments, one stored at -80°C, the other was fixed. Both segments were labelled clearly describing time and date of procedure, the animal number, and experiment code. The specimens were accessioned by giving them a number identifying each specimen for each experiment.

II.v.c. Fixation

Fixation is carried out in order to preserve tissues permanently in life-like state. Fixation is carried out as soon as possible after removal of the tissues to prevent autolysis. There is no perfect fixative and formaldehyde is the closest. A variety of fixatives are available for use, depending on the aldehydes including formaldehyde and glutaraldehyde. Tissue is fixed by cross-linkages formed in the proteins. This

cross-linkage does not harm the structure of proteins greatly and the antigenicity is not lost. Formaldehyde is good for immunoperoxidase techniques. It penetrates tissue well, but is relatively slow. The standard solution is 10% neutral buffered formalin. A buffer prevents acidity that would promote autolysis and cause precipitation of formol-heme pigment in the tissues.

II.v.d. Tissue Processing:

Once the tissue has been fixed, tracheal segments embedded in paraffin, which is similar in density to tissue, can be sectioned at anywhere from 3 to 10 microns, usually 6-8 routinely. The technique of getting fixed tissue into paraffin is called tissue processing. The main steps in this process are dehydration and clearing.

Wet fixed tissues cannot be directly infiltrated with paraffin. First, the water from the tissues must be removed by dehydration. This is usually done with a series of alcohols, say 70% to 95% to 100%. Sometimes the first step is a mixture of formalin and alcohol.

The next step is called "clearing" and consists of removal of the dehydrant with a substance that will be miscible with the embedding medium (paraffin). The commonest clearing agent is xylene. Finally, the tissue is infiltrated with the embedding agent, almost always paraffin wax. Paraffins can differ in melting point, depending upon the way the histologist likes them and upon the climate i.e. warm or cold.

The above processes are usually automated using an instrument that moves the tissues around through the various agents on a pre-set time scale. Tissues that come off the tissue processor are in cassettes and are manually put into the blocks by a technician who must pick the tissues out of the cassette and pour molten paraffin over them. This "mounting" process is very important, because the tissues must be aligned, or oriented, properly in the block of paraffin.

II.v.e. Sectioning

Once the tissues have been embedded, they must be cut into sections that can be placed on a slide. This is done with a microtome (figure 19). The Microtome is a knife that has a mechanism for advancing the paraffin block across it. Usually this distance can be set, for most paraffin embedded tissues at 6 to 8 microns. Sectioning tissues is

a rather difficult process that require skill and practice. Sections with common artefacts including tearing, ripping, "venetian blinds", holes and folding were excluded. Once sections are cut, they are floated on a warm water bath that helps remove wrinkles. Then they are picked up on a glass microscopic slide. Each slide had 4 section of the biopsy. The glass slides are then placed in an oven at 37°C, overnight to help the section adhere to the slide.

II.v.f. Haematoxylin and Eosin staining:

The embedding process must be reversed in order to get the paraffin wax out of the tissue and allow water-soluble dyes to penetrate the sections. Therefore, before any staining can be done, the slides are "deparaffinized" by running them through xylenes to alcohols to water. There are no stains that can be done on tissues containing paraffin.

Haematoxylin and eosin (H&E) staining were used to assess the tracheal pathology, cell infiltration, OAD and presence and severity of fibrosis. Haematoxylin will not directly stain tissues, but needs a "mordant" or link to the tissues. This is provided by a metal cation such as iron, aluminum, or tungsten. The variety of haematoxylins available for use is based partially on choice of metal ion used. They vary in intensity or hue. Haematoxylin, being a basic dye, has an affinity for the nucleic acids of the cell nucleus. A progressive haematoxylin staining was used. The slide was dipped in the haematoxylin until the desired intensity of staining was achieved.

Eosin on the other hand is an acidic dye with an affinity for cytoplasmic components of the cell. Eosin is much more forgiving than haematoxylin and is less of a problem in the lab. The only potential problem encountered is overstaining, especially with decalcified tissues.

II.vi. Histologic Assessment:

We attempted to quantify the viability of the heterotopic allografted trachea by measuring epithelial thickness and by subjectively assessing and evaluating the epithelial and submucosal layers histologically. The magnitude of the mononuclear cell infiltrate (MNC) was measured using counting squares. The presence and severity

of fibrosis was assessed by measuring submucosal thickness (SMF). Microscopic slides were made from transverse sections of the explanted trachea and stained with hematoxylin and eosin. Assessment was carried out by two blinded observers one of whom was a Consultant Histopathologist.

Epithelial thickness and epithelial regeneration: The thickness of the epithelial layer was measured between the basement membrane and the lumen. The OAD score was as follows: 0 = no OAD, 1 = 1-5μm (mild), 2 = 5-10μm (moderate), 3 = 10-20μm (moderate/severe), and 4 = >20μm (severe, complete obliteration).

The epithelial cell lining was scored according to an established grading system (253) as follows: 0, no epithelium, single nonconfluent epithelial cells; 1, confluent single layer nonciliated epithelium; 2, confluent multilayer nonciliated epithelium; and 3, normal mucociliary epithelium.

The intensity of the mononuclear cell infiltrate was counted within the mucosa, submucosa and muscularis using cell counting chambers or squares. The grading was based on the number of cells/chamber. It was graded from 0 to 4+: grade 0= no cells, grade + = 1-5 cells/chamber, grade ++ = 5-10 cells/chamber, grade +++ = 10-15 cells/chamber grade ++++ = <15 cells/chamber.

The presence and intensity of fibrosis was assessed using an empirical score based on the thickness (in μ) of the submucosal fibrotic layer and graded as follows: 1-5μ = mild, 5-10μ is moderate, 11-19 is moderate/severe and >20μ is severe.

II.vii: Results: (see table 1)

All 27 recipient animals survived the transplant operations. There was 100% survival throughout the follow-up period and until the sacrifice procedure. No intra-abdominal sepsis was encountered in any of the groups. Obliterative Airway Disease (OAD), epithelial lining, mononuclear cell (MNC) infiltrate and submucosal fibrosis were graded, for each group, on H& E stained donor trachea.

OAD was absent in isografts (group 1) at 7 days, 14 days (figure 20) and 28 days (figure 21). It was moderate in group 2 (figure 22), moderate-severe in group 3 (figure 23) and severe (complete obliteration of the lumen) in group 4 (figure 24 and 25).

Epithelial regeneration: The epithelial layer was absent (grade 0) in groups 3, 4 at 14/28 days of sacrifice (Figure 23. 24. 25). Subgroup 1a, 1b, and 1c animals showed

complete regeneration with normal epithelial lining (figure 20-21) i.e. epithelial score of grade 3 at 7/14/28 days. Subgroup 2d and 2e, had an epithelial score of 1.

Mononuclear cell infiltrate: There was some degree of MNC infiltrate in all sections in the mucosa, submucosa and muscularis layers, it was mild in subgroup 1b and 1c, moderate in subgroup 1a. It was moderate-severe in groups 2, 3, and 4 animals (figure 22).

Fibrosis: The thickness of submucosal fibrous layer varied between the groups being mild in group 1 (less than 5μ), moderate/severe in groups 2 (10-20μ) and severe in group 3 and 4(>20μ).

II.viii: Discussion:

In this study, we successfully designed a reproducible heterotopic tracheal transplant rat model with omenotexy using the above mentioned rat strains. This model replicated the histopathological lesions obliterative bronchiolitis seen in clinical lung transplantation and has several attractive features. The donor and recipient operations are technically simple and can be carried out without the need for special equipments or expertise. The model was designed to transplant across major and minor histocompatability antigens which will facilitate studies of immuno-pathogenesis.

In this study, we have been able to assess the model with different rat strain combinations diverse in their MHC antigen compatibility in an attempt to select the most suitable combination for future experiments as well as to understand the role of the major MHC and minor antigens on OAD and other immunological processes.

Theoretically, disparity of MHC antigen i.e. both class I and class II MHC loci will result in severe rejection and the worst outcome with severe OAD, extensive inflammatory cell infiltration, epithelial loss and extensive fibrosis. These histological findings were confirmed in this study. It is also worth noting that all the histological assessment of the tracheal allografts was significantly worse in the PVG RT1^u/Lewis combination (mismatched at both MHC class I and II loci as well as minor antigens) which strongly suggests an immunological pathogenesis to OAD. As expected, isografts i.e.transplant between animals within the same strain resulted in no rejection and no OAD at all, normal epithelium and minimal MNC infiltrate. This have been noticed in other heterotopic isografts models (98).

The only potential problem with isografts is ischaemia following implantation of the trachea into the greater omentum. Hence part of the study was aimed to identify the appropriate duration of follow-up after implantation to achieve normal epithelial regeneration and reversing the effects of ischaemia completely. It has been suggested that tracheal revascularization without surgical reconnection of the bronchial circulation will take between 12-15 days (255). In addition, other experimental work has clearly shown that implantation for 3 days resulted in residual ischaemia with ecchymosis in the graft mucosa. The findings at 7 days were similar to 3 days, while at 10 and 14 days the grafts were intact and had normal mucosa (98, 256). The most detailed well conducted study addressing effect of ischaemia on allografts and isografts was carried out by Boehler and associates from Toronto (257) who, on a similar rat model, carried out detailed analysis of isografts as well as allografts in order to characterise the evolution of transplant induced fibrous airway disease. They also studied time course effect on isografts and allografts. In brief, their results suggested that ischaemia affect epithelial integrity in isografts and allografts in a similar fashion, this usually last 72 hours. Epithelial regeneration is then evident but by seven days, isografts have almost normal epithelium which is complete by 14 days and continue to show normality up to one year, while allograft without immunosuppression showed, by 7 days, severe lymphocytic infiltration associated with epithelial oedema, exfoliation, thrombosis and vasculitis. This process involved the epithelium as well as the peritracheal connective tissue. Epithelial loss was complete by day 14 and was associated with the appearance of granulation tissue. This replaced the epithelial layer and this resulted in reduced patency of tracheal lumen. Airway obliteration was progressive between the second and third week and by day 21, the lumen is almost obliterated with fibrotic tissue with minimal cells. The lumenal granulation tissue become less cellular and vascular with increasing collagen deposition over the next few weeks ultimately developing a picture of obliterative airway disease similar to OB in humans. The results from this study showed remarkable similarity to these published in that, subgroup 1a (isografts at 7 days sacrifice, see results) had mild MNC with almost complete epithelial regeneration on histological examinations that seemed to improve with time. Hence tracheal segments removed from isografts and examined at 14 and 28 days post implantation have

normal tracheal histology with mild/minimal MNC infiltration. The results in allografts without immunosuppression was equally similar in that OAD as well as mucosal and submucosal fibrosis, expensive mononuclear cell infiltrate was seen on 14 days and complete obliteation was evident by 28 days. Following results from this study, we decided that in the next experimental phase, the duration of tracheal implantation until sacrifice operation will be 14 and 28 days in all transplant groups hence minimising the effect of early ischaemia and at the same time allowing for the development of the rejection process in allograft transplants to be well established.

It was interesting to note that the groups in which the disparity was in minor histocompatability antigens only (PVG RTI^u/AO) showed minimal epithelial regeneration with moderate OAD but less MNC infiltrate compared with the PVG RTI^u/Lewis combination. This observation suggests that transplantation even across minor antigen barriers without immunosuppression may result in severe rejection and ultimately OAD. Thus minor antigens may play a significant role in acute and chronic rejection in clinical lung transplantation. It was rather unexpected to find the outcome of transplant between strains diverse only in minor histocompatable antigens have severe OAD although the intensity of the rejection process and the OAD was less than other allograft groups. This may indicate that localised antigens on vascular endothelium and bronchial epithelium plays a major role in the rejection process and that all minor antigens may have a very important immunological influence post transplantation.

The group with disparity in MHC class I and II (PVG RTI^u/PVC) showed histological features of OB manifested as lack of epithelial regeneration, moderate/severe OAD and moderate MNC infiltrate. However, these changes were similar (slightly less severe) to the PVG RTI^u/Lewis group in whom desparity is for both MHC and minor antigens. This is not surprising for two reasons. Firstly, it is known that OAD relies on a host T-cell response that includes CD8+ cells, directed against allo-class I-bearing donor cells within the graft (258). Secondly, disparity in MHC, particularly in class II antigens, causes OAD with severe immunological responses. This is because the bronchial epithelium, as well as vascular endothelium, are known to express class II antigen. Furthermore, animal experiments suggested that MHC class II antigen expression could be induced on the bronchial epithelium and vascular endothelium of

lung allografts during acute rejection. As the bronchial epithelium and vascular endothelium of lung allografts become MHC class II-positive, they are likely to be the targets for low-grade T-cell mediated rejection, resulting in the development of bronchiolitis obliterans and occlusive vascular disease in lung allografts (58).

Similarly, some reports have suggested that OB following heart-lung transplantation is a form of chronic allograft rejection that is related to augmented expression of class II major histocompatibility complex antigens on the airway epithelium and mediated by activated T cells (63).

Chapter II: Tables

Table (1) Summary of results.

Groups	OAD	Epith	SMF (μ)	MNC
group 1:				
subgroup a	**0**	**3**	**4**	++
subgroup b	**0**	**3**	**5**	+
subgroup c	**0**	**3**	**5**	+
group 2:				
subgroup d	**1**	**1**	**12**	+++
subgroup e	**2**	**1**	**14**	+++
group 3:				
subgroup f	**3**	**0**	**20**	+++
subgroup g	**3**	**0**	**21**	+++
group 4:				
subgroup h	**4**	**0**	**22**	+++
subgroup i	**4**	**0**	**24**	++++

Abbreviations for tables 1 and 2

Obs 1 = observer 1, obs 2 = observer 2

OAD = Oblitertive Airway Disease: 0= no OAD, 1= mild, 2= mild/moderate, 3= moderate/severe, 4= severe

Epith = epithelium grade (see methods)

SMF = submucosal fibrosis: Mild=1-5μ, moderate 5-10μ, moderate /severe=10-20, severe= >20μ

MNC = mononuclear cell infiltrate: see methods

Table (2) Scoring results of Individual animals (both observers)

Groups	OAD		Epith		SMF (μ)		MNC	
	obs 1	obs 2	obs 1	obs 2	obs 1	obs 2	obs 1	obs 2
group 1:								
a1	0	0	3	3	4	5	++	++
a2	0	0	3	3	5	5	+++	++
a3	0	0	3	3	5	4	+++	++
b1	0	0	3	3	5	5	++	+
b2	0	0	3	3	5	4	+	+
b3	0	0	3	3	4	5	++	+
c1	0	0	3	3	4	4	+	+
c2	0	0	3	3	5	5	++	+
c3	0	0	3	3	5	4	+	+
group 2:								
d1	1	2	1	1	12	14	+++	+++
d2	2	1	1	2	11	11	+++	++
d3	1	2	1	1	10	12	+++	+++
e1	1	2	1	2	15	14	++++	+++
e2	2	2	1	1	14	13	++	+++
e3	1	1	2	2	15	12	+++	+++
group 3:								
f1	3	3	0	0	20	18	+++	++
f2	3	4	0	0	20	22	+++	+++
f3	3	3	0	0	21	20	+++	+++
g1	3	4	0	0	18	19	+++	++
g2	3	3	0	0	21	22	+++	+++
g3	3	3	0	0	22	21	++	+++
group 4:								
h1	4	4	0	0	22	20	+++	++++
h2	4	4	0	0	23	20	++++	+++
h3	4	4	0	0	21	22	+++	++++
i1	4	4	0	0	20	22	+++	++++
i2	4	4	0	0	23	21	++++	++++
i3	4	4	0	0	18	24	++++	++++

Chapter II: Figures (17-25)

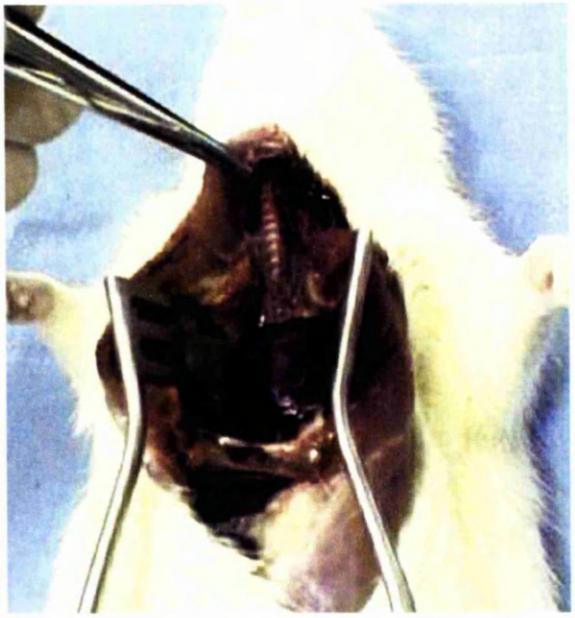


Figure 17: Donor operation. The whole trachea of Lewis donor rat is exposed ready for resection

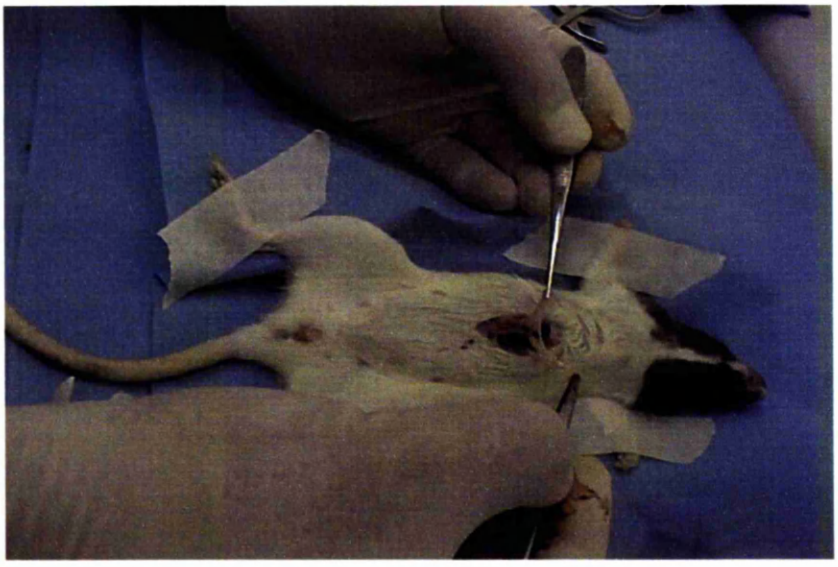


Figure 18: Recipient operation

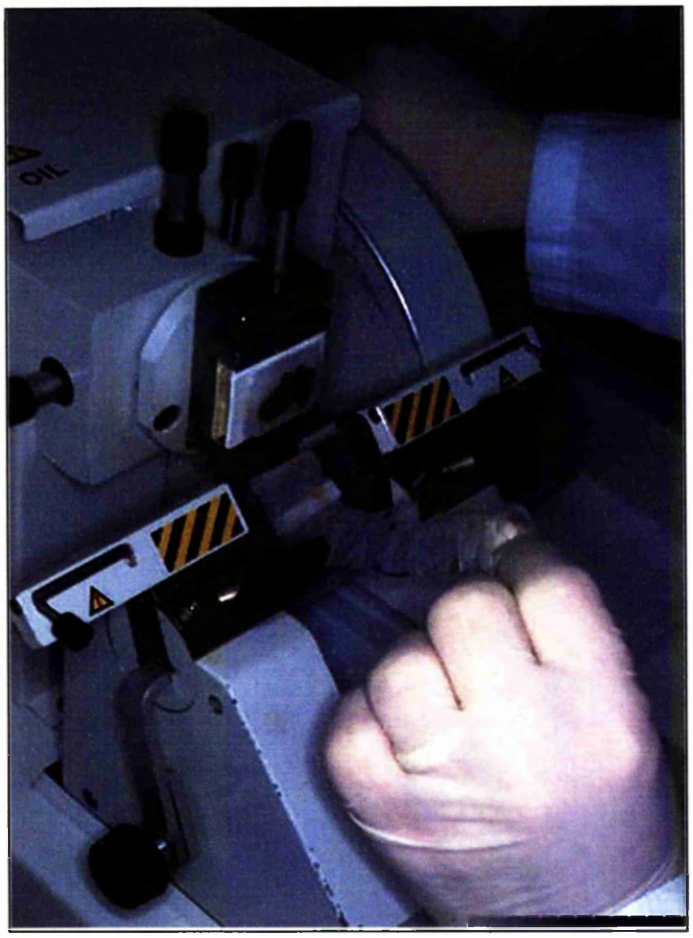


Figure 19: Tissue sectioning
Using microtome

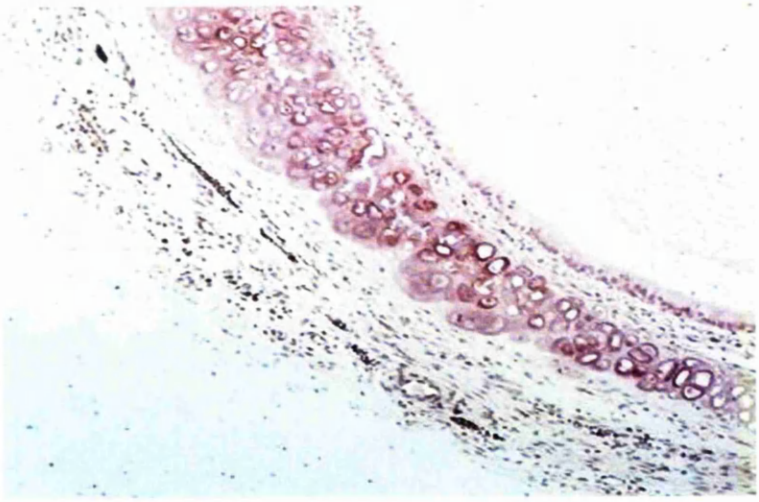


Figure 20: Isograft 14 days (LP)

Section of trachea showing normal
Epithelial healing

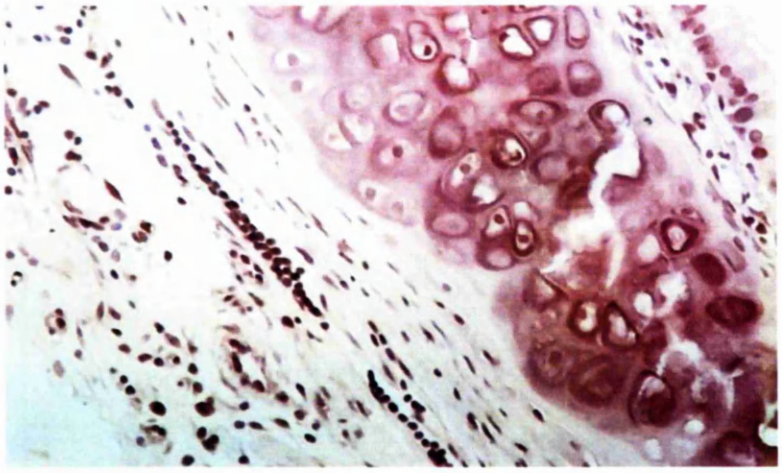


Figure 21: Isograft 28 days (HP)

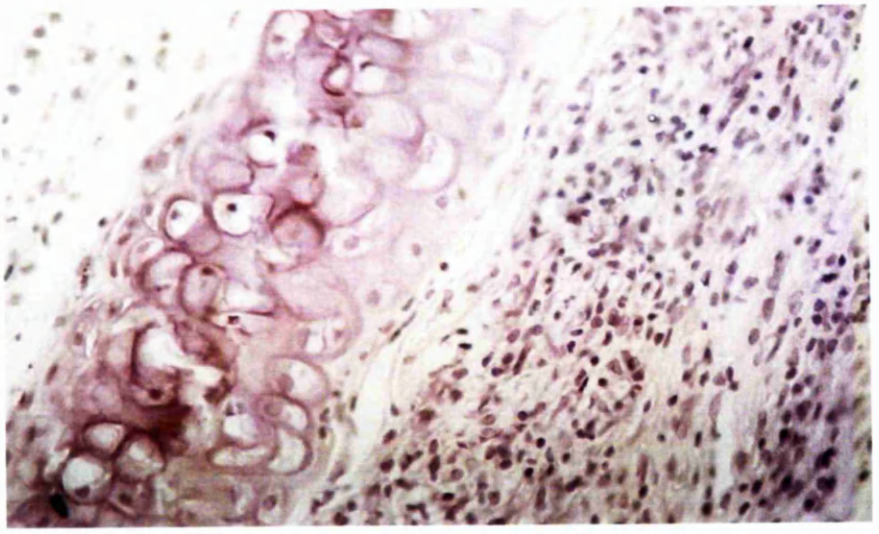


Figure 22: AO-PVG RT1u allograft

Extensive MNC infiltrate and epithelial degeneration

Figure 23: PVC-PVG RT1u allograft
Note extensive OAD and extensive MNC infiltrate

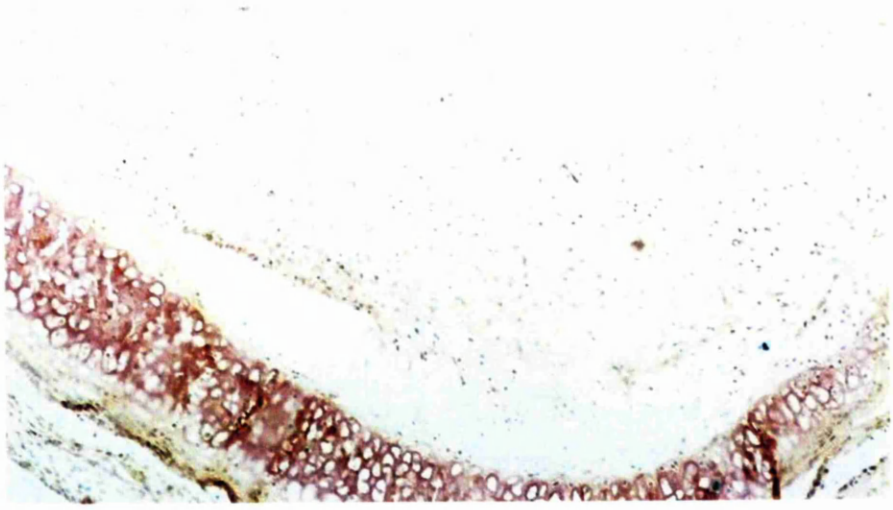


Figure 24: Lewis-PVG RT1u allograft (LP)
Note complete obliteration of tracheal lumen

Figure 25: Lewis-PVG allograft (HP)
Complete obliteration of tracheal lumen

CHAPTER III

Tracheal allograft transplantation in rats: the role of immunosuppressive agents on development of obliterative airway disease

I presented material from this chapter at the International Transplant meeting at Florence, Italy in 1998.

This paper was subsequently published in Transplantation Proceedings, 1998, Volume 30, Issue 5, PP 2207-2209.

III.i. Introduction:

Obliterative Bronchiolitis (OB) is the most common late pulmonary complication among lung and heart-lung transplant recipients (47, 259). Early pathology of OB demonstrates injury of the bronchiolar epithelium with submucosal lymphocytic infiltrate, and the subsequent development of granulation tissue with acute and chronic inflammatory cells, which eventually leads to formation of scar tissue. In severe cases this eventually result in total occlusion of the entire lumen of the bronchiole.

The aetiology of obliterative bronchiolitis in lung transplants is still uncertain. Various factors have been implicated as possible causes, among which are lack of bronchial circulation and lymphatic drainage (93), infections (91) and rejection (94). Chronic rejection has been considered to be the most likely cause in several clinical reports (96, 97) and animal studies (95). The clinical studies, however, are not conclusive regarding the aetiology of obliterative bronchiolitis because of the large number of confounding variables.

We used a technically simple heterotopic tracheal transplant model that was also successfully used by other units (98, 100, 260). This is a model of rapidly progressing obliterative airway disease (OAD) in rats with histological features characteristic of OB in human lung allograft recipients. As rejection is considered to be the most likely cause of OB (261), this study was designed to assess the efficacy of different, clinically available immunosuppression regimes on the prevention of OAD. We also performed detailed histopathological analysis in order to assess the severity of OAD in each treatment group.

III.ii. Material and Methods:

The heterotopic tracheal transplant rat model with omentopexy was used (chapter II). Recipient procedures were carried out on male (300g) inbred PVG RT1^u rats (Biological Services Unit, Manchester University, Manchester UK). Complete tracheal segments were harvested from Lewis rats, divided into two segments and implanted into the greater omentum of PVG RT1^u recipient rats.

III.ii.a. Donor Procedure

See donor procedure, chapter II; page 65.

III.ii.b. Recipient Procedure

See recipient procedure, chapter II; pages 65-66.

III.ii.c. Animal Groups and Drug Administration:

There were seven experimental groups consisting of six animals each as follows:

Group 1 No immunosuppression (served as a control)

Group 2 Mycophenolate Mofetile (MMF) 40 mg/kg/day by gavage

Group 3 Tacrolimus (TAC) 0.1 mg/kg/day injected subcutaneously

Group 4 TAC and MMF in combination (Tac plus MMF), using the same dose for each drug as in groups 2 and 3 when used individually.

Group 5 CyA 5mg/kg/day injected subcutaneously

Group 6 CyA and MMF in combination (CyA plus MMF), using the same doses of drugs as in groups 2 and 5 drugs when used individually.

Group 7 Vitamin E supplement at a dose of 100mg/kg/day orally

In each animal group of 6 rats, the experiments were terminated at 14 and 28 days of drug treatment (subgroups a & b) apart from animals in group 7, those were treated with Vitamin E for 28 days.

Drug administration:

Cyclosporin A (iv solution, Novartis® Pharma, UK) was diluted to 10 mg/ml and given subcutaneously (SC) at a dose of 5mg/Kg/day.

Tacrolimus (Prograf, IV preparation, Fujisawa® UK) was diluted to 1 mg/ml and was given SC at a dose of 1mg/kg/day. Both CyA and tacrolimus were freshly prepared before use and injected SC daily, beginning on the day of the operation and continuing until the animals were sacrificed at either 14 days (3 animals) or 28 days (3 animals).

Mycophenolate mofetile (Roche® UK) was administered by gavage at 40mg/kg/day for 14 days (3 animals) or for 28 days (3 animals). In groups where recipients were

treated with combinations of agents, in groups 4 (CyA plus MMF in 6 animals) and 6 (TAC plus MMF in 6 animals) were carried out using similar regimes as for the individual drugs.

Finally, one group of 6 rats had treatment using oral **Vitamin E** at 100mg/kg/day for 28 days. No antibiotics were given to recipients.

III.ii.d. Retrieval of transplanted tracheas:

See retrieval of transplanted tracheas, chapter II, page 66.

III.ii.e. Histologic Assessment

See histological assessment, chapter II; pages 68-69.

III.iii. Results: (see tables 3 and 4)

All 42 recipient animals survived the transplant operations. There was 100% survival throughout follow-up period and until the sacrifice procedure. No intra-abdominal sepsis was encountered in any of the groups. Obliterative Airway Disease (OAD), epithelial lining, mononuclear cell (MNC) infiltrate and submucosal fibrosis were graded, for each group, on H& E stained donor trachea.

OAD and epithelial lining: OAD was severe (score 4) in the no immunosuppression group (figure 26), moderate/severe (score 3) in Vitamin E (group 7, figure 36), moderate (score 2) in the MMF treated recipients at 14 and 28 days (group 2, figure 33) and was absent in the rest (figures 27, 28, 29, 30, 31, 32).

The epithelial layer was completely degenerated (grade 0) in no treatment controls (figure 26), Vitamin E and MMF groups (figures 33 and 36). Grade 1 (cuboidal epithelium) in group 3a (treated with TAC for 14 days)(figure 31), and in the TAC plus MMF animals (group 4 a & b, figures 29 and 30). Normal ciliated pseudo-stratified columnar epithelium (grade 3) was seen in recipients treated with CyA 28 days(group 5b, figure 34), Tac 28 days (group 3b, figure 32), and in the CyA plus MMF (groups 6 a & b, figures 27 and 28) recipients.

Mononuclear cell (MNC) infiltration: MNC infiltration was most pronounced (++++) in controls (groups 1 a & b), was moderately severe (+++) in the Tac group 3b and

MMF (group 2a). Moderate (++) in the CyA group 5a, MMF group 2b), CyA plus MMF group 6a and Tac plus MMF group 4a and Vitamin E (group 7) transplants. Mild (+) infiltration was observed in the Tac group 3a, CyA group 5b , CyA plus MMF group 6b and Tac plus MMF group 4b.

Submucosal fibrosis: The intensity of fibrosis was assessed by measuring the submucosal fibrous layer. It was severe (>20 μ) in controls, moderate /severe (15-20 μ) in the Vitamin E group , moderate (10-15 μ) in both MMF only groups, mild/moderate (5-10μ) in Tac 14 days (3a), Tac+MMF 14 and 28 days (4a & 4b)and CyA+MMF 28 (6b), mild (1-5 μ) in Tac 28 (3b), CyA 14 & 28 days (5a & 5b) and CyA+MMF 14 (6a) group. (see table 2)

III.iv. Discussion:

Obliterative Bronchiolitis remains the most common cause of morbidity and mortality following clinical lung transplantation (Ltx), affecting nearly 50% of patients at 4 years (47, 90). Although a variety of factors have been implicated as possible aetiological factors for OB, immunological factors are considered to be the most likely causes. We used this simple, well tested animal model to try to understand the role of different, clinically available immunosuppressive agents on the incidence, development and severity of OAD.

Acute rejection has been shown by various studies to be extremely important for the development of OB in clinical Ltx (262). In this study, we have shown that adequate immunosuppression regimes using CyA or tacrolimus, in doses similar to that used clinically and given regularly, resulted in almost complete regeneration of the lining epithelium, absence of OAD, minimal fibrosis and MNC infiltrate.

Subclinical rejection has been considered to be a likely cause of OB in long term lung and heart/lung transplant recipients and in order to exclude subclinical rejection some authors have advocated a regular lung biopsy protocol. However, the lack of adequate rejection diagnosis with routine transbronchial biopsy (TBB) due to the low diagnostic yield (263) resulted in less stringent TBB protocols for follow-up lung transplant patients, so the majority of follow-up protocols rely on domiciliary spiromerty parameters. It is possible that the difficulty in diagnosing subclinical acute

rejection in stable patients may result in a persistent rejection that, with the passage of time, may cause immunological injury to the respiratory epithelium ultimately resulting in OB.

The optimum level of immunosuppression in Ltx is not known. Due to the nephrotoxicity associated with long term CyA therapy, the tendency is to reduce the doses of CyA and accept lower levels during the follow-up period. Furthermore, there are no data regarding the appropriate dose and level for CyA in lung transplant follow-up. Consequently, differences in level of immunosuppression during follow-up among different centres make it impossible to correlate incidence and outcome of OB. In addition, infection has been implicated as a cause of OB and unscrutinised augmentation in immunosuppression may cause deterioration rather than improvement in the grade of OB.

In this study, it was interesting to note that CyA and Tacrolimus in appropriate doses, used individually or in combination with MMF, were effective in preventing OAD while MMF alone was rather ineffective. A similar observation was noticed by the Stamford group (100). MMF treatment using the similar dose of 40mg/Kg/day orally resulted, at 28 days of therapy, in 63% airway narrowing compared 96% narrowing in the no treatment group.

Vitamin E (α- tocopherol) has been shown to have a protective effect against fibrosis most likely related to its antioxidant effect. It has also been suggested that it may have a net inhibition of Transforming growth factor-β1 (TGF-β1) and α2(I) procollagen mRNA levels. TGF-β1 is known to play a major role in connective tissue healing and remodelling following necrosis and inflammation through the synthesis of extracellular matrix components (232, 264). Vitamin E may also down-modulate basal levels of TGF-β1 mRNA suggesting that it may potentially interfere with the initiation and progression of the fibrosclerotic process (265). In this study, it was interesting to find poor epithelial regeneration, mild MNC infiltrate and significant OAD in animals on Vitamin E supplement, but submucosal fibrosis score was less severe compared with control animals, suggesting that down regulation of TGF-β1 may have played a part in reducing the severity of the fibrotic response.

We used 14 and 28 days regimes in order to counteract the theoretical risk of incomplete regeneration of epithelium after de-vascularisation at 2 weeks (253). It

was interesting that none of the grafts showed deterioration in epithelial regeneration, or increased severity of OAD, and in one group (tacrolimus alone), the additional 2 weeks resulted in improvement in epithelial regeneration from cuboidal/squamous metaplasia to normal epithelium. We can conclude that in the context of this experiment, one protocol with 3-4 weeks treatment is adequate.

In similar experiments (253) surgical steel wires were used to keep a normal length of tracheal segments. It was perceived that the trachea shrink to half their normal length exposing the surface to high percentage of cartilage which is thought to inhibit epithelial regeneration. On the other hand, implanting a foreign body in the omentum will potentially create a more intense inflammatory reaction with intense angiogenesis. It may also result in a higher rate of infection that may interfere with the interpretation of results and outcome. In this study, we did not use steel wires and none of the animals had abdominal sepsis (despite using no prophylactic antibiotics and clean rather than a sterile surgical technique). All animals survived the duration of the experiment. Furthermore, we noticed excellent epithelial regeneration within 2 weeks of implantation and the epithelium was normal ciliated columnar epithelium in a number of experiments indicating that stenting of the trachea is probably unnecessary.

In summary, The results of this study are compatible with the notion that an ongoing rejection response contribute to the development of changes charasteristic of OB and that adequate immunosuppression with Tacrolimus or CyA alone or in combination with MMF can control these changes. The major implication is that some lung transplant recipients are under immunosuppressed because of the potential nephrotoxicity of agents currently used. The use of agents in synergistic combinations should be explored.

Table (3) Histology score for individual animal's trachea by each observer.

Animal	OAD(1)	OAD(2)	Epith1	Epith2	SMF(1)	SMF(2)	MNC(1)	MNC(2)
1a1	4	4	0	0	21	19	4+	4+
1a2	4	4	0	0	20	23	3+	3+
1a3	4	4	0	0	20	18	4+	4+
1b1	4	4	0	0	22	20	4+	4+
1b2	4	4	0	0	19	20	3+	4+
1b3	4	4	0	0	17	18	4+	3+
2a1	2	3	0	0	12	12	3+	3+
2a2	3	2	0	0	12	11	2+	3+
2a3	3	2	0	0	11	12	3+	3+
2b1	2	1	0	0	10	10	2+	1+
2b2	2	2	0	0	10	11	1+	2+
2b3	2	2	0	0	11	10	2+	2+
3a1	0	0	2	2	7	6	1+	1+
3a2	0	0	3	3	6	6	2+	1+
3a3	0	0	2	3	6	5	1+	2+
3b1	0	0	3	3	4	3	3+	3+
3b2	0	0	3	2	2	3	2+	3+
3b3	0	0	2	3	5	4	3+	2+
4a1	0	0	2	1	6	6	1+	1+
4a2	0	0	1	2	7	6	2+	2+
4a3	0	0	2	2	4	5	1+	0+
4b1	0	0	2	2	10	9	1+	1+
4b2	0	0	2	1	7	8	1+	1+
4b3	0	0	1	2	9	8	2+	2+
5a1	0	0	1	1	4	3	1+	1+
5a2	0	0	1	1	5	4	2+	2+
5a3	0	0	2	2	4	5	2+	2+
5b1	0	0	2	3	3	2	1+	1+
5b2	0	0	3	3	4	3	1+	1+
5b3	0	0	3	3	3	5	0+	0+
6a1	0	0	3	2	5	6	2+	2+
6a2	0	0	2	3	5	5	1+	2+
6a3	0	0	3	3	4	3	2+	3+
6b1	0	0	3	3	7	6	1+	1+

6b2	0	0	3	3	8	7	1+	2+
6b3	0	0	3	3	5	6	2+	1+
7a1	3	4	0	0	18	16	1+	1+
7a2	3	3	0	0	19	17	2+	3+
7a3	3	3	0	0	15	15	2+	2+

Abbreviations:

Animals: Code for individual animal

OAD1= Score for observer 1, OAD2 score for observer 2.

OAD2 Score code is summarised in methodology.

Epith1= score for epithelial regeneration by observer 1

Epith2= Score for epithelial regeneration by observer 2

Epithelial score is summarised in methodology.

SMF(1)= Score for submucosal fibrosis by observer 1.

SMF(2)= Score for SMF by observer 2.

SMF score is summerised in methodology.

MNC(1)= Score for mononuclear cells by observer 1.

MNC(2)= Score for MNC by observer 2.

MNC score is summarised in methodology.

Animal groups:

1a = Control 14

1b = Control 28

2a = MMF 14

2b = MMF 28

3a = Tac 14

3b = Tac 28

4a = Tac+MMF 14

4b = Tac+MMF 28

5a = CyA 14

5b = CyA 28

6a = CyA+MMF 14

6b = CyA+MMF 28

7 = Vitamin E

Table (4)

Summary of the results for the 7 groups.

Groups	Drug Regime	OAD	Epithelium	SMF	MNC
1a	Control 14	yes (4)	A grade 0	22	4+
1b	Control 28	yes (4)	A grade 0	20	4+
2a	MMF 14	yes (2)	A grade 0	12	3+
2b	MMF 28	yes (2)	A grade 0	10	2+
3a	TAC 14	no (0)	SM grade 2	6	1+
3b	TAC 28	no (0)	N grade 3	3	3+
4a	TAC+MMF 14	no (0)	SM grade 2	6	2+
4b	TAC+MMF 28	no (0)	SM grade 2	9	1+
5a	CyA 14	no (0)	N grade 1	4	2+
5b	CyA 28	no (0)	N grade 3	3	1+
6a	CyA+MMF 14	no (0)	N grade 3	4	2+
6b	CyA+MMF 28	no (0)	N grade 3	6	1+
7	Vitamin E	yes (3)	A grade 0	16	2+

OB = Oblitertive bronchiolitis, SMF submucosal fibrosis (μ), MNC mononuclear cell infiltrate, A = absent, SM Squamous metaplasia, C= cuboidal epithelium, n = normal epithelium.

Chapter III: Figures (26-36)

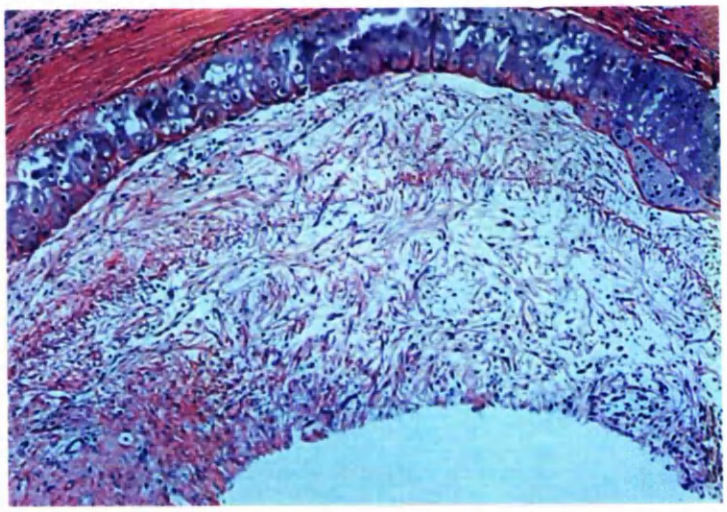


Figure 26

No treatment allograft group, 28 days

Loss of epithelium and severe OAD, extensive SMF and moderate MNC infiltrate

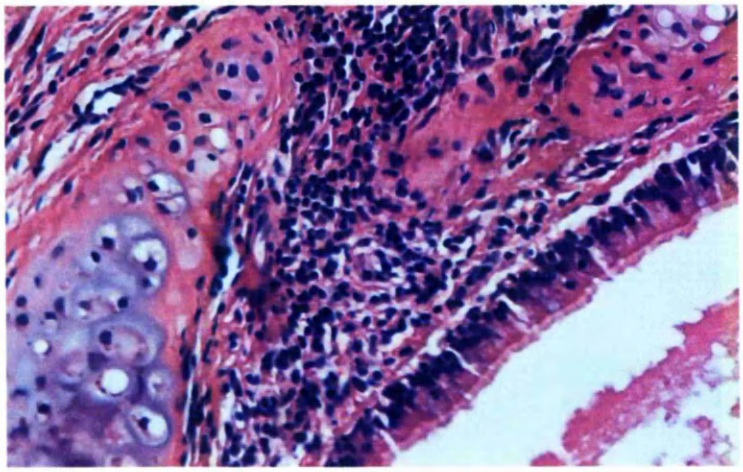


Figure 27

Cyclosporine-MMF treatment at 14 days

Normal epithelial regenration with moderate MNC infiltration

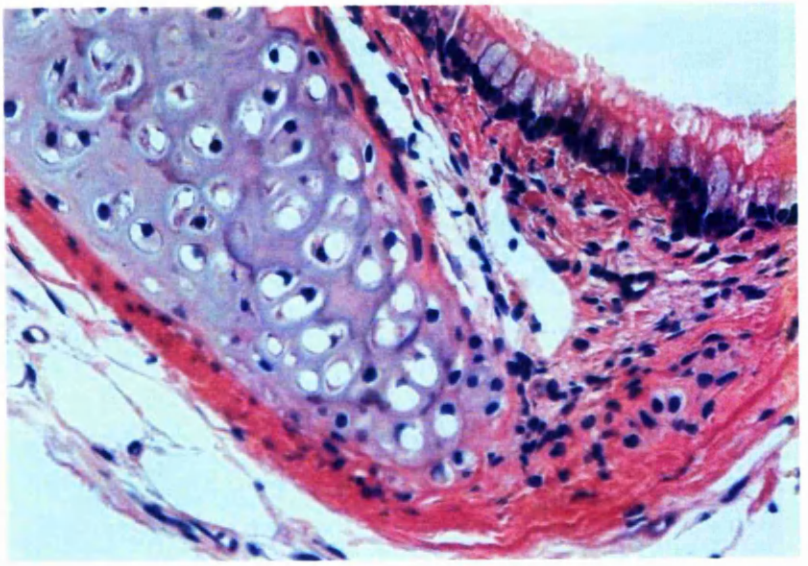


Figure 28
Cyclosporine and MMF treatment at 28 days

Normal epithelium, no OAD, normal SML and minimal MNC infiltrate

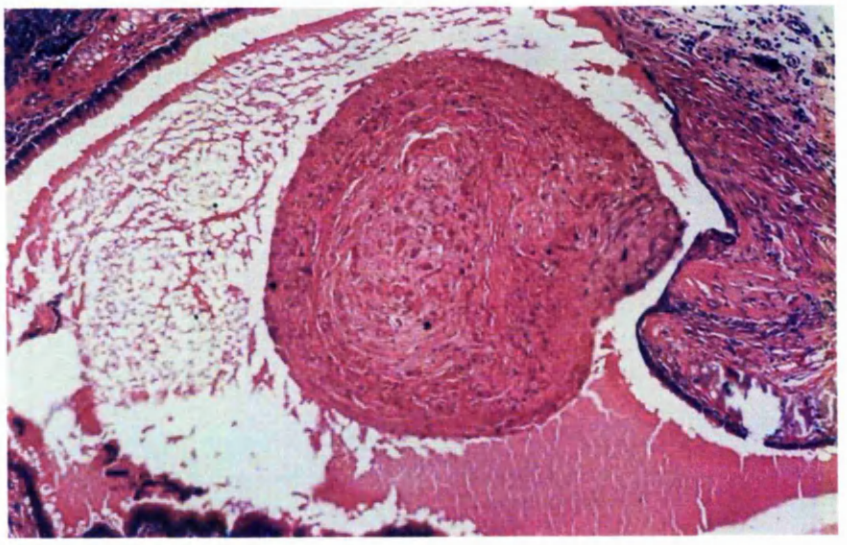


Figure 29

Tacrolimus and MMF at 14 days

Note normal epithelial layer with moderate MNC infiltrate

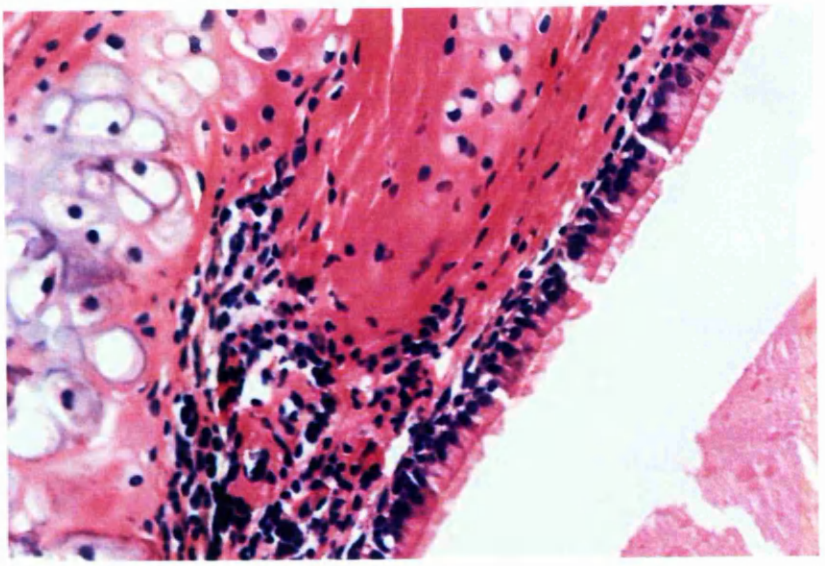


Figure 30

Tacrolimus and MMF at 28 days

Normal epithelial layer, no OAD with mild MNC infiltrate

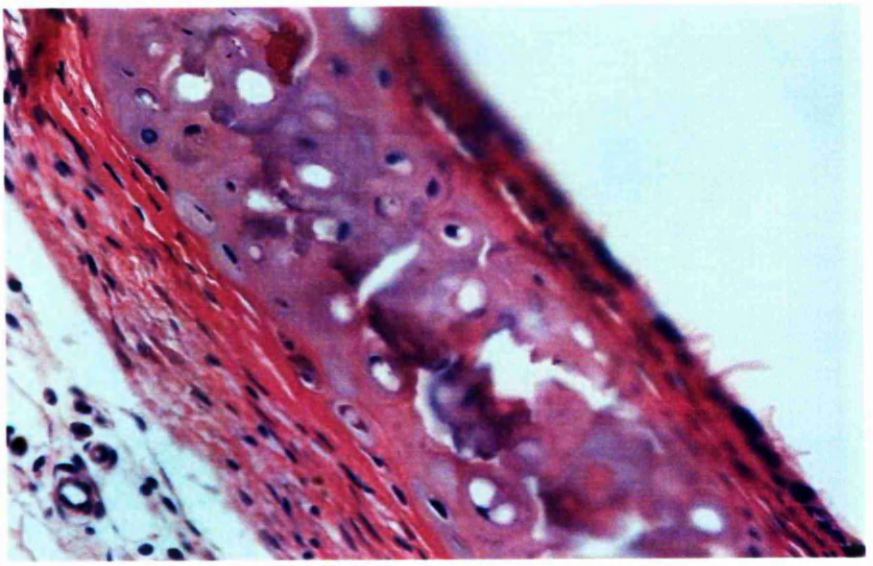


Figure 31

Tacrolimus at 14 days

Cuboidal epithelium, no OAD, minimal SMF and mild MNC infiltrate

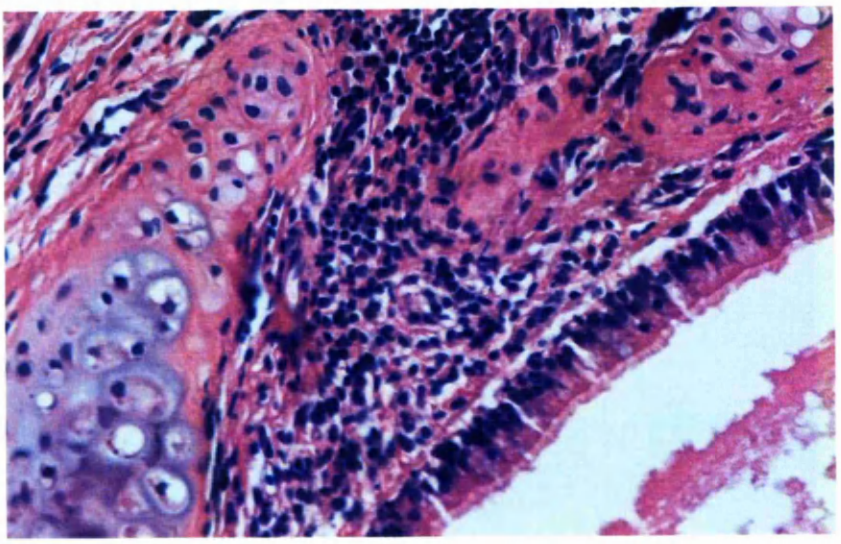


Figure 32

Tacrolimus 28 days treatment group

No OAD, normal epithelial degeneration, moderate/severe MNC infiltrate

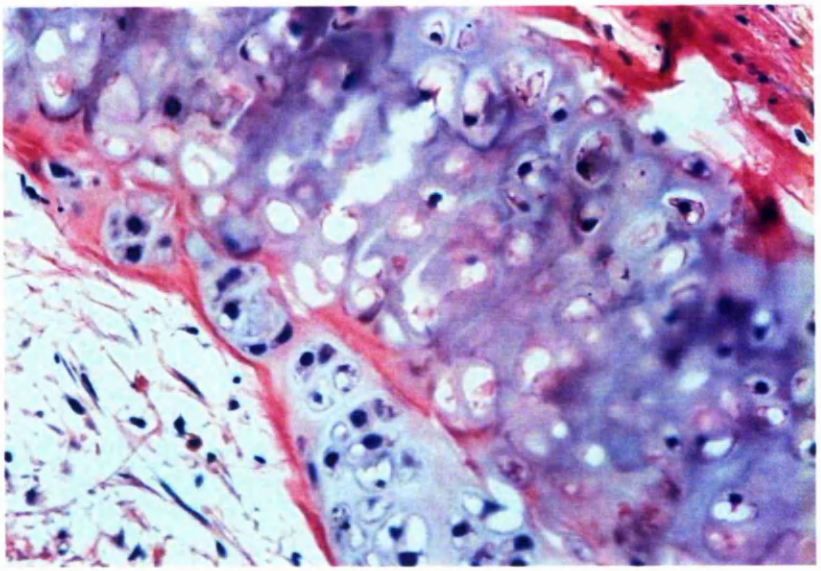


Figure 33

MMF treatment group day 28

Moderate OAD, loss of epithelium and minimal MNC infiltrate

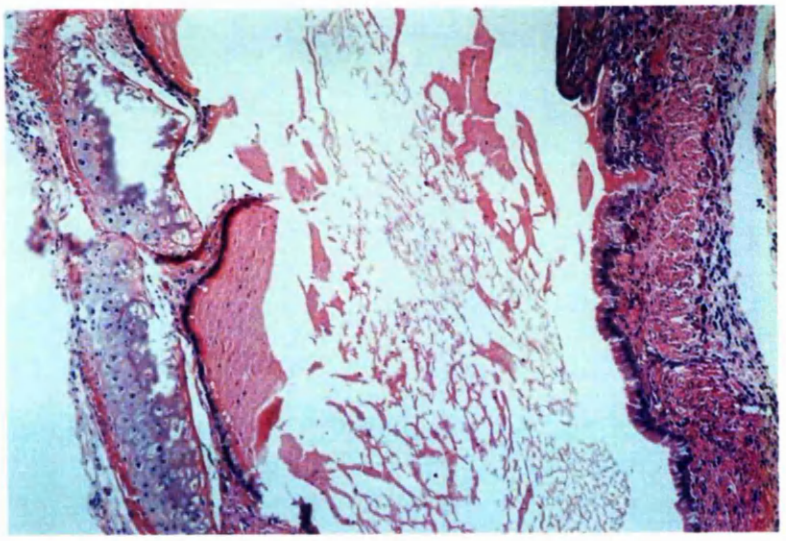


Figure 34

Cyclosporine 28 days treatment group

Normal epithelium, no OAD, mild MNC infiltrate

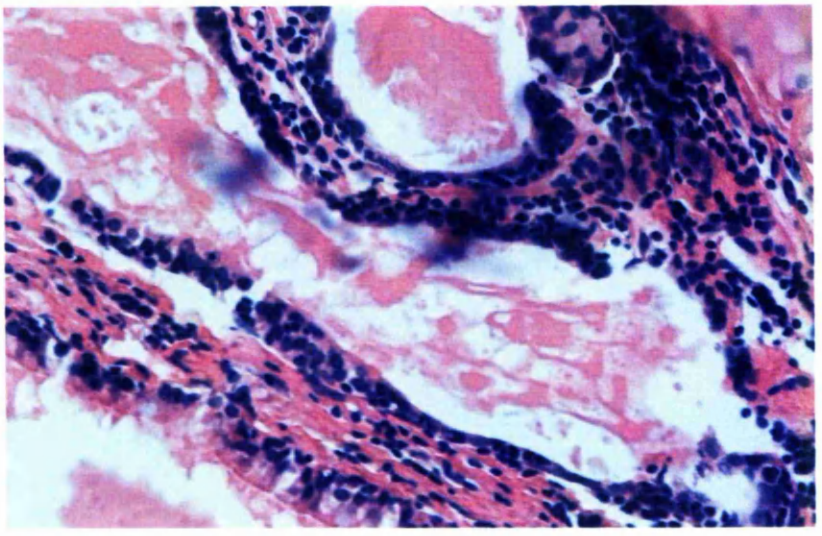


Figure 35

Cyclosporine 14 days treatment group

Normal epithelium, no OAD, moderate MNC infiltrate

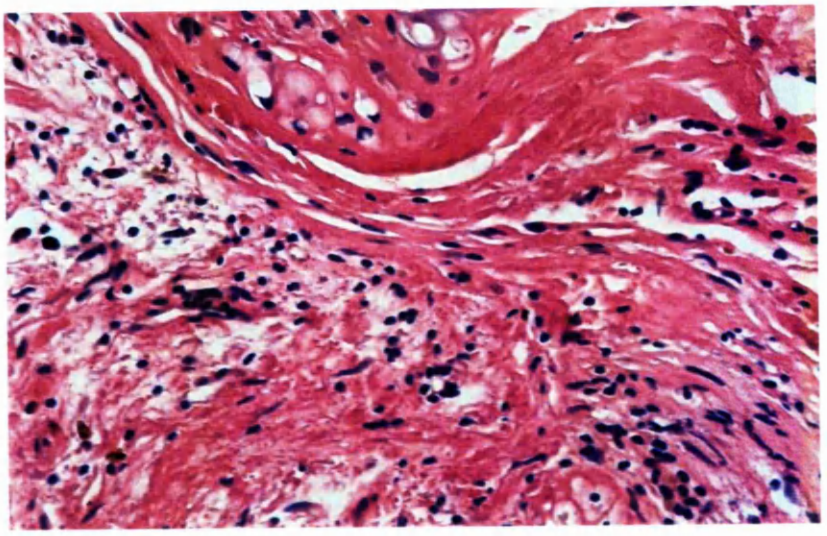


Figure 36

Vitamin E group at 28 days

Moderate OAD, absent epithelial layer, moderate SMF and mild/moderate MNC infiltrate

CHAPTER IV

TGF-β, Immunosuppression and Obliterative Airway Disease in the Heterotopic Tracheal Rat Transplantation model.

Tracheal Allograft Transplantation: The impact of different immunosuppressive regimes on Transforming Growth Factor beta expression and development of obliterative airway disease.

Material from this chapter was submitted as a manuscript for publication in the Journal Transplant Immunology August 2002.

IV.i. ABSTRACT

RT1u Rats were transplanted with tracheal allografts from Lewis rats and treated with immunosuppressive agents for 14 or 28 days. The histological appearance of the grafts at these times and the presence of transforming growth factor-beta (TGF-β) were scored.

Increased TGF-β expression was noted in tracheal biopsies with severe obliterative airway disease (OAD) compared with biopsies that had no or mild OAD. It was evident that mycophenolate mofetil had a beneficial effect on OAD. It was associated with reduced expression of TGF-β in the graft as well as reduced fibrosis score and reduced development of OAD, especially when combined with tacrolimus or cyclosporine. This combination immunosuppression may be of benefit in the prevention of OB in clinical lung transplantation.

IV.ii. INTRODUCTION

Obliterative bronchiolitis (OB) is the most common late pulmonary complication following clinical lung transplantation among lung and heart-lung transplant recipients (3, 90) causing morbidity and mortality affecting around 50% of patients at 4 years (92). The pathological changes of OB includes injury to the bronchiolar epithelium with submucosal lymphocytic infiltration. Granulation tissue subsequently develops, with the presence of acute and chronic inflammatory cells, eventually leading to formation of scar tissue. In severe cases this may culminate in total occlusion of the entire lumen of the bronchiole (93).

The aetiology of obliterative bronchiolitis in transplanted lungs is unclear. Various factors have been implicated such as lack of bronchial circulation or lymphatic drainage (94), infections (121) and rejection (52, 95, 96). Chronic rejection has been identified as the most likely cause of OB in several clinical reports (97, 249, 266) and animal studies (98, 99).

More recently, different growth factors (102, 104, 267, 268) have been implicated in the pathogenesis of OB, particularly the ubiquitous transforming growth factor-beta (TGF-β). TGF-β potently promotes fibrosis by enhancing the synthesis of

extracellular matrix components (103). The repair process following lung allograft injury whether it is due to ischaemia, rejection or infection replaces lung parenchyma with fibrous tissue leading to pulmonary dysfunction (104). TGF-β has a major role in co-ordinating tissue repair, hence its over-expression may result in an exaggerated or aberrant tissue repair process which ends ultimately in tissue fibrosis (104).

In this study we used a technically simple tracheal allograft transplant model (99, 260, 269). This is a model of rapidly progressing obliterative airway disease (OAD) in rats with histological features characteristic of OB in lung allograft recipients. The aim of this study was to investigate the role of growth factors, particularly TGF-β, in the pathogenesis of OB. Because the immunological processes of acute and chronic rejection are considered to be a major contributor to OB (96, 249), we investigated the influence of immunosuppressive agents currently in clinical use on the development and severity of OB. We performed detailed immunohistochemical analysis of TGF-β expression in tracheal biopsies in order to assess the influence of this growth factor on the incidence and severity of OB and to correlate TGF-β expression with immunosuppressive drug treatment.

IV.iii. MATERIAL AND METHODS: (See chapter III, page 82)

The heterotopic tracheal transplant rat model with omentopexy was used. Adult (300g) male rats of the PVG.RT1^u and Lewis (RT1^l) inbred strains, supplied by the Biological Services Unit (BSU) Manchester University, were used. Complete donor tracheal segments were harvested from Lewis rats, divided into two segments and were implanted into the greater omentum of PVG.RT1^u recipient rats. In this combination the donor and recipient are different at both major and minor histocompatibility loci.

IV.iii.a. Donor Procedure.

See donor procedure, chapter II; page 65.

IV.iii.b. Recipient Procedure.

See recipient procedure, chapter II; pages 65-66.

IV.iii.c. Animal Groups and Drug Administration:

See animal groups and drug administration, chapter III; pages 83-84.

IV.iii.d. Retrieval of Transplanted Tracheas

See chapter II, page 66,

IV.iii.e. Histologic Assessment:

See histological assessment, chapter II; pages 69-70.

IV.iv. Immunohistochemical Staining procedure:

TGF-β immunoperoxidase staining of rat tracheal transplants was carried out on all sections.

IV.iv.a. Immunohistochemical staining for TGF-β:

Immunohistochemistry was performed on paraffin-wax embedded heterotopic allograft tissues. 7.5μ thick sections were mounted on poly-L lysine coated slides (Menzel-Glaser, Germany). Slides were de-waxed, rehydrated in alcohol, immersed in 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity and then incubated in 10% normal rabbit serum for 20 minutes. Mouse anti-human TGF-β antibody (Serotec Ltd, Oxford, UK) was diluted in 0.1% bovine serum albumin (BSA, Sigma, Poole, Dorset, UK) at a concentration of 10μg/ml overnight at 4° C. After washing the slides 3 times in Tris buffered saline (TBS), a secondary reagent, rabbit anti-mouse horseradish peroxidase conjugated antibody (Dako, High Wycombe, UK) was added and incubated for 2 hours at room temperature in a humidified box. Slides were washed 3 times in TBS and developed in 0.05% 3,3'Diaminobenzidine (Sigma, Poole, Dorset, UK) in TBS plus 0.002% hydrogen peroxide, washed in tap water then counter stained in Harris's haemotoxylin for 1 min. Slides were then washed in running tap water, dehydrated in alcohol and Citroclear before being mounted in DPX (Raymond Lamb, London, UK).

IV.iv.b. Assessment of TGF-β staining.

The standard semiquantitative method for evaluating immuno-histochemical staining

described previously (270) was used. The grading was carried out by two independent observers one of whom is a Consultant Histopathologist. Both observers were blinded to treatment identity. The grading of TGF-β staining was carried out on each section on the basis of the **distribution** of TGF-β stain as well as the **intensity** of staining of the cells and matrix. Most of the positive staining was in the thickened mucosa, submucosa and was mainly intracellular. The *intensity* of the staining was graded as follows: 0 being absent, 1 considered mild, 2 mild/ moderate, 3 moderate, 4 moderate/severe and 5 was severe. The *distribution* of staining in the lung biopsies and as follows; <5% score 0, 5-20% score 1, 20-49% score 2, 50-75% score 3 and >75% score 4.

Prior to staining the tracheal biopsies, positive control slides from human tonsillar tissue for TGF were stained at the same session (figure 49). All the slides then compared to the positive control. The scoring of TGF was carried out by two independent observers, one of whom is a Consultant Histopathologist, and was carried out on sections from all the animals in the study (table 5)

IV.v. RESULTS (See summary of results, table 6)

All recipient animals were healthy and survived the experimental procedures without any evidence of intra-abdominal sepsis during the experiment. Tracheal sections were stained with H&E and immunohistochemical staining for TGF-β. The scores for the epithelial lining, mononuclear cell infiltrate, fibrosis and obliterative airway disease (OAD) are *seen in the results section, chapter III* and are summarised in tables 4, page 90 and table 6, page 108.

TGF-β staining: ***S***ummarized as follow: also see (table 1)

Normal controls: No TGF staining (figure 37)

No immunosuppression groups 1a&1b: The TGF staining was severe in intensity (score 4) as well as distribution (score 4). Total 9 (figure 38)

MMF 14 (2a): Distribution was >20% (score 1), intensity was mild/moderate (score 2). Total 3 (figure 47).

MMF 28 (2b): Distribution was>20% (score 1), intensity was mild/moderate (score

2). Total 3. (figure 40)

Tac 14 (3a): Distribution was around 40% (score 2), intensity was mild (score 1). Total 3

Tac 28 (3b): Distribution was 45% (score 2), intensity was mild/moderate (score 2). Total of 4 (figure 41)

Tac+MMF (4a): Distribution was 40% (score 2), intensity was mild (score 1). Total 3

Tac+MMF (4b): Distribution was 10% (score 1), intensity was mild (score 1). Total 2 (figure 44).

CyA 14 (5a): Distribution was 60% (score 3), intensity moderate (score 3). Total 6 (figure 42).

CyA 28 (5b): Distribution was 60% (score 3), intensity was mild/moderate (score 2). Total 5 (figure 43).

CyA+MMF (6a): Distribution 10% (score 1), intensity was mild/moderate (score 2). Total 3 (figure 46).

CyA+MMF (6b): Distribution was 5-10% (score 1), intensity was mild (score 1). Total 2 (figure 45).

Vitamin E (7): Distribution was 40% (score 2), intensity mild/moderate (score 2). Total 4 (figure 48).

IV.vi. Discussion:

Immune mediated damage to lung allografts, and the subsequent induction of growth factor expression, in particular TGF-β, has been implicated in the development of OB (102, 271). In this study, using a reproducible animal model of transplant OB, the transplantation of tracheal segments was used to examine the relationship between immunosuppression, the expression of TGF-β and the development of OB.

Untreated isografts showed normal histological features 14 and 28 days after transplantation. By contrast, untreated allografts rapidly developed OAD, were denuded of living epithelium, were heavily infiltrated with mononuclear cells and had submucosal fibrosis, associated with the presence of significant TGF-β staining (Table 1, groups 1a and 1b).

The administration of MMF alone reduced but did not prevent the development of

OAD or the loss of epithelium. However, the intensity of the mononuclear cell infiltrate was reduced, and submucosal fibrosis was only moderate. In the MMF treated recipient rats, we observed a lower expression of TGF-β (Table 1, groups 2a, and 2b) compared to controls. The effect of MMF monotherapy at 40 mg/kg/day on OAD was noticed previously (100, 260). In those studies, there was a 47-63% airway narrowing compared with 98% in the untreated controls. Thus MMF on its own failed to prevent acute rejection or to abolish OAD but it reduced the severity of OAD.

MMF is a new antimetabolite immunosuppressive agent that is rapidly metabolised to the active form mycophenolic acid (MPA) in the liver. MPA inhibits purine synthesis through the classical pathway by inhibiting the enzyme inosine monophosphate dehydrogenase (IMPDH). This inhibitory effect is more effective on the proliferation of T and B lymphocytes (272). MMF induces apoptosis of activated T-lymphocytes hence reducing T lymphocyte populations responsible for graft rejection and OAD. It also suppresses the expression of adhesion molecules by depleting guanosine nucleotides. This may decrease the recruitment of lymphocytes and other cells into the site of rejection. It has also been shown that MMF therapy has a suppressing effect on the production by iNOS of nitric oxide (NO), hence reducing the tissue damage mediated by peroxynitrites (272). MMF has an inhibitory effect of fibroblasts proliferation (273). This is relevant to its action in reducing the severity OAD and interstitial fibrosis as fibroblasts are pivotal to the process of fibrogenesis.

In regard to the association between MMF therapy and reduced expression of TGF-β in tracheal biopsies, it is uncertain whether this is a direct or an indirect effect (cause or a result). MMF may possess a direct inhibitory effect, on the other hand, it may be related to its suppressive effect on fibroblasts, T and B lymphocytes and smooth muscle cells, or even due to its effect on reducing the incidence and severity of acute rejection (acute rejection augments TGF-β expression). Future studies assessing the molecular effect of MMF on TGF-β, other cytokines and growth factors is warranted.

CyA monotherapy has shown to be extremely effective in reducing acute rejection as well as OAD. This has been seen in other similar studies (260). Normal epithelial lining was observed as well as reduced mononuclear infiltrate and minimal/mild fibrosis scores (table 6). The only drawback to CyA monotherapy was that the

observed TGF-β score was higher when compared with Tacrolimus monotherapy and combination regimes. This may be relevant in the clinical scenario as other factors that cause tissue damage such as ischaemia, infection or even reperfusion injury may be present and may augment TGF production or activation in that environment, triggering aggressive OB. It was interesting that in the combined CyA/MMF groups, the effect of CyA on TGF-β expression was neutralized. It is also worth noting that, in this study, MMF is most effective when used in conjunction with the calcineurin inhibitors CyA or Tac as their combined effect has been additive without demonstrable increase in toxicity (274). This suggests that combining MMF with CyA in clinical lung transplantation may be most beneficial in reducing the incidence of OB, not only through reduction of the incidence of acute rejection (which is a major risk factor for the subsequent development of OB), but also by counteracting the known effect of CyA in augmenting TGF-β expression in allografts by several cell types (275, 276). There is some clinical evidence suggesting that this combination may reduce the incidence of acute rejection and clinical OB (128). Recent clinical studies suggest that MMF in combination with other agents has some beneficial effect against acute rejection and OB with some improvement in long term survival (113, 129, 130, 277).

In this study, Tac alone or in combination with MMF was effective in preventing OB and at the same time was not associated with a significant increase in expression of TGF-β. As the majority of lung transplant patients have, until recently, received CyA as the primary immunosuppressive agent, data from patients on long term Tac therapy is not available. Tacrolimus use has been focused on patients with established OB (rescue therapy) in which it seems to be marginally beneficial (115-117). The results of treatment of OB with augmented immunosuppression using other agents like ATG (112) have, however, been generally disappointing. The development of OB is a slow and irreversible process that can, at best, only be contained and very rarely reversed. This may explain why Tacrolimus rescue has only been marginally beneficial in the clinical setting. It is possible that the primary use of a Tacrolimus-based immunosuppressive regime in lung transplantation, particularly if combined with MMF (to widen its immunosuppressive properties) has some theoretical advantages. However this has yet to be established, although some emerging reports

do suggest a significant benefit (27, 278, 279).

Vitamin E was included in the study because it was shown to reduce fibrosis in other experimental models possibly acting as a potent TGF-β antagonist (265). In this study, Vitamin E administered alone offered no protection against the development of OAD or epithelial cell loss, but there was some reduction in submucosal fibrosis and TGF-β expression (Table 1, group 7). Thus Vitamin E may be a useful adjunct to the suggested combination of MMF/CyA or Tacrolimus immunosuppressive regimens.

The optimal level of immunosuppression in lung transplantation has not been determined. CyA and Tacrolimus therapy, potentially, have serious, life threatening side effects such as nephrotoxicity, infection and malignancy. In clinical practice there is a tendency to reduce the dose of these agents in long term patients without routine monitoring for acute rejection with transbronchial biopsy (TBB). However, no monitoring carries the risk of missing subclinical rejection, a process that is now considered to be a major cause of OB. This has led to a call for more aggressive lung biopsy protocols. However, TBB is less effective in diagnosing lung rejection than cardiac biopsy is in discovering heart transplant rejection, due to (a) the patchy nature of lung rejection and the difficulty in obtaining representative samples and (b) the difficulty in differentiating rejection from other causes of lung injury. As a result, most transplant units have adopted less stringent biopsy protocols for long term follow up of lung transplant patients.

An alternative to a more aggressive diagnostic approach would be to define the most effective immunosuppressive protocols and ensure that all patients receive adequate and balanced immunosuppression. In that regard, it appears from this study that protocols combining either Tacrolimus or CyA with MMF are likely to provide a better protection of lung allografts against acute rejection and OB. Supplementation with Vitamen E may have a further protective effect.

Table(5): TGF Scores for each animals by observers 1 & 2.

Animal	TGF-D(1)	TGF-D(2)	TGF-I(1)	TGF-I(2)
1a1	4	4	4	4
1a2	4	3	4	4
1a3	3	4	3	4
1b1	4	4	4	3
1b2	4	4	4	4
1b3	3	3	4	4
2a1	1	1	2	2
2a2	2	1	3	2
2a3	1	2	2	3
2b1	2	2	2	1
2b2	1	1	1	2
2b3	1	2	2	2
3a1	2	2	1	1
3a2	3	2	2	1
3a3	2	2	1	2
3b1	2	1	1	1
3b2	1	2	2	2
3b3	3	3	1	2
4a1	2	1	1	1
4a2	2	2	1	1
4a3	2	2	1	1
4b1	1	1	1	2
4b2	2	1	2	1
4b3	1	1	1	1
5a1	3	2	3	3
5a2	2	3	2	3
5a3	3	3	3	2
5b1	2	3	3	2
5b2	3	3	2	2
5b3	3	2	1	2
6a1	1	1	2	2
6a2	1	1	2	1
6a3	2	1	1	2

6b1	1	0	1	2
6b2	2	1	1	1
6b3	1	1	2	1
7a1	2	2	2	3
7a2	3	2	3	2
7a3	2	3	1	2

Abbreviations: Animals: individual animal in each experiment

TGF-D1: Distribution score for TGF-β staining by observer 1 based on scoring system (see methods, chapter 5).
TGF-D2: Distribution score for TGF-β staining by observer 2
TGF-I(1): Intensity score of TGF-β in biopsies by observer 1. (see methods in chapter 5).
TGF-I(2): Intensity score of TGF-β in biopsies by observer 2.

Animal groups:

1a = Control 14

1b = Control 28

2a = MMF 14

2b = MMF 28

3a = Tac 14

3b = Tac 28

4a = Tac+MMF 14

4b = Tac+MMF 28

5a = CyA 14

5b = CyA 28

6a = CyA+MMF 14

6b = CyA+MMF 28

7 = Vitamin E

Table (6) Summary of histology and immunohistochemical staining score of treated tracheal allografts.

Grou-ps	Drug Regime[1]	OAD[2]	Epithelium[3]	SMF[4]	TGF-β[5] **Distrib.**	TGF-β[6] **Intensity**	MNC[7]
1a	Control 14	4	**Absent/0**	**21**	4	4	**17** ++++
1b	Control 28	4	**Absent/0**	**20**	4	4	**15** ++++
2a	MMF 14	**2**	**Absent/0**	**12**	**1**	**2**	**12** +++
2b	MMF 28	**2**	**Absent/0**	**10**	**1**	**2**	**11** ++
3a	TAC 14	**0**	**SM / 2**	**6**	**2**	**1**	**3** +
3b	TAC 28	**0**	**Normal/3**	**3**	**2**	**2**	**7** ++
4a	TAC+MMF 14	**0**	**SM /2**	**6**	**2**	**1**	**2** +
4b	TAC+MMF 28	**0**	**SM /2**	**9**	**1**	**1**	**4** +
5a	CyA 14	**0**	**Normal/3**	**4**	**3**	**3**	**2** +
5b	CyA 28	**0**	**Normal/3**	**3**	**3**	**2**	**0**
6a	CyA+MMF 14	**0**	**Normal/3**	**4**	**1**	**2**	**10** ++
6b	CyA+MMF 28	**0**	**Normal/3**	**6**	**1**	**1**	**5** +
7	Vitamin E	**3**	**Absent/0**	**18**	**2**	**2**	**10** ++

Abbreviations:

1) **Drugs. MMF= Mycophenolate mofetile. TAC= Tacrolimus. CyA= Cyclosporin A.**
2) **OAD= obliterative airway disease. (see score in material and methods)**
3) **Epithelium: Absent= epithelium completely destroyed. SM= squamous metaplasia. Normal= normal pseudo-stratified columnar ciliated epithelium.**
4) **SMF= Submucosal fibrosis (μm) (see text).**
5) **TGF= Distribution of staining based on % of stain in slide.**
6) **TGF = Intensity of staining, graded 0-5 (see material and methods) .**
7) **MNC= mononuclear cell infiltrate, no. of cells/chamber(see text).**

Chapter IV: Figures (37-48)

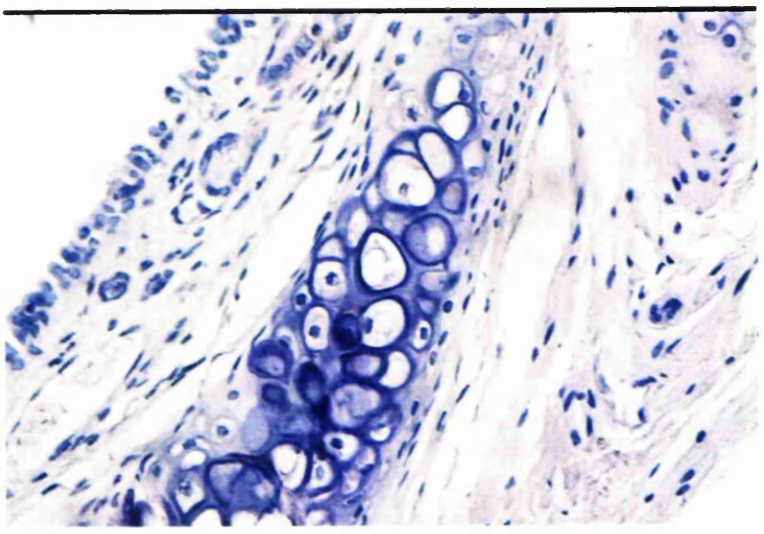


Figure 37

Tracheal section, normal control

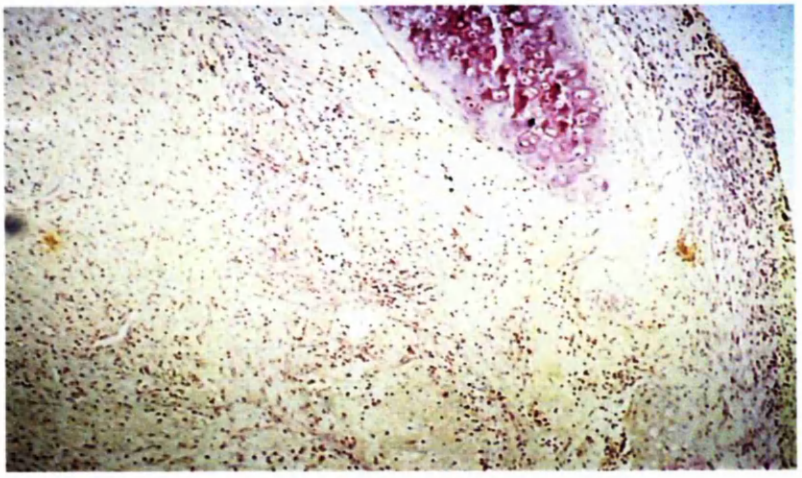


Figure 38
No immunosuppression 14 days.
TGF score: distribution grade 4, intensity grade 5

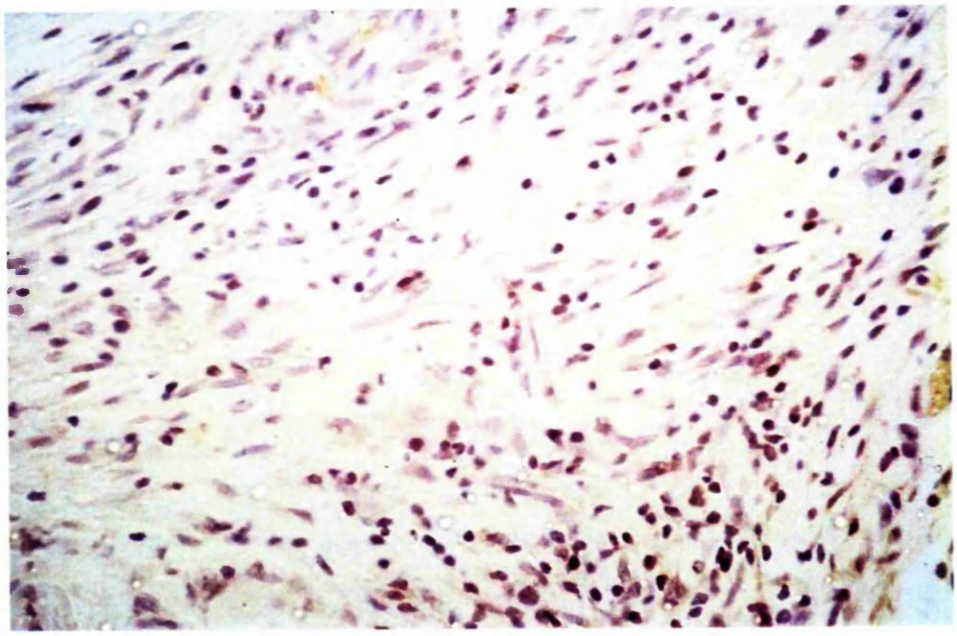


Figure 39

No treatment group. Note complete obliteration of tracheal lumen

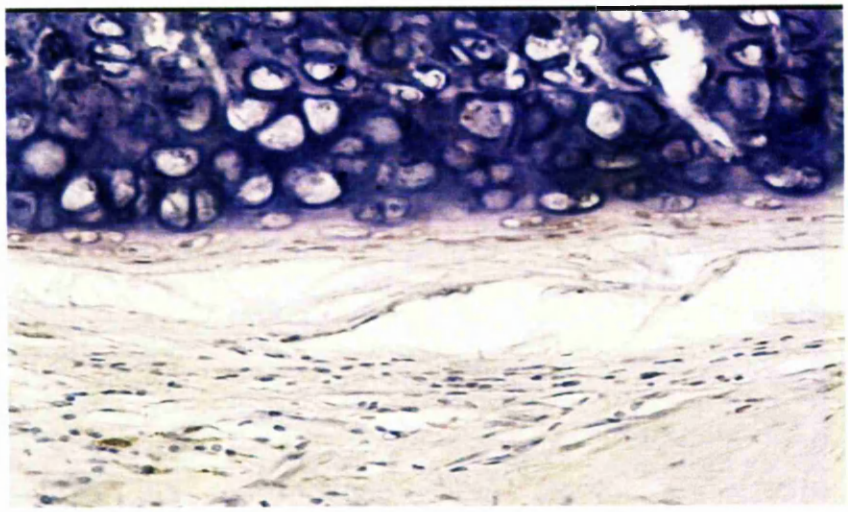


Figure 40

Treatment with MMF at 28 days
TGF Staining score: distribution grade 2, intensity grade 1

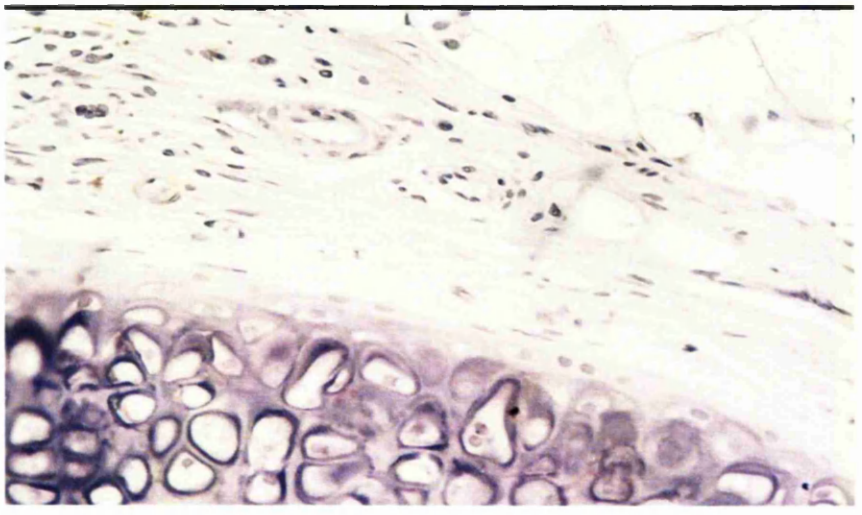


Figure 41

Treatment with tacrolimus at 28 days
TGF staining score: distribution grade 2, intensity grade 2

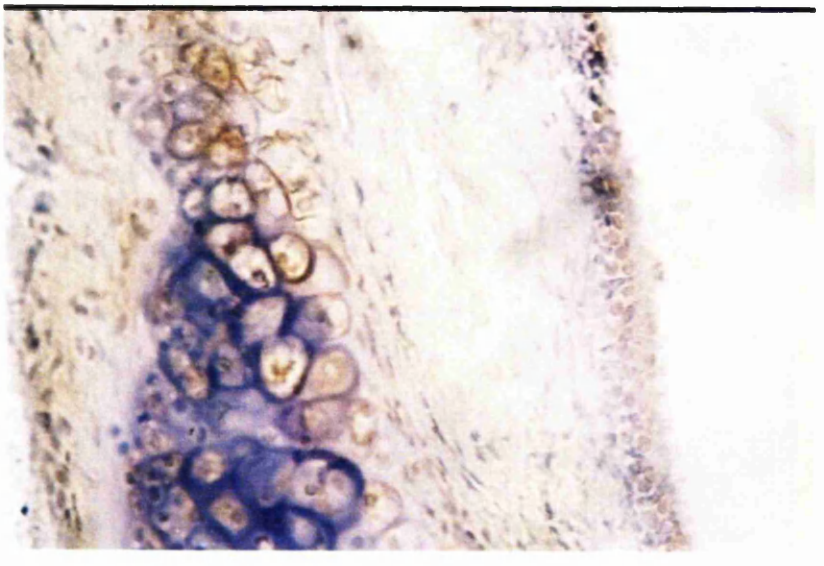


Figure 42

Treatment with cyclosporine at 14 days
TGF staining grade: distribution score 3, intensity grade 3

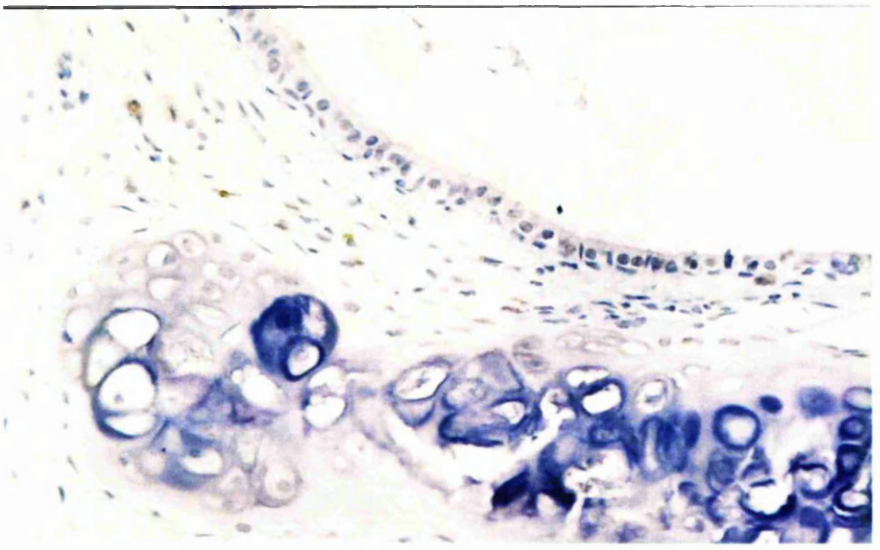


Figure 43

Cyclosporine treatment 28 days
TGF staining score: distribution grade 2, intensity grade 2

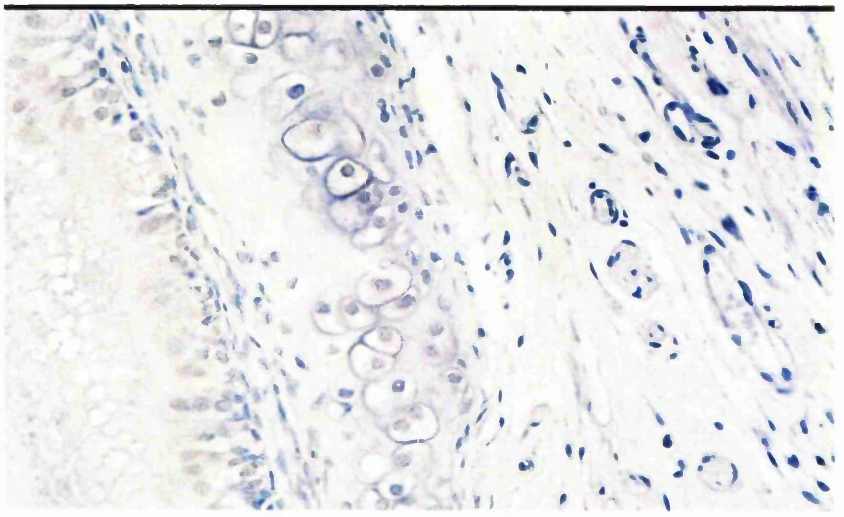


Figure 44
Treatment with tacrolimus + MMF at 28 days
TGF staining grade: distribution score 0, intensity grade 0

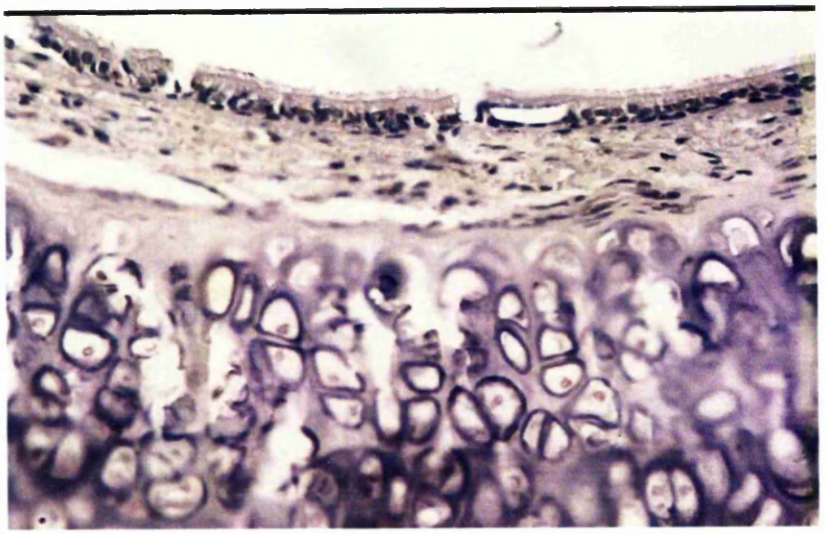


Figure 45

Treatment with cyclosporine and MMF 28 days
TGF staining score: distribution grade 1, intensity grade 1

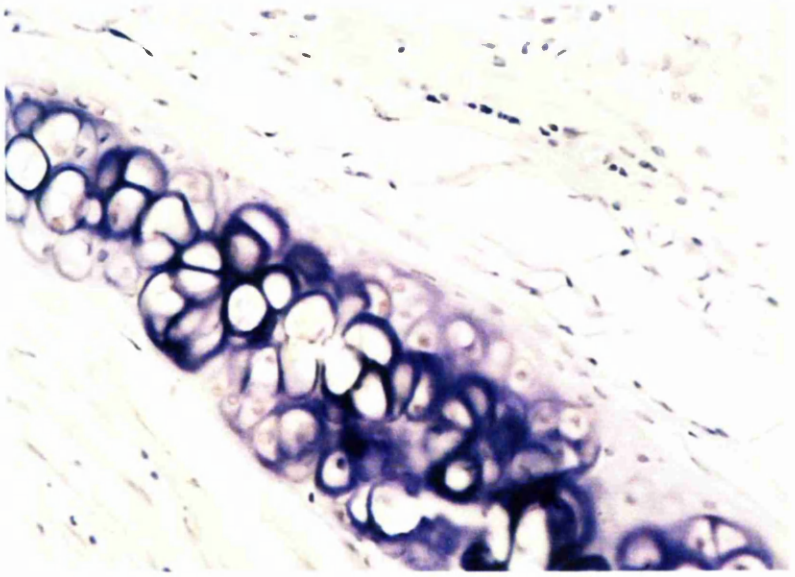


Figure 46

Treatment with cyclosporine and MMF 14 days
TGF staining: distribution grade 2, intensity grade 1

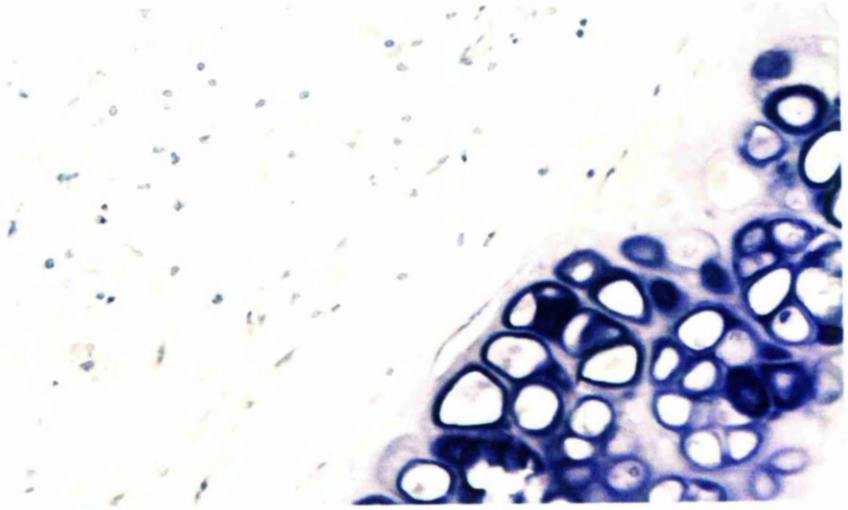


Figure 47

Treatment with MMF
TGF staining: distribution grade 2, intensity grade 1

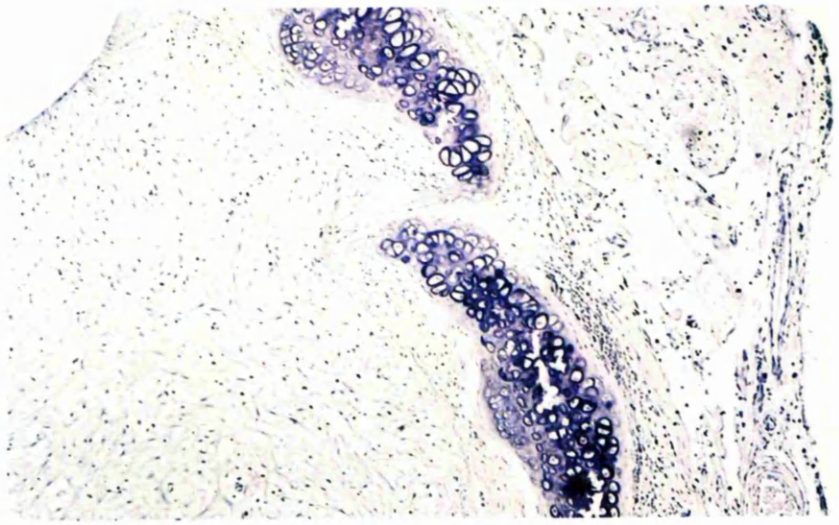


Figure 48

Treatment with Vitamin E at 28 days
TGF staining: distribution grade 3, intensity grade 2

Chapter V:

In a randomised double blind multi-institutional clinical study of Mycophenolate Mofetile (MMF) versus Azathioprine (Aza) in lung transplantation, does Mycophenolate Mofetile have any beneficial effect on acute and chronic rejection/OB? Does MMF has a suppressive effect on TGF-β and other growth factors?

V.i. Introduction:

It is a common fact that chronic rejection/OB is the leading cause of morbidity and death following lung transplantation with a reported prevalence of 30-60% in the first three years (92, 107). This fibro-proliferative disorder is believed to be a manifestation of chronic rejection with chronic decline in lung function in most cases (91, 280, 281). Its early histological manifestation is characterised by lymphocytic bronchitis and eosinphilia (102, 282). This results in submucosal fibrous thickening, extensive inflammatory cell infiltrate and mesenchymal cell accumulation. The epithelium becomes necrotic and this attracts fibroblasts and connective tissue products causing fibro-collagenous occlusion of the respiratory and terminal bronchiole (93, 283, 284). This inflammatory/healing process results in increased expression of TGF-β in the tissue which in turn causes further recruitment of inflammatory cells and fibroblasts to the bronchioles, TGF-β also stimulates the synthesis of extracellular matrix (105). This will finally cause an exaggerated/intense healing process and more fibrosis. Current management strategies are largely ineffective after the fibrosis is established (285).

Treatment of OB has been based on augmentation of immunosuppression. With the recent advances of immunosuppressive therapies, newer drugs have been investigated using established OB models (51, 269, 274, 286). An increasing number of lung transplant centres have carried out non randomised studies (128, 129, 278, 279) as well as randomised clinical studies (130, 287, 288).

Successful outcomes have been reported using traditional immunosuppressants like cytolytic therapy (112) and methotrexate (289), as well as newer agents like Tacrolimus (115, 116, 288) and MMF (114, 130). Some of these studied have shown a halt in the decline of the pulmonary function tests with stabilisation of clinical symptoms of OB. Recently, our unit's policy has been to switch the patient to the Tacrolimus/MMF combination when early signs of OB become apparent or the patient developed early acute rejections.

An update from the large multicentre randomised trial of MMF versus Azathioprine (130) showed improved intermediate term survival in the MMF patients. Animal studies of OB models (260, 269, 274) has also suggested that MMF has been

beneficial in the prevention of OB particularly in combination with calcineurin inhibitors. MMF is a newer antimetabolite immunosuppresive agent. It is rapidly hydrolysed into its active form mycophenolic acid (MPA) which is known to inhibit inosine monophosphate dehydrogenase hence selectively inhibiting the proliferation of T and B lymphocytes (290). Our most recent animal study (chapter 3, manuscript submitted for publication) suggested that in the MMF therapy group, there was less severe OAD and that was mirrored by other animal studies (100, 260). However, in our animal model, we found an association between MMF therapy and reduced expression of TGF-β in the tracheal biopsies which may, at least in part, be the mechanism by which MMF reduces the severity of OAD in this model.
This current study was carried out on a subgroup of patients who have taken part in the MMF versus azathioprine randomised clinical study in lung transplantation and were transplanted at this centre. The aims of this project is to assess the effect of MMF on the expression of different growth factors and in particular TGF-β in the lung biopsy sections taken routinely and urgently post lung transplantation. We also aimed to compare the histological and immunohistochemical observations with the clinical data of these patients.

V.ii. Patients and methods:

Seventeen patients were enrolled into the "multicentre randomised study comparing MMF versus azathioprine in primary lung transplantation" from the Wythenshawe transplant centre and they all were included in this study. Informed consent was obtained from the Patients as well as Permission to use biopsy material and data from these patients case notes were obtained from Roche Pharmaceutical (Roche® UK). The clinical data collected included demographics of donors and recipients, ischaemic time (IT), number of rejection episodes/patient, number of biopsies/patient, infection episodes/patient, latest FeV1, presence or absence of OB, duration of follow-up and mortality.

V.ii.a. Immunosuppression Protocol

In the study protocol, recipients were treated with triple immunosuppression therapy consisting of cyclosporine, azathioprine or MMF, and corticosteroids,

All lung transplant recipients had 500x2 mg of methylprednisolone per-operatively. All patients received a short induction course(3 days) of rabbit antithymocytic globulin (RATG, Institute Pasteur-Merieurx, France) starting from day one. Postoperative steroids were given at a dose of 125mg tds in the first 24 hours and then oral steroid therapy started at a dose of 1mg/kg/day tapered to 0.3 mg/kg/day at the 3rd week. Acute rejection episodes were treated by pulse steroid therapy (10 mg/kg/day for 3 days). Cyclosporine was started on day one orally or IV aiming to achieve a trough level of 250-350 ng/ml in the first 3-6 months. The level was reduced depending on rejection history and renal function aiming at a level between 150-300 ng/ml.

The study was a randomised double blind double dummy multi-institutional trial. Study medication consisted of either Aza at dose of 2mg/kg/day tailored to patients white blood count level or MMF at a dose of 2gms per day. Study medication (MMF or Aza) was started as soon as possible within the first 72 hours post-transplant. The protocol of the study was adhered to throughout the study period (3 years).

V.ii.b. Immunohistochemical staining for TGF, EGF and bFGF:

Rehydrating and Dehydrating paraffin embedded tissue on slides.

A. Rehydrating:

1. Sections were placed in square staining racks. First, they were placed in Citroclear for 10 minutes until all the wax has dissolved then moved to fresh Citroclear for 1-3 min.
2. The rack was drained onto a paper towel to avoid carry over into alcohol.
3. Then the sections were placed in 100% Alcohol for 1-3 min. (agitated gently to ensure all slides were washed) then the process was repeated in the fresh 100% alcohol, then 90%, 70%, 50%, respectively.
4. The sections were washed quickly in distilled water. They were not allowed to dry out if performing Immunochemistry (submerged in PBS or TBS).
5. Slides were then ready to be stained.

After performing staining, slides were dehydrated so that they could be mounted with a cover slip. This enabled the slides to be stored for future analyses.

B. Dehydrating:

1. The slides were washed quickly in distilled water.
2. They were placed in 50% alcohol for 1-3 min (agitated gently to ensure all slides were washed) and then the process was repeated through to 70%, 90%, 100%, and 100% alcohol.
3. The rack was drained onto a paper towel to avoid carry over into Citroclear.
4. Then, it was placed in a fresh Citroclear for 1-3 min.
5. Each slide was drained separately and mounted with a cover slip by adding a drop of DPX mountant onto a cover slip and slowly lowering the slide to avoid air bubbles. The DPX was allowed to set overnight and then the slides were ready to be viewed under the microscope.

Stock solutions: DAB (Diaminobenzadine) Stored in fridge To make up : 1.5g DAB in 200ml TBS aliquot in 20mls. Store in freezer.

Lung biopsy staining using immunohistochemistry

Antibodies used:

Mouse anti human TGF-beta monoclonal antibodies (clone MCA797) 1mg/ml. (Serotec Ltd.)
Working solution of 1/ 200 (5μg/m)
Monoclonal anti-epidermal growth factor (EGF) antibody clone EGF-10 2mg/ml (Sigma- Aldrich Ltd.)
Working solution of 1/400 (5μg/ml)
Rabbit anti-basic fibroblastic growth factor (FGF-2) polyclonal antibody (lot no. 19102369). 1mg/ml (Chemicon International)
Working solution of 1/100 (10μg/ml)
Swine anti rabbit peroxidase conjugated secondary antibody (Dako Ltd) use at 1/50 of stock.
Goat anti swine peroxidase conjugated secondary antibody (Dako Ltd) used at 1/50 of stock.

Reagents used

3% Hydrogen peroxide, H_2O_2 (Sigma)

Proteinase –K 2mg/ml (Sigma)

1% Bovine Serum Albumin,BSA (Sigma)

Phosphate Buffered Saline, PBS (Sigma)

Tris Buffered Saline, TBS (Sigma)

Normal Rabbit Serum NRS (Sigma)

Normal Swine Serum NSS (Sigma)

Normal Human Serum NHS (Sigma)

DAB (Diaminobenzadine) (Sigma)

Positive control tissue for TGF-beta and EGF – Human Inflamed Tonsil.

Positive control tissue for bFGF – Human Ovarian Cancer.

V.ii.c. Histological examination and diagnosis of acute and chronic rejection (OB)

All transbronchial biopsies were reviewed by one of the three consultant histopathologists in the Department of Pathology, Wythenshawe Hospital to assess rejection, infection and the development of OB. Biopsies were classified according to ISHLT guidelines (86). This latest classification takes into consideration the relative activity of the inflammatory infiltrate in the TBB. In those cases of co-existent acute and chronic rejection, the pathology report will reflect these processes. Bronchiolitis obliterans as a pathological term refers to dense eosinophilic hyaline fibrous plaques in the lamina propria of the terminal and the respiratory bronchioles which cause partial or complete luminal compromise in the bronchioles of the lung allograft. (figure 10). This fibrous tissue may be associated with disruption of the smooth muscles and may also extend into the interstitial layer. Foamy histiocytes are frequently present in the distal airspaces. The study duration was three years. Long term follow-up of these patients was essential for evaluating the effect of MMF on incidence and severity of OB as OB is essentially a long term sequel of lung transplantation and is usually seen after the first year following transplantation, although occasionally it has been seen earlier (291).

Routine and urgent trans-bronchial biopsies (TBB) were stained with H & E staining for histological diagnosis and with immunohistochemical staining for expression of three different growth factors. We analysed the expression of TGF-β, fibroblast growth factor (FGF) and epidermal growth factor (EGF). The slides were coded

before evaluation. Assessment of staining was carried out using a semiquantitative method for evaluating immunohistochemical staining described previously (270, 292). The standard immunohistochemical grading was carried out by two independent observers and in a blinded fashion. It was based on the presence and absence of TGF-β, FGF and EGF staining, its extend (distribution) and intensity of positive staining for each specimen. The intensity of the staining was graded on a scale from 0 (negative) to 5 (strongly positive) (intensity grade 5 for TGF, figures 51 and 52). The distribution of staining in the lung biopsies was measured in percentage terms and as follows; <5% score 0 (for TGF, figure 54), 5-20%, score 1, 20-49% score 2, 50-75% score 3 and >75% score 4 (for TGF, figure 53).

Prior to staining, a positive control slide from human tonsillar tissue for TGF and FGF (figures 49, 59) or ovarian tissue for EGF were stained at the same time. Negative controls slides were carried our for TGF (figure 50) and bFGF (figure 60). All the slides were then compared to the positive control.

V.ii.d. Statistical Analysis:

Clinical data from each group were analysed using non-parametric testing. The variables were age, sex, diagnosis, type of lung transplant, number of biopsies, acute rejection episode/100 patient days, infection episodes/patient, incidence of OB, duration of follow-up and mortality between the two groups.

A SPSS statistics package licensed through University of Manchester was used in this thesis. Statistical analyses were carried out using non-parametric tests, continuous variables using the Mann-Whitney test, categorical variables using X^2 or the Fisher exact test. Survival analysis was carried out using the Kaplan Meier methods. Similar statistics were used for immunohistochemical data. A positive result was considered when the *p*- value was 0.05.

V.iii. Results:

Demographic data of donor and recipients characteristics, ischaemic time (IT), rejection data, infection data, type of procedure are **summarised in table (8)**

There were no significant differences between the MMF group versus Aza groups regarding age, sex, and infection episodes. Regarding to type of procedure, there were

more bilateral lung transplants in the MMF arm (4/8 vs 0/9, p=NS). Also ischaemic time was longer in the MMF group with lower number of rejection /patient, lower incidence of OB. The difference did not reach significance. There was slightly higher FeV1 value in the MMF group (p=NS). Survival data from the 2 groups were similar. Data of the immunohistochemical analyses of biopsies stained with TGF-β, bFGF and EGF were as follows: (summarised in tables 10 and 11)

TGF-β: Lung Biopsies showed no significant difference in the distribution or intensity of TGF-β staining score between the MMF group and the AZA group. The mean score=3.6(d) and 3.7(i) vs 3.4 and 4.0. Figures 51, 52, 53 and 54).

bFGF: MMF group: There was a significant reduction in the distribution of bFGF staining as well as its intensity compared to the AZA group (figures 57 and 58). The mean score= 3.4(d) and 1.4(i) vs 4.6 and 2, p=0.01 and 0.04.

EGF: MMF group: There was a non-significant reduction in the distribution of EGF staining but NOT in its intensity when compared to the AZA group (figures 55 and 56). The mean score= 2.4(d) and 1.3(i) vs 3.1 and 1.3. (p=NS)

V.iv. Discussion:

Obliterative bronchiolitis is the commonest cause of morbidity and mortality following lung transplantation (249). The principles of management of OB are based in general on the strategies of prevention, stabilisation and reversal. With regard to prevention, it is vital to have a clear understanding of the aetiology of OB to plan a prevention strategy. However, the aetiology of OB is still not precisely known. Early acute rejection and lymphocytic bronchitis are considered to be the strongest known risks (102, 249, 262, 283). CMV infection is also considered to be an aetiological factor (91, 293) but a systematic review in a recent publication suggested that the role of CMV and other infections and HLA matching was less clear and requires further studies (249). Based on the currently available evidence, the prevention of acute rejection seems to be the most logical strategy for preventing OB. This strategy has not been investigated in a large randomised clinical trial. With the advent of newer immunosuppressive agents, some encouraging small studies suggesting reduced or delayed incidence of OB in lung transplant patients have

emerged (127, 294) but none have yet been confirmed in a large randomised clinical trial. The only large international study to date is the MMF vs Azathioprine study. This study completed recruitment in 1999 and interim one year analysis was presented and published recently (130). Meaningful long term results are awaited.
In as far as stabilisation of pulmonary function is concerned, a number of reports have suggested some success using a variety of immunosuppressive agents. Cytolytic therapy using ATG, used to treat intractable acute rejection, has also been used with some success in OB. A study of 15 patients with OB treated with ATG showed that nearly half the patients were stabilised on treatment and in 2 patients (13%) reported an improvement (112). Aerosolised cyclosporine was also successfully used in 9 patients with established OB resulting in stabilising pulmonary function (295). Total lymphoid irradiation (TLI) (111, 296), methotrexate (289) as well as steroids (297) have also been successful in stabilising some OB patients in small clinical studies. Newer agents have also been used successfully in halting the progression of BOS is small clinical studies, most notably tacrolimus (115, 116, 279) and MMF (113, 277), while Rapamycin (Sirolimus) has been shown to be effective in experimental studies (51, 260).
With regard to reversal of lung function and improvement of airflow obstruction, this has not yet been achieved consistently with any form of drug therapy.
As discussed earlier, in order to identify the agent most likely to succeed in prevention of OB, we need to understand the basic aetiology and pathogenesis of OB at the cellular/molecular level. Then the chosen agent must be tested in a basic animal model of OB without the complexity of clinical lung transplantation where a number of variables, known and unknown, are interacting. Finally it is important to identify whether this agent is effective through the suggested or hypothesised mechanisms.

In the previous study (chapter IV), we have identified that acute rejection and increased TGF-β expression is the most likely aetiological factors for OB in a basic rat model (269) as well as a large clinical study (102). For the purpose of prevention, MMF was chosen in this study based on its effectiveness in reducing acute rejection incidence in a variety of solid organ transplants including lungs (277), hearts (298), kidneys (299) and liver (300) as well as in animal models (with or without calcineurin inhibitors). MMF is rapidly metabolised to its active form

mycophenolec acid (MPA), in the liver. MPA inhibits purine synthesis through the classical pathway by inhibiting the enzyme inosine monophosphate dehydrogenase (IMPDH). IMPDH is a rate limiting enzyme in the *de novo* synthesis of guanosine nucleotides. This inhibitory effect is more pronounced in T as well as B lymphocytes as these cells are more dependent on this pathway than any other cell type (272). MPA can induce apoptosis of activated T-lymphocytes hence reducing a T lymphocytes population responsible for graft rejection. It also suppresses glycosylation and expression of adhesion molecules by depleting guanosine nucleotides. This may decrease the recruitment of lymphocytes and other cells into the site of rejection. Depletion of guanosine nucleotides may also deplete tetrahydrobiopterin, a co-factor for inducible nitric oxide synthase (iNOS). MPA also suppresses the production by iNOS of nitric oxide (NO) leading to reduced tissue damage mediated by peroxynitrites (272). Finally, in the OAD rat model (chapter V), MMF was associated with reduced expression of TGF-β in tracheal biopsies, although whether this was a direct cause or an indirect effect is not yet clear. MMF is most effective when used in conjunction with calcineurin inhibitors like CyA or Tac as their combined effect is additive without demonstrable increase in toxicity (274). Chronic rejection is emerging as the major limiting factor for long term survival of most solid organ transplant patients. In heart, kidney and liver, it is usually associated with proliferative and obliterative arteriopathy of the graft and is thought to be due to proliferation of arterial smooth muscle cells and recruitment of lymphocytes and other mononuclear cells. It usually starts in small and medium size arteries and arterioles and finally affect the whole arterial tree (301). Chronic rejection in renal allografts is also associated with glomerulosclerosis and interstitial fibrosis. A similar picture of chronic rejection in lung allografts prevails: vascular changes are associated with lymphocytic bronchitis and bronchiolitis with fibro-proliferative lesions affecting the bronchioles. The epithelium becomes necrotic and this attracts and stimulates fibroblasts, and the production of procollagen and other connective tissue products causing fibro-collagenous occlusion of the respiratory and terminal bronchioles (93, 283), the ultimate feature of OB.

The pathogenesis of chronic rejection is complex, probably mediated by both cellular

and humoral mechanisms. Genes for several growth factors including TGF-β are expressed in the arterial wall (301, 302). Activated epithelium as well as other factors stimulate smooth muscle proliferation in the neo-intima. There is some collagen deposition in the arterial wall as well as interstitial sites. Collagen formation is associated with proliferation of fibroblasts and increased expression of TGF-β and FGF that in turn induce procollagen gene expression. Therefore the inhibition of TGF-β, FGF and EGF expression as well as suppression of proliferation of fibroblast is desirable. It is important to remember that CyA as well as tacrolimus and rapamycin augment TGF-β expression by human lymphocytes and probably other cell types (275, 303, 304). With regard to smooth muscle cell proliferation, CyA does not inhibit the proliferation of smooth muscle cells and fibroblasts while MPA in modest concentrations, suppresses the proliferation of arterial smooth muscle cells and fibroblasts. This has been demonstrated in humans (305, 306) as well as rat smooth muscle cells (307). The inhibition of fibroblast proliferation by MMF (273) is relevant to the prevention interstitial fibrosis as fibroblasts are pivotal to the process of fibrogenesis. It is interesting to note that TGF-β is a potent fibroblast chemo-attractant as well as a stimulator of fibrogenesis (194, 308-312) and it is possible, at least theoretically, that MMF may possess directly or indirectly a suppressive effect on TGF-β expression. This may explain our finding in the rat model (see chapter 3).

An interim analysis of data from the multi-institutional, randomised study of MMF versus azathioprine in lung transplantation in which 317 patients received MMF or azathioprine in randomised blind fashion was published. This one year analysis suggested improved survival in the MMF arm of about 10% compared to the AZA group. The incidence of OB was low in both groups (3.8 in AZA vs 3.1 in MMF: p=NS). With regard to reported rejection episodes, it was 51.3 in the AZA group against 50.9 in the MMF group (p=NS). The final analysis will be at end of the third year (130).

Clinical data from our study at Wythenshawe from 17 patients are summarised in the results section. The 2 groups are similar regarding donor and recipient characteristics apart from more CF patients in the MMF arm. This is the main reason why more bilateral lung transplants were in this arm. These differences did not reach

significance because of small sample size.

What was interesting among those 17 study patients was that although the number of biopsies were the same in each group, there was a strong trend towards more rejection episodes in the AZA arm (p=0.06). This is contradictory to the interim analysis data from the whole 317 study population at one year (130). The possible reason for this discrepancy, in my view, is that rejection episodes in this study were based on histological assessment of TBB biopsies while in the main study, rejections were based on either the clinical or histological picture (or both). Lower incidence of acute rejection has been reported with MMF therapy. Zuckermann and associates reported significantly lower rejection episodes (10%) in the MMF patients against 43% in the AZA patients (p=0.001). In that study, freedom from rejection in MMF group was 17/21 while in the AZA group was 3/17 (277). This is comparable to the study where 3/8 MMF patients had any rejection compared to 7/9 in AZA group.

With regard to OB, this is a small study and the results need to be interpreted with caution. However, this is one of the few studies that has 3 year follow-up data. Survival was similar in the 2 groups but incidence of OB was higher in the AZA (4/9) compared to MMF group (1/8) p=NS. It was also interesting to note that all patients with OB had at least one episode of acute rejection in the first 3 months. This also supports the concept that acute rejection is a major risk factor for OB and that OB is an immunological phenomenon.

In the previous chapter, we found a correlation between OAD and intensity of TGF-β staining. This finding confirmed previous clinical observations correlating TGF-β expression with lung fibrosis and OB in clinical lung transplantation (102, 271, 292). In this clinical study, we aimed to investigate a previous observation (chapter IV) that MMF may inhibit growth factors and in particular TGF-β as part of its suppressive effect on fibroblasts, epithelial cells and smooth muscle cells (305, 306). In this clinical study, there was no significant difference between the groups with regard to the intensity and distribution of TGF-β and EGF staining. By contrast, the distribution and to a lesser extent, the intensity, of staining of bFGF was reduced in the MMF group compared to the AZA group. The inhibition of distribution of staining of bFGF was more pronounced than its intensity. Only data from bFGF distribution was found to be statistically significant. The discrepancy between the

animal study and these findings was not surprising as the clinical scenario following lung transplantation is rather complex with many conflicting variables compared to the controlled conditions of the animal experiment. Furthermore, the dose of MMF in the rat model(40mg/kg/day) was nearly twice that used in the clinical study. Those may explain why no significant inhibition of TGF-β expression was found in this study. It is also important to remember that there were other associated factors such as ischaemia, rejection or infection in the biopsies of the lung transplants, which are known to influence the expression of growth factors including TGF-β. Hence, it is impossible to exclude the effect of factors such as infection on the expression of these growth factors in the clinical setting. If biopsies associated with infection or rejection were excluded in this study, the sample size would become too small which makes statistics less meaningful.

In regard to the inhibition of bFGF, MMF may have a direct inhibitory effect, or it may be an indirect suppressive effect on this growth factors expression through its suppressive effects on fibroblasts, epithelial cells and T and B lymphocytes. These cells are the main source of cytokines and growth factors at the area of inflammation, ischaemia, infection and rejection (302).

The effect of bFGF on OB in lung transplantation is not known. However, bFGF has been shown to be a potent mitogen for fibroblasts, airway smooth muscle cells and endothelial cells (313). It promotes cellular proliferation and differentiation. It has also been implicated in experimental arterial atherosclerosis (314, 315). Although there are no data to relate bFGF and lung transplantation, chronic rejection or OB, its known action in asthma and atherosclerosis may well suggest a significant role in the pathogenesis of chronic rejection or OB. This new finding is very interesting and worth further experimental as well as clinical studies.

Table (7)

MMF versus AZA in lung transplantation

Recipient characteristics and results.

Name	Sex	AGE	TxDate	OP *	Mort Date	Rej	Bx	last FeV	Rand	Alive/ days	cause of Death	OB	Infection CI=**
LM	F	54.7	13/11/97	s	13/06/98	1	2	0.3	aza	212	infection, OB	1	CI
DG	F	57.7	05/12/97	sx2	09/09/99	0	3	0.58	mmf	643	hyperinflat-ion, bleed	1	none
RS	M	22.6	21/12/97	d	01/06/98	2	3	4.1	mmf	162	infection (cap)	0	B.capacia
JG	M	51.9	28/12/97	s		2	2	1.87	aza	1509	well	0	none
CK	F	50.9	24/02/98	s		0	2	2.37	mmf	1451	well	0	none
PW	M	54.1	28/03/98	sx2	26/01/99	2	2	0.8	aza	304	OB	1	CI
JD	F	47.8	05/05/98	s	25/05/99	0	1	1.29	aza	385	ca of lung	0	none
KB	M	31.7	22/05/98	d	14/03/99	1	5	1.19	mmf	296	infection	0	appendex
WT	M	47.4	24/05/98	s		0	1	1.6	mmf	1362	well	0	sinusitis,CI
JS	F	60.1	11/06/98	s		3	3	1.08	aza	1344	well, OB	1	none
EM	F	53.7	07/07/98	s		4	4	0.79	aza	1318	OB	0	CI,OB
SN	M	55.6	28/08/98	d		0	2	2.36	mmf	1266	well	0	pacreatitis
VW	M	61.8	28/10/98	s		1	1	2.93	aza	1205	well	0	PE,none
MO	F	21.5	04/12/98	d		2	4	2.44	mmf	1168	well	0	CMV
BC	M	60.5	23/03/99	s	24/05/99	0	2	1.33	mmf	62	stent/bleed	0	CI,stent
PF	M	62.6	31/03/99	s	12/10/99	1	3	0.4	aza	195	poor graft, OB	1	CI
JT	M	55.6	31/03/99	s	13/09/00	0	1	0.84	aza	532	Pancost tumour	0	CI

Abbreviations:

* = OP: operation, s for single lung, Sx2 single lung carried out twice, d is for bilateral sequential lung transplant

** = Chest infection

Table (8)

MMF versus AZA Study. Demographic data

	Age	Sex	IT	Procedure: SL/BL	Diagnosis
AZA	55.3	5/4	4.28	9/0	IPF=4 Emphy=5
MMF	43	5/3	4.95	4/4	IPF=2 Emphy=3 CF=3

Aza: Azathioprine, MMF: Mycophenolate mofetile.
IT: Ischaemic time.
SL: Single lung transplant, BL: bilateral sequential lung transplant
IPF: Idiopathic pulmonary fibrosis
Emphy: Emphysema
CF: Cystic fibrosis

Table (9)

MMF versus AZA study. Summary of results

	No of Biopsies	No of Rej = >1a	A/D	OB (y/n)	FEV_1(L)	Infective episode	Survival (days)
AZA	21	14	4/5	3/9	1.14	3/9	778
MMF	22	4	4/4	1/8	2.0	5/8	802

No of rej => 1a: number of rejections greater than 1a

A/D: alive/dead

OB: Obliterative bronchiolitis.

FEV_1 : Forced expiratory volume in first second.

Table 10:

Intensity and distribution scores for TGF, EGF and bFGF in all lung biopsies.

Biopsy	Rand[1]	TGF/D[2]	TGF/I[3]	EGF/D[4]	EGF/I[5]	FGF/D[6]	FGF/I[7]
B98/4891	M	3/4(3)	4/4(4)	1/2(1)	1/1(1)	4/3(4)	1/1(1)
B98/2436	M	4/4(4)	4/4(4)	1/2(2)	1/1(1)	3/4(4)	2/3(3)
B98/2740	A	4/5(4)	4/5(4)	1/2(2)	1/1(1)	5/5(5)	4/5(4)
B98/265	A	4/5(4)	3/4(3)	3/4(4)	1/2(2)	4/5(5)	4/4(4)
B98/2123	A	4/4(4)	2/3(2)	1/2(1)	1/2(1)	4/4(4)	2/1(1)
B98/1802	M	2/3(3)	3/4(4)	2/3(3)	1/2(2)	3/4(4)	1/2(2)
B98/586	M	4/4(4)	3/4(4)	3/4(4)	1/2(1)	4/5(5)	1/1(1)
B99/41	M	3/4(4)	2/3(2)	4/5(5)	2/2(2)	4/5(5)	2/3(3)
B98/2435	A	4/5(4)	4/4(4)	1/2(2)	4/4(4)	4/4(4)	2/3(2)
B98/5214	M	4/4(4)	4/3(4)	2/2(2)	1/1(1)	1/2(2)	1/1(1)
B99/2421	A	4/4(4)	4/3(4)	5/5(5)	2/3(2)	5/5(5)	2/3(3)
B98/5213	M	3/3(3)	4/4(4)	1/1(1)	1/2(1)	2/3(3)	2/2(2)
B99/2683	A	4/5(4)	4/4(4)	4/5(4)	2/3(2)	5/5(5)	3/4(3)
B98/3966	A	3/3(3)	3/4(3)	1/1(1)	1/2(1)	3/4(3)	2/3(2)
B97/7753	M	4/4(4)	4/3(4)	4/4(4)	2/3(2)	4/4(4)	3/4(4)
B98/7561	M	3/4(4)	4/4(4)	2/3(3)	1/1(1)	2/3(3)	3/3(3)
B99/1131	M	4/4(4)	3/4(4)	1/0(0)	0/0(0)	3/4(4)	2/2(2)
B98/58	M	4/4(4)	4/5(4)	2/3(3)	3/3(3)	3/4(4)	4/4(4)
B98/1708	A	3/4(3)	3/4(3)	5/5(5)	1/1(1)	4/4(4)	3/4(3)
B98/3083	M	4/4(4)	4/3(4)	2/1(2)	1/1(1)	3/3(3)	2/3(3)
B98/506	A	4/4(4)	4/4(4)	4/5(4)	1/2(2)	5/5(5)	4/5(4)
B98/243	A	5/5(5)	4/4(4)	5/495)	3/4(3)	5/5(5)	4/4(4)
B98/4119	A	4/4(4)	4/3(4)	2/3(2)	1/1(1)	4/5(4)	4/4(4)
B98/206	A	4/4(4)	4/5(4)	1/2(2)	2/2(2)	5/5(5)	4/5(4)
B98/5238	A	5/5(5)	4/5(4)	2/3(3)	1/1(1)	5/5(5)	3/4(3)
B97/7463	A	4/4(4)	4/5(4)	5/5(5)	1/1(1)	5/5(5)	3/4(3)

1 Randomisation: A = azathioprine, M = MMF.

2 TGF-β/D; Distribution: Percentage of staining per slide. (see methodology)

3 TGF-β/I; Intensity: The intensity of staining per slide. (see methodology).

4 EGF/D; Distribution: same for TGF-β .

5 EGF/I; Intensity: same for TGF-β .

6 bFGF/D; Distribution: same as for TGF-β.

7 bFGF/I; Intensity: same as for TGF-β .

Table 11:

TGF, bFGF and EGF Staining score for individual lung biopsies.

Lung Biopsy	Code	TGF	EGF	Rej/B	FGF
B98/4891	M	+++ I4	+ I1	1/5	++++ I2
B98/2436	M	++++ I4	++ I1	2/3	++++ I3
B98/2740	A	++++ I4	+ I1	0/1	+++++ I3
B98/265	A	++++ I3	++++ I2	2/4	+++++ I4
B98/2123	A	++++ I2	+ I1	2/2	++++ I1
B98/1802	M	+++ I4	+++ I2	2/3	++++ I2
B98/586	M	++++ I4	++++ I2	0/3	+++++ I4
B99/41	M	++++ I2	+++++ I2	2/4	+++++ I3
B98/2435	A	++++ I4	+ I1	2/2	++++ I2
B98/5214B	M	++++ I4	++ I1	0/2	++ I1
B99/2421	A	++++ I4	+++++ I2	0/1	+++++ I4
B98/5213	M	++++ I4	+ I2	0/1	+++I2
B99/2683	A	++++I4	++++ I2	1/2	+++++ I3
B98/3966	A	+++ I3	+ I1	3/3	+++ I2
B97/7753	M	+++++ I4	++++ I2	0/3	+++++ I4
B98/7561	M	++++ I4	+++ I1	2/4	++++ I3
B99/1131	M	+++++ I4	no section	1/5	++++ I3
B98/58	M	++++ I4	+++ I3	2/3	++++ I4
B98/1708	A	+++ I3	++++ I4		++++ I3
B98/3083	M	++++ I4	+++ I1	1/5	++++ I3
B98/506	A	++++ I4	++++ I2	2/2	+++++ I4
B98/243	A	+++++ I4	+++++ I3		+++++ I4
B98/4119	A	++++ I4	++ I1	2/4	++++ I3
B98/206	A	++++ I4	++ I1	2/2	+++++ I4
B98/5238	A	+++++ I4	+++ I1	2/4	+++++ I3
B97/7463	A	++++ I4	++++ I1	1/2	+++++ I3

KEY:- +++++ High proportion of the tissue section stained (large distribution through tissue)

\+ Low proportion of the tissue section stained (only small areas in tissue)

I4 Strong dark brown Peroxidase Staining of tissue (intensity of staining high)

I1 Paler brown Peroxidase staining of the tissue (intensity staining Low)

Chapter V: Figures (49-60)

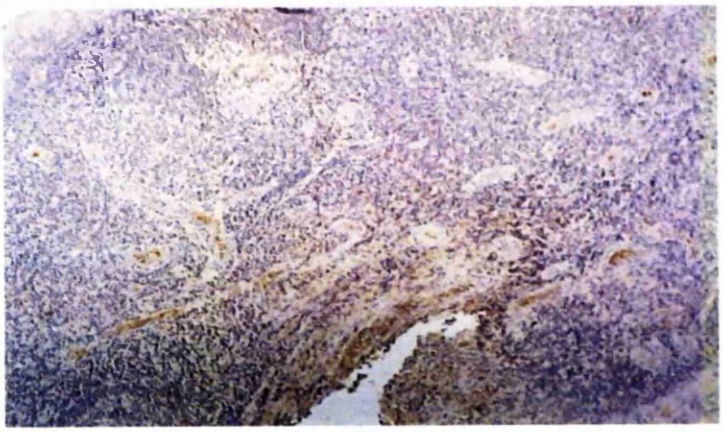


Figure 49
Positive control for TGF-β, tonsillar tissue

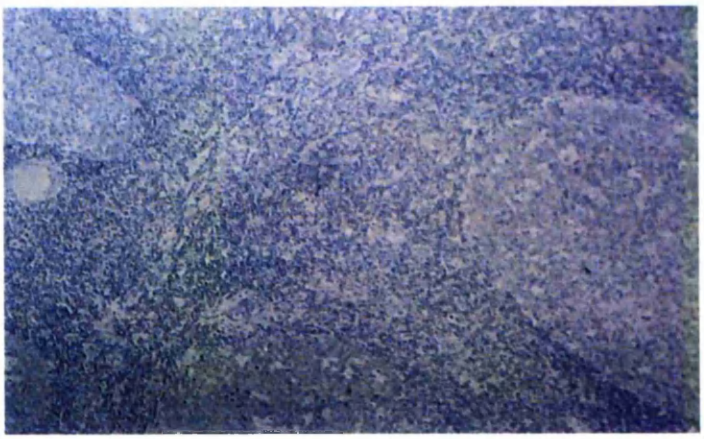


Figure 50
Negative control for TGF, tonsillar tissue

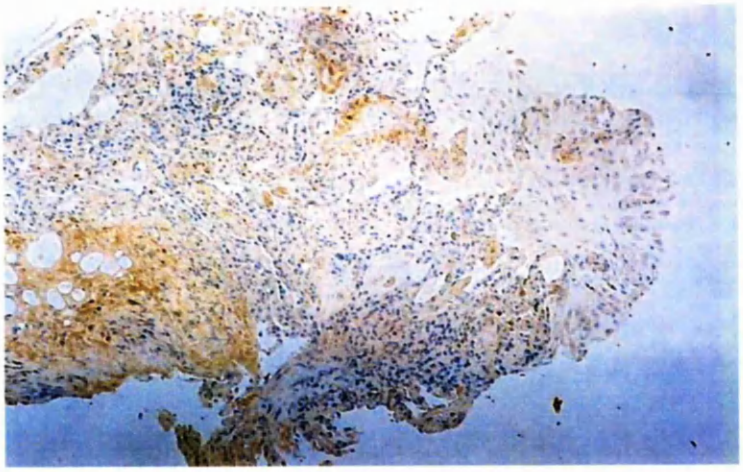


Figure 51
Lung biopsy with positive TGF staining. Intensity grade 5 (x10)

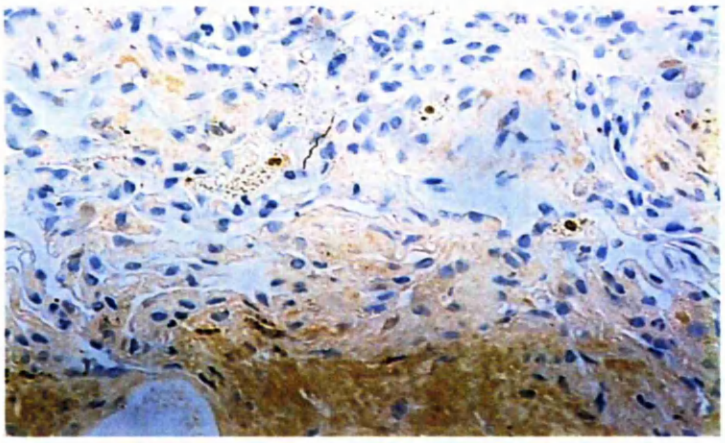


Figure 52
TGF staining intensity, grade 5 (x40)

Figure 53

Lung biopsy showing TGF staining.
Distribution of grade 4 (75-100%)

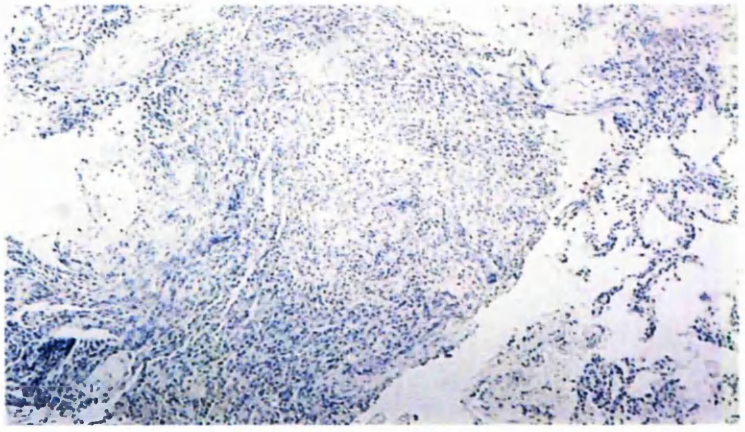


Figure 54

TGF distribution grade 0 (less than 5%)

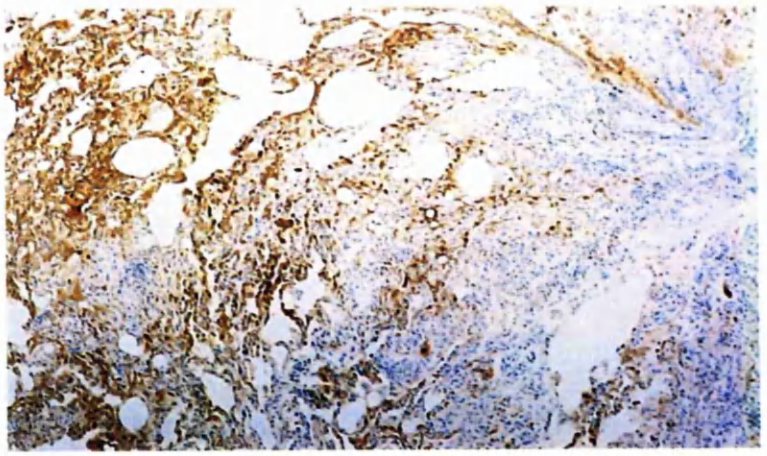


Figure 55

EGF distribution grade 3 (50-75%)
EGF staining intensity grade 5

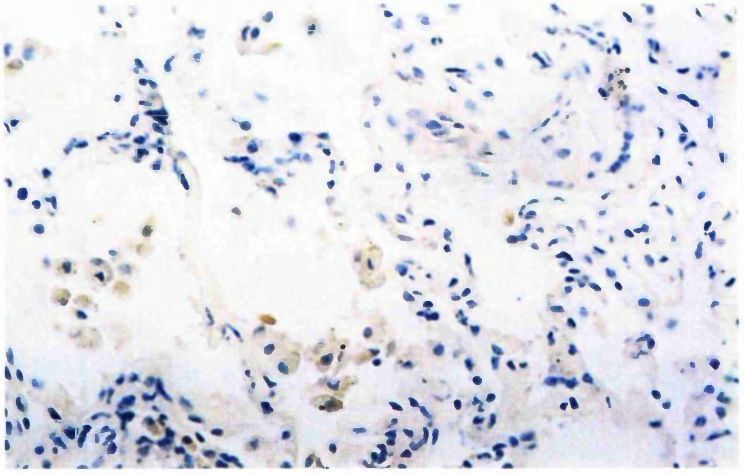


Figure 56

EGF staining (X40) showing intensity of grade 3
and distribution of grade 3

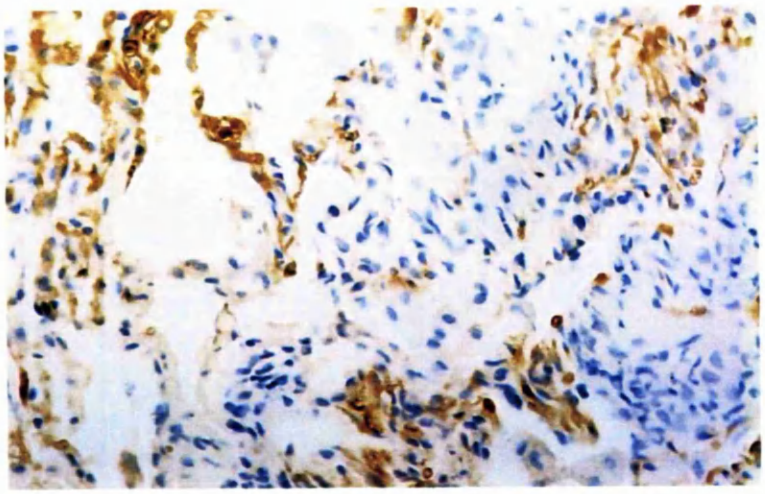


Figure 57

FGF staining (x40) showing positive distribution of grade 4
(50-75%) and intensity of grade 4

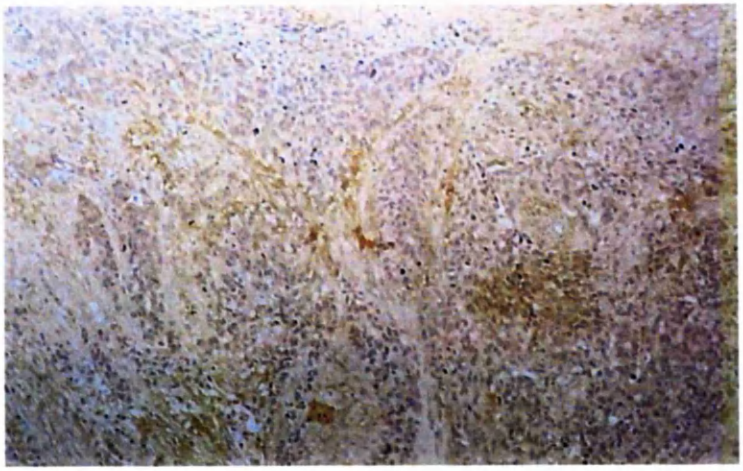


Figure 58
FGF staining showing distribution grade 4 (75-100%)
and intensity of grade 5

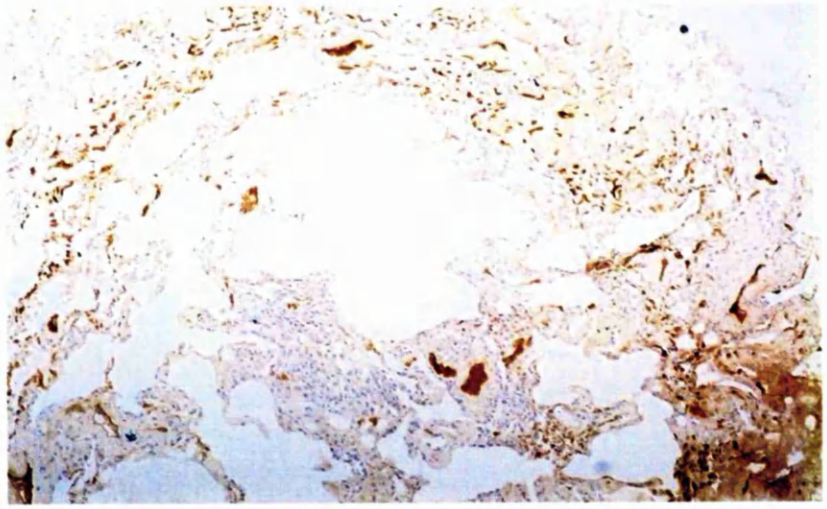


Figure 59

Positive control for bFGF (ovarian cancer)

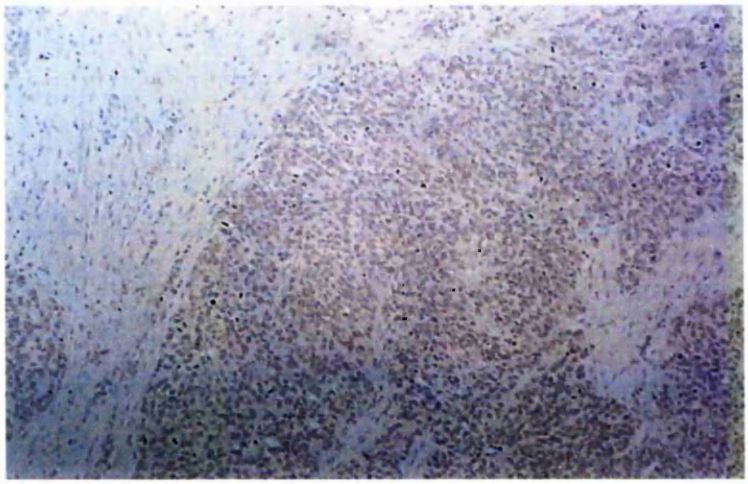


Figure 60

Negative control for bFGF (tonsilar tissue)

Chapter VI

General Discussion and Conclusions

Lung transplantation is an established therapy for end stage pulmonary failure (110). It offers a significant improvement in the quality of life, as well as prolongation in survival. However, long-term survival of lung transplant patients is hampered by the development of bronchiolitis obliterans syndrome or BOS (109, 316) which is the main cause of long term mortality and morbidity. The incidence of OB is between 30-60% in the first 3 years (91, 92, 107). Despite the improvement in the management of acute rejection and pulmonary infections in the last decade, the incidence of OB has remained more or less unchanged. There are no preventive therapeutic strategies yet that have reduced, significantly, the incidence of OB or reversed its pathology once it has started. This work was designed to unravel some aspects of the cellular and molecular processes that take place in the bronchioles of patients with OB and in the transplanted trachea of the OAD rat model. In so doing it was hoped to establish an improved immunosuppressive regime that may prevent or reduce the incidence of OB.

Previous experimental work studying the cellular and molecular processes implicated in OB and fibrosis have suggested an association with various cytokines and growth factors (235, 317-319). These studies were based on models for lung injury and OAD as well as human lung transplant patients. These studies aimed at identifying the pathological processes that take place in response to lung injury, and the resulting complex biological processes that lead to pulmonary fibrosis and ultimately OB. Recently, El-Gamel and associates from this unit and in collaboration with Manchester University reported an important observation when he demonstrated a correlation between increased TGF-β expression in lung biopsies and pulmonary fibrosis and OB in lung transplant recipients (102, 135, 292). From his extensive work on different aspects of OB and lung fibrosis in lung transplant patients he concluded that the risks of lung allograft fibrosis were increased with frequent acute rejection episodes, pulmonary eosinophilia, homozygous recipient TGF-β high producer genotype and the use of cardiopulmonary bypass. The risk for developing OB was associated with recurrent acute rejection, lymphocytic bronchiolitis, pulmonary eosinophilia and heavy expression of TGF-β in lung biopsies. It was interesting to note that TGF-β positive staining preceded the histological confirmation of OB by 6-18 months. He also found that 2 mismatches at the HLA-A locus was a

risk factor for OB. He reported elevated concentrations of plasma TGF-β levels in lung transplant recipients, particularly in those with fibrotic lung diseases in comparison to a normal population. The increased expression of TGF-β in lung transplant recipients was related to homozygous Arginine/Arginine at codon 25 of the TGF-β gene leader sequence, which is associated with high TGF-β producers. Individuals with homozygous Arginine/Arginine at codon 25 were associated with marked pre and post transplant fibrosis, when compared with those of the Arginine/Proline genotype (low TGF-β producers) (292).

In order to further investigate the role of TGF-β in OB, I set up a heterotopic transplant OB rat model in order that this hypothesis could be tested under standardised experimental conditions. Furthermore, I aimed to test two newer immunosuppressive agents individually and in combination, and Vitamin E supplements, on OAD and expression of TGF-β in tracheal biopsies.

OB is considered as a form of chronic rejection (50). Chronic rejection is a well established entity in solid organ transplantation and has been extensively researched in other organs (302, 320-322). The histological features of chronic rejection in general include persistent perivascular and interstitial inflammation that lead onto fibrosis, thinning of the tunica media, intimal injury that leads to intimal thickening and smooth muscle proliferation which ultimately leads to allograft atherosclerosis. This process affects arteries of most transplanted organs, like the coronary arteries in the heart, arteries of the kidneys and liver (320).

In addition to the general picture, there are organ specific changes. In the kidneys, these changes consist of interstitial fibrosis, tubular atrophy and glomerulosclerosis (323). The changes in the liver are typically hepatocellular ballooning and vanishing bile ducts (321). In the heart, it manifests itself typically as vasculitis affecting large and small arteries as well as veins (324, 325), the typical clinical picture of accelerated coronary artery disease and interstitial fibrosis leading clinically to myocardial ischaemia/ infarction with systolic and diastolic cardiac dysfunction. In the pancreas, it manifests as vascular changes, acinar cell loss, fibrosis, the presence of foamy cells and glycosaminoglycan deposition (322).

In the lungs, in addition to the picture of vasculitis seen in other transplanted organs, the immunological response is directed mostly against the donor airway epithelium.

There is an increase in the expression of MHC class II antigens on bronchiolar epithelium and the presence of lymphocytic infiltration of the allograft (53, 101, 261). From the histological perspective, this fibro-proliferative disorder results in airway ulceration (252), lymphocytic bronchitis and eosinophilia (102, 282). There is, in addition, submucosal fibrous thickening, extensive inflammatory cell infiltrate and mesenchymal cell accumulation. Peripheral lymphocytes show a distinct phenotypic profile characterised by disappearance of CD-19 B cells, a decrease in the CD4/CD8 ratio and an increase in cytotoxic effector cells (326). The necrotic epithelium will attract fibroblasts and connective tissue products causing fibro-collagenous occlusion of the respiratory and terminal bronchioles (93, 283, 284). This inflammatory/healing process results in an increase in growth factor expression in which TGF-β is pivotal to the inflammatory/healing processes in the tissues. Augmented TGF-β expression in turn leads to further recruitment of inflammatory cells, fibroblasts and mesenchymal cells to the bronchioles. TGF-β also stimulates the synthesis of extracellular matrix and inhibits its degradation (105). This eventually causes an exaggerated/ intense healing process and more fibrosis. The healing of inflamed bronchioles attracts chronic inflammatory cells, fibroblasts and certain cytokines and growth factors. This milieu may promote fibrosis and scar formation. In severe cases this will result in total occlusion of the bronchioles leading to OB or BOS.

Although the exact cause of OB is still uncertain, there is substantial evidence to suggest an immunological cause as acute rejection and lymphocytic bronchitis are considered the strongest risk factors (249). The potential strategies for the management of OB are generally based on prevention, stabilisation and reversal. Prevention clearly is the best strategy as treatments for OB are largely ineffective after the fibrosis is established (285). Prevention, however, relies on understanding the actual cause of OB and its pathogenesis or identifying the patients at most risk. Based on current evidence, a preventive strategy has to be based on prevention of acute rejection and reduction of the pathological processes that take place during the development of OB. Conclusions obtained from this study as well as other recent scientific work suggest that the new immunosuppressant MMF combined with calcineurin inhibitors offers the multiple advantages of better and more effective

control of acute rejection, wider immunosuppressive activity with its effects on T and B lymphocytes and macrophages, and its direct or indirect suppressive effect on vascular smooth muscle cells, suppression of activated fibroblasts and adhesion molecules as well as suppression of growth factors such as TGF and FGF.

It was interesting that our second experiment showed a correlation between TGF-β expression in the tracheal biopsies and OAD. This relationship proved to be more complex when immunosuppression agents were used to control rejection and OAD. This was not surprising as growth factors particularly TGF-β are ubiquitous molecules that have complex functions that vary depending on the local environment and the stimuli that they are subjected to. There is evidence that TGF-β is overexpressed in the tissues during rejection. TGF-β has an immunosuppressant property and its expression by several cell types is augmented in CyA therapy (275) as well as by Tac and rapamycin (303). On the other hand TGF-β promotes collagen, pro-collagen and fibroblast migration and its suppression in allografts is desirable to prevent or dampen the intensity of chronic rejection. Hence in the experimental model, there is complex relationship between acute rejection and OAD which represents chronic rejection. To the recipient, TGF-β is beneficial in acute rejection and harmful in chronic rejection, so that the addition of agents like CyA has complicated the eventual immunohistochemical picture. Hence, in the rat experiment, TGF-β was moderately overexpressed in the CyA treatment group despite adequate control of acute rejection and OAD. By contrast, in the no treatment group TGF-β over-expression was rather severe while it was mild in MMF + CyA and Tac groups with or without MMF. I suspect that if the CyA treatment were carried out for a longer duration in the experimental model (>28 days) and the transplanted trachea was subjected to another insult (like CMV infection), this scenario (infection +immunosuppression) may result in an accelerated cascade of local inflammation. This would result in an inflammatory cell infiltrate with fibroblasts, smooth muscle cells, procollagen and tissue matrix in addition to the release of a number of cytokines, growth factors and adhesion molecules. In that milieu, the over-expression of TGF-β in the affected tissues will result in exaggerated/ aberrant healing that results in vasculitis, fibrosis and OAD.

A well known method of assessing the role of TGF-β in the experimental rat model is

to treat a group of animals with anti-TGF-β antibody and assess the outcome in the same way in our experiment. This is an avenue for further exploration in the future development of research is this important area of chronic rejection and OAD.

One of the important outcomes of our study is identifying the effect of MMF on TGF-β and OAD. When the study was carried out, MMF was a new, novel immunosuppressant and very little was known about its effect on acute rejection as well as on chronic rejection and OB. Since then a number of human as well as animal studies have shown its efficacy in both acute as well as chronic rejection (99, 113, 129, 130, 260, 277, 298-300, 327). Recently, an extensive review was carried out by AC Allison and EM Eugui on mechanisms of action of MMF, which in summary suggests that, as an inhibitor of IMPDH, it selectively inhibits the proliferation of T and B lymphocytes. It induces apoptosis of activated T lymphocytes, it suppresses the formation of NO by inhibition of iNOS, it suppresses primary antibody responses, it suppresses fibroblasts, arterial smooth muscle cells, adhesion molecules and possibly some growth factors (272). Finally, although on its own it was less effective than CyA in control of rejection and OAD (99, 260, 269), the combination with CyA achieved both control of acute rejection as well as reduced TGF-β over-expression which is vital in the control of chronic lung rejection, fibrosis and OB. The combination with a calcineurin inhibitor is additive with more efficacy and lower toxicity for both agents (274). Some authors even suggest a synergistic effect between CyA and MMF (328). In summary, our findings in the animal study as well as an extensive literature search suggests that MMF and a calcineurin inhibitor is the ideal immunosuppressive combination for the ***prevention*** of OB following lung transplantation.

The randomised clinical study (chapter V) is interesting despite the small number of patients enrolled from this centre. The results clearly shown that there was less rejection in the MMF arm when compared with the standard AZA arm of the study. This was nearly significant($p=0.06$) despite the small number of patients and biopsies. There was also a trend toward less OB in the MMF arm. Although there was no significant difference in the intensity of TGF-β and EGF staining between the groups, there was a significant difference in the expression of bFGF which was a new unexpected finding. This was mainly in the distribution of the bFGF staining rather

than in its intensity. There has been no published work to correlate bFGF to chronic lung rejection and OB. The basic fibroblast growth factor (bFGF) was found to increase in BAL of patients with atopic asthma with further increases occurring in response to acute allergen exposure (313). This was thought to support the hypothesis that bFGF is implicated in airway modelling in asthma in which a chronic structural change occurs which includes subepithelial fibrosis, airway smooth muscle hyperplasia/hypertrophy and angiogenesis. bFGF is implicated because it is a potent mitogen for fibroblasts, airway smooth muscle cells and endothelial cells. bFGF was associated with arterial atherosclerosis in experimental human studies (329) as well as animal studies (314, 315, 330, 331). bFGF is known to have a potent mitogenic effect on endothelial cells and smooth muscle cells, promoting cellular proliferation and differentiation. It has also been implicated in adaptive arterial remodelling (332) and in promoting angiogenesis during wound healing. A recent study suggested that it is essential to the mediation of the stimulatory effect of lipoprotein (a) and LDL on endothelial cell migration and proliferation (333). In experimental arterial grafting, it has been shown to play a major role in the proliferation of smooth muscle cells (SMC) at anastomotic sites with bFGF blockers inhibiting myointimal hyperplasia (334). Kohno and associates suggested that SMC migration is inhibited by bFGF neutralising antibodies and not neutralising antibodies for TGF-β. They concluded that migration of SMC in atherosclerosis, at least in part, is through the release of bFGF and this can be inhibited by bFGF neutralising antibodies and Vitamin E (335). In experimental studies on aortic PTFE interposition grafts, the bFGF level increases and that leads to myointimal hyperplasia and smooth muscle cell proliferation at anastomotic sites which can be blocked by bFGF blocking agents (314). It has also been suggested that thrombin induced myointimal hyperplasia through bFGF and PDGF production by smooth muscle cells. Most interestingly, Cucina and colleagues suggested that bFGF and TGF are regulated by nicotine and they suggest that this is the key role in the development and progression of atherosclerosis in smokers (330).

The above evidence strongly relate bFGF to arterial atherosclerosis as well as bronchial myo-proliferative disorder which are both similar processes to chronic rejection in the allograft vasculature as well as bronchial obliteration. This may be a

very important observation that for the first time links bFGF with bronchiolitis obliterans.

With regard to EGF, MMF did not suppress this growth factor significantly when compared with azathioprine. Yet there have been no known relationships between EGF and chronic pulmonary rejection. However, from the elegant work of Pekka Hayry and associates on chronic rejection (302), it seems that the role of individual growth factors is rather complex and interrelating. The authors believe that TGF-β have a central role as it acts as a master switch. It initially up-regulates IL-1, TNF, IL-6, bFGF and PDGF. As the activated macrophages are downregulated by TGF-β, the resulting pattern is one of switching off activated cells and switching on resting cells. TGF-β also has a direct stimulatory effect on PDGF and an inhibitory effect on EGF (264) hence it is believed that TGF-β function is stimulatory in the early phase of allograft atherosclerosis when PDGF is up-regulated (301, 307) but it is inhibitory later when EGF is produced (336).

The inhibitory effect of MMF on growth factors could be explained via its suppressive effect on fibroblast proliferation in the area of inflammation. Alternatively, it could be a possible direct effect. Whether the effect is direct or indirect, it indicates that MMF has a specific suppressive function on chronic rejection by suppressing fibrogenesis. Future studies are necessary to investigate the direct and indirect effects of MMF on all growth factors and more particularly on TGF-β, PDGF and bFGF. Contrary to the animal experiment, the clinical study indicated that there was no significant difference in the TGF-β expression between the MMF and AZA groups. However, the results of the clinical experiment need to be interpreted with caution because, firstly the number of patients and biopsies were small so firm conclusions should not be drawn. Secondly, the MMF dose used clinically was lower than that used in the animal model. It is a known fact that the MMF effect on fibroblasts and other dividing cells is dose dependent. Thirdly, there were also other accompanying pathologies in the clinical study (but not the animal study) like rejection and infection that were present in the lung biopsies. This will have a significant effect on TGF-β expression regardless of MMF use. Finally, in the clinical setting, there are a number of confounding variables, known and unknown that can complicate the cellular, molecular and the immunohistochemical picture.

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