Role of IL-17 and IL-11 in Immunopathology of Chronic Rejection Post-Lung Transplantation

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Abstract

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Lung transplantation is a therapeutic option for patients affected with end-stage lung diseases. However, several complications may arise following the procedure, such as Bronchiolitis Obliterans (BO). This condition, characterized by small airway fibrosis, remains a major cause of morbidity and mortality in patients following lung transplantation. It is thought to be a manifestation of chronic rejection within the airways, with hallmark inflammation and fibroproliferation. TGF-β and other cytokines, including IL-1, IFN-γ and PDGF, have been implicated in the pathogenesis of fibrosis, mostly in animal models. IL-11 and IL-17 are novel profibrotic cytokines that induce fibroblasts and epithelial cells to produce excess extracellular matrix. They have recently been identified as having a role in tissue remodelling and induction of tissue fibrosis. We hypothesize that IL-11 and IL-17 are involved in chronic lung rejection (Bronchiolitis Obliterans) and that their expression could be a predictive and prognostic marker of chronic lung rejection.

The objectives of the study were to investigate the expression of IL-11 and IL-17 (mRNA and protein) in endobronchial biopsies from lung transplant patients and to define the correlation between the expression of IL-11 and IL-17 and the development of chronic rejection. To achieve our objectives, twenty lung transplant patients underwent endobronchial biopsies and bronchoalveolar lavage. The clinical data of these patients were obtained. Endobronchial biopsy samples from ten healthy non-smoking subjects were used as normal controls. Cytospins were either fixed in paraformaldehyde or snap frozen for immunocytochemistry (ICC). Immunohistochemistry (APAAP technique) was used to examine the presence of IL-11 and IL-17 immunoreactive cells. In situ

hybridization was performed to identify the cell phenotype expressing IL-11 and IL-17 mRNA.

Results showed that tissues obtained from lung transplant patients with evidence of BO had a significant increase in the expression of IL-17 immunoreactive cells and IL-17 mRNA positive cells when compared to samples obtained from stable lung transplant recipients and control subject tissues. In contrast, there was a significant increase in the expression of IL-11 immunoreactive cells and IL-11 mRNA positive cells in tissue samples obtained from stable lung transplant recipients when compared to those obtained from transplant recipients with BO and control subjects. These results suggest that IL-17 can be used as a marker for chronic lung rejection and may also be a potential molecular target to modulate the development of chronic rejection. The results further demonstrate that IL-11 is not associated with chronic lung rejection and its presence may, in fact, indicate a good prognosis.

Extrait

Rôle des IL-17 et IL-11 en immunopathologie concernant le rejet chronique des greffés pulmonaires

La greffe de poumon est une option thérapeutique pour des patients atteints de maladies pulmonaires en phase terminale. Cependant, plusieurs complications peuvent survenir à la suite de ce procédé, notamment la Bronchiolite Oblitérante (BO). Cette maladie, caractérisée par la fibrose des petites voies aériennes, demeure la cause principale de morbidité ainsi que de mortalité chez les patients qui ont subi une greffe pulmonaire. On pense que c'est une manifestation de rejet chronique à l'intérieur des voies aériennes, accompagnée d'inflammation et de fibroprolifération caractéristiques. TGF-B et d'autres cytokines, incluant IL-1, IFN-y et PDGF, sont en cause dans la pathogénie de la fibrose, en grande partie dans la recherche chez les animaux. IL-11 et IL-17 sont des nouvelles cytokines profibrotiques qui induisent des fibroblastes et des cellules épithéliales à produire un surplus de matrice extra-cellulaire. On leur a récemment découvert un rôle dans le remodelage de tissu et dans l'induction de fibrose de tissu. Nous émettons l'hypothèse que IL-11 et IL-17 aient une implication dans le rejet chronique du poumon (BO) et que leurs présences puissent être un indicateur prognostique annonçant un rejet chronique pulmonaire.

La présence des IL-11 et IL-17 (ARNm et protéine) dans les biopsies endobronchiques chez les patients greffés, et la corrélation de cette présence des IL-11 et IL-17 en rapport avec le rejet chronique étaient les objectifs de cette étude. Dans le but d'atteindre nos objectifs, 20 patients ayant subi une greffe pulmonaire ont été soumis à des biopsies endobronchiques et à un lavage broncho-alvéolaire. Nous avons eu accès aux données cliniques de ces patients. Les échantillons de biopsie de dix sujets non-fumeurs et en santé ont été utilisés comme tissu contrôle. Les cytospins ont été fixées par le paraformaldehyde ou préparées pour l'immunocytochimie. L'immunohistochimie (technique APAAP) a été utilisée pour identifier la présence des cellules

immunoréactives pour IL-11 et IL-17. L'hybridation in situ (ISH) a été utilisée afin d'identifier la cellule phénotype exprimant l'ARNm IL-11 et IL-17.

Chez les greffés pulmonaires souffrant du syndrome de Bronchiolite Oblitérante (SBO), les résultats indiquent une augmentation marquée de la presence des cellules immunoréactives pour IL-17 et des cellules positives pour l'ARNm IL-17 en comparaison des échantillons obtenus des greffés pulmonaires stables et des tissus de contrôle. En contraste, il y avait une augmentation marquée dans la présence des cellules immunoréactives pour IL-11 et des cellules positives pour l'ARNm IL-11 dans les échantillons obtenus des tissus des greffés pulmonaires stables en comparaison des patients souffrant du SBO et des tissus de contrôle. Les résultats suggèrent que IL-17 peut être utilisé comme indicateur de rejet chronique pulmonaire et peut également être une cible moléculaire potentielle afin de moduler le développement du rejet chronique. De plus, les résultats démontrent que IL-11 n'est pas associé au rejet chronique pulmonaire et que, de fait, sa présence révèle un bon pronostic.

Figures

Abbreviations

AD- Anodal duration

AJRCCM- American Journal of Respiratory and Critical Care Medicine

ALG- antilymphocyte globulin

APAAP- Alkaline Phosphatase-AntiAlkaline Phosphatase

APC-antigen presenting cells

AR-acute rejection

ATG-antithymocyte globulin

BAL- bronchoalveolar lavage

BO- Obliterative bronchiolitis

BOS- Bronchiolitis obliterans syndrome

CC- Clara cell

CD- cell determinants

cDNA- complementary deoxyribonucleic acid

COPD- chronic obstructive pulmonary disease

CMV-cytomegalovirus

CNTF- ciliary neurotrophic factor

CR- chronic rejection

CsA- cyclosporine A

EBB- endoscopic bronchial biopsies

Fc-fragment crystallizable

FEV- forced expiratory volume

FGF- fibroblast growth factor

G-CSF- granulocyte colony stimulating factor

GI- Gastrointestinal

(GM)-CSF- granulocyte macrophage colony stimulating factor

HLA- human leukocyte antigen

HM- heart murmur

HSV- herpes simplex virus

ICAM- Intercellular adhesion molecule

ICC- immunocytochemistry

IFN- interferon

IgG- immunoglobin G

IL- interleukin

iNOS- inducible nitric oxide synthase

ISH- In situ hybridisation

LAM- lymphangioleiomyomatosis

LIF- leukaemia inhibitory factor

LTR- lung transplant recipient

Lys- lysine

MAPK- mitogen-activated protein kinase

MCP- monocyte chemoattractant protein

Met- methione residue

MHC- major histocompatibility complex

MMP- metalloproteinase

mRNA- messenger deoxyribonucleic acid

NF- nuclear factor

NGF- nerve growth factor

NO- nitric oxide

OCT- ornithine carbamoyltransferase

OSM- oncostatin M

PDGF- platelet- derived growth factor

PF- Purkinje fibers

PGE- prostaglandin E

PIV- peripheral intravenous (line)

PMA- phorbol 12- myristate 13 –acetate

RA- Rheumatoid arthritis

RANTES- regulated upon activation, normal T-cell expressed and secreted

Rh- Rhesus blood factor

RSV- respiratory syncytial virus

RT-PCR- reverse transcription polymerase chain reaction

SCF- stem cell factor

SEM- scanning electron microscopy

SSc- Systematic sclerosis

Tac- tacrolimus

TBS- tris buffered saline

TCR- T-cell receptor

TGF- transforming growth factor

Th cell- T helper cell

THF- tetrahydrofuran

TNF- tumor necrosis factor

TPO- thrombopoietin

TRAF- Tumor Necrosis Factor receptor associated factors

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Chapter 1. Introduction

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1.1 Lung transplantation:

1.1.1 History

Lung transplantation was first attempted in humans during the 1960s (1, 2). During that time, powerful and selective anti-rejection drugs were not available, and subsequently, poor healing of the airway was common, resulting in high mortality rate due to post surgical complications stemming from lung graft rejection, and infection (3-5). The advent of the anti-rejection drug, cyclosporine, and improved surgical techniques in the early 1980s resulted in more successful heart-lung, and isolated lung transplantation (6). To date, approximately 9,500 patients have undergone single or bilateral lung transplantation (6).

1.1.2 Indication

Indications for lung transplantation are an important consideration, the most common in many centers, being chronic obstructive pulmonary disease (COPD) (6, 7). Other indications include pulmonary fibrosis, primary pulmonary hypertension (on the decline due to better medical therapy), sarcoidosis, pulmonary lymphangioleiomyomatosis (LAM), Eisenmenger's syndrome (primarily for atrial septal defect and patent ductus arteriosis), and re-transplantation (8-10).

1.1.3 Outcome

Lung transplantation has evolved into an accepted and effective treatment for end-stage pulmonary disease (2, 3). Although survival rates following lung and heart-lung transplantation have improved in recent years, there is, nonetheless, a significant decrease in survival rate in the years after transplantation (2, 3). This is demonstrated by a marked decrease in survival rates, documented in the years following a primary

lung transplant; which showed a drop in survival from 72% after the first year, to 52% after the third year (2, 3). While refinements in surgical techniques have enhanced post-operative outcomes, improved preoperative management, encompassing organ preservation, ischemia-reperfusion injury, infectious disease complications, and immunosuppression, have significantly increased the success of this procedure (11).

1.1.4 Complications

Obliterative bronchiolitis (OB), representative of chronic rejection, is considered the major long-term complication and one of the major barriers to the success of lung transplantation. Clinically, it unfolds in an obstructive pattern, leading to a progressive decline in lung function (12). Pathologically, it is a fibroproliferative process leading to occlusion of small airways (13). At present, obliterative bronchiolitis affects up to 50% of survivors, showing initial signs 3 months after a primary lung transplantation (14). Various immunologic and non-immunologic forms of injury have been thought to contribute to the risk of OB, including acute rejection, which has been shown to be a high risk factor for OB (15). Cytomegalovirus (CMV) pneumonitis, late acute rejection, HLA mismatches at the A loci, and total HLA mismatches have also been shown to be risk factors (12, 15, 16).

The second most common cause of early post-operative death in lung-transplant patients is infection (15); with the infection rate of lung transplant recipients exceeding that of any other solid organ transplants.

1.1.5 Immunosuppression

Clinical transplant immunosuppression aims not only to prevent host immune responses against antigens on the transplanted organ, thereby avoiding rejection, but also to prevent undesirable complications of immunodeficiency (e.g. infection and malignancy) (17). Immunosuppression drugs are either small molecules or proteins.

Representative of the first class of small molecule drugs is glucocorticosteroids. They are the oldest immune response-modifying drugs, used as early as the 1950's to suppress inflammation and immune-mediated diseases (18). Their immunosuppressive and anti-inflammatory effects are complex and dose-related. In addition, they exert a wide range of effects on many phases of immune and inflammatory responses (18).

Another class of small-molecule drugs, the purine analogs, were developed by the Nobel prize-winning team of Gertrude Elion and George Hitchings (19), and derived principally as anticancer agents. More recently, several other small-molecule agents have been discovered such as cyclosporine (CsA), tacrolimus (Tac), and rapamycin (17).

The second type of immunosuppressive drugs consists of those that are protein based. Designed to target specific cell membrane receptors or their corresponding ligands, they are typically antibodies or are derived from antibody molecules. These agents are highly selective and potent, including such drugs as antilymphocyte globulin (ALG) and antithymocyte globulin (ATG). Another drug of the same group, OKT3, is a monoclonal antibody that reacts with the T-3 complex by blocking the function of T cells. This drug binds to all mature T cells in the peripheral blood (20).

1.2 Chronic rejection in Lung transplantation:

1.2.1 Definition

Chronic rejection (CR) is defined as an indolent but progressive form of primarily immunologic injury to the allograft, which slowly compromises organ function (21). Although the clinical symptoms of CR are dependent on the function of the specific organ allograft, the most common histologic manifestation is a progressive narrowing

of the muscular arteries, which, when occurring in lungs, is termed bronchiolitis obliterans (21).

1.2.2 Etiology

The main obstacle to the long-term success of lung transplantation remains chronic rejection, which occurs in up to two-thirds of patients (14). Chronic rejection (CR) usually begins within weeks to months after transplantation, often during severe and/or persistent acute rejection (AR) episodes (14). It can also insidiously develop years after transplantation, particularly in inadequately immunosuppressed patients with late-onset AR (13). In general, the incidence of CR increases with time after transplantation and eventually affects a majority of solid organ allografts, with the exception of liver allografts. Five years following transplantation, CR only affects approximately 5% of liver recipients, but up to 80% of lung allograft recipients (13).

Various immunologic and non-immunologic forms of injury have been thought to contribute to the risk of OB and a high risk factor for chronic rejection is shown to be acute rejection. Cytomegalovirus (CMV) pneumonitis, late acute rejection, HLA mismatches at the A loci, and total HLA mismatches have also been shown to be risk factors (22).

1.2.3 Pathogenesis

Two working hypotheses have evolved to explain the pathogenesis of chronic rejection (23, 24). First, the process is thought to be primarily an antigen-dependent phenomenon influenced by early immunologic injury, such as acute rejection and continuing host alloresponsiveness (23). The second hypothesis is that alloantigen-independent factors, such as prolonged ischemia, surgical manipulation, reperfusion injury, recurrent infection, and aspiration may lead to the progressive changes that occur (24).

Some studies suggest that CR develops because of excessive scar formation in the allograft in response to the tissue injury. Evidence supporting this contention includes the correlation between high intragraft TGF-β production and increased risk of late graft failure (25).

1.3 Cytokines in Lung transplantation

1.3.1 General overview

Cytokines are low-molecular-weight soluble proteins that transmit signals between cells. It is now clear that cytokine production is not limited to lymphoid and myeloid cells, and that cytokines produced by epithelial and mesenchymal cells amplify inflammatory responses in the lungs and other organs. Cytokines are produced in "cascades" in which the initial cytokine signals are amplified many-fold by target cells, such as epithelial cells, fibroblasts, and endothelial cells. Cytokines function in networks in which feedback is provided by many points, thereby co-ordinating and regulating cytokine and cellular responses.

The quality of organ function achieved post transplantation is a powerful predictor for the duration of organ survival. Organs that manifest acute non-immune-mediated injury have an increased rate of acute rejection and decreased long-term survival. This may reflect the tendency of grafts injured in this way to become inflamed, which increases T-cell triggering and activation (26). Gene expression, including chemokines and major histocompatibility complex antigens, is highly inducible by acute injury (26). It has long been recognized that T-helper cells influence the immune system through secretion of cytokines, which in turn have dramatic effects on other immune regulatory cells (26). The activation of T-cells begins with the recognition of antigens (i.e., transplanted organ). The antigen is presented to the T cells by antigen-presenting cells (APC), such as dendritic cells.

This is the first of two signals necessary for T-cell activation. The second signal involves proteins on the surface of the APC that interact with cell surface molecules on the T cell, providing a costimulatory signal. Once this occurs, the T cell is fully activated and upon activation, the T cells produce cytokines that have various effects on both T cells and other inflammatory cells within the vicinity (26). Naive helper T cells (Th0), exposed to different stimuli, can differentiate into either Th1 or Th2 cells (27). The predominant Th cells vary according to the disease process. For example, a predominance of Th2 cells is seen in allergic conditions, while Th1 cells drive the response in allo- and autoimmune mechanisms (27). Each type of helper cell has a characteristic pattern of cytokine production as Th1 predominantly produces interleukin (IL-2) and IFN-gamma, and Th2 predominantly produces IL-4 and IL-10 (27).

1.3.2 Animal models

Animal models have been used to investigate the role of cytokines in the pathogenesis of chronic rejection, post solid organ transplants.

Kallio and coworkers (28) investigated the role of complement activation in obliterative bronchiolitis. In their investigation, they used a tracheal allograft model in rats (graft from a genetically dissimilar individual of same species). Compared with syngeneic grafts (grafts from genetically identical rats), the allografts developed airway occlusion and progressive loss of respiratory epithelium associated with increased deposition of complement components C3 and C5b-9. Treatment with complement receptor type 1, an inhibitor of the classic and alternative complement pathways, decreased the obliterative changes by 40%, and decreased the deposits of C5b-9 and IgG, neutrophil chemoattractant interleukin-8, and expression of ICAM-1.

Treatment also produced increased staining for Th 2 cytokines. The authors conclude that blocking the activation of complement attenuates the development of obliterative bronchiolitis.

Growing evidence that the angiotensin system plays a prominent role in the pathogenesis of fibrosis, encouraged Maclean and coworkers (29) to examine the role of angiotensin-converting enzyme in a rat heterotopic tracheal transplant model (organ transplanted into a different location than the native organ) with bronchiolitis obliterans. In allograft rats, the obliterated portion of the trachea showed heavy staining for angiotensin-converting enzyme on day 21 after transplantation. Rats started on Captopril, an inhibitor of angiotensin-converting enzyme, showed 45% less airway obstruction. The authors concluded that angiotensin-converting enzyme is found in the fibroproliferative lesion in a model of bronchiolitis obliterans, and inhibiting the enzyme limits the development of airway obstruction (29), in a rat heterotopic tracheal transplant model.

1.3.3 Human Model

Human studies had been used to investigate the role of cytokines in the pathogenesis of bronchiolitis obliterans.

Charpin and coworkers (30) investigated whether insulin-like growth factor-1, a stimulator of collagen synthesis by fibroblasts, is involved in the development of obliterative bronchiolitis. They studied nine patients who had undergone lung transplantation. Three patients developed bronchiolitis obliteran syndrome at 8, 14, and 17 months after the surgery. Compared with the six patients who did not develop the syndrome, the affected patients developed 3 to 13-fold increases in insulin-like

growth factor in their bronchoalveolar fluid at 7, 13, and 17 months before the diagnosis was made. The binding protein for insulin-like growth factor was also highly expressed. The authors conclude that insulin-like growth factor-1 contributes to the fibrotic process in obliterative bronchiolitis and may serve as an early marker (31).

Ali and colleagues measured the expression of TNF-α in bronchoalveolar lavage (BAL) cells by quantitative reverse transcription polymerase chain reaction (RT-PCR) methodology (32). TNF-α gene expression was not correlated with the identification of acute rejection histology on transbronchial biopsies. However, increased TNF-α gene expression was correlated with increasing degrees of airflow obstruction in all participants and with increasing airflow obstruction over time in individual patients.

In a related study, Lu and associates found statistical correlation between specific genetic polymorphisms of TNF-α and interleukin-6 and BOS, in lung transplant recipient (33). These 2 studies extend the existing data implicating macrophage-derived cytokines in the pathogenesis of OB/BOS.

1.3.4 Pro-fibrotic cytokines

1.3.4.1 Interleukin-11 (IL-11)

Interleukin-11 (IL-11) is an IL-6 like cytokine, as they share a number of functional properties. However they are regulated differently and they do not have significant sequence homology. Both human and murine genomic sequences have been mapped to chromosome 19 and 7 respectively (34, 35).

IL-11 is expressed in several tissues and a variety of cells of mesenchymal origin (49). The expression of IL-11 can be modulated by a number of inflammatory cytokines and hormones and is induced by IL-α and TNF-α (36). Heparine and

extracellular matrix proteins can also modulate the expression of IL-11 in respiratory epithelial cells and fibroblasts can be regulated by TGF-B and TGB-2 via calmodulin dependent and PKC-independent pathways (37).

The resulting cDNA for IL-11 encodes a predicted protein of 199 amino acids with a 21 amino acid leader sequence (38). The molecular weight of recombinant human and murine IL-11 is 19,144 and 19,154 daltons (34), respectively. Mature human and murine IL-11 proteins share 88% homology. IL-11 is rich in proline residues (12%) and lacks cysteine residues along with any potential for disulfide bonds (34).

IL-11 is among several growth factors that use the gp130 receptor common subunit for receptor function (39). Other factors that use this signaling mechanism include IL-6, oncostatin M (OSM), leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF) (39). A recent study has cloned the IL-11 receptor α-chain (IL-11Rα) and shown that the extracellular region shares sequence similarity with the a-chains of IL-6 (40). The genomic structure of the human IL-11Rα consists of 12 exons and 12 introns within a 9 kb genomic region and is located on chromosome 9. There are two isoforms of IL-11Rα which, differ in their cytoplamic domain. One isoform shows similarity to the human IL-6 and has a short cytoplasmic domain (IL-Rα1) (40). The other isoform demonstrates similarity to the human CNTF receptor and completely lacks the cytoplasmic domain (40). The functional significance of these two different receptor isoforms has yet to be determined.

1.3.4.1.1 Biological effects of IL-11

Various stages and lineages of hematopoiesis can be stimulated by IL-11, acting synergistically with other early and late acting growth factors. IL-11 can act

together with the IL-4 (41), IL-7 (42), IL-12 (43), IL-13 (44), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (45), and to induce the proliferation of primitive stem cells, as well as multipotential and committed progenitor cells from various sources including bone marrow (46) and peripheral blood in different culture systems (45). It is believed that IL-11 acts in conjunction with other cytokines to increase commitment of primitive stem cells into the multilineage progenitor compartment (47). In vivo studies have shown that IL-11 increases the cycling rates and absolute number of myeloid progenitors in both the bone marrow and spleen of normal mice (48); however, it showed no effects on peripheral leukocyte counts when administered to normal rodents (49) and primates (43).

IL-11 can modulate the differentiation and maturation of myeloid progenitor cells. In addition, it can also act synergistically with SCF to stimulate murine bone marrow cells to produce myeloid colonies composed of mostly granulocytes and myeloid blasts (42). Interestingly, when IL-11 is added to the same cells in combination with IL-4 or IL-13, the proportion of granulocytes and myeloid blasts is greatly reduced in the colonies while the number of macrophages is greatly increased (44). In addition, combination treatment of IL-11, SCF and G-CSF in rats, results in a significant increase in peripheral neutrophil and granulocyte macrophage counts (16).

IL-11 can act with IL-3, SCF, or thrombopoietin (TPO) to stimulate various stages of megakaryocytopoiesis and thrombocytopoietin in both human (50) and murine (51) bone marrow cells. In vivo studies with IL-11 show enhanced stimulation of megakaryocytopoiesis in rodents, primates, and human (50, 52). Treatment with anti-IL-3 antiserum results in a 28% reduction in colony formation, whereas anti-TPO antiserum results in a 90% reduction in colony formation,

suggesting that effects of IL-11, megakaryocytopoiesis and thrombocytopoiesis are partly mediated by TPO activity (50).

It has been shown that the addition of IL-11 or IL-6 to a mixture of B lymphocytes, CD4+/45RA-T lymphocytes, monocytes, and pokeweed mitogen leads to an increase in DNA synthesis and immunoglobulin production by B lymphocytes (53). After adding anti-IL-6 antiserum to the mixture, the increase in immunoglobulin secretion was only partially blocked, suggesting that IL-11 promotes the differentiation of B lymphocytes by selective stimulation of CD4+/45RA-T cells and monocytes in a manner similar to IL-6. A component of the IL-11 effect on B lymphocyte differentiation may be through the stimulation of IL-6 production. In addition, B lymphocytes can be generated in primary cultures of bone marrow cells from 5-fluorouracil-treated mice, after the addition of IL-11 in combination with SCF or IL-4 (54). IL-11 and IL-4 can also reverse the inhibitory effect of IL-3 on early B-lymphocyte development (54).

It has been demonstrated that bronchial and alveolar epithelial cells produce large amounts of IL-11; and up-regulation of IL-11 production by these cells in response to pro-inflammatory cytokines, respiratory syncytial virus (RSV), and retinoic acid, suggests that IL-11 plays an important role in inflammatory processes of the lung (55). IL-11 and IL-11Ra are also found in the epithelium of the GI tract where in vitro studies have shown that IL-11 can directly interact with the GI epithelial cells and inhibit the proliferation of intestinal crypt stem cell lines (56, 57). Therefore, it has been postulated that IL-11 has been shown to enhance GI absorption of iron in rats, which does not appear to be related to the effects of IL-11 on erythropoiesis (58).

IL-11 is able to stimulate the production of hepatic acute phase proteins both in vitro and in vivo. Like IL-6, IL-11 stimulates the production of type 1 (a1-acid glycoprotein, complement component C3, haptoglobin, and hempexin) and type 2 (thiostatin and fibrinogen) acute phase proteins in rat hepatoma cells and hepatocytes (59).

Furthermore, IL-11 is also able to directly inhibit the process of adipogenesis and suppress the lipoprotein lipase activity in differentiated adipocytes (60, 61). IL-11 also inhibits the differentiation of adipocytes induced by insulin and dexamethasone, as well as the accumulation of adipose in a cell line derived from the adherent layer of human, bone marrow culture.

In addition, IL-11 acts as an inducible cytokine in connective tissue. Stimulation of human articular chondrocytes and synoviocytes by IL-1 or TGF-β enhances the expression of IL-11 mRNA and protein secretion. IL-11 does not induce catabolic response of chondrocytes, such as the production of stromelysin activity, a matrix metalloproteinase, nor the proliferation of chondrocytes and synoviocytes. Also, it does not induce the synthesis of the tissue inhibitor metalloproteinase-1 and it is thought that this effect balances IL-1β-induced extracellular matrix breakdown by stromelysin and has a protective effect on connective tissue.

Another effect of IL-11 is its ability to inhibit macrophage production of IL-1, IL-12, and nitric oxide (62). This biologic function is an important mechanism in preventing cell damage after radiation treatment.

1.3.4.1.2 IL-11 in Disease

Human IL-11 has been shown to be expressed and have a broad spectrum of activity in multiple tissues; however, its role in human disease is poorly understood. This deficit of knowledge is partly because of a lack of sensitive bioassays. Epidemiologic studies of asthma have demonstrated a close correlation between respiratory viral infections and asthmatic exacerbations (63). A recent investigation has defined IL-11 as an important mediator in the pathogenesis of virus-induced asthma (64). This study demonstrated that RSV, PIV, and RV, in contrast to AD, CMV< HSV, and bacteria, were all able to impressively stimulate cultured lung stromal cell production of IL-11 in the nasal secretions of children with viral upper respiratory tract infections, and this presence of nasal IL-11 clinically correlated with wheezing (55, 63).

Another study looked at the histologic changes in the lung when IL-11 was chronically present in the respiratory tract (65, 66). This was done by generating and evaluating the airways of transgenic mice in which the Clara cell 10kD protein (CC10) promoter was used to target IL-11 to the respiratory tree. In the airways, the transgene (+) animals manifested airway remodelling, peribronchiolar nodules and alveolar air space enlargement. The sub-epithelial response in these animals was characterized by increased interstitial collagen deposition and heightened stromal cellularity with the local accumulation of fibroblasts, myofibroblasts, and smooth muscle cells. The physiologic study of the transgene (+) mice showed increased airway resistance and airway hyperresponsiveness to methacholine, when compared to transgene (-) mice. This study demonstrated that chronic expression of IL-11 in airways results in several changes that mimic the pathologic and physiologic features

of airway inflammation, further suggesting that IL-11 is important in the pathogenesis of such airway abnormalities.

1.3.4.2 Interleukin-17 (IL-17)

Interleukin-17 (IL-17) was first identified as a rodent cDNA transcript, termed CTLA8, that had been isolated from an activated T-cell hybridoma. It bore a striking 58% identity to a predicted open-reading frame, HSVS13, in the T-lymphotropic herpesvirus, *Herpesvirus samiri* (67). Initial characterization recognized that this factor could promote the production of other cytokines and chemokines such as IL-6, IL-8, and granulocyte colony-stimulating factor (G-CSF) from a variety of epithelial, endothelial, and fibroblastic cell types and led to its proposed nomenclature as an IL (67, 68). IL-17 was striking in its uniqueness. It bore no resemblance to other known ILs, and moreover, beyond the viral ortholog, it bore no clear resemblance to any known protein or structural domain. Its uniqueness was further exemplified by the isolation of a receptor that binds IL-17 and is required for signaling (69). IL-17 receptor (IL-17R) was not recognized to be related to any of the other known cytokine receptors, and remarkably, in spite of a relatively large size of 860 amino acids, it also did not possess similarity to any other known protein nor any recognizable domains. Thus, the IL-17 system appeared to be a distinct and potent signaling system involved in the control of the immune response.

1.3.4.2.1 Biological effects of IL-17

The biological actions of IL-17 are pro-inflammatory in character (70). It increases the local production of chemokines such as IL-8, monocyte chemoattractant protein-1 (MCP-1), and growth-related ongogene (Gro), thereby promoting the

recruitment of monocytes and neutrophils (71). Furthermore, it stimulates the production of the hematopoietic cytokines G-CSF and granulocyte macrophage (GM)-CSF, who promote the expansion of these myeloid lineages (72). Other actions such as the stimulation of IL-6 and PGE_2 production enhance the local inflammatory environment. In addition, IL-17 also drives T-cell responses, notably through the induction of the costimulatory intercellular adhesion molecule (ICAM) (71).

IL-17 has long been considered a molecule produced by activated memory T cells (73). Studies designed to establish whether IL-17 could be classified according to the Th1/Th2 paradigm have been unable to provide clear categorisation. Although individual T-cell clones can be derived, which produce interferon-γ (IFN-γ) and IL-17, many IL-17-producing clones appear to produce neither IFN- γ nor IL-4 (74). The significance of the observation that IL-17 was produced by activated CD4+CD45RO memory T cells has perhaps, not been fully appreciated. One interpretation of this observation is that there exists within the memory T-cell compartment a population of T cells that were originally activated by unknown stimuli to produce IL-17. Having acquired this differentiated, polarized state, these cells are able to re-express IL-17 when subsequently re-stimulated with relatively non-specific stimuli such as phorbol 12-myristate 13-acetate (PMA) and ionomycin. This interpretation has been given strong support by the recent observation that splenocytes from T-cell receptor (TCR) transgenic mice can be driven to produce IL-17 when primed with cognate peptide in the presence of microbial lipopeptides (75). The study demonstrated that this outcome was not observed when cells were stimulated with peptide and the known Th1 driver, IL-12 (75). Characterization of the cytokine expression profile of individual T cells within the population by cytometric single-cell analysis revealed that the IL-17-expressing cells represent a distinct population from the traditional Th1 profile and

are characterized by the production of IL-17, GM-CSF, and TNF-α. This represents the first known physiological stimuli capable of directing the development of IL-17-expressing T cells. These data fit well with other observations that there is abundant IL-17 in various conditions that are impacted by microbial pathogens, including Helicobacter pylori-infected gastric mucosa and synovial fluid of patients with Lime arthritis (75).

1.3.4.2.2 Structure of IL-17

The apparent uniqueness of the sequences of IL-17 and its receptor has made it difficult to appreciate whether the IL-17 system is truly unrelated to other known signaling systems. To better understand the character of the IL-17, the crystal structure of one of the family members, IL-17F, has recently been determined. Unexpectedly, the structure of IL-17F reveals that the protein adopts a cysteine knot fold and suggests that the family may have a relationship to the cysteine knot superfamily of proteins (76). IL-17F folds in a manner quite similar to that of nerve growth factor (NGF) and the other neurotrophins (76). In addition, IL-17F and NGF exist as dimmers and the IL-17 family does not have any appreciable sequence identity with the neurotrophins.

The cysteine knot superfamily is a diverse family and includes other proteins, such as the endocrine glycoprotein hormones (e.g., chorionic gonadotropin), the platelet-derived growth factors (PDGFs), and the TGF-ß family, which also display limited sequence similarity (76). Although IL-17 folds in a manner highly analogous to the cysteine knot superfamily, one of the cononical cysteine pairs, the pair that links through the ring formed by the other two pairs and thereby forms the "knot," is not present. Instead, a third cysteine bridge is formed by a spatially distinct pair of

cysteines. Thus, although the members of IL-17 family clearly adopt a cysteine knot fold, actual evolutionary membership within the family cannot be assumed.

Following the isolation of mouse, rat, human, and viral homologs of IL-17, viral IL-17 was used to identify a mouse IL-17R that bound to all ortholog forms (67). In contrast to a relatively restricted expression of IL-17, IL-17R was found to be ubiquitously expressed in all cell types examined. The mouse IL-17R is a type 1 membrane protein that contains 864 amino acids and eight putative N-linked glycosylation sites and the human homolog of mouse receptor exhibits 69% sequence homology. Direct binding assays using 125I-labeled human hIL-17 on cells transfected with IL-17R have indicated that IL-17 binds to its receptor with relatively low affinity with a Ka value of approximately 2×10^7 to 2×10^8 M^{-1} (67). This is a substantially low affinity for IL-17R, considering the low concentrations of IL-17 required for its biological activity, suggesting the potential presence of additional components of the IL-17R. However, in human fibroblast cell line HFF, an antibody to IL-17R blocks IL-6 production in response to IL-17 Fc ligand, indicating that binding to its receptor is necessary to generate an IL-17-specific response in these cells (67).

Initial studies of the new members of the IL-17 family suggest that they will use distinct, cognate receptors. IL-17E has been identified as a high-affinity ligan for a newly recognized receptor termed IL-17Rh1 (also termed EVI27 and IL-17BR) (77). This receptor has also been suggested to interact with IL-17B although apparently with lower affinity. The comparison of IL-17R and IL-17Rh1 reveals conservation of several cysteines within the extracellular domains, suggesting they share similar structure. There are also conserved elements within the intracellular domain, suggesting that these receptors likely engage similar intracellular machinery.

This is supported by the observation that IL-17E, like IL-17, is able to induce the activity of NF-B and similar downstream effector molecules. IL-17Rh1 was first shown as EVI27, a protein encoded, whose expression was up-regulated as a result of retroviral integration in BXH2 murine myeloid leukemias. IL-17F, which has similar activity to IL-17, also appears able to interact with the IL-17 (78).

IL-17 is produced by activated T cells and mediates its pro-inflammatory effects via its receptor, IL-17R, which is ubiquitously expressed on all cell types. However, the exact mechanisms of IL-17 signalling are still not fully elucidated. In chronic diseases including RA, IL-17 results in tissue damage, directly by matrix degradation (79, 80) or indirectly by recruiting activated inflammatory cells and inducing other pro-inflammatory cytokines including IL-1ß and TNF-α to the inflamed tissue (80). The matrix degradation results from up-regulation of inducible nitric oxide synthase (iNOS) and NO in chondrocytes through a tyrosine kinase-dependent cascade, which is protein kinase A (PKA)- and, to a lesser extent, protein kinase C (PKC)-dependent (81). IL-17 activates all three subgroups of MAPKs, which are the p44 and p42 extracellular signal-regulated kinases (ERK1 and ERK2), stress-induced Jun NH2-terminal kinases (JNK), and p38 (82). The IL-17-induced activation of JNKs (JNK1 and JNK2) results in an up-regulation of iNOS and cyclooxygenase-2 (COX-2) genes (81). NF-B activation by IL-17 was seen in human fibroblasts, intestinal epithelial cells, and chondrocyte in culture. The mechanisms by which IL-17 activates NF-B are not resolved but appear to be dependent on TNF receptor-associated factor (TRAF)-6 (71).

1.3.4.2.3 IL-17 in disease

Rheaumatoid arthritis (RA) is characterized by chronic inflammation and progressive destruction of RA synovium and of bone and cartilage (83). In this disease, T cells infiltrate into the synovial membrane, and tissue pathogenesis occurs through a complex cell-cell interaction among T cells, antigen-presenting cells, endothelial cells, and synovium. The structural changes associated with RA are caused, in part, by contribution from increased NO production (84). Additionally, bioactive IL-17 is detected in RA and osteoarthritis (OA) synovial fluid and serves to stimulate the production of iNOS and NO levels and other catabolic enzymes in human chondrocytes, thereby resulting in decreased chondrocyte proliferation and proteoglycan synthesis. IL-17 together with (concomitantly or concurrently) IL-1ß and TNF, stimulate osteoblasts to secrete cytokines such as GM-CSF and IL-6, which in turn, regulate osteoclast and chondrocyte-mediated resorption and hence, bone and cartilage destruction (85). This interleukin, also directly plays a destructive role in disease progression, by inducing matrix metalloproteinases (especially MMP-1) in synoviocytes, which initiate tissue damage by proteolytic degradation of collagens and proteoglycans (86).

IL-17 can also play a pro-inflammatory role in the airways by recruiting and activating neutrophils (100). Exacerbations of obstructive airway diseases, including bronchial asthma and chronic obstructive pulmonary disease (COPD), have resulted in increased neutrophilic granulocytes that are not associated with any detectable infection. The neutrophil recruitment by IL-17 is mediated in part by CXC chemokine release or by induction of endogenous tachykinins that act on natural killer (NK)-1 receptors, to mediate neutrophil recruitment (47). In addition to neutrophil recruitment, IL-17 can also stimulate neutrophil activity in the airways, because it

stimulates release of neutrophil-activating cytokines IL-6 and IL-8 from bronchial epithelium and fibroblasts (68). The IL-17-induced release of IL-6 and IL-8 is potentiated by IL-1ß in bronchial epithelial cells, which in turn, can be regulated by IL-17, because increased IL-1ß is present in obstructive airway diseases (87).

Overproduction of IL-17 has been associated with several chronic disease conditions, suggesting that it play a role in these diseases. Several studies point to the association among IL-17, transplant rejection, systemic sclerosis, psoriasis, and promotion of tumor growth (71).

Blocking the effects of IL-17 using IL-17R:Fc inhibits proliferative responses of T cells to allo-antigens and also significantly prolongs major histocompatibility complex (MHC)-mismatched, and, nonvascularized and vascularized cardiac allograft survival in animal models (88). Systemic multiple sclerosis (SSc) is a connective tissue disease of unknown etiology characterized by fibrosis of the skin, lung, and gastrointestinal tract and by microvascular abnormalities of the skin and visceral organs. Increased IL-17 expression is seen in CD4+ T cells in the lymphocytes from peripheral blood and fibrotic lesions of the skin and the lung of affected patients (89). Similarly, a presence of IL-17 is seen in CD4+ and CD8+ T-cell clones derived from biopsies from lesional psoriatic skin but not in non-lesional control biopsies (90). Lastly, the expression of IL-17 is seen in >50% of ovarian, endometrial and cervical cancers (91). Furthermore, in selected cervical cancer cell lines, IL-17 exhibited angiogenic effects, as tumors from IL-17-transducted cell lines resulted in increased tumor size, possibly because of IL-17-mediated, increased expression of IL-6 and macrophage recruitment (91).

1.4 General hypothesis

Since IL-11 and IL-17 are mainly produced by $CD4^+$ T cells and stimulate the release of fibroblast-derived mediators, we hypothesized that IL-11 and IL-17 are involved in chronic lung rejection (Bronchiolitis Obliterates) and their expression could be a predictive or prognostic marker of chronic lung rejection.

1.5 Aims of the study

A) To measure the different cell counts in Bronchial alveolar lavage (BAL) and endobronchial biopsies from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome.

B) To examine the expression of IL-11 and IL-17 (m-RNA and protein) in endobronchial biopsies from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome.

C) To assess the correlation between the expression of IL-11 and IL-17 and the development of chronic lung rejection.

Chapter 2. Material and Methods

Chapter 2. Material and Methods

2.1. Patient's characteristics

STUDY POPULATION AND INCLUSION CRITERIA

Twenty lung transplant recipients were studied and two groups were selected based upon the patient's condition following the transplantation procedure.

Group 1 consisted of ten lung transplant patients, characterized as being clinically stable, 58-1585 days after transplantation. Their best postoperative lung function mean was (95% confidence interval). Their forced expiratory volume in one second (FEV_1) following transplantation corresponded to 98.9±1.9% of normal and, clinically and histologically, there was no evidence of acute allograft rejection in the patients within this group. Additionally, there was no airway infection present, one month prior to the study.

Group 2 consisted of post lung transplantation patients, afflicted with BOS. Ten patients resided in this group, and were diagnosed as having BOS. They were studied 252-1518 days after transplantation.

The diagnosis of BOS was based on a decline in FEV_1 of at least 20% from their best postoperative lung function mean percentage, as well as their best FEV_1 after transplantation, which corresponded to 52.2±9.5%. Other complications that could cause deterioration in lung function such as airway infection, acute bronchitis or muco pus on bronchoscopic assessment of the airways was not detected. Patients were afebrile, had no new chest crepitations or consolidations, had no change in leukocyte counts, and presented no changes in their chest radiographs. In addition, Gram staining showed no bacteria present, Cytomegalovirus cellular inclusions were not detected in any of the BAL fluid or biopsy samples, and histological diagnosis was confirmed.

Group 3 consisted of ten normal non-smoking volunteer subjects.

Immunosuppressive therapy for Group1 and 2 involved receiving a standard long-term maintenance regimen of cyclosporin (to achieve a blood level of 200-350 μg/l by EMIT assay; Syva, California, USA) azathioprine (1-2 mg/kg/day) and prednisolone (0.15-0.25 mg/kg/day).

Lung function tests used for analysis were performed in all the patients immediately before bronchoscopy. A computerised rolling seal spirometer (Sensor Medics 922, California, USA) was used to measure flow-volume loops. As part of the routine follow up after transplantation, regular spirometric tests were performed with the best forced expiratory volume in one second (FEV_1) after surgery being used as the benchmark standard against which to assess later development of BOS.

2.2. Bronchoscopy, bronchoalveolar lavage and biopsies

Bronchoalveolar lavages (BAL), were performed under sedation using a fiber optic endoscope and a total of 180 ml warmed (37°C) phosphate buffered saline solution was instilled in the right middle lobe or lingula in three 60 ml aliquots with immediate aspiration at a negative pressure of approximately -80 mm Hg after each instillation. Additional tissue samples for research purposes were collected from EBB specimens taken from the lower lobe subcarnae using alligator forceps. (Olympus FB 15C, Tokyo, Japan). An average of 6 samples was collected. Normal controls underwent the same procedure of BAL and EBB on a single occasion and ten ml pooled BAL fluid was retained for microbiological testing, the rest being immediately transported to the laboratory at 4°C for processing and analysis.

The BAL fluid total cell count was determined using a Neubauer counting chamber with unfiltered fluid. Cytospin preparations were made using a Shandon

Cytospin III centrifuge with 200 μl, unfiltered BAL fluid spun at 850 rpm for 10 minutes, and samples were stained with "Quik Diff".

BAL fluid cell differential counts were performed on duplicate cytospin preparations by counting 1000 cells.

The supernatants were obtained by filtering the pooled BAL fluid through a 200 μm nylon mesh, centrifuging at 1500 rpm for 10 minutes, and then transfer into 1.5 ml tubes to be stored at -80°C until they were to be used. EBB and TBB specimens were snap frozen in a liquid nitrogen chilled isopentane slurry, embedded with ornithine carbamyl transferase (OCT), and stored at -80°C.

2.3 Immunocytochemistry

Immunocytochemistry staining for neutrophils (elastase), CD68 marker for macrophage and CD3 marker for lymphocytes were performed using a monoclonal mouse anti-human neutrophil elastase antibody (Dako, Denmark), monoclonal mouse anti-human CD68 antibody (Dako, Denmark) and monoclonal mouse anti-human CD3 antibody (Dako, Denmark) respectively, and visualized using an Alkaline phosphatase antialkaline phosphatase method (APAAP method).

Isotype control immunoglobulins (IgG_1, Dako, Denmark) were used as negative controls. EBB specimens were processed into 10% formalin for two hours and embedded in paraffin using an automated tissue processor (Histokinette, Shandon).

Serial consecutive 3 μm sections were cut from the post-processed tissue and de-paraffinised in xylene, re-hydrated through graded ethanol solutions, and washed with TBS. The quality of the sections obtained was then assessed using standard haematoxylin and eosin staining.

The sections were then incubated for 20 minutes with 0.015% normal horse serum in TBS to prevent non-specific binding of immunoglobulins to tissue, covered with the specific antibody (diluted 1/600 in TBS) at 4°C in a moist chamber overnight, and then washed again with TBS. The sections were incubated with biotinylated horse anti-mouse antibody (Vector Laboratories, California, USA) for 30 minutes, treated with 0.12% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity, and then incubated for 30 minutes with a preformed avidin and biotinylated horseradish-peroxidase macromolecular complex. (VectaStain Elite ABC kit, Vector Laboratories Burlingame, California, USA). Diaminobenzidine was used as a substrate within this process (Dako, Denmark). EBB sections were then counterstained with haematoxylin and mounted in synthetic mounting medium.

In order to minimise any subjective variation in the interpretation of the slides, all staining was done by one person, and the slides were then coded and assessed by a blind observer. The total positive cells in the lamina propria of the airway wall and lung parenchyma in the EBB sections, respectively, were counted using a computerised image analyser (Video Pro 32, Leading Edge, Sydney, Australia) at a final magnification of ×400. The results were expressed as positive cells/mm^2 lamina propria or positive cells/mm^2 alveolar septa.

In the process of immunocytochemistry for IL-17 and IL-11, slides were immunostained with a goat anti-human IL-17 polyclonal Ab (R&D systems, Minneapolis, MN, USA) at a 1μg/mL concentration and with a goat anti-human IL-11 monoclonal Ab (R&D systems, Minneapolis, MN, USA) at a 1μg/mL concentration, respectively. Incubation with the first Ab was followed by incubations with a biotinylated rabbit anti-goat Ab (1/500; Sigma Chemicals, St Louis, MO) and then with an alkaline phosphatase-conjugated streptavidin solution (1/100; Sigma Chemicals, St

Louis, MO). Following this process, the slides were developed in Fast Red (1mg/mL; Sigma Chemicals, St Louis, MO) and were then counterstained in haematoxylin. For negative control preparations, the primary antibody was replaced by TBS or the irrelevant isotype, and species-matched primary Ab.

2.4. *In situ* hybridization

In situ hybridization (ISH) is the cellular localization of specific nucleic acid sequences (DNA or RNA), using a labeled complementary strand. The general principle of mRNA ISH is based on the fact that labeled single-stranded RNA (riboprobes) are hybridized intracellularly to mRNA, under appropriate conditions, thereby forming stable hybrids. The demonstration of mRNA within a cell provides valuable information about gene expression, and indicates possible synthesis of the corresponding protein. Although a number of techniques are based on the same principle of complementary sequence hybridization, such as Northern blots or polymerase chain reaction, the advantage of ISH over other techniques is that signal can be localized to a particular cell type within the tissue. We used *in situ* hybridization in order to detect the mRNA expression of IL-11 and IL-17 cytokines, within bronchial tissue.

Generation of single stranded RNA, complementary to mRNA requires the insertion of cDNA into an expression vector with promoter regions that recognize RNA polymerases. Using the appropriate restriction enzymes the vector is linearized immediately downstream of the insert. PGEM vectors contain a multiple cloning site flanked by two promoters, which allow transcription of the insert in alternate directions, thereby producing fragments that are either anti-sense (complementary to mRNA), or sense (with identical sequences to mRNA). To synthesize a single-

stranded RNA probe, the cDNA which is attached to the promoter site must be transcribed in the presence of nucleotides and the appropriate RNA polymerase (SP6, T7 or T3 polymerase). RNA probes are labeled with either radioactive or non-radioactive agents.

In preparation for *in situ* hybridization, biopsies underwent fixation in order to preserve the tissue in a morphologically stable state while retaining the maximal accessible mRNA within the cells. Tissue was placed immediately in freshly prepared 4% paraformaldehyde for 2 hr, followed by three washes with 15% sucrose in DEPC treated 0.1 M phosphate- buffered saline (PBS), pH 7.4 (first two washes for 1 hr at room temperature and then overnight at 4° C). Biopsies were then placed in OCT (optimal cutting temperature) embedding medium, snap frozen in isopentane precooled in liquid nitrogen, and stored at -80° C until further use.

In preparation for *in situ* hybridization cryostat sections, 10 μm thick, were cut from the each block and mounted on poly-L-Lysine (PLL)-coated microscope slides, in order to maximize tissue retention on the slides throughout the various rigorous treatments involved in *in situ* hybridization. Poly-L-lysine gives the slides a positive charge and provides firm adhesion of the tissue. They were then air dried for 1 hr, and left to incubate at 37° C overnight to allow for dehydration and maximum adherence of tissue. Fixation in freshly prepared paraformaldehyde maintains tissue morphology while allowing cellular penetration of the probe and thus efficient hybridization.

In this thesis, both anti-sense and sense probes were generated for IL-11 and IL-17 mRNA. The IL-11 and IL-17 probe were generated and inserted into a pGEM vector, linearized by SPH1 and EcoR1 restriction enzymes and transcribed in the presence of ^{35}S-UTP with T7 or Sp6 polymerase for the antisense and sense probes, respectively.

To perform *in vitro* transcription of radio-labeled probes, 1.0 μg/ml of linearized plasmid template, (either IL-11 or IL-17), was added to 10μl mixture of 5X transcription buffer (200mM Tris-HCl; pH 7.5, 30 mM $MgCl_2$, 10mM spermidine, 5 mM NaCl), 100 mM diethylthreitol, 25 U/μl of ribonuclease inhibitor, nucleotide mixture (2.5 μM of ATP, GTP, CTP), 25 mCu of ^{35}S-UTP and 10 units of RNA polymerase (T7, Sp6 or T3) and heated to 37°C in a waterbath for 60 minutes. The polymerase catalyzes transcription from its corresponding promoter on the vector and the nucleotides bind to the cDNA in complimentary fashion. RNase-free DNase is added to separate the RNA probe from the cDNA template, leaving single-stranded RNA fragments. To extract the probe from unincorporated nucleotides, 10 μg/ml of tRNA, 4M NaCl and phenol-chloroform (1:1, v/v) were added. After microfugation at 12 000g, the aqueous phase was removed and subjected to a second extraction with an equal volume of chloroform. The aqueous phase was treated with 100μl of 7M ammonium acetate (2.5 M final concentration) and 750 μl of cold (stored at –20°C or for two hours at -80°C. This mixture was microfuged at 4°C, the aqueous phase removed, except the last 50μl, which was placed in a speed vacuum centrifuge for dissipation. The purified RNA probe (the remaining pellet), was then dissolved in 20 μl of DEPC-treated H20. Incorporation of the radiolabel was assessed using a β–emissions counter by placing a 1 μl sample of the probe into 5 ml of scintilating fluid. Measurements were made in total counts per million (cpm) where 35S labeled probes were always $\geq 1.0 \times 10^6$ cpm.

In order to increase the efficiency of hybridization and to render target sequences more accessible to the probe, slides underwent prehybridization treatment allowing cell membrane permeabilization. Initially slides were immersed in 0.1M glycine/PBS and then in a 0.3% Triton-X-100/PBS solution, which degrades the cell

surface proteins, for 5 minutes. Slides were then placed in a 1μg/ml proteinase K solution, dissolved in 1M Tris-HCl pH 8.0 and 0.5M EDTA and PBS, for 20 minutes at 37°C to degrade intramembrane proteins. Further permeabilization and fixation was performed by placing the slides in a freshly prepared solution of 4% PF/PBS for 5 minutes. Pre-hybridization also includes steps for reducing non-specific binding. These were carried out by immersing the slides in high salt solutions of *1)* 0.25% acetic anhydride and 0.1M triethanolamide, and *2)* 0.1M N-ethylmalamide and 0.1M iodoacetamide, both dissolved in distilled water, and used at a temperature of 37°C for 10 minutes and 20 minutes respectively. Immersion in a mixture of 50% ionizing formamide in 4X standard saline-citrate (SSC) for 15 minutes at 37°C was used to regulate tissue equilibrium and stabilization. Finally, slides were dehydrated in increasing concentrations of ethanol (70%, 90% and 100%) for 5 minutes each and left to dry for a period of at least 2 hours.

In order to allow the hybridization of cRNA and cytoplasmic mRNA to take place, the radiolabelled probe was incubated with the pretreated tissue within optimal incubation conditions. A hybridization mixture was prepared consisting of hybridization buffer (50% deionized formamide, 5X Denhardt's solution, 10% dextran sulphate, 0.5% sodium pyrophosphate, and 0.5% SDS and 100mM dithiothreitol (DTT). To this buffer, 0.75 X 10^6 cpm/section of radiolabelled riboprobe was added as well as 10mM of DTT. Each tissue section was incubated with 15μl of this probe mixture preheated to 37°C. The sections were then covered with suitably sized dimethyldichlorosilane-coated coverslips. Slides were placed in a humid chamber and incubated overnight at 42°C allowing hybridization to take place.

Post hybrization washes are important as they determine the stringency of the *in situ* procedure allowing diffusion of material trapped in the section, and selecting for a

'good fit' between the probe and target mRNA. Once the coverslips were removed by gently immersing each slide in 4X SSC, slides were washed in three changes of 4X SSC at 42°C for 20 minutes each with gentle agitation. These are considered stringent conditions since both the temperature and salt concentrations were relatively high thus effective at reducing non-specific binding of the probe. The slides were then treated with 20μg/ml of RNAse, dissolved in 4M NaCl, 1M Tris and 0.5M EDTA, for 30 mins at 42°C. This was done in order to eliminate the excess unhybridized single-stranded cRNA, minimizing non-specific interactions. Subsequently, slides were put through decreasing concentrations of SSC washes of 2X, 1X, 0.5X and 0.1X SSC for 20 minutes at 42°C each, and then dehydrated in 70%, 90%, and 100% ethanol solutions with 0.3% acetic anhydride, for 10 minutes each at room temperature. The slides were then left to air dry at room temperature for one hour.

To visualize hybridization signals slides were dipped in liquid emulsion in a dark room under red light and left overnight to dry in complete darkness. Exposure took place in a light-proof sealed black box at 4°C for a period of 10-15 days. Following this exposure period, sides were developed under red light using Kodak D-19 developing solution at 16-18°C for 3.5 minutes with gentle agitation. This reaction was arrested by placing the slides in water. Fixation of the development was performed by immersing the slides in Rapid Fixer solution for 5 minutes at 16-18°C and then rinsed for 20 minutes in water. Using a straight razor blade, excess emulsion was removed and special care was taken to clean the underside of the slide beneath the tissue. Tissue sections were then counter-stained in hematoxylin, a blue basic dye that combines with acid substances and therefore stains the nuclei, creating a contrast for histological analysis with light field microscopy. Slides were then dehydrated in increasing concentrations of ethanol (70-100%) and finally immersed in xylene.

Subsequently, slides were coverslipped using entellyn which adheres the coverslip to the slide. When analyzing the slide under light microscopy, a positive signal is observed as a cluster of small black silver grains overlying the cell.

Proper controls are necessary in *in situ* hybridization experiments to assess the specificity of the probe and the procedures used. In this study we used negative controls such a sense probe or RNase pretreatment. The sequence of a sense probe is identical to that of mRNA and therefore replacing the anti-sense with a sense probe in the hybridization mixture should not result in a positive signal. Another control performed was to pre-treat the tissue with 100μg/ml of RNase A at 37°C prior to the prehybridization step, digesting all single stranded RNA. Loss of signal by RNAse pretreatment shows that the probe was interacting with digestable nucleic acids.

2.5 Statistical analysis

Differences between groups were determined using the Mann-Whitney U test and were considered significant when p values were less than 0.05. Data are expressed as means ± 1 SEM.

Chapter 3 Results

Chapter 3 Results

3.1. Inflammatory cells Immunoreactivity

3.1.1. Neutrophils

NEUTROPHILS IN BAL FLUID

All lung transplant recipients without BOS, lung transplant recipients BOS, and 20 normal controls underwent an adequate BAL procedure. The most striking feature of the BAL fluid data was a significant increase in neutrophil's immunoreactivity in the BOS group ($p<0.05$) versus both normal controls and lung transplant recipients without BOS (Figure 1).

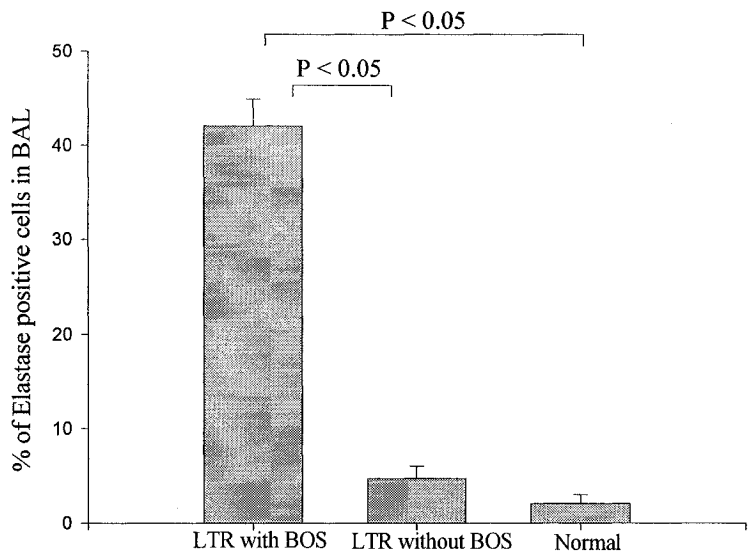

Figure 1: Number of Elastase positive cells (Neutrophil) in BAL from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome.

Neutrophils in Endoscopic bronchial biopsy

Neutrophil elastase staining was obtained from lung transplant recipients without BOS, from lung transplant recipients BOS, and normal controls. Neutrophil's immunoreactivity in the BOS group ($p<0.05$) versus both normal controls and lung transplant recipients without BOS is shown in Figure 2.

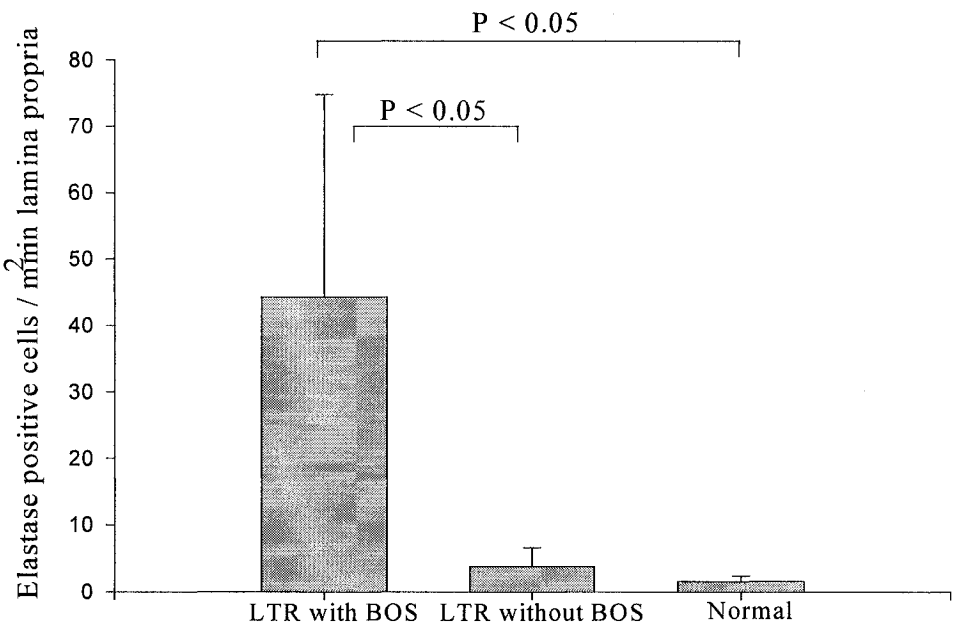

Figure 2: Number of Elastase positive cells (Neutrophil) in endobronchial biopsies from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome.

3.1.2. Macrophages

Reciprocally, the immunoreactivity of macrophages was lower in subjects with BOS than in normal controls ($p<0.05$) and in transplant recipients without BOS ($p<0.05$). This occurred both in BAL and EBB (Figures 3 A and B).

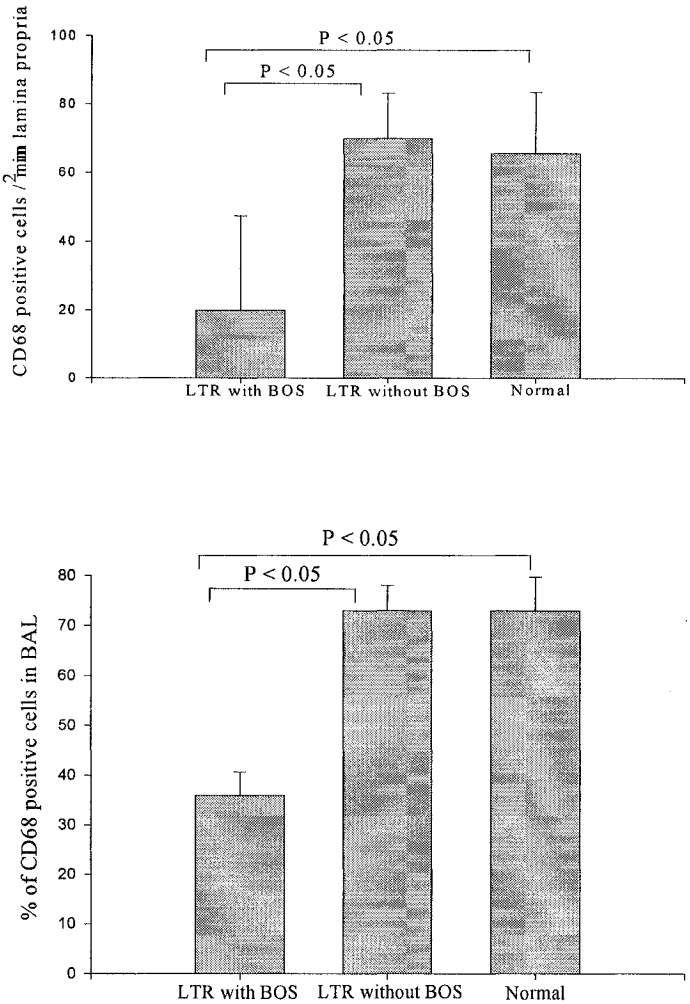

Figure 3: A) Number of CD68 positive cells (Macrophage) in endobronchial biopsies from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome (Top figure). **B)** Number of CD68 positive cells (Macrophage) in BAL from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome (bottom figure).

3.1.3. Lymphocytes

The immunoreactivity of lymphocyte positive cells was high in all groups, however the increases were not statistically significant (figures 4 and 5).

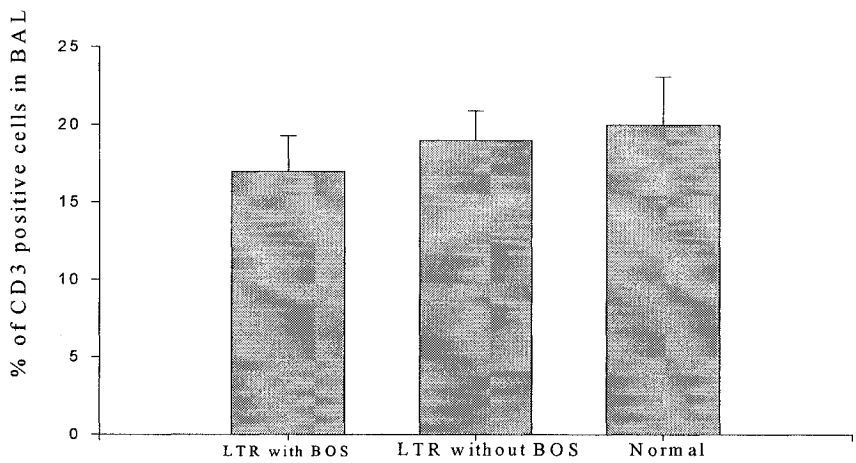

Figure 4: Number of CD3 positive cells (T cell) in BAL from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome.

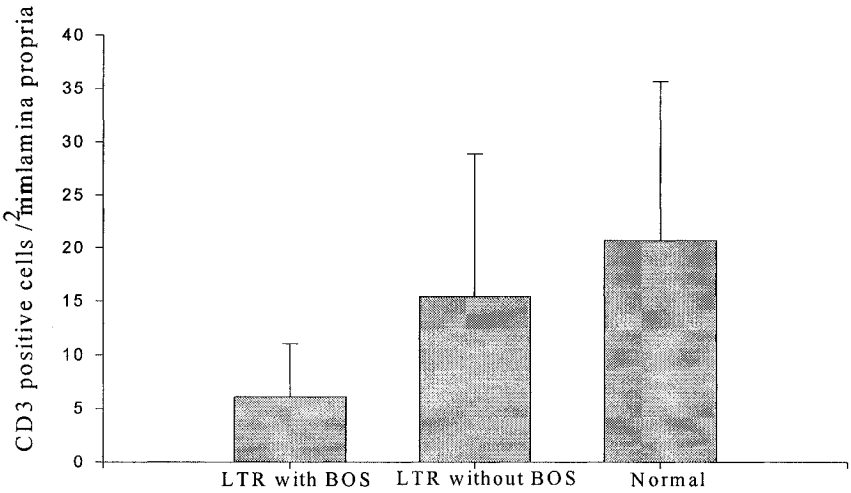

Figure 5: Number of CD3 positive cells (T cell) in endobronchial biopsies from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome.

3.2. Expression of IL-17 Immunoreactivity in EBB:

Cells positive for IL-17 immunoreactivity were found in EBB of normal, stable lung transplant and lung transplants with bronchiolitis obliterans (Figure 6). The number of cells expressing IL-17-immunoreactive protein was significantly higher in EBB ($p<0.05$) recovered from lung transplants with bronchiolitis obliterans compared to normal control and stable lung transplants. The morphology of the majority of IL-17-immunoreactive cells was consistent with T-lymphocytes particularly in lung transplants with bronchiolitis obliterans.

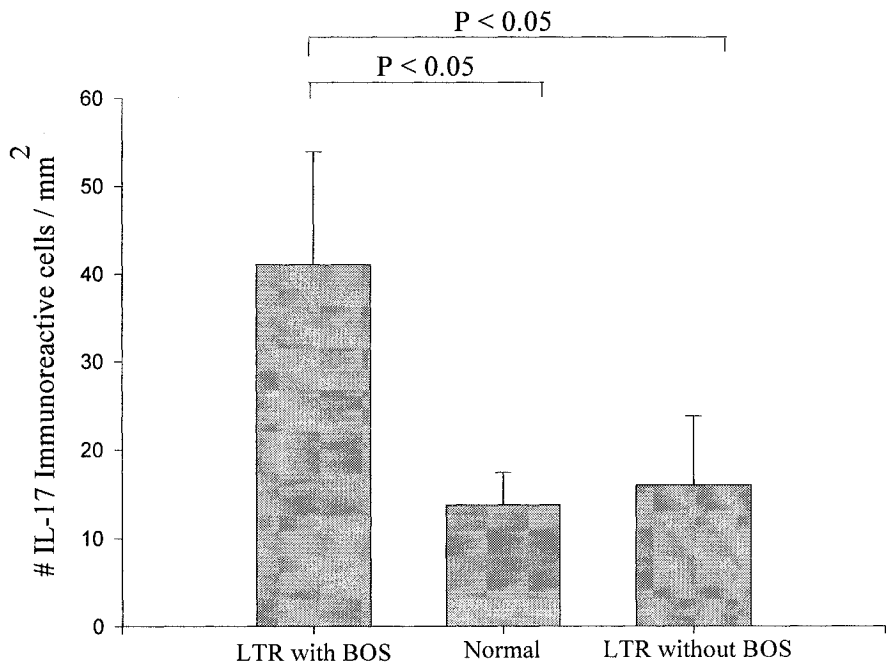

Figure 6: Number of IL-17 immunoreactive cells in bronchial biopsies from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome.

3.3. Expression of IL-17 mRNA

To assess local cytokine production at the site of lung tissue, ISH of cytokine mRNA in EBB was performed. The results of IL-17 mRNA hybridization, expressed as the number of positive cells/ mm^2 shown in (Figure 7), were significantly higher in EBB ($p<0.05$) recovered from lung transplants with bronchiolitis obliterans, when compared to those of normal control and stable lung transplants.

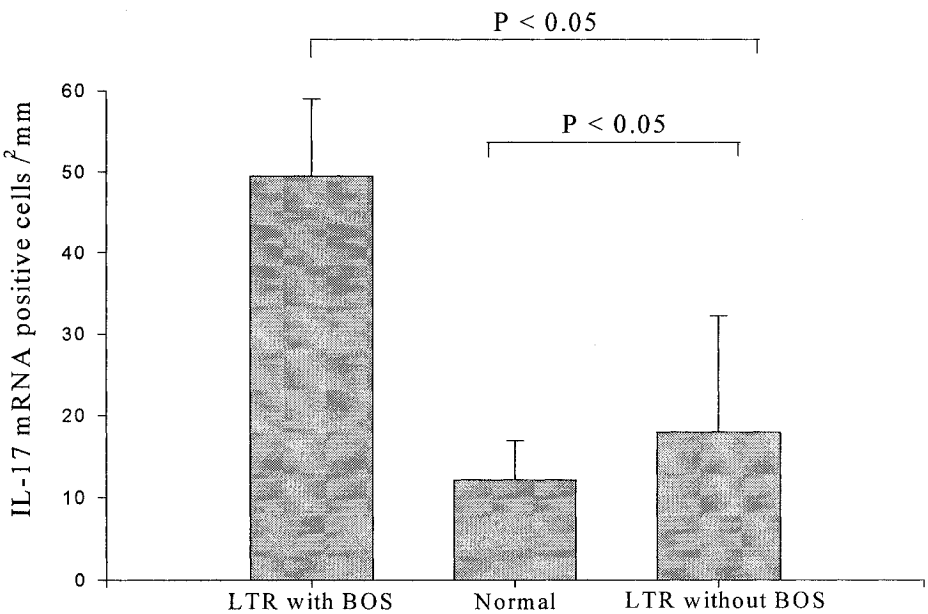

Figure 7: IL-17-mRNA expression in bronchial biopsies from normal subjects and in lung transplant recipient with or without bronchiolitis obliterans syndrome.

3.4. Expression of IL-11 Immunoreactivity

Cells positive for IL-11 immunoreactivity were found in EBB of normal, stable lung transplant and lung transplants with bronchiolitis obliterans (Figure 8). As well, the number of cells expressing IL-11-immunoreactive protein was significantly higher in EBB ($p<0.05$) recovered from stable lung transplants compared to normal control and lung transplants with bronchiolitis obliterans. The morphology of the majority of IL-11-immunoreactive cells was also consistent with T-lymphocytes and was particularly true in stable lung transplants.

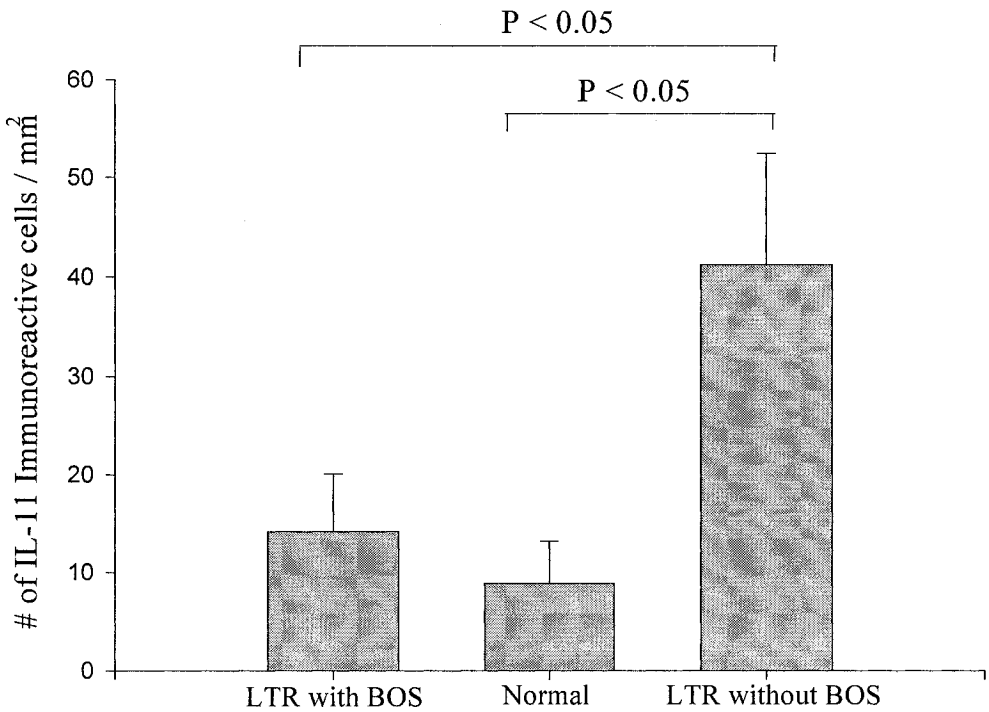

Figure 8: Number of IL-11 immunoreactive cells in bronchial biopsies from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome.

3.5. Expression of IL-11 mRNA

To assess local cytokine production at the site of lung tissue, ISH of cytokine mRNA in EBB were performed. The results of IL-11 mRNA hybridization, expressed as the number of positive cells/ mm^2 shown in (Figure 9), were significantly higher in EBB ($p<0.05$) recovered from stable lung transplants compared to normal control and lung transplants with bronchiolitis obliterans.

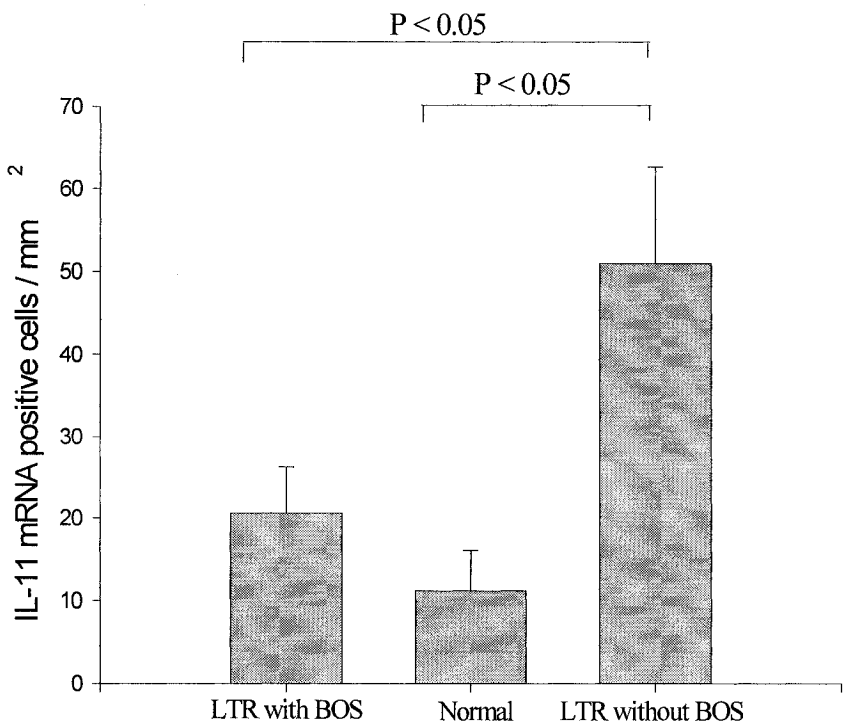

Figure 9: IL-11-mRNA expression in bronchial biopsies from normal and in lung transplant recipient with or without bronchiolitis obliterans syndrome.

Chapter 4 Discussion

Chapter 4 Discussion

4.1 Expression of IL 17 and IL 11 by Bronchial tissue post lung transplant.

Lung transplantation currently provides great hope for patients with end stage lung disease wishing to increase their life expectancy and quality of life. Current 5-year survival rates dictate that these procedures continue to be regarded as palliative rather than as curative interventions. The major obstacle to improved long-term outcomes remains the problem of chronic allograft rejection/bronchiolitis obliterans syndrome (BOS). Although it is widely accepted that the histopathology of BOS is characterised by excessive fibrous tissue obliterating small airways, the exact pathways leading to end stage BOS are far from being elucidated. In particular, the question of whether there is an association between well described tissue fibrosis and the Bronchoalveolar Lavage (BAL) neutrophilia that accompanies end stage BOS, is yet to be answered. Additionally, specific cytokine profiles that may offer clues to underlying pathological mechanism are yet to be determined. It is in this setting that we hypothesized that novel cytokines, IL-11 and IL-17, may be important in BOS development as they have been linked to fibroproliferative responses and neutrophil recruitment, respectively.

In this study, we investigated the expression of IL-11 and IL-17 in the airways of lung transplant recipients that had developed BOS, and compared this to stable lung from transplant recipients and normal control subjects. Interestingly, IL-11 expression was increased in the airways of both stable lung transplant recipients and BOS subjects. The levels being significantly greater in the stable lung transplant recipients. Conversely, IL-17 expression was dramatically increased in BOS airways. This data is strengthened by the fact that our findings were similar using both immunocytochemical and *in situ* hybridization techniques. IL-11 and 17 immunostaining within EBB tissue confirms translation of the mRNA into immunoreactive protein, whereas the presence of increased mRNA in the airway wall confirms the presence of

an ongoing active process. In addition, our results are in keeping with previous reports in other chronic inflammatory airway disorders, such as asthma, that have linked IL-11 to sub-epithelial fibrosis and myofibroblast accumulation, and IL-17 to macrophage and neutrophilic activation.

Although fibrosis and neutrophilic inflammation dominate the current immunopathological picture of BOS, very little is known regarding the exact underlying mechanisms driving these processes. This is so even if we accept that sub-optimally suppressed alloreactivity remains primarily responsible for the development of BOS. Our finding that IL-17 expression was primarily associated with T-lymphocytes supports previous evidence that IL-17 is primarily produced by T lymphocytes (92). Indeed, it has been shown that IL-17 production from T cells is mainly restricted to the memory T cell subset which maybe especially important in the setting of lung transplantation and persistent alloresponses (92). Although we did not perform CD4 and CD8 co-localisation studies, other investigators have shown that both of these T cell subtypes can be a source of IL-17 in their activated memory phenotype state. In addition, it has now been shown that IL-17 acts largely on epithelial and phagocytic cells resulting in marked increases in IL-8 production (93, 94). Hence, it is possible that this cytokine may be an important link between any ongoing alloresponses and persistent neutrophilic inflammation in the lung allograft complicated by BOS.

Similarly, our results have confirmed previous reports regarding the potential for IL-11 to be widely expressed in epithelial cells and in a wide variety of inflammatory cells. As this increased expression was particularly marked in stable lung transplant recipients but not in those who had developed BOS, it could be postulated that BOS subjects most likely differ from stable lung transplant recipients in the "driving signals" for IL-11 expression. In addition, the increased number of CD3+ and CD68+ cells in the airways of BOS subjects compared to

stable lung transplant recipients, suggests a potential link between these cells (or their subsets) and activating signals for IL-11 expression.

Given our divergent findings regarding both IL-11 and IL-17 in lung transplant recipients, it would be particularly useful to investigate whether the pattern of IL-11 and IL-17 expression in the airways of lung transplant recipients is mirrored by a similar pattern of expression their respective receptors and signal transduction pathways. Although, this information would support the role of these cytokines being important in chronic inflammation and fibrosis after lung transplantation, there still the paradox; we showed an increased of IL-11 expression in lung transplant recipient without BOS, whereas IL-17 was increased expression in airways of lung transplant recipients with BOS. One possible interpretation of these results is that IL-11 is a necessary but insufficient to drive BOS associated fibrogenic pathways and IL-17 may be marker of airways injury that have already been irreversibly damaged after lung transplant. Another explanation is that in lung transplant recipients with BOS, IL-11 and IL-17 expression may have increased dramatically just prior to BOS and decreased differentially post BOS development, so that our initial interpretation of our cross–sectional findings may be significantly flawed by the lack of temporal information. In either case, however, our findings clearly require further systematic investigation.

We also need to compare our results with previous studies, which examine the role of IL-11 and IL-17 in other inflammatory airway diseases, specifically asthma, and other diseases associated with chronic inflammation and connective tissue such as Rheumatoid arthritis and Scleroderma. Increased expression of IL-17 has been linked to CD4 helper T cells, to IL-6 and IL-8 release by bronchial epithelial cells, and to bronchial influx of neutrophils in experimental allergic asthma and in asthmatic airways. In Rheumatoid arthritis, increased IL-17 expression has been linked to both CD4 T helper and CD8 T cells, as well as to directly contributing to extracellular matrix degradation by inducing nitric oxide synthase and metalloproteinases in

chondrocytes; indirectly leading to a pro-inflammatory state by stimulating IL-1, IL-6 and TNF production.

The potential role of IL-17 in chronic inflammatory disease, including BOS post lung transplantation, is particularly exciting given that the large-scale sequencing of both the human and other vertebrate genomes has revealed that this cytokine and its cognate receptor belong to a "newly recognised" family of cytokines that can modulate immune function which have also been highly conserved throughout vertebrate evolution. The restriction of IL-17 production to T cells of the CD45RO+ activated memory phenotype and the strong link with neutrophil recruitment and activation suggests an important link between the adaptive immune system that remembers past encounters with antigen and the innate immune system which is primed for immediate host defence.

The conceptual links between IL-17 and the potential amplification of an allo-response that may lead to the development of BOS post lung transplantation is supported by experimental evidence. In a study by *Loong CC et al*, (95), it was demonstrated that IL-17 expression was strongly associated with the early development of human and experimental renal allograft rejection and that IL-6, IL-8 and neutrophilia tended to occur several days after the increases in IL-17 expression. In addition, *Tang JL et al,* (96) have shown that specific antagonism of IL-17 can inhibit acute vascular rejection in a mouse model of heart transplantation but was not sufficient to block neo-intimal formation in this model, suggesting that other factors are also critically important in the remodelling and fibrosis that often accompanies chronic inflammation.

Given the reported links between IL-17 and several other key cytokines including IL-6 and TNF, it is not surprising that IL-17 has also been reported to regulate IL-11 production to some degree. The exact extent of this relationship however remains to be elucidated, including the possibility of any negative feedback pathway and the potential links with other regulatory

cytokine loops. These relationships would be intriguing to map out given both our findings and the relatively recent results from IL-11 and IL-13 transgenic mouse models linking these cytokines with airway inflammation and remodelling (97). Two aspects of the IL-11 transgene model that are particularly interesting in light of our results, were the tendency for IL-11 to be most readily detected in the tissues with the most severe remodelling, and the tendency for IL-11 to inhibit inflammation to some degree while stimulating fibrosis. It could be speculated, therefore, that IL-11 elaboration in chronically inflamed airways is, in part, an attempt at airway healing.

In conclusion, the results of our cross-sectional study not only clearly demonstrate a potential role for IL-11 and IL-17 driven pathways in the airways of lung transplant recipients that are chronically inflamed, and therefore, susceptible to the development of BOS, but have also raised several questions as to the extent of this role, the specifics of the underlying mechanistic pathways, and the amenability of specific blockades to influence clinical outcomes following lung transplants. Furthermore, our results have contributed to the growing evidence from clinical studies linking these cytokines to chronic inflammatory disease and the increasing information from basic science investigators regarding the potentially critical role of these cytokines in modulating immune, inflammatory and fibrogenic processes.

4.2 Limitation of the study

The cross sectional nature of this study is clearly limited in delineating whether the associations described are cause or effect. Nevertheless, the dramatic differences seen within the 3 groups does suggest that examining the expression of these cytokines in a longitudinal study of lung transplant recipients may prove very useful. The prospective nature of such a longitudinal study would also allow for the better control of potential confounding factors such as variations in immunosuppression regimens and airway infection and the simultaneous examination of other key fibrogenic and inflammatory cytokines. Given the well described redundancy in cytokine pathways it is essential to place any increased expression of IL-11 and IL-17 in the airways of lung transplant recipients, within the context of other key fibrogenic and inflammatory cytokines such as TGF-β, FGF, IL-4, IL-13, IL-8 and GM-CSF. In addition, it has been shown that IL-11 and IL-17 can regulate the secretion of many of these cytokines in inflammatory tissues and so one will need to consider both the potentially direct and indirect effects of these cytokines in any overall analysis of their role in chronic inflammatory and fibrogenic pathways.

4.3 Future Study

The future directions of this work should in the first instance, address some of the issues raised in the limitations of our study. In particular, a properly conducted prospective cohort study of lung transplant recipients from the time of lung transplantation, and with frequent sampling time points, would greatly improve our understanding of the role of these specific cytokines in the airways of lung transplant recipients. Should such a longitudinal study confirm, and add weight to our cross-sectional findings, it would logically follow that further investigations would be warranted, and should include efforts to further explore the underlying

mechanistic pathways that may be involved in using in vitro systems and animal models. Another avenue of interest would revolve around providing specific blocs to IL-11 and IL-17 cytokines within the airways of lung transplant recipients; in order to assess their overall contribution to BOS and other key processes following post lung transplantation. With regards to the latter, one option would be to synthesise specific antagonists of the IL-17R which would hopefully have minimal cross-reactivity with other cytokine receptors given the relatively unique sequence of this receptor.

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