INNATE IMMUNE RESPONSES IN OBLITERATIVE BRONCHIOLITIS AFTER LUNG TRANSPLANTATION – THE ROLE OF STATINS AND HYPOXIA-INDUCIBLE FACTOR-1

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ACADEMIC DISSERTATION

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Helsinki 2015
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To my family
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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>AR</td>
<td>acute rejection</td>
</tr>
<tr>
<td>AZA</td>
<td>azathioprine</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BOS</td>
<td>bronchiolitis obliterans syndrome</td>
</tr>
<tr>
<td>CCL</td>
<td>CC chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CINC-1</td>
<td>cytokine-induced neutrophil chemoattractant</td>
</tr>
<tr>
<td>CLAD</td>
<td>chronic lung allograft dysfunction</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine A</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>DA</td>
<td>Dark Agouti</td>
</tr>
<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>extracorporeal membrane oxygenator treatment</td>
</tr>
<tr>
<td>FEV1</td>
<td>forced expiratory volume in one second</td>
</tr>
<tr>
<td>FoxP3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>GER</td>
<td>gastroesophageal reflux</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia inducible factor</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high-mobility group box-1</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon- γ</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP-10</td>
<td>interferon-gamma induced protein-10</td>
</tr>
<tr>
<td>IPF</td>
<td>idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>IRI</td>
<td>ischemia-reperfusion injury</td>
</tr>
<tr>
<td>ISHLT</td>
<td>International Society for Heart and Lung Transplantation</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N(omega)-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LT</td>
<td>lung transplantation</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MMF</td>
<td>mycophenolate mofetil</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NRAD</td>
<td>neutrophilic reversible allograft dysfunction</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PGD</td>
<td>primary graft dysfunction</td>
</tr>
<tr>
<td>OAD</td>
<td>obliterative airway disease</td>
</tr>
<tr>
<td>OB</td>
<td>obliterative bronchiolitis</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RAS</td>
<td>restrictive allograft syndrome</td>
</tr>
<tr>
<td>TBLB</td>
<td>transbronchial biopsy</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th cell</td>
<td>T-helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-a</td>
<td>tumor necrosis factor-a</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel Lindau</td>
</tr>
<tr>
<td>WF</td>
<td>Wistar Furth</td>
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</table>
ABSTRACT

Lung transplantation is the only effective treatment for selected patients with end-stage lung diseases and limited life expectancy and poor quality of life. Chronic lung allograft dysfunction (CLAD) is the major life-limiting factor after lung transplantation and bronchiolitis obliterans syndrome (BOS) is the most common subtype and best-characterized form of CLAD. BOS is a clinical diagnosis and it is defined as deterioration of lung allograft function in the absence of any other identifiable cause. Pathologically, BOS presents as obliterative bronchiolitis (OB) and it is characterized by peribronchial inflammation, epithelial damage, and obliteration of small and medium-sized bronchioli by fibrotic plaques. BOS is the leading cause of morbidity, lung allograft loss, and mortality after the first post-operative year. No specific treatment is available for clinical BOS at the moment.

Perioperative injury of the lung allograft leads to the activation of an immediate innate immunity response. Innate immunity plays a crucial role in the modulation of adaptive immunity. Activation of adaptive immunity responses results in acute rejection of the allograft. Repetitive rejection episodes are the best characterized risk factor for BOS. At present, immunosuppressive treatment mainly focuses on suppressing the adaptive immunity and this appears to be inadequate to prevent BOS. In this study, we hypothesized that inhibiting innate immune activation through different pathways influences the development of experimental OB. To test our hypothesis, we investigated different factors and pathways leading to experimental OB using both rat and mouse heterotopic tracheal allograft models.

In these models, the donor trachea is excised and transplanted into the subcutaneous pouch of the mouse or into the greater omentum of the recipient rat. There is a severe ischemic phase in the tracheal graft after transplantation before tracheal neo-vascularization develops. In this study, both the syngrafts and allografts showed severe epithelial injury and innate immune activation early after transplantation. It is likely that lack of alloantigens led to the resolution of the inflammatory response through activation of dominant tolerogenic T cells in syngrafts. The absence of alloimmune activation resulted in regeneration of the tracheal epithelium and the tracheal lumen remained completely open. However, in the presence of alloantigens, early ischemic injury induced both innate and adaptive immune responses followed by Th17 activation and afterwards by a sustained Th1 immune response. This was accompanied by infiltration of the allograft with proinflammatory effector cells that target the tracheal epithelium and lead to progressive fibroproliferation and total tracheal occlusion. Interestingly, recipient treatment with simvastatin, a cholesterol-lowering drug with lipid-independent immunomodulatory properties, enhanced early epithelial recovery after transplantation in the allografts. It also inhibited the infiltration of inflammatory cells and the expression of lymphocyte chemokines and proinflammatory cytokine mRNA. Most importantly, simvastatin inhibited the development of experimental OB in the absence of other immunosuppression.

The cellular responses to hypoxia are regulated by transcription factors called hypoxia inducible factors (HIFs). HIF-1 is a principle regulator of hypoxic adaptation, regulating gene expression involved in glycolysis, erythropoiesis, angiogenesis, proliferation, and stem cell function under hypoxia. In addition, HIF-1 plays an important role in inflammatory responses of myeloid cells.
but these effects may be either pro- or anti-inflammatory depending on the setting. In this study, we used the heterotopic mouse tracheal allograft model and fully major histocompatibility complex (MHC) mismatched recipients to investigate the effect of myeloid cell-targeted gene deletion of HIF-1α or its negative regulator pVHL in the recipients of tracheal allografts on the development of experimental OB. We found that continuous HIF-1 expression in myeloid cells improved epithelial recovery, reduced inflammatory cell accumulation, and increased regulatory FoxP3 mRNA expression in mouse tracheal allografts. Importantly, these effects led to better preservation of tracheal epithelium and a decrease in the development of experimental OB suggesting a protective role of HIF-1 in this constellation.

The results of this study suggest that the early ischemic injury and the following innate immune response play major roles in the development of OB. The role of statins should also be thoroughly evaluated in the treatment of lung transplant patients. In addition, it seems that despite the shortcomings of the murine heterotopic allograft model many advantageous features favour its use in OB investigation also in the future, until a significantly better method is presented.
INTRODUCTION

Lung transplantation (LT) is the only effective treatment for selected patients with end-stage lung diseases and limited life expectancy and poor quality of life. Chronic lung allograft dysfunction (CLAD) is a major life-limiting factor after lung transplantation. This review concentrates on the bronchiolitis obliterans syndrome (BOS), which is the most common subtype and best-characterized form of CLAD. BOS is a clinical diagnosis and it is defined as a deterioration of lung allograft function in the absence of any other identifiable cause, such as acute rejection, infection, or anastomotic complication (Estenne et al. 2001). According to the International Society for Heart and Lung Transplantation (ISHLT), the diagnosis of BOS requires a permanent 20% decrease in forced expiratory volume in one second (FEV1) in spirometry compared to stable post-transplant baseline values (Cooper et al. 1993, Estenne et al. 2001). However, it is still quite difficult to diagnose BOS reliably even with modern techniques. Pathologically, BOS presents as obliterative bronchiolitis (OB) and it is characterized by peribronchial inflammation, epithelial damage, and obliteration of small and medium-sized bronchioli by fibrotic plaques (Yousem et al. 1996).

Although the results of lung transplantation have markedly improved during recent decades, there are still major unsolved problems considering BOS. BOS is the leading cause of morbidity, lung allograft loss, and mortality after the first post-operative year (Yusen et al. 2013). Even with modern immunosuppressive treatment almost 50% of the recipients developed BOS five years after transplantation and within 10 years the percentage is still 76% (Yusen et al. 2013). Present immunosuppressive treatment modalities mainly concentrate on suppressing the adaptive immunity, especially T cell function and this appears to be inadequate. There is no specific treatment for OB/BOS and the exact etiology and pathogenesis of OB are not yet fully elucidated.

The perioperative period is associated with significant immune activation. A cytokine storm following donor brain death, donor infection, and possible aspiration episodes all contribute to the development of lung allograft injury. In addition, cold and warm ischemia during the preservation, transportation, and implantation of the lung transplant may enhance the injury (Christie, Kotloff et al. 2005; Daud et al. 2007). These events lead to the activation of an immediate innate immunity response. Innate immunity plays a crucial role in the modulation of adaptive immunity as it precedes and prepares the ground for adaptive immunity responses (Palmer et al. 2003, Land 2007). Activation of adaptive immunity responses causes acute rejection of the allograft. Repetitive rejection episodes might eventually lead to the development of BOS (Estenne et al. 2002, Hopkins et al. 2004).

We hypothesized that innate immune activation plays a central role in the development of OB. Using rat and mouse heterotopic tracheal allograft models, we investigated whether inhibition of innate immune activation through different pathways could influence the development of experimental OB. A special emphasis was placed on the heterotopic tracheal allograft model itself, innate immune response, the role of simvastatin treatment, and HIF-1-α expression.
REVIEW OF THE LITERATURE

1. Chronic lung allograft dysfunction

Chronic lung allograft dysfunction (CLAD) is a term for all cases of chronic deterioration of lung allograft function (Verdeleden 2014). Previously bronchiolitis obliterans syndrome (BOS) was considered the only form of CLAD, but several new subtypes of CLAD have been identified during the recent years (Sato et al. 2011). These include neutrophilic reversible allograft dysfunction (NRAD), fibrous BOS, and restrictive allograft syndrome (RAS) (Verleden et al. 2014, Sato et al. 2011). Patients with NRAD have neutrophilia in the airways, which may respond to azithromycin therapy (Verleden et al. 2013). On the other hand, patients with BOS have less inflammation and more fibrosis in the airways and due to this there is no response to azithromycin or other immunosuppressive treatment (Sato 2013). RAS is defined as a fibrotic process in lung parenchyma and produces more aggressive functional decline than conventional BOS (Verleden et al. 2014, Sato et al. 2011). The RAS phenotype of CLAD is considered when a patient shows an irreversible decline in lung capacity and meets certain criteria: FEV1 should be < 80% and total lung capacity (TLC) should be < 90% of the post transplant baseline values in spirometry (Sato et al. 2011). However, definition and diagnostic criteria of all CLAD subtypes are not yet fully established and universally accepted (Woodrow et al. 2010). A precise definition of CLAD is also to be determined (Meyer et al. 2014) and further investigation of the mechanisms and risk factors of the development of CLAD subtypes is needed. This review concentrates on BOS as it is the best-characterized form of CLAD and the experimental models we used were developed to study BOS.

2. Clinical lung transplantation

The first experimental lung transplantations (LT) were performed in the 1940s and 1950s, but the first human LT was performed by Dr. James Hardy and his surgical team at the University of Mississippi in 1963 (Hardy et al. 1963). However, early results were poor and post-operative mortality was universal due to surgical complications and lack of efficient immunosuppressive drugs. These problems resulted in a decline in lung transplantation activity until a proper immunosuppressive drug, cyclosporine A, was introduced. Dr. Cooper and his team performed the first successful single (1983) and double (1985) LT at the University of Toronto, Canada (Cooper et al. 1989). Together with improvements in the treatment of rejection, donor care, surgical techniques, intensive and other postoperative care, and antimicrobial therapy, LT was established as routine treatment for end-stage pulmonary diseases in the late 1980’s (Higenbottam et al. 1990).

2.1. Indications for lung transplantation in adults

According to the recent ISHLT Consensus document, patients who have chronic, end-stage lung disease and are considered for lung transplant operation should meet the criteria shown in Table 1. (Weill et al. 2015):
Table 1. General criteria for adult lung transplantation according to the ISHLT Consensus Document (modified from Weill et al. 2015)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;50% risk of death from lung disease within 2 years without transplant operation</td>
<td></td>
</tr>
<tr>
<td>&gt;80% chance to survive at least 3 months after operation</td>
<td></td>
</tr>
<tr>
<td>&gt;80% likelihood of 5-year survival after operation with adequate graft function</td>
<td></td>
</tr>
</tbody>
</table>

Usually, these patients are not amenable for medical treatment or conservative surgical/endoscopical treatment, i.e., endobronchial valve replacement or lung volume reduction surgery. The main goal of LT is to provide survival benefit. However, this treatment also significantly improves the quality of life of these patients. According to recent studies, especially physical health and every day functioning of the lung transplant patients is improved significantly during the first postoperative year and their physical quality of life is comparable to healthy population one year after the operation (Copeland et al. 2013; Singer et al. 2013). Unfortunately, psychological wellbeing of these patients may remain abnormal despite the operation (Copeland et al. 2013).

The main indications for adult LT are listed in Table 2. Two thirds of lung transplant recipients are 45 - 65 years old (Yusen et al. 2013). In the past 30 years, the median age of recipients has increased from 45 to 55 years. Only 10% of the recipients were over 65 and 3 % over 70 years old (Yusen et al. 2013).

Table 2. Indications for adult primary lung transplantation performed 1995-2012. Data from the Registry of the ISHLT 30th report (modified from Yusen et al. 2013)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic obstructive pulmonary disease (COPD)</td>
<td>34%</td>
</tr>
<tr>
<td>Interstitial lung diseases (ILD)</td>
<td>24%</td>
</tr>
<tr>
<td>Bronchiectasis associated with cystic fibrosis (CF)</td>
<td>17%</td>
</tr>
<tr>
<td>a1-antitripsin deficiency emphysema</td>
<td>6%</td>
</tr>
<tr>
<td>Idiopathic pulmonary artery hypertension (PAH)</td>
<td>2%</td>
</tr>
<tr>
<td>Others</td>
<td>17%</td>
</tr>
</tbody>
</table>
2.2. Contraindications for lung transplantation in adults

There are several absolute contraindications for LT and these are shown in Table 3.

**Table 3. Absolute contraindications for lung transplantation according to the ISHLT Consensus Document (modified from Weill et al. 2015)**

<table>
<thead>
<tr>
<th>Contraindication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreatable other major organ dysfunction (e.g. heart, liver, kidney and brain)</td>
</tr>
<tr>
<td>unless combined organ transplantation can be performed</td>
</tr>
<tr>
<td>Active malignancy in the last 5 years (except non-melanoma carcinoma of skin)</td>
</tr>
<tr>
<td>Uncorrected atherosclerotic disease with end-organ ischemia or dysfunction</td>
</tr>
<tr>
<td>Coronary artery disease not amenable to revascularization</td>
</tr>
<tr>
<td>Acute medical instability (e.g. sepsis, myocardial infarction, liver failure)</td>
</tr>
<tr>
<td>Uncorrectable bleeding diathesis</td>
</tr>
<tr>
<td>Chronic infection with highly virulent and/or resistant microbes</td>
</tr>
<tr>
<td>Evidence of active <em>Mycobacterium tuberculosis</em> infection</td>
</tr>
<tr>
<td>Severe chest wall or spinal deformity</td>
</tr>
<tr>
<td>Body mass index &gt; 35.0</td>
</tr>
<tr>
<td>Non-adherence to medical therapy (current or prolonged episodes)</td>
</tr>
<tr>
<td>Absence of an adequate social support system</td>
</tr>
<tr>
<td>Severely limited functional status with poor rehabilitation potential</td>
</tr>
<tr>
<td>Substance abuse or dependence (e.g. alcohol, tobacco, marijuana etc.)</td>
</tr>
<tr>
<td>Psychiatric or cognitive conditions associated with limited co-operation</td>
</tr>
</tbody>
</table>

There are also several relative contraindications for LT, such as age. There is basically no absolute age limit for lung transplantation and an operation may be considered for highly selected patients older than 65 years with no other severe comorbidities. However, patients > 75 years are unlikely to benefit from the operation in most cases (Weill et al. 2015). Chronic medical conditions without end-organ failure are not contraindications for transplantation (e.g. hepatitis B/C, HIV, diabetes mellitus), but these patients should be operated in centers with expertise in care of these patients (Weill et al. 2015). Other relative contraindications for lung transplantation are shown in Table 4.

**Table 4. Relative contraindications for lung transplantation according to the ISHLT Consensus Document (modified from Weill et al. 2015)**

<table>
<thead>
<tr>
<th>Relative contraindication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt; 75 years (age &gt; 65 years and associated low physiologic reserve and/or other relative contraindications)</td>
</tr>
<tr>
<td>Body mass index 30.0 - 34.9</td>
</tr>
<tr>
<td>Progressive or severe malnutrition</td>
</tr>
<tr>
<td>Severe symptomatic osteoporosis</td>
</tr>
<tr>
<td>Extensive prior chest surgery with lung resection</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
</tr>
<tr>
<td>Chronic extrapulmonary infection expected to worsen after transplantation</td>
</tr>
<tr>
<td>Atherosclerotic disease with the risk of end-organ disease after lung transplantation</td>
</tr>
</tbody>
</table>
Review of the Literature

2.3. Survival of lung transplant recipients

The ISHLT Registry contains data of over 47,000 adult lung transplants that were performed by June 30, 2013 (Yusen et al. 2014). Although survival after LT has improved over the past 25 years, long-term survival remains a challenge. In the latest ISHLT report from 1994–2011, the median survival was 5.6 years and survival at 1, 5, and 10 years was 79%, 53%, and 31%, respectively (Yusen et al. 2013). The half-life of a lung transplant is limited to approximately 5 years (Trulock et al. 2007). Fortunately, the overall survival of lung transplant patients has increased steadily during 2004 through 2011 (Yusen et al. 2013), and may be even better in single dedicated centers. Verleden et al. has reported up to 75% 5-year survival after transplantation in their unit (Verleden et al. 2007). However, according to the latest ISHLT report, improvement in survival is achieved during the first year after transplantation and unfortunately, long-term survival after the first year has not improved (Yusen et al. 2013). It should be noted that patients who have the most urgent need for a transplant due to their critical condition (i.e., pre-operative ventilator, and/or extracorporeal membrane oxygenator), have worse outcome after operation (Christie et al. 2011).

2.4. Complications and co-morbidities

Immediate: Primary graft dysfunction (PGD). PGD is a clinical diagnosis defined as severe impairment of oxygenation and diffuse radiological changes within the first 72 hours after lung transplantation (Christie, Carby et al. 2005). It is considered to result predominantly from ischemia-reperfusion injury (IRI) that was previously used to describe this clinical situation. Donor brain death, aspiration episodes, donor infection, cold ischemic preservation, and mechanical ventilation all contribute to the development of PGD (Christie, Kotloff et al. 2005; Daud et al. 2007). PGD is the leading cause of morbidity and mortality immediately after transplantation (Christie, Kotloff, et al. 2005, Daud et al. 2007). To diagnose PGD, other immediate complications, such as venous anastomotic obstruction, cardiogenic edema, pneumonia and hyperacute rejection have to be excluded (Christie, Kotloff, et al. 2005). Hyperacute rejection may occur within minutes or hours after transplantation, caused by preformed donor-specific antibodies.

Early: Infections are the leading cause of death during the first post-operative year (Christie et al. 2010). The risk for lung transplant infection is always increased, because of direct exposure to microbes by inhalation, impaired clearing mechanisms, and immunosuppressive medication. Severe infection or recurrent infections may contribute to the development of acute rejection (Khalifaf et al. 2004; Kumar et al. 2005). Approximately 10-15% of lung transplant patients suffer from different types of airway complications such as stenosis of the bronchial anastomosis (Machuzak et al. 2015).

Acute cellular rejection remains a common complication, especially during the first 6 months after transplantation (Bando et al. 1995). Acute rejection (AR) develops within days to weeks and according to the ISHLT Registry, it occurs at least once in 33% of the patients within the first year (Yusen et al. 2013). However, the severity of the manifestation of AR varies significantly. Many cases are clinically silent. Development in antibody detection techniques such as the detection
of donor specific anti-HLA antibodies (DSA) has revealed that antibody-mediated AR is more common than previously thought (Girnita et al. 2004; Cooper et al. 2011).

Late: Permanent immunosuppressive medication presents risks for lung transplant recipients. The required immunosuppression is associated with an increased risk for the development of renal dysfunction, bone-marrow depression, malignancies, hypertension, metabolic disorder, and osteoporosis. Within 5 years after transplantation almost 25% of recipients develop significant renal dysfunction with a major rise of creatinine levels, or require dialysis or renal transplantation. One or more of these renal complications were experienced by circa 40% of recipients during the first 10 years after the primary operation (Yusen et al. 2013).

BOS is a major life-limiting factor after lung transplantation. It is the leading cause of morbidity, lung allograft loss, and mortality after the first post-operative year. Within five years after transplantation almost 50% of recipients have developed BOS and within 10 years the percentage was 76% (Yusen et al. 2013). Patients with BOS have markedly inferior survival at already 5 years after operation compared to patients with no BOS (Valentine et al. 1996). A median survival after diagnosis of BOS is reported to be 3 - 4 years (Verleden et al. 2009).

2.5. Donor organs

According to the Registry of the ISHLT, the number of LTs per year worldwide is approximately 2100 and is the fastest growing field of solid organ transplantation (Christie et al. 2008). However, the number of LTs and its increase are limited due to the shortage of suitable donor organs. According to the report of the US Organ Procurement and Transplantation Network and the Scientific Registry of Transplant Recipients, donor lungs are harvested from only 15% of multiorgan donors (Chang and Orens 2006). Due to this, careful patient selection for the transplant operation is warranted. Different scoring systems seem to help determining proper allocation of the allografts (Osaki et al. 2009). On the other hand, shortness in the number of suitable donor lungs has also led to acceptance of less optimal/marginal donor lungs in some centers (Zych et al. 2014) and different strategies have been adopted to increase the amount of usable grafts (Valenza et al. 2014).

Some of the methods of extension of the donor pool are summarized in Table 5. Initial results are quite promising, but these methods are not yet widely accepted.
Table 5. *The most important methods of extension of the lung donor pool*

<table>
<thead>
<tr>
<th>Donor Comments</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Donors with extended criteria age &gt; 55 years, smoking history of &gt; 20 pack/year, less than optimal gas exchange in otherwise normal lungs <em>(Botha 2006)</em></td>
<td></td>
</tr>
<tr>
<td>2. Donation after circulatory death harvesting of organ after cardiac arrest <em>(Mason et al. 2008)</em></td>
<td></td>
</tr>
<tr>
<td>3. Lung resection large donor-recipient body size mismatch <em>(Puri and Patterson 2008)</em></td>
<td></td>
</tr>
<tr>
<td>4. Split lung transplantation the left lung is split into two separated lobes and implanted to the same recipient, the inferior lobe to the left and superior lobe to the right chest cavity <em>(Couetil et al. 1997)</em></td>
<td></td>
</tr>
<tr>
<td>6. Living lobar harvesting of the right and the left inferior lobes from two different healthy donors <em>(Puri and Patterson 2008)</em></td>
<td></td>
</tr>
</tbody>
</table>

Young adults (age 18-29 years) comprised 30% of donors in the recent ISHLT Registry *(Yusen et al. 2013)*. Only 1% of the donors were over 65 years old, but the mean age of donors has been increasing over the years *(Yusen et al. 2013)*.

However, the major limitation in the number of LTs has been the low amount of recipients in Finland. According to the Scandiatransplant registry, the number of patients listed for LT in Finland has been the lowest in Scandinavia during the past 10 years, as shown in Figure 1 *(Scandiatransplant Registry 2015, www.scandiatransplant.org)*. While the lack of referred patients may be explained in part by the very low prevalence of cystic fibrosis in Finland, this alone does not explain this low number of listed patients in Finland.

![Figure 1. The number of patients/million people listed for lung transplantation in Scandinavian countries during 2005 - 2014. From Scandiatransplant Registry 2015.](image-url)
3. **Bronchiolitis obliterans syndrome**

3.1. **Definition, clinical manifestation, and diagnosis**

Bronchiolitis Obliterans Syndrome (BOS) is a pulmonary manifestation of chronic lung allograft rejection (Estenne et al. 2002). The common manifestations of chronic rejection in allografts are chronic inflammatory and fibroproliferative processes. BOS can be diagnosed on clinical grounds during routine surveillance. The narrowing of airways causes shortness of breath, which is usually the first symptom. As the disease progresses, patients may suffer from continuous cough, wheezing and frequent respiratory tract infections (Verleden et al. 2009).

BOS is defined as lung allograft deterioration secondary to persistent airflow obstruction in the absence of other conditions that may alter lung allograft function, such as acute rejection and infection. It is characterized by progressive and irreversible decline in forced expiratory volume in 1 second (FEV1) compared to stable post-transplant baseline values. According to the ISHLT, the diagnosis of BOS requires a permanent decrease of 20% in FEV1 without other explaining factors (Cooper et al. 1993, Estenne et al. 2002). Clinical course in the development of BOS is variable. The median time from transplantation to the diagnosis of BOS is 16-20 months (Verleden et al. 2009).

One of the major clinical problems is the lack of reliable and repeatable methods to diagnose BOS. It is staged into four categories according to severity as shown in Table 6. Although the diagnosis of BOS is purely based on lung function measurements, transbronchial biopsies (TBB) are recommended for differential diagnosis, for example in the evaluation of acute rejection (Swanson et al. 2000). Although the specificity of TBB is reasonable high, patchy distribution of changes in the airway wall makes TBB quite insensitive (Kramer et al. 1993). According to previous reports, its sensitivity for BOS may be as low as 31% for each biopsy specimen, but it can be significantly increased by multiplying the amount of tissue specimens (Kramer et al. 1993; Reichenspurner et al. 1996). Unfortunately, lack of changes in TBB samples does not exclude BOS (Meyer et al. 2014). Bronchoalveolar lavage (BAL), and measurement of exhaled nitric oxide fraction can be also used as supportive diagnostic methods (Zheng et al. 2000; Gabbay et al. 2000). Chest imaging is an integral part of the evaluation and especially exclusion of other processes. Typical radiographic features of BOS are air trapping and bronchiectasis (Ikonen et al. 1996; de Jong et al. 2006). However, routine chest radiography is insensitive and non-specific for diagnosing BOS, but high-resolution computed tomography may detect typical changes more accurately (Meyer et al. 2014).

<table>
<thead>
<tr>
<th>BOS Grade</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>FEV1 &gt; 90% of stable post-transplant baseline value</td>
</tr>
<tr>
<td>0-p (probable)</td>
<td>FEV1 81-90%</td>
</tr>
<tr>
<td>1</td>
<td>FEV1 66–80%</td>
</tr>
<tr>
<td>2</td>
<td>FEV1 51-65%</td>
</tr>
<tr>
<td>3</td>
<td>FEV1 &lt; 50%</td>
</tr>
</tbody>
</table>
3.2 Pathology

Obliterative bronchiolitis (OB; bronchiolitis obliterans) is the histopathological manifestation of BOS and a histologic hallmark of chronic rejection (Stewart et al. 2007). The diagnosis of OB, but not of BOS, requires histological proof by TBB, open lung biopsy, or autopsy. Pathologically OB is restricted to small non-cartilagenous airways or respiratory bronchioles, and characterized by submucosal fibrosis of small airways that partially or totally occludes the airway lumen (Stewart et al. 2007). This scar tissue may be eccentric or concentric, it may be associated with breaks in the smooth muscle wall, and may also extend into the peribronchial interstitium (Yousem et al. 1996). OB is graded as present or absent, irrespective of the presence of inflammatory activity (Stewart et al. 2007).

3.3 Risk factors

There are several reported risk factors for BOS. Acute rejection (AR) is considered to be the most important risk factor (Meyer et al. 2014). However, the data available result mainly from retrospective analyses or the ISHLT Registry. In Table 7, suggested risk factors for BOS are listed.

Table 7. Suggested risk factors for BOS (modified from Meyer et al. 2014)

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute rejection</td>
<td>Multiple and severe ARs increase the risk (Yousem et al. 1991; Bando et al. 1995)</td>
</tr>
<tr>
<td>Lymphocytic bronchitis/bronchiolitis (LB)</td>
<td>Precursor of BOS, especially the highest grade of LB (Stewart et al. 2007; Glanville et al. 2008)</td>
</tr>
<tr>
<td>Primary graft dysfunction (PGD)</td>
<td>Severe PGD increases the risk of BOS (Daud et al. 2007)</td>
</tr>
<tr>
<td>Gastroesophageal reflux (GER) and microaspiration</td>
<td>Repeated airway injury may lead to BOS (King et al 2009)</td>
</tr>
<tr>
<td>Infection (viral, bacterial, fungal)</td>
<td>For example CMV and Pseudomonas aeruginosa may increase the risk. Activates immune responses. On the other hand, severe infection leads often to reduced and suboptimal immunosuppressive therapy (Bando et al. 1995; Reichenspurner et al. 1996; Botha et al. 2008).</td>
</tr>
<tr>
<td>Persistent BAL neutrophilia</td>
<td>Associated with the development of BOS (Henke et al.1999; Scholma et al. 2000)</td>
</tr>
<tr>
<td>Autoimmunity (collagen V sensitisation)</td>
<td>Associated with the development of BOS (Burlingham et al. 2007)</td>
</tr>
<tr>
<td>Antibody-mediated rejection</td>
<td>HLA mismatches and the development of anti-HLA class I and II antibodies are associated with BOS (Jaramillo et al. 1999; Palmer et al. 2002; Morrell et al. 2009; Safavi et al. 2014)</td>
</tr>
</tbody>
</table>
4. Pathogenesis of obliterative bronchiolitis

4.1. Lung allograft injury

Several factors contribute to the development of epithelial and endothelial damage during and after transplantation in the clinical setting, including donor brain death, cold preservation and warm ischemia, ischemia-reperfusion injury, surgical techniques, infection, and rejection (Carden and Granger 2000, de Perrot et al. 2003). In the donor, brain death leads to the activation of innate immune responses (Rostron et al. 2010). In addition, donors are often mechanically ventilated before brain death and lungs are exposed to aspiration and infections, which may contribute to a proinflammatory milieu. During the storage and operation lungs are temporarily without blood circulation, before new arterial and venous connections are established. After transplantation, ischemia-reperfusion injury and lytic induction therapy induce release a variety of pro-inflammatory factors, including macrophage-derived and epithelial-derived cytokines and chemokines (de Perrot et al. 2003). These factors, among others, contribute to an innate immune response that leads to bronchial epithelial damage, injury of the subepithelial structures, and injury of the vascular vessel endothelium in the allograft. This might be one of the key initiating events in the development of OB (Yousem et al. 1996).

The allograft airway blood supply is damaged during the lung transplant operation. This probably contributes to angiogenic remodelling. There is no clear evidence that lack of revascularisation of bronchial arteries would enhance the development of BOS (Langenbach et al. 2005). On the other hand, the loss of proper microvasculature most likely contributes to the development of OB (Luckraz et al. 2006; Babu et al. 2007; Jiang et al. 2011). However, also angiogenesis and neo-vascularisation are detected at the injured airways at the same time (Luckraz et al. 2006). These results refer to the complexity of vascular changes in the allograft and although vascular injury and remodelling have been linked to the development of OB, the exact relationship is not yet fully defined. To prevent or at least alleviate these events, different strategies for organ preservation and perfusion have been developed (Keshavjee et al. 1989; Thabut et al. 2001; Cypel et al. 2011). Unfortunately, none of these methods are able to prevent tissue damage completely.

Prolonged allograft ischemia and ischemia-reperfusion injury may increase the risk of acute rejection, and lead to the development of BOS (Fiser et al. 2002). However, in experimental heterotopic airway models of OAD, where allografts suffer from severe ischemia, OAD does not develop without alloimmune activation and injury (Koskinen et al. 1997).

4.1.1. Innate immunity

Innate immunity is the major contributor to acute inflammation induced by microbial infection or tissue injury (Akira et al. 2006). Innate immunity is developed during the evolution of man and predominantly does not in itself generate a memory response. It consists of the plasma complement system and circulating inflammatory cells that lead to inflammatory responses via different cascades. Innate immunity is an integral part of the host’s first-line defence against invading pathogens (i.e. bacteria, viruses, and fungi), but it is also activated during tissue injury of any kind (Land 2007). Many cell types are involved in the process. For example, neutrophils, monocytes, natural killer cells, dendritic cells and macrophages mediate the innate immune response (Janeway and Medzhitov 2002). On the other hand, both the epithelium and
endothelium also contribute to innate immune responses in normal lung (Grossman and Shilling 2009). It is generally accepted that innate immunity has an integral role in the alloimmune response to foreign MHC antigens (Palmer et al. 2003). Although innate immune cells do not recognize alloantigens, in the presence of tissue injury or pathogens, innate immune activation induces the maturation and migration of antigen presenting cells (APCs, such as dendritic cells) to secondary lymphoid tissues, leading to alloantigen-specific T and B cell activation. Without innate immune activation, alloantigen-specific adaptive immune activation is significantly decreased (Walker et al. 2006).

4.1.2. Innate immune cells

Innate immunity consists of several cell types, each contributing to the inflammatory response. Monocytes are circulating phagocytic cells that are able to internalize and ingest pathogens and different particles. A significant part of the bone marrow, splenic, and blood myeloid cells are monocytes and they rapidly migrate into sites of inflammation and give rise to dendritic cells or macrophages. It has been shown recently that monocytes migrate into a lung allograft before neutrophils and monocytes actually regulate the transendothelial migration of neutrophils (Kreisel et al. 2010). Macrophages produce chemokines and other inflammatory mediators and they are the most efficient phagocytes of the innate immune cells and the host’s first-line defense against invading pathogens. Both macrophages and DCs also play a key role in the development of adaptive immune response as they serve as APC.

Neutrophils are bone marrow-derived cells and also capable of phagocytosis and opsonisation of internalized bacteria and particles. They are amongst the first-responders to inflammation and an important source of chemokines and other mediators of inflammation (Morita et al. 2001). They also play a role in the development of IRI after transplantation (Kreisel et al. 2010). Neutrophils migrate to the site of inflammation/injury within minutes guided by chemotaxis. Neutrophils can adhere to the vascular wall with the help of adhesion molecules and transmigrate through the endothelium to the site of inflammation.

Natural killer cells (NK cells) are a subset of lymphocytes which can directly destroy virally-infected and tumour cells in an alloantigen-independent manner. They express germ-line encoded receptors which are able to recognize the loss of self-MHC class I expression in the virally-damaged or otherwise transformed cells (missing self theory, Ravetch and Lanier 2000). They are the major source of interferon-γ (IFN-γ) production, but they also produce other immunosuppressive or proinflammatory cytokines and chemokines. As they lack antigen-specific cell surface receptors (Vivier et al. 2011), they have been considered to be a component of the innate immunity system. However, recent findings have shown that NK cells also play a role in adaptive immune responses and they may be able to create an immunological memory (Sun et al. 2009; Vivier et al. 2011).

4.1.3. Pattern recognition receptors

Pattern recognition receptors (PRRs) are germ-line encoded receptors. They recognize and react to the ligation of different pathogen-associated molecular patterns (PAMPs) and also endogenous host derived damage-associated molecular patterns (DAMPs). During tissue injury, PAMPs and DAMPs are formed and released (Aderem and Ulevitch 2000; Janeway and Medzhitov 2002).
PAMPs consist of molecules such as bacterial lipopolysaccharides, lipoproteins, peptidoglycan, and viral RNA and DNA (Aderem and Ulevitch 2000). On the other side, DAMPs are molecules derived from the host’s own damaged cells. These include, i.e., fibrinogen, hyaluronic acid, heat-shock proteins, and high-mobility group box-1 (HMGB1) (Smiley et al. 2001; Yu et al. 2006). After proper ligation, PRRs activate down-stream signaling pathways. There are several distinct classes of PRRs in mammals (Akira et al. 2006). Toll-like receptors (TLRs) and complement receptors (CRs) are the best characterized subgroups of PPRs.

4.1.4. The Toll-like Receptor System

Toll-like receptors (TLR) play a major role in immunity against different pathogens. Dendritic cells (DC), mononuclear phagocytes, polymorphonuclear phagocytes, T lymphocytes, endothelial, and epithelial cells are equipped with TLRs (Land 2007). They are detected both on the cell surface and in intracellular compartments and at the moment there are 11 identified functional human TLRs (Farrar et al. 2013). Together these are able to recognize a basically unlimited number of different PAMPs. In addition, TLR2 and -4 also recognize DAMPs (Roach et al. 2005; Yu et al. 2006; 2010). Except for TLR3, all other ten TLRs signal through the adaptor protein called myeloid differentiation factor 88 (MyD88) (Takeda and Akira 2004). Stimulation of MyD88 is followed by downstream signaling cascades and production of proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6 and chemokines (Akira et al. 2006; Janeway and Medzhitov 2002). Stimulation also leads to upregulation of antigen presentation by APCs.

4.1.5. The Complement System

The complement system is a major player in the generation of the innate response. This system consists of a large amount of small proteins in the blood. Normally, these proteins are in inactive form. Complement is activated through three different pathways (Carroll 2004). The classical pathway is mainly antibody-mediated, but a wide range of molecules, including DAMPs, can activate the alternative pathway and the lectin pathways (Farrar et al. 2013, Wood and Goto 2012). Activation of any of these pathways leads to the activation of protein C3, which is the crucial trigger of the complement cascade (Farrar et al. 2013). Activation of this cascade causes chemotaxis and vasodilatation in tissues. At the end of the cascade, three different groups of complement effector molecules are formed. These effector molecules mediate killing (membrane attack complex) and phagocytosis (opsonisation) of pathogens. They also serve as ligands for complement receptors, which are found on leukocytes and parenchymal cells and this signaling mediates and promotes inflammation (Farrar et al. 2013). Complement activation is markedly enhanced in IRI and AR (Pratt et al. 2000; Farrar et al. 2006). In the presence of alloantigens complement signaling also enhances antigen uptake and stimulates B- and T-lymphocytes. Furthermore, the complement system is an effector in the development of antibody-mediated immune responses where interaction of the complement and antibody are essential for the process (Colvin and Smith 2005). This complex signaling system may alter the magnitude of the inflammatory response depending on the prevailing microenvironment. However, the understanding of these mechanisms is still limited. There seems to be cross talk between complement receptors and TLRs via intracellular signaling (Damman et al. 2011). DAMPs are supposed to play a central role in connecting these two systems (Farrar et al. 2013), shown in Figure 2.
4.1.6. Innate immune response and adaptive immunity

Both TLRs and complement receptors are expressed by immature APC. Activation of APCs such as DCs and macrophages provides a link between innate and adaptive immune responses (Land 2007). Although innate immunity responses precede and prepare the ground for the adaptive immune responses they are also crucial in the modulation of adaptive immune responses and due to this, in the development of rejection. (Palmer et al. 2003; Land 2007). An innate immune response is immediate, but its potency is limited, and generally the innate immunity response itself cannot reject the allograft in the absence of adaptive immune activation (Wood and Goto 2012).

4.2. Alloantigens, T cell activation, and allograft recognition

Rejection is the major obstacle to successful transplantation. After transplantation, the recipient’s immune system considers the donor organ as foreign and tries to remove it from the system. Perioperative injury to the lung allograft initiates innate immune responses that trigger the host’s adaptive immune cells to infiltrate the allograft. The presented foreign alloantigens are then recognized by the adaptive immune cells of the host eliciting acute allograft rejection (Rogers and Lechler 2001). The major histocompatibility complex (MHC) antigens are called major alloantigens. There are two different kinds of MHC molecules. The MHC class I molecules are found on the surface of almost every nucleated cell and they present peptide antigens originating from cytosolic proteins. The MHC class II molecules are expressed only on the APCs, such as dendritic cells, macrophages, B cells and certain activated endothelial and epithelial cells. The MHC class II molecules present peptide antigens derived from extracellular proteins. Human MHC is termed human leukocyte antigen (HLA).

Alloantigens are presented by APCs to recipient’s T cells. In this process, the T cell receptor (TCR) complex on the surface of a T cell interacts with the alloantigen which is presented by the MHC molecule. Each TCR recognizes a specific antigen. TCR engagement with an alloantigen-MHC complex is the primary requirement for T cell activation (signal 1). A co-stimulatory signal is also needed (signal 2). These two signals activate different pathways leading to upregulation...
and secretion of the T cell growth factor IL-2. IL-2 and other proproliferative cytokines (signal 3) initia clonal expansion of activated T cells (Whitelegg and Barber 2004). Antigen presentation occurs mainly in the allograft and in secondary lymphoid organs, i.e., lymph nodes and spleen.

Alloantigen-presentation can take place by three different mechanisms. In direct recognition, T cells recognise intact donor MHC molecules expressed by donor APC (Afzali et al. 2008). The indirect pathway is defined by the recognition of processed donor-derived peptides presented by recipient APC (Whitelegg and Barber 2004; Afzali et al. 2008). Semi-direct recognition occurs, when recipient APC catch intact donor MHC molecules by exchange of cell membrane fragments from the donor APC (Afzali et al. 2008). All of these pathways are active early after transplantation. They are summarized in Figure 3. The direct pathway leads to a strong alloimmune response rapidly after transplantation, and it has been suggested that acute allograft rejection is mainly mediated through this mechanism. The significance of the semi-direct allorecognition in rejection is not fully elucidated yet (Wood and Goto 2012). Direct and semi-direct allorecognition decrease afterwards, probably due to the decline of donor-derived APCs in the allograft. During the later postoperative course, the indirect allorecognition pathway is thought to be the most important pathway for late rejection episodes and chronic rejection (Illigens et al. 2009). It is also linked to the development of alloantibodies (Rocha et al. 2003).

Figure 3. The direct (A), indirect (B), and semi-direct (C) antigen presentation lead to the activation of recipient T cells. DC; dendritic cell, APC; antigen presenting cell. TCR; T cell receptor. Modified from Whitelegg and Barber 2004.
4.3. **Adaptive immunity, alloimmune response and rejection**

After activation, T cells start to differentiate, and multiple factors contribute to this process. The status of the microenvironment, the nature of the antigens leading to alloreognition, and additional costimulatory signals guide T cell development (Wood and Goto 2012). The adaptive immune responses may be proinflammatory or anti-inflammatory in nature and this process seems to be directed in part by the innate immune activation (Land 2007; Liu and Zhao 2007; Wood and Goto 2012). T cells can be divided into CD4+ T cells and CD8+ T cells. The CD4+ T cells function primarily as helper cells for other immune cells, whereas the CD8+ T cells identify foreign MHC class I molecules on target cells and are able to destroy them. Several classes of CD4 T cells have been characterized. Each of these subsets is characterized by their own set of unique transcription factors and every subset has also distinct cytokine profiles. The CD4 Th1 cells primarily induce cell-mediated immunity that is characterized by the production of proinflammatory cytokines such as IFN-γ, TNF-α, via production of transcription factor T-bet (Lehmann et al. 1997; Stinn et al. 1998; Miossec et al. 2009). These cytokines further activate monocytes and macrophages leading to the production of cytokines such as IL-2 and IL-12 (Afzali et al. 2008). The CD4 Th2 cells produce cytokines such as IL-4, IL-5, IL-10, and IL-13 (Miossec et al. 2009; Rocha et al. 2003), via production of transcription factor GATA 3. The Th2 immune response is associated with mucosal, allergic, and humoral immunity. The CD4 Th3 cells are responsible for mucosal immunity in the gut. IL-9 secreting CD4 Th9 cells are involved in mast cell activation and recruitment and T follicular helper cells are involved in B cell maturation in lymph nodes (Wood and Goto 2012). The CD4 Th17 cells mediate the production of cytokines such as IL-17 and IL-23, via production of transcription factor RORγt (Yuan et al. 2008). Th17 response is associated with host pathogen defense, autoimmune reactions and it has been also shown to play a central role in allograft rejection (Yuan et al. 2008; Miossec et al. 2009). The CD4+ T cells may also become regulatory in nature and thus inhibit and limit immune activation in antigen-dependent manner and they may be identified by the expression on transcription factor FoxP3 (Long and Wood 2009) and the secretion of cytokines such as IL-10 and TGF-β. Differentiation pathways for naïve CD4+ T cells are shown in Figure 3.

The activation and differentiation of T cells leads to the activation of an effector phase, which is defined by coordinated activity of CD4+ and CD8+ T cells, B cells, mast cells, macrophages, NK cells, polymorphonuclear cells, and their regulatory counterparts (Rocha et al. 2003). During the process, these cells migrate to the transplanted organ from the secondary lymphoid organ. The effector phase contributes to allograft rejection and, without proper immunosuppression, actions of the effector cells finally destroy the allograft.

Earlier it was believed that the Th1 response is mainly responsible for rejection. However, it has been recently reported, that Th2 and Th17 pathways also have a role in the development of rejection, especially of chronic rejection (Yuan et al. 2008; Chadha et al. 2011). It is also reported that a Th1 response might inhibit a Th2 response and vice versa (Miossec et al. 2009) and both responses can suppress the Th17 pathway (Wynn 2005). Different pathways also overlap and the balance can change along with the change of local polarizing conditions (Miossec et al. 2009). Recognition of alloantigens leads eventually to the development of life-long antigen-specific memory T cells (Jones et al. 2006). It has become clear that allograft rejection is a complicated process involving the balance between multiple proinflammatory and regulatory pathways and
that innate immune activation plays a central role in guiding this process (Land 2007; Wood and Goto 2012).

**Figure 4. Differentiation pathways for naïve CD4 + T cells and hallmark cytokines of each subtype.**

**4.4. Immunomodulation**

There are different populations of T cells that have regulatory activity and are capable of preventing transplant rejection. In addition to regulatory T cells, also B cells, macrophages, myeloid derived suppressor cells, dendritic cells, and mesenchymal stromal cells are reported to have tolerogenic activity (Wood et al. 2012). The microenvironmental conditions eventually dictate whether these cells contribute to rejection or promote tolerance (Wood et al. 2012). Tregs can suppress host immune responses against antigens (Liu and Zhao 2007) and contribute to the regulation of rejection. Although TLR-mediated signaling is mainly responsible for innate and adaptive immune responses, it may also regulate the function of Tregs (Liu and Zhao 2007). The balance of Tregs and CD4+Th cells is an important factor that dictates the nature of an alloimmune response. If the balance shifts towards tolerogenic Tregs, the development of rejection may decrease. It is not fully understood, which factors contribute to the development of a tolerogenic response. One of the common features of regulatory T cells is that these cells produce the cytokine IL-10 that favours the development of tolerogenic response (Rubtsov et al. 2008). It is also reported that the development of T cells towards Treg phenotypes requires the presence of TGF-β and a change in the microenvironment is an important factor directing immunomodulation (Afzali et al. 2007; Wood et al. 2012).

**4.5. Antibody-mediated rejection**

Antibody-mediated immunity as a significant contributor to lung allograft rejection has gained more interest in the recent years. Naïve B cells are able to recognize different antigens that are circulating in the blood or bound to the surfaces of microbes. Although some B cells are able to activate independently, the majority of B cells requires the help of T cells (Takemoto et al. 2004;
Wood and Goto 2012). However, B cells themselves can sometimes act as APCs and interact with T cells due to their expression of MHC class II and co-stimulatory molecules (Tarlinton et al. 2008). Recognition leads to clonal expansion and differentiation of B cells and during this process, B cells mature to antibody secreting plasma cells (Colvin and Smith 2005). These antibodies are specific for the recognized alloantigen and they are the effector molecules of the humoral immunity. They are able to neutralize antigens and mark them for the phagocytes. B cells also express complement receptors and they are able to recognize complement-coated cells (Carroll 2004). After the effector phase, some of the plasma cells transform to memory cells, capable of a quick response to the same antigen. If the immune system is to be exposed to the same antigen, again these cells contribute to the development of a rapid and strong alloimmune response. This immunological memory is highly beneficial against invading pathogens. However, in transplantations, immunological memory is harmful and may lead to acute rejection and PGD. If the recipient has preformed alloantibodies before the transplant operation, these antibodies may cause hyperacute antibody-mediated rejection (Glanville 2010).

4.6. Rejection and development of OB

An allograft suffers different injuries after transplantation. The number and severity of these injuries contribute to alloimmune activation and rejection episodes via innate immunity (McDyer 2007). Genetic factors of the recipient may also have an important role in determining the quality and magnitude of the immune responses (Lu et al. 2002) and the patient may be sensitized to the donor alloantigens prior to transplantation, further promoting a robust alloimmune activation. Together, all these factors contribute to the development of rejection episodes. While the current immunosuppression suppresses adaptive T and B cell responses, its use is accompanied by side effects and toxicity related to the immunosuppression. Furthermore, infections and other causes of graft injury such as aspiration may induce innate immune activation and thereby adaptive immune responses leading to allograft rejection and injury via either cell-mediated or antibody-mediated mechanisms. The rejection response may be clinically evident and as stated before, acute rejection is the leading risk factor for BOS (Yousem et al. 1991; Bando et al. 1995). However, many patients develop BOS without evidence of clinical acute rejection. It is likely that these patients had clinically silent smouldering alloimmune activation and allograft injury.

In the long run, repeated bronchial epithelial damage and injury of the subepithelial structures lead to activation and proliferation of fibroblasts and myofibroblasts. This leads to excessive fibroproliferation, due to ineffective epithelial regeneration and aberrant tissue repair (Yousem et al. 1996). Non-alloimmune mechanisms activate the innate immune response and thereby the adaptive immune response. This is why syngenic grafts do not develop BOS even if they suffer from aspiration, for example. Only mild obliterative changes are observed in experimental models of OB when the allograft lumen is lined with epithelium supporting the notion that continuous epithelial injury is central for the development of OB (King et al. 1997, Koskinen et al. 1997).
5. Immunosuppression after lung transplantation

Immunosuppression after lung transplantation remains a difficult issue. Optimal treatment should maintain a balance between infection and rejection. To prevent both acute and chronic allograft rejection, multi-drug therapy must be used to achieve control of multiple immune pathways. The current clinically used immunosuppressive medication primarily targets host T cell activation in different ways.

The so called “triple drug” immunosuppression is the cornerstone of anti-rejection therapy. It consists of a calcineurin inhibitor (cyclosporine or tacrolimus), an antimetabolite (azathioprine or mycophenolate mofetil/ mycophenolic acid), and corticosteroids (Korom et al. 2009). Calcineurin inhibitors inhibit the calcineurin pathway and prevent transcription of IL-2 genes involved in T cell activation. Antimetabolic agents have an effect on nucleotide metabolism and inhibit T and B cell proliferation. At the moment multiple combinations of different medications are possible. According to the ISHLT Registry, the combination of tacrolimus and mycophenolate mofetil (+ corticosteroid) is the most common immunosuppressive therapy for lung transplant patients (Christie et al. 2012). Other widely used combinations are tacrolimus and azathioprine, and cyclosporine (CsA) and mycophenolate mofetil (Christie et al. 2012). Mammalian target of rapamycin inhibitors, like sirolimus and everolimus, may be used to replace the calcineurin inhibitor or antimetabolite in some patients (Korom et al. 2009). In addition, at the time of transplantation, many centers use so-called induction agents, e.g., monoclonal antibodies (e.g. daclizumab, a blocker of the α-subunit of the interleukin-2 receptor) or antithymocyte globulin to deplete the recipient immune system in the immediate post-transplant period to prevent acute rejection (Ailawadi et al. 2008). The use of any type of induction therapy has increased in recent years, but there is no established protocol for the use or choice of these therapies (Christie et al. 2010). According to the ISHLT report, the amount of patients with acute rejection was highest with CsA-based regimens and lowest with tacrolimus-based regimens during the years 2004-2009 (Christie et al. 2010).

6. Treatment of BOS

Surgical techniques and immunosuppressive therapies in the treatment of lung transplant recipients have greatly developed since the early 1980s. However, no treatment for BOS is available. Present immunosuppressive medication is targeted mainly to the suppression of adaptive immune responses, but this does not suffice, as most lung transplant recipients experience late allograft dysfunction of some degree in the form of BOS (Belperio et al. 2003; Yusen et al. 2013). When BOS is fully developed, there is no efficient way to stop or reverse the process. The main therapy for clinical BOS is augmentation of immunosuppression or changing immunosuppressive medications within therapeutic classes, for example, converting CsA to tacrolimus (Meyer et al. 2014). More intensive immunosuppression, such as steroid boluses have also been used, but long-term high-dose corticosteroid treatment should be avoided because of its severe side effects and increased risk of infection (Meyer et al. 2014). All these treatments may stabilize lung function or delay the progression for a while in some patients, but the benefits and efficacy of these therapeutic modalities are inconsistent. There is also no clear evidence supporting one drug or a drug combination over another (Belperio et al. 2003; Meyer et al. 2014). Furthermore, intensified immunosuppression may not have any clear effect on BOS, if
there are no signs of ACR, antibody-mediated rejection, or neutrophilia in BAL samples (Meyer et al. 2014).

Therapies modulating immune response, such as extracorporeal photopheresis, and total lymphoid irradiation have been used with varying results in some lung transplant recipients with BOS, but the beneficial effects are based on retrospective cohorts and case-reports, and no prospective randomized data exists to support the use of these modalities (Slovis et al. 1995; Diamond et al. 1998). Anti-reflux surgery could be beneficial for the patients with proven gastroesophageal reflux (GER) at the onset of BOS, but again the results are contradictory at best (Cantu et al. 2004; Burton et al. 2009). Patients with neutrophilic reversible allograft dysfunction have neutrophilia in the airways, which may respond to azithromycin therapy (Verleden et al. 2013).

As a last option, lung retransplantation should be considered for patients with severe BOS. Nowadays the survival after retransplantation is comparable to first-time transplant patients in experienced centers, and evaluation for retransplantation is recommended for end-stage BOS recipients in the latest ISHLT guideline (Meyer et al. 2014). However, retransplantation is not appropriate for the majority of these patients and the evaluation process should be highly selective (Meyer et al. 2014).

7. Novel and experimental methods in the treatment of BOS

At the moment, it seems that the best way to deal with BOS is to try to prevent its development. During the transplantation procedure, injury to the lung transplant during brain death, procurement, surgery and IRI should be minimized. This might decrease the innate immune response and altogether these actions could also have an impact on the development of the adaptive immune response, rejection, and the development of BOS. Therefore, prevention of initial allograft injury may be one of the most important factors in the treatment of lung transplant patients and novel therapeutic options are needed.

7.1. HMG-CoA reductase inhibitors (statins)

Statins are a group of drugs that inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and effectively lower blood cholesterol. Statins inhibit the conversion of HMG-CoA to L-mevalonic acid, thereby preventing the synthesis of important isoprenylated proteins, which control diverse cellular functions. The so-called pleiotropic effects of statins are lipid-independent and have important vasculoprotective and immunomodulatory properties (Bonetti et al. 2003; Wang et al. 2008). Statins improve vascular endothelial cell function (Koh 2000; Endres et al. 1998; Tuuminen et al. 2011; 2013). Especially, statins are reported to have lots of highly varied anti-inflammatory effects, i.e., inhibition of cell adherence and movement, T cell proliferation, and alteration of cytokine production (Johnson et al. 2003). The inhibition of inflammation may be partially explained by HMG-CoA independent lymphocyte function-associated antigen (LFA)-1 inhibition on T cells (Weitz-Schmidt et al. 2001). This may lead to reduced adhesion and activation of lymphocytes (Weitz-Schmidt et al. 2001).
Endothelial dysfunction is characterized by impaired synthesis, and release and activity of endothelial-derived nitric oxide (NO). It has an impact on vascular relaxation and the reduction of NO production leads to vasoconstriction. NO also inhibits platelet aggregation and vascular smooth muscle cell proliferation and plays a role in the interaction of endothelium and leukocytes (Harrison 1997, Laufs and Liao 2000). Statin treatment is known to increase NO bioavailability through stabilization of endothelial NO synthase (eNOS) mRNA (Laufs et al. 2000). Both eNOS and inducible NOS (iNOS) are produced by pulmonary epithelial cells and may have both pro- and anti-inflammatory properties. Increased NO expression was reported during infection and BOS in endobronchial biopsies and exhaled air after lung transplantation (Gabbay et al. 2000). High levels of exhaled NO are also seen during eosinophilic airway inflammation, such as exacerbation of asthma (de Jongste et al. 2005). In contrast, NO stimulates the activity of thioredoxin, a known scavenger of reactive oxygen species (Haendeler et al. 2002). We have previously demonstrated that blocking NO production with aminoguanidine accelerates while supplementation of the NO pathway with L-arginine slows the development of OAD and decreases the rate of epithelial loss (Kallio et al. 1997).

Accumulating experimental evidence supports a beneficial role for statins also in the improvement of airway epithelial cell function during inflammation, and different pulmonary diseases (Johnson et al. 2003; Murata et al. 2005, Ahmad et al. 2011, Liu et al. 2014) but there is only little data on statins in lung transplantation and the development of BOS at the moment (Johnson et al. 2003; Li et al. 2006).

### 7.2 Hypoxia-inducible factor

Severe inflammation usually causes hypoxia in the target tissue. Metabolic demands of the cells increase during inflammation, and on the other hand, a reduction of metabolic substrates (oxygen for example) occurs at the same time (Eltzschig and Carmeliet 2011). During this event both the innate immune response and hypoxic response are activated (Rius et al. 2008). The cellular response to hypoxia is regulated by transcription factors called hypoxia-inducible factors (HIF) (Semenza et al. 1991; Semenza 2000). HIFs are the principle regulators of hypoxic adaptation, regulating gene expression involved in glycolysis, erythropoiesis, angiogenesis, proliferation, and stem cell function under hypoxia (Intiayz and Simon 2010). On the other hand, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is an essential mediator for inflammatory responses and the NF-κB pathway primes the HIF-1 pathway through transcriptional regulation of HIF-1α (Rius et al. 2008). This interplay increases HIF-1 mediated adaptive functions especially during hypoxic inflammation (Bruning et al. 2012).

HIF-1 is a master regulator of cellular hypoxic adaptation (Semenza 2000). It is a heterodimer transcription factor constituting of HIF-1α and HIF-1β subunits that is known to regulate the expression of multiple genes (Intiayz and Simon 2010). Both HIF subunits are produced at a constant rate and while HIF-1β is stable, HIF-1α is extremely fragile at normal oxygen concentrations and is rapidly degraded via an ubiquitin-mediated pathway (Salceda and Caro 1997). HIF-1α has an essential role in inflammatory responses of myeloid cells (Cramer...
et al. 2003; Doedens et al. 2010). Leukocytes of myeloid origin – including neutrophils, monocytes/macrophages and dendritic cells - are essential for both innate and adaptive immune responses. Cramer et al. have shown that HIF-1α is an important in the regulation of myeloid cell aggregation, invasion and motility (Cramer et al. 2003). Furthermore, the overexpression of HIF-1α in macrophages leads to enhanced phagocytosis (Anand et al. 2007). HIFs also support the innate immune functions of dendritic cells, mast cells and epithelial cells (Nizet and Johnson 2009). On the other hand, HIF-1 acts as an adaptive and survival factor for ischemic tissue (Semenza 2000; Prabhakar and Semenza 2012).

The Von Hippel Lindau protein (pVHL) is crucial for the execution of HIF proteolysis. Absence or genetic inactivation of VHL leads to stabilization of both HIF isoforms, HIF-1α and HIF-2α even under normoxic conditions (Jaakkola et al. 2001; Krieg et al. 2000). These both have different cell type-specific functions, which however, may partially overlap. For example, macrophages and neutrophils are able to express both HIF-1α and HIF-2α, but HIF-2α regulates mainly the key neutrophil functions (Poitz et al. 2014; Thompson 2014). pVHL has also functions not involving HIFs, as it can regulate intracellular junctions and also NF-κB signaling among other things (Calzada et al. 2006; Yang et al. 2007). However, most of the pathological alterations detected in VHL knock-out mice are developed due to constant activation of HIF signaling (Kapitsinou and Haase 2008).

The role of HIFs and VHL in solid organ transplantation is not fully understood, yet. Perioperative ischemia contributes to graft dysfunction and ischemia promotes sustained HIF-1 expression. HIF-1α plays an important role in the regulation of myeloid cell aggregation, invasion, and motility (Cramer et al. 2003). On the other hand, HIF-1 acts as an adaptive and survival factor for ischemic tissue (Semenza 2000; Prabhakar and Semenza 2012). Therefore, the final effect of HIF-1 might be dualistic and in any case, multifactorial.

8. Mouse and rat tracheal transplantation as a model for OB

There are several experimental animal models to investigate the development of acute and chronic rejection after lung transplantation (Hele et al. 2001; Sato et al. 2009). These are listed in Table 8.

<table>
<thead>
<tr>
<th>Model</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthotopic lung transplantation</td>
<td>Murine models: a single lung is transplanted to anatomical position in the recipient</td>
</tr>
<tr>
<td>Orthotopic tracheal transplantation</td>
<td>Murine models: trachea is transplanted to anatomical position in the recipient</td>
</tr>
<tr>
<td>Intrapulmonary tracheal transplantation</td>
<td>Murine and pig models: tracheal is transplanted inside the lung tissue</td>
</tr>
<tr>
<td>Heterotopic tracheal transplantation</td>
<td>Murine models: trachea is transplanted into the omentum (rat) or into a subcutaneous pouch (mouse)</td>
</tr>
<tr>
<td>Large animal lung transplantation</td>
<td>e.g. pigs; mimics human transplant operation</td>
</tr>
</tbody>
</table>
Hertz and colleagues were the first to describe the murine heterotopic tracheal transplant model in 1993 (Hertz et al. 1993). In this model, the donor trachea is inserted into a subcutaneous pouch in the recipient and the model is highly reproducible (Hertz et al. 1993). Later, this model was introduced to the rat (Huang et al. 1995). The difference between these two models is that in rats the tracheal graft is wrapped in to the greater omentum during laparotomy. Using mouse and rat tracheal allografts, it is possible to achieve accelerated development of OAD, histologically mimicking human OB (Hele et al. 2001; McDyer 2007; Sato et al. 2009).

Several factors contribute to the development of epithelial and endothelial damage during and after transplantation in the clinical situation (Carden and Granger 2000, de Perrot et al. 2003). Instead, in a non-vascularised rat and mouse tracheal transplantation model, revascularisation of the trachea is slower and this limits the immediate evaluation of reperfusion injury, but prolonged ischemia causes severe injury to the epithelium. In these models, there is an ischemic phase in the tracheal graft for the first 2 - 3 days after transplantation, before the tracheal neovascularisation develops (Hele et al. 2001, McDyer 2007). Due to prolonged ischemia, the respiratory epithelium undergoes ischemic damage in both syngeneic and allogeneic grafts (Hele et al. 2001). In syngrafts, the epithelium recovers, the lumen remains completely open, and the trachea is lined with normal epithelium in 30 days after transplantation. In allografts in non-immunosuppressed recipients, the epithelium sustains progressive damage leading to total loss of epithelium. Allografts develop a strong alloimmune response, that peaks at 10 days, culminating in the occlusion of the tracheal lumen at 30 days (Hele et al. 2001).

We chose to use the murine heterotopic tracheal allograft model in all experiments of this study, because our group has 20 years of experience with this model (the first study published by Koskinen et al. in 1997). Furthermore, the transplantation operation is rather simple, the learning curve is short, and almost all the operations are uneventful. Due to these reasons, the model is reproducible and the expenses are bearable.
AIMS OF THE STUDY

We hypothesized that innate immune activation plays a central role in the development of OB. We investigated whether inhibition of innate immune activation through different pathways could influence the development of experimental OB. To test our hypothesis, we investigated different factors and pathways leading to obliterative airway disease (OAD) using both rat and mouse tracheal allograft models. A special emphasis was placed on the heterotopic tracheal allograft model itself, the innate immune response, the role of simvastatin treatment, and the role of HIF-1 and VHL expression in the innate immune cells in the development of OAD.

The specific aims of the study were:

**Study I**: to characterize the innate and adaptive immune responses in OAD.

**Study II**: to dissect the role of simvastatin treatment in the development of OAD.

**Study III**: to investigate the effect of myelomonocytic cell-targeted deletions of HIF-1a or its negative regulator VHL on the development of OAD.
METHODS

1. **Heterotopic rat tracheal transplantations**

Specific pathogen-free inbred male Dark Agouti (DA; AG-B4, RT1a) and Wistar Furth (WF; AG-B2, RT1u) rats weighing 200-300 g and of 2-3 months of age (Scanbury, Sollentuna, Sweden) were used. The rats received care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Academy Press (ISBN 0-309-15400-6, revised 2011). Permission for animal experimentation was obtained from the Provincial State Office of Southern Finland.

A 3-cm long segment of the donor trachea was excised just above the bifurcation, perfused with PBS containing 10 000 IU/ml penicillin and 1000 μg/ml streptomycin, and stored in the same solution at +4°C until transplantation. In the recipient, the trachea was wrapped into the greater omentum and the abdomen was closed using absorbable continuous 3-0 sutures. Isoflurane (Isoflur, Baxter, Deerfield, IL) and buprenorphine (Temgesic, Schering-Plough, Kenilworth, NJ) were used for anesthesia and perioperative analgesia and buprenorphine was also used for postoperative pain relief.

Syngeneic tracheal grafts were transplanted from DA donors to DA recipients and fully-MHC mismatched allografts from DA donors to WF recipients into the recipient greater omentum, and the grafts were removed at 3, 10 and 30 days after transplantation. Non-transplanted DA tracheas were used as normal controls.

2. **Heterotopic mouse tracheal transplantations**

Allogeneic tracheal transplantations were performed from specific pathogen-free fully MHC-mismatched Balb/C (Scanbur, Sollentuna, Sweden) donors to mixed Sv129/C57B1/6/CB.20 recipients with HIF-1α or VHL gene deletion in the myeloid-cell lineage. Gene deletion confined to neutrophils and macrophages was achieved by mating the Sv129/C57B1/6/CB.20 mice containing loxP sequences on either side of the target gene (HIF-1α or VHL) with Sv129/C57B1/6/CB.20 mice carrying the LysMCre recombinase (Clausen et al. 1999). The result is mice that are deficient in either HIF-1α or VHL alleles in all myeloid cells (later defined as mHIF-1alpha⁻/⁻ or mVHL⁻/⁻). This was controlled by genotyping the animals used in the experiments. Littermates (LM) served as negative controls. All mice were male, weighed 25-30 g and were 2-3 months of age.

A 1.5-cm long segment of the donor trachea was excised just above the bifurcation, perfused with PBS containing 10 000 IU/ml penicillin and 1000 mcg/ml streptomycin, and stored in the same solution at +4°C until transplantation. In the recipient, the trachea was set into the abdominal subcutaneous pouch and the wound was closed using absorbable continuous 6-0 sutures. Tracheal allografts were heterotopically transplanted from Balb/C donors to MHC-mismatched recipients with HIF-1alpha (mHIF-1α⁻/⁻) or VHL (mVHL⁻/⁻) deficiency in myelomonocytic cells.
3. Drug regimens

3.1. Rat-experiments (studies I and II):

Cyclosporine A (study I): CsA (Novartis, Basle, Switzerland) was dissolved in Intralipid (KabiVitrum, Stockholm, Sweden) to a final concentration of 1 mg/ml. CsA was administered daily at a dose of 0, 1.0 or 1.5 mg/kg s.c. from the operation day until sacrifice. The doses were based on our previous dose-response study with this animal model (Koskinen et al. 1997).

Simvastatin (study II): Simvastatin (kindly supplied to us by MSD Finland, Helsinki, Finland) is a lipophilic member of the statin group. Simvastatin diluted in polyethylene glycol (PEG, molecular weight 300, Sigma-Aldrich, Steinhein, Germany) was administered at doses of 0.1, 0.5, 2, 5, to 20 mg/kg daily via nasogastric tube, throughout the study. Polyethylene glycol (PEG) was used as vehicle in control animals. In the simvastatin study, no immunosuppression was administered to avoid drug interactions.

L-NAME (study II): In order to clarify whether simvastatin mediates its effects via NO-activity, N(omega)-nitro-L-arginine methyl ester (L-NAME) was used to inhibit nitric oxide synthase (NOS) action. L-NAME was administered in drinking water after transplantation at a concentration of 1g/L.

3.2. Mouse-experiments (study III):

Tacrolimus: Animals received tacrolimus 0.75 mg/kg daily subcutaneously. According to our previous studies with this animal model this dose leads to partial occlusion of the tracheal allografts (Hollmen et al. 2008). Again, the idea was to try to delay the development of OAD rather than to prevent it totally. By this method, we were able to investigate if the role of HIF-1 in myelomonocytic cell is protective or deleterious in the development of OAD and if potential beneficial effects are still detected with mild immunosuppression.

4. Histological evaluation

For histological evaluation, sections were stained with Mayer’s hematoxylin and eosin (H&E). Histological changes in the respiratory epithelium were evaluated as the percentage of circumference not covered by epithelium. Luminal occlusion was evaluated as the reduction in luminal area using NIH Image program version 1.59 (U.S. National Institutes of Health, National Technical Information Service, Springfield, VA). All analyses were done in blinded review by two independent observers.

5. Immunohistochemistry

Immunohistochemistry was performed using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, United States) on serial frozen sections (4-6 μm). The specimens were counterstained with hematoxylin and cover slips were aquamounted (Aquamount; BDH Ltd., Poole, UK). The reaction was revealed by 3-amino-9-ethylcarbazole (AEC; Vector Laboratories). The number
of cells was recorded by counting positive staining cells/cross section using a grid and x400 magnification and moving the grid across the tracheal cross section in two perpendicular lines. The negative controls showed no immunoreactivity. Antibodies and dilutions used are shown in Table 9 (studies I-II) and Table 10 (study III). All analyses were performed in a blinded manner by two independent observers.

Table 9. Antibodies used in the studies I and II

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalogue number</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cells</td>
<td>554835</td>
<td>5 μg/ml</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>554854</td>
<td>5 μg/ml</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>ED1+ macrophages</td>
<td>22451D</td>
<td>5 μg/ml</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>ICAM adhesion molecule</td>
<td>ab33894</td>
<td>10 μg/ml</td>
<td>Abcam</td>
</tr>
<tr>
<td>Ki-67+ proliferative cells</td>
<td>NCL-Ki67p</td>
<td>8-12 mg/ml</td>
<td>Novocastra Laboratories Ltd</td>
</tr>
<tr>
<td>MHC class II</td>
<td>MCA46R</td>
<td>10 μg/ml</td>
<td>Serotec</td>
</tr>
<tr>
<td>MPO+ neutrophils</td>
<td>ab9535</td>
<td>20 μg/ml</td>
<td>Abcam</td>
</tr>
<tr>
<td>OX-62+ dendritic cells</td>
<td>MCA 1029G</td>
<td>10 μg/ml</td>
<td>Serotec</td>
</tr>
<tr>
<td>RECA-1</td>
<td>MCA970</td>
<td>50 μg/ml</td>
<td>Serotec</td>
</tr>
<tr>
<td>VCAM adhesion molecule</td>
<td>MMS-141P</td>
<td>1 μg/ml</td>
<td>Covance</td>
</tr>
</tbody>
</table>

Table 10. Antibodies and dilutions used in the study III

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalogue number</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO+ neutrophils</td>
<td>ab9535</td>
<td>20 μg/ml</td>
<td>Abcam</td>
</tr>
<tr>
<td>CD11b+ macrophages</td>
<td>557394</td>
<td>5 μg/ml</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>553043</td>
<td>5 μg/ml</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>553027</td>
<td>5 μg/ml</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD11c+ dendritic cells</td>
<td>ab33483</td>
<td>10 μg/ml</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

6. RNA isolation and quantitative real-time PCR

Total RNA was isolated from tissue samples dissected from tracheas using RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed with High-RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR (qRT-PCR) was performed on a RotorGene-6000 (Corbett Research, Doncaster, Australia) using 2X DyNAmo Flash SYBR Green Master mix (Finnzymes, Espoo, Finland). The amount of mRNA used for reverse transcription was approximately 100 nanograms. Measurement of the PCR product was performed at the end of each extension period. The mRNA quantities of the factors seen in Table 11 were measured from each group. Stability of the housekeeping genes 18sRNA, β-actin, GAPDH, and TBP was determined using geNorm application version 3.2 (PrimerDesign, Southampton, UK), and the most stable gene was chosen for normalization. Data was normalized against GAPDH (study I, phase 1 and study II), TBP (study I, phase 2), or 18sRNA (study III). The number of mRNA copies of the gene of interest was calculated from a corresponding standard curve using RotorGene (Corbet Research) software.
Table 11. The mRNA quantities of the factors shown in the table were measured from each group

<table>
<thead>
<tr>
<th>Molecule</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>innate immune ligands</td>
<td>hyaluronic acid synthase (HAS) 1, 2, 3</td>
</tr>
<tr>
<td></td>
<td>TLR 2, 4</td>
</tr>
<tr>
<td></td>
<td>HMGB1, NF-kb</td>
</tr>
<tr>
<td>inflammatory cytokines</td>
<td>TNF-α, IL-1β, LFA1, CINC-1, MCP-1, CCL20</td>
</tr>
<tr>
<td>dendritic cell maturation markers</td>
<td>CD86, CD83</td>
</tr>
<tr>
<td>secondary lymphoid chemokine and its receptor</td>
<td>CCL21, CCR7</td>
</tr>
<tr>
<td>Th1-related transcription factor and cytokines</td>
<td>T-bet, IFN-γ, IL-12 (subunit p35), IL-2, IP-10</td>
</tr>
<tr>
<td>Th2-related transcription factor and cytokines</td>
<td>GATA-3, IL-4</td>
</tr>
<tr>
<td>Th17-related transcription factor and cytokines</td>
<td>RORc, IL-6, IL-23, IL-17A, IL-17F, IL-17E</td>
</tr>
<tr>
<td>T-regulatory transcription factor and cytokines</td>
<td>Foxp3, IL-10</td>
</tr>
<tr>
<td>lymphocyte adhesion molecules</td>
<td>LFA-1</td>
</tr>
<tr>
<td>T cell differentiation factors</td>
<td>KLF2</td>
</tr>
<tr>
<td>costimulatory signaling</td>
<td>CD40, CD40L</td>
</tr>
<tr>
<td>endothelial peptides</td>
<td>ET-1</td>
</tr>
<tr>
<td>heme oxygenase enzymes</td>
<td>HO-1</td>
</tr>
<tr>
<td>growth factors</td>
<td>transforming growth factor (TGF)-β</td>
</tr>
<tr>
<td></td>
<td>platelet derived growth factor (PDGF) – a</td>
</tr>
<tr>
<td></td>
<td>connective tissue growth factor (CTGF)</td>
</tr>
</tbody>
</table>

7. Statistical Methods

All data are expressed as mean ± SEM. An appropriate parametric or non-parametric test was used for statistical evaluation, depending on the situation. Parametric Student’s T test was used for two group comparisons. ANOVA-test with Dunnett’s correction was used for parametric multiple group comparison. Analyses were made using SPSS for Windows version 15.0 (SPSS Inc., Somers, NY, IL). Non-parametric Mann-Whitney U-test was used for two group comparisons (study III). P ≤ 0.05 was regarded as statistically significant.
RESULTS

1. The development of obliterative airway disease in rat tracheal allografts (studies I and II)

First, we decided to validate the heterotopic allograft model for the aims of this study. We compared the histological changes of respiratory epithelium and the degree of luminal occlusion of tracheal syngrafts and allografts at 3, 10, and 30 days after transplantation. During the ischemic phase at 3 days, both syngrafts and allografts showed a marked loss of epithelium, and no significant difference was seen in the extent of epithelial loss between syngrafts and allografts (Fig. 1A, B, E). In syngrafts, epithelium rapidly recovered and trachea was already lined with epithelium at 10 days (Fig. 1A, C). At 30 days, the respiratory epithelium of syngrafts was fully recovered, and the grafts were filled with mucus, a sign of normal epithelial function (Fig. 1A, D). In allografts, there was an almost total loss of epithelium already at 10 days and no epithelial lining was detected in any of the allografts at 30 days (Fig. 1A, F, G).

![Figure 1](image-url)  
**Figure 1.** The development of epithelial injury in rat tracheal allografts. Heterotopic rat tracheal transplantation model. For histological evaluation, the tracheal sections were stained with Mayer’s hematoxylin and eosin (H&E). The histological changes in respiratory epithelium were evaluated as the percentage of circumference not covered by epithelium (A). Illustrative photomicrographs of tracheal epithelium (B-G) of the syngrafts and the allografts at day 3, 10, and 30. Original magnification x80. Data is expressed as mean ± SEM. n=6 per group. *P<0.05, **P<0.01, ***P<0.001. E, epithelium; L, lumen. Modified from study I.

In syngrafts, no tracheal occlusion occurred, and the overall histology resembled that of non-transplanted tracheas (Fig. 2A-D). In allografts, the luminal area was reduced by a progressive fibroproliferative lesion that almost totally occluded the lumen at 30 days (Fig. 2A, E-G).
2. Alloantigen dependent Th1 and Th17 responses emerged during the development of obliterative airway disease in non-immunosuppressed recipients (study I)

After the validation, we focused on the mechanisms of the development of OAD in this model. Allografts were compared to syngrafts, while syngrafts were compared to normal tracheas. As syngrafts have no foreign MHC antigens when compared to allografts, they may be used for the evaluation of the magnitude and mechanisms of innate immune response caused by ischemia and the operation itself without a superimposed alloimmune response as seen in the allografts. In syngrafts, significantly increased infiltration of ED1+ macrophages and CD8+ T cells was observed at 3 and 10 days and of CD4+ T cells at 10 days, when compared to normal nontransplanted tracheas. Innate immune activation was observed during the ischemic period in both syn- and allografts at 3 days. In the allografts, a more prominent infiltration of these cells and OX62+ dendritic cells was observed especially at 10 days (Fig. 3A-J).
Figure 3. The increased influx of inflammatory cells after ischemia was strongly alloimmune related. Here is shown the number (×100) of MPO+ neutrophils, ED-1+ macrophages, CD4+ T cells, CD8+ T cells, and OX-62+ DCs in cross-sections of normal tracheas and of syngrafts and allografts at day 3 (A-E) and at day 10 (F-J). Data is expressed as mean ± SEM. n=6 per group. *P<0.05. Modified from study I.

In syngrafts, the mRNA expression of proinflammatory, but also of tolerogenic cytokines was significantly upregulated (Fig. 4A, D, E, H), whereas Th1 and Th17 priming factors were downregulated (Fig. 4C, F, G). In allografts, prominent mRNA expression of proinflammatory cytokines was seen (Fig. 4A). Adaptive Th17 alloresponse was increased after the ischemic period at 3 days (Fig. 4C). Adaptive immune response modulated the expression of cytokines mainly towards Th1 at day 10 (Fig. 4F). However, the expression of tolerogenic Treg transcription factor FoxP3 was also significantly increased at that point (Fig. 4H).
Figure 4. Adaptive immune response modulates the expression of cytokines mainly towards Th1 during the development of OAD in non-immunosuppressed recipients. qRT-PCR analysis was performed and mRNA levels of innate and adaptive immunity cytokines and receptors were measured from normal tracheas and from syngrafts and allografts at day 3 (A-D) and 10 (E-H). Data is expressed as mean ± SEM. n=6 per group. mRNA data normalized against 18S rRNA. *P<0.05. Modified from study I.
3. **Cyclosporine A treatment reduced Th1, Th2, and Th17 responses and decreased the development of obliterative airway disease in tracheal allografts (study I)**

In the second phase of the study, we wanted to evaluate the effects of CsA treatment in tracheal allografts. CsA treatment prevented the loss of epithelium and the development of OAD in a dose-dependent fashion (Fig. 5A-B). Furthermore, CsA suppressed Th1, Th2, and Th17 responses but also inhibited the mRNA expression of tolerogenic factors CCR7 and Foxp3.

![Graphs showing loss of epithelium and luminal occlusion](image)

**Figure 5.** Cyclosporine A treatment decreased the loss of epithelium and the development of OAD in a dose-dependent fashion in allografts. CsA was administered daily at a dose of 0, 1.0 or 1.5 mg/kg s.c. from the day of operation until sacrifice. For histological evaluation, tracheal sections of allografts at 3 and 10 days were stained with Mayer's hematoxylin and eosin (H&E). The histological changes in the respiratory epithelium were evaluated as the percentage of circumference not covered by epithelium (A). Luminal occlusion was evaluated as the reduction in luminal area (B). Data is expressed as mean ± SEM. n=6 per group. *P<0.05. Modified from study I.

4. **Simvastatin treatment inhibited the development of obliterative airway disease (study II)**

First, we investigated whether simvastatin treatment inhibits the development of OAD 30 days after transplantation and whether the effect is dose-dependent. In the treatment groups, simvastatin doses were 0.1, 0.5, 2, 5 or 20 mg/kg daily, with the treatment commenced on the day of transplantation and continued throughout the study. Simvastatin treatment in the absence of background immunosuppression failed to prevent the total loss of epithelium at 30 days regardless of the dose. The lowest dose of simvastatin, 0.1mg/kg, had no effect on tracheal occlusion (Fig. 6A,C).

Simvastatin treatment with doses of 0.5, 2, 5, and 20 mg/kg daily attenuated tracheal occlusion at 30 days, when compared to vehicle-treated controls (Fig. 6A,D-G) and this effect of simvastatin was not dose-dependent.
Simvastatin treatment inhibits the development of OAD. Heterotopic rat tracheal transplantation model. Allograft recipients received simvastatin 0.1, 0.5, 2, 5 or 20 mg/kg or vehicle daily for a study period of 30 days. No immunosuppression was administered to avoid drug interactions. For histological evaluation tracheal sections were stained with Mayer’s hematoxylin and eosin (H&E). Luminal occlusion was evaluated as the reduction in luminal area (A). Illustrative photomicrographs of tracheal cross-sections (B-G), original magnification x40. Data is expressed as mean +/- SEM. n=6 per group. * P<0.05. Modified from study II.

5. Simvastatin treatment enhanced the proliferation and regeneration of the epithelium (study II)

Since the effect of simvastatin was dose-independent, for the second phase of the study we chose to investigate the beneficial effects of the clinically relevant dose of 0.5 mg/kg/d and a suprachanical dose of 20 mg/kg/d of simvastatin at the 3 and 10 day time-points. Both doses delayed the development of OAD similarly in the first phase of the study and we wanted to evaluate, if there were some differences in the mechanisms of epithelial recovery and in the immunological responses between these two doses.

Neither the lower nor the higher dose of simvastatin prevented the loss of epithelium due to ischemic injury at 3 days, but both doses enhanced epithelial recovery during the alloimmune response at 10 days (Fig. 7A). Immunohistochemical analysis revealed that early epithelial recovery was linked to a significantly increased number of proliferating Ki-67+ cells in the tracheal epithelium in both groups at 3 days (Fig. 7B).
**Figure 7.** Simvastatin treatment enhances the proliferation and regeneration of the epithelium in rat tracheal allografts. Allograft recipients received simvastatin 0.5 or 20 mg/kg or vehicle daily. Tracheal sections of allografts at 3 and 10 days were stained with Mayer’s hematoxylin and eosin (H&E) for histologic evaluation and immunohistochemically for ki-67+ proliferating epithelial cells. The histological changes in the respiratory epithelium were evaluated as the percentage of circumference not covered by respiratory epithelium (A). The number of positively staining ki-67+ cells was counted as positive cells in the tracheal respiratory epithelial lining using x400 magnification (B, grade; 0-3, 0 = no positive cells, 3 = nearly all positive cells). Data is expressed as mean +/- SEM. n=6 per group. * P<0.05. Modified from study II.

6. **Simvastatin treatment inhibited adaptive T cell alloimmune activation (study II)**

Both doses of simvastatin markedly reduced IL-23 mRNA and lymphocyte chemokine CCL20 production, and the infiltration of CD4+ and CD8+ T cells into allografts already at 3 days. At 10 days, a time-point focused on the adaptive immune response phase, simvastatin significantly attenuated the production of proinflammatory cytokines IL-1β, TNF-α, MCP-1, and IP-10, as well as Th17 polarizing cytokines IL-6 and IL-17e, and inhibited allograft infiltration by inflammatory cells. However, we did not detect any major differences in the mRNA expression of chemokines or proinflammatory cytokines between the two simvastatin doses at 3 and 10 days.

7. **Protective effects of simvastatin on inflammation and obliterative airway disease were partially mediated through nitric oxide synthase (study II)**

Some of the beneficial effects of the statin therapy are mediated via induced NO-activity. Therefore, in the third phase of the simvastatin study, L-NAME was used to inhibit NOS activity in simvastatin treated animals for a period of 30 days. We wanted to dissect whether the beneficial effect of simvastatin therapy is NOS-dependent and again we chose to evaluate the same 3, 10, and 30 day time-points. Co-administration of L-NAME alongside with simvastatin markedly increased the loss of epithelium of the allografts compared to the simvastatin-only therapy group at day 3. A total loss of epithelium was also seen in all L-NAME treated allografts at 30 days. Co-administration of L-NAME with simvastatin resulted in a markedly increased development of luminal occlusion compared to simvastatin-only receiving animals at 30 days but did not negate the beneficial effect entirely.
8. The development of obliterative airway disease was delayed in mVHL\(-/\) recipients of tracheal allografts (study III)

The effect of HIF-1\(\alpha\) on the development of OAD was investigated by studying HIF-1\(\alpha\) upregulation (VHL\(-/-\)) and HIF-1\(\alpha\) downregulation (HIF \(-/-\)) in monocyte/macrophages using recipients with HIF-1\(\alpha\) or VHL gene deletion in the myeloid cell lineage. We chose to evaluate the same 3, 10, and 30 day time-points as in previous studies. We decided not to compare mHIF-1\(\alpha\)/- and mVHL\(-/-\) to each other, because mVHL\(-/-\) leads to stabilization of both HIF isoforms, HIF-1\(\alpha\) and HIF-2\(\alpha\), but on the other hand, mHIF-1\(\alpha\)/- leads only to HIF-1\(\alpha\) deficiency. At day 3, during the ischemic phase, all tracheal allografts showed equally prominent respiratory epithelial cell injury (Fig. 8A). In mVHL\(-/-\) recipients, the epithelium was fully recovered at 10 days and remained partly preserved at 30 days (Fig. 8A), whereas a total loss of epithelium was seen in mHIF-1\(\alpha\)/- recipients and in littermates (LM) at 30 days (Fig. 8A). In mVHL\(-/-\) recipients, the airway occlusion was significantly decreased at 30 days in the absence of immunosuppression (Fig. 8B, E).

**Figure 8.** Development of OAD was delayed in mVHL\(-/-\) recipients (VHL-ko, knockout) of tracheal allografts. Tracheal allografts were heterotopically transplanted from Balb/C donors to MHC-mismatched recipients with HIF-1\(\alpha\) (mHIF-1\(\alpha\)/-) or VHL (mVHL\(-/-\)) deficiency in myelomonocytic cells. No immunosuppressive or any other treatment was given. The histological changes in the respiratory epithelium were evaluated as percentage of circumference not covered by respiratory epithelium (A). Airway occlusion was evaluated as the reduction in luminal area (B). Illustrative photomicrographs C-E (original magnification x 80) stained with Mayer’s hematoxylin and eosin (H&E). Data is expressed as mean +/- SEM. n=6 per group. * P<0.05. mHIF-1\(\alpha\)/- and mVHL\(-/-\) were not compared to each other.
9. The inflammatory response was reduced and T regulatory cell transcription factor was increased in tracheal allografts in mVHL-/- recipients (study III)

After the histological evaluation, we analysed the number of inflammatory cells in the tracheal cross-sections at 3 and 10 days. There was no difference between the three groups at day 3. However, the number of CD11b+ macrophages and CD4+ T cells was significantly reduced in tracheal allografts transplanted to mVHL-/- recipients at 10 days. Based on the histological and immunohistochemical results we decided to concentrate on mVHL-/- recipients to define the mechanisms of the delayed OAD development. qRT-PCR analysis was performed from the tracheal samples at the peak of the alloimmune response at 10 days. At that point, the mRNA expression of T regulatory cell transcription factor FoxP3 was significantly increased (Fig. 9A). Meanwhile, the mRNA expression of the Th17 related cytokine IL-6 and of MCP-1 was significantly reduced in mVHL-/- at the same time-point (Fig. 9B).

Figure 9. The mRNA expression of T regulatory cell transcription factor (FoxP3) was increased in tracheal allografts of mVHL-/- recipients (VHL-ko, knockout). Tracheal allografts were heterotopically transplanted from Balb/C donors to MHC-mismatched recipients with HIF-1α (mHIF-1α-/-) or VHL (mVHL-/-) deficiency in myelomonocytic cells. No immunosuppressive or any other treatment was given. qRT-PCR analysis was performed from the tracheal samples 10 days after transplantation (A-B). Data is expressed as mean ± SEM. n=6 per group. * P<0.05.

10. The development of OAD in tracheal allografts was not alleviated in mHIF-a+/ recipients in the presence of T cell immunosuppression (study III)

In the second phase of our study, we investigated how changes in HIF-1 activity affect the development of OAD in tracheal allografts in the presence of low-dose tacrolimus preventing T cell signal transduction and IL-2 production. In all allografts, an almost total loss of epithelium was seen at 30 days despite immunosuppression. However, OAD in tracheal allografts was significantly increased in mHIF-1α+ recipients compared to LMs at 30 days. There was no difference in the degree of preserved epithelium or occlusion between mVHL-/- and LM.
1. Rat and mouse heterotopic allograft models

According to Grossman and Shilling “the ideal model for studying lung alloreactivity would be one that is not technically prohibitive and that closely resembles both the natural pulmonary physiology, as well as pathophysiology of lung transplantation rejection both acute and chronic” (Grossman and Shilling 2009). Although new animal models of OB investigation are constantly being developed, an ideal model has not yet been presented (Grossman and Shilling 2009). As an idea, murine orthotopic single LT model could be the solution for the problem. Both rat and mouse orthotopic lung transplant models have been developed (Matsumura et al. 1995; Okazaki et al. 2007). The theoretical benefits of these models are clear. There is airflow into the transplanted lung, the graft is vascularized, and chronic lesions build up in the small airways during the follow-up (Matsumura et al. 1995). A single LT model in the mouse is a quite promising setup considering OB investigation (Okazaki et al. 2007) and it also allows different knockout modifications. However, orthotopic lung transplantations are technically far more demanding procedures compared to other more simple models, especially in the mouse. Due to this, the reproducibility of the OB lesions in both mouse and rat orthotopic lung transplant models is inconsistent (Hirschburger et al. 2007) and an orthotopic model over a major MHC mismatch is lacking. Additionally, chronic lesions seen in the grafts in the rat model are not typical OB lesions (Matsumura et al. 1995). Therefore, we still lack the optimal experimental model for studying OB development.

Because the the simplicity, both the rat and mouse heterotopic tracheal allograft models are widely used and accepted methods to investigate OAD. The use of these models has several advantages. Tracheal transplantation is technically a simple and reproducible method, and no baseline immunosuppression is required for the allografts to survive (Hele et al. 2001). Unlike most orthotopic lung transplant models, our model is performed over a major MHC mismatch (Hertz et al. 1993; Hele et al. 2001). In addition, allografts develop obliteratorive changes in an accelerated fashion (Hertz et al. 1993; Koskinen et al. 1997) and results of previous studies using this model have many similarities with investigations in humans (Hele et al. 2001; McDyer 2007; Sato et al. 2009). However, pathological changes of human OB are not seen in large airways and there is no airflow in heterotopically transplanted rodent tracheas, which may affect the epithelial function and pathophysiology. The graft circulation also differs from human lung allografts. In this model, the capillary network develops into the graft and the blood supply comes from the systemic circulation. Therefore, the grafts develop adequate circulation early after transplantation (Hele et al. 2001; McDyer 2007). Furthermore, tracheal allografts contain less lymphoid tissue, which may have an effect on immunogenicity. These limitations must be kept in mind when extrapolating our results. Despite the model’s shortcomings many advantageous features of the heterotopic allograft model favour its use in OB investigation, until a significantly better model is presented.

In our study we specifically brought out new features of the rat heterotopic allograft model. Our results shed light on the mechanisms and relations of innate and adaptive responses and help us to better understand the development of OAD in this particular model. Better understanding of the model will allow us and other investigators to plan future experiments even more carefully.
In addition, we might be able to draw more accurate conclusions from the results of forthcoming studies.

2. **Early ischemia and innate immune response in tracheal allografts**

Ischemia is currently unavoidable in transplantation procedures. The inflammatory response to ischemia is predominantly an innate immune response (Carden and Granger 2000; Linfert et al. 2009). Innate immunity is a genetically programmed reaction to tissue injury and the magnitude of the response is defined by the prevailing microenvironment and extent of injury and/or pathogen load. In our study, ischemia-induced changes were similar in syn- and allografts, suggesting alloantigen independency in early inflammation and underlining the role of innate immune activation in the early postoperative period. Although we can control adaptive immune responses to a certain extent with our current immunosuppressive therapies, we have very little control over innate immunity. On the other hand, the suppression of innate immunity also could attenuate the adaptive immune response and the development of CLAD. Due to the nature of transplant surgery, it is unlikely that one can totally prevent the expression of PRR ligands (PAMPs and DAMPs) during tissue injury. However, decreasing the activation and downstream signaling of PRRs might reduce innate immune responses. In our study, an increase in the mRNA levels of TLR2, a well described PRR (Barton and Medzhitov 2003), was seen in the syngrafts early after transplantation. Prevention of TLR activation could have an impact on the innate immune response. Nowadays, the investigation of TLR signaling pathways is highly active in the field of cancer research, as TLRs are reported to have both stimulatory and inhibitory effects in the development of malignancies (Wang et al. 2014). However, these mechanisms are not yet fully understood and further investigation of innate immunity activation is needed. The aim is to be able to manipulate TLR signaling pathways in a controlled matter. Regulation of MyD88, a key TLR signal adaptor could be one option. According to Walker et al., the absence of MyD88 reduces the adaptive immune response (Walker et al. 2006). On the other hand, reduced innate response might also be achieved with selective inhibition of TLR4-mediated cytokine production, including TNF-α and IL-6 (Li et al. 2006).

Although syngrafts showed severe epithelial injury and innate immune activation early after transplantation in our study, they recovered completely. It is likely that the absence of alloantigens guided the differentiation of naïve T cells towards a more regulatory phenotype in syngrafts compared to allografts. The observed increase in the mRNA expression of Treg-related FoxP3, IL-10, and TGF-β in syngrafts supports this hypothesis. Eventually, the regulatory response led to cessation of inflammation and allowed regeneration of the tracheal epithelium. On the other hand, in the presence of alloantigens, early ischemic injury and innate immune response were followed by robust adaptive immune responses in the allografts. In this model, the early ischemic injury is very extensive and not affected by our immunosuppressive therapies (Hertz et al. 1993, Hele et al. 2001, study I). The fate of the syn- and allografts is highly predictable and as mentioned before, very reproducible. Due to these facts, the beneficial effects of any pre-, intra-, or early postoperative therapeutical method or agent can very likely be detected in the transplanted grafts. Therefore this model seems to be useful also in the future for investigating the control of the innate immune response.
3. Adaptive immune responses and the development of obliterative airway disease

The innate immune response is not usually able to reject an allograft on its own and adaptive immune activation is required. In our study, the presence of foreign antigens in the allografts subsequently led to increased mRNA expression of proinflammatory cytokines as well as increased influx of inflammatory cells. These actions were followed by Th17 activation leading eventually to a sustained Th1 immune response. This was accompanied by infiltration of the allograft with proinflammatory effector cells that target the tracheal epithelium and contribute to epithelial injury. This is probably one of the most important initial events in the development of OAD (Yousem et al. 1996). The activation of adaptive immune responses also seems to increase tolerogenic activity in the allografts. Increased Foxp3 expression has been observed in endomyocardial biopsies of heart transplant patients as well as in the urine of kidney transplant patients suffering from acute rejection (Muthukumar et al. 2005; Dijke et al. 2007). Increased Treg activity may therefore be a sign of a systemic regulatory response against overactive inflammation. However, the proinflammatory cytokine milieu was more dominant in allografts compared to syngrafts in our study and probably overrode the regulatory component. Continuous alloimmune response led to irreversible epithelial injury followed by progressive fibroproliferation and eventually total occlusion of the tracheal lumen.

Most of the present immunosuppressive medication is targeted on the suppression of adaptive immune responses. Cyclosporine A inhibits T cell activation and proliferation. In our present study, CsA treatment prevented the development of OAD in a dose-dependent fashion, as previously shown (Koskinen et al. 1997, study I). Furthermore, CsA suppressed Th1, Th2 and Th17 responses. Since high doses of CsA can prevent OAD in this model, the T cell-mediated alloimmune activation is central for OAD development in this setting. It should be noted that CsA trough levels required to prevent OAD in our model are above 350 ng/ml (Koskinen et al. 1997), which is a toxic level in humans. CsA also prevented the tolerogenic signaling of T cells and this may be one of the reasons why despite enabling lung transplantation and preventing acute rejection, CsA-based immunosuppression has not affected the development of CLAD. CsA has also been linked to increased expression of the innate immune activators TLR2 and 4 (Lim et al. 2005). In our study, no apparent effect on innate immune activity was observed with CsA treatment. However, we did not observe any significant differences between innate immune activation of syn- or allografts. It is also unlikely that CsA could modulate the very early innate immune activation in the allografts in this model as there is no proper circulation in the allograft for the first 2-3 days after transplantation (Hele et al. 2001).

4. Simvastatin treatment and the development of obliterative airway disease

Previous reports state that statins have different anti-inflammatory effects in a murine model of allergic asthma and emphysema (McKay et al. 2004; Lee et al. 2005). Statins have important vasculoprotective and immunomodulatory properties (Bonetti et al. 2003; Wang et al. 2008). Especially, statins are reported to have lots of highly varied anti-inflammatory effects, i.e., inhibition of cell adherence and movement, T-cell proliferation and alteration of cytokine production (Johnson et al. 2003). They also inhibit MHC class II expression and downregulate...
alloantigen responses (Robertson et al. 2009). According to these reports, statins attenuate the innate immune response in tissue injury. However, there is only scarce data on statin treatment and lung transplantation at the moment. A previous retrospective study by Johnson et al. showed that patients treated with statins had better survival rates and less OB than patients who were not on statin therapy. However, this was not a controlled study but rather a retrospective analysis (Johnson et al. 2003). The possible role and mechanisms of action of statins in the prevention of OB remain unclear.

Due to the severe ischemic tracheal graft injury, there is an intense innate response in this model, as described earlier. Although statins do not reduce IRI in our model, we can investigate the changes in the subsequent inflammatory activation. It has been reported that simvastatin downregulates the epithelial release of proinflammatory and airway remodeling factors, which contribute to the airway injury (Murphy et al. 2008). In our study, simvastatin treatment led to increased regeneration of epithelium in the allografts. Statins are also reported to have vasculoprotective properties, which preserve the stability of endothelial cytoskeleton and decreased microvascular permeability and leucocyte extravasation (Lee et al. 2004; Tuuminen et al. 2013). These beneficial effects might explain why simvastatin inhibited allograft infiltration of macrophages, and dendritic cells, and also the expression of lymphocyte chemokine and proinflammatory cytokine mRNA in the allografts, referring to a decreased innate immune response. Probably due to this, reduced numbers of CD4+ and CD8+ T cells and an inhibition of the adaptive immune response were detected and the development of OAD was delayed. According to our results, simvastatin seems to regulate the adaptive immune activation via a reduced innate immune response. According to Watts et al., statins also reduce growth factor expression in a fibroblast model and this may have an effect on airway remodelling (Watts et al. 2005). However, we did not detect any beneficial effect of simvastatin on the mRNA expression of fibrosis related growth factors.

In our study, the effect of simvastatin was partially mediated by increased NO-activity. Restoration of NO-activity has been shown to effectively inhibit smooth muscle cell proliferation in models of pulmonary hypertension (Zuckerbraun et al. 2010)) and we previously reported that upregulation of NO production by L-arginine resulted in reduced OAD (Kallio et al. 1997). Thus, it is likely that simvastatin may also have inhibited smooth muscle cell proliferation through induction of NO-activity in our study. This is supported by our finding that statins reduced tracheal luminal obliteration even at later stages when total loss of epithelium was observed. L-NAME-mediated NOS inhibition resulted in accelerated epithelial loss in simvastatin-treated animals. This suggests that simvastatin promotes epithelial function at least partially through induction of NO-activity.

Preserved epithelium was linked to inhibition of OAD development in the heterotopic tracheal allograft model (Koskinen et al. 1997). The delayed loss of epithelium in the simvastatin group probably slowed down the fibroproliferative response and thereby the development of OAD in our study. However, although our results are promising, simvastatin treatment could only delay OAD development. Since no epithelium was left in the allografts at 30 days, it is clear that the simvastatin-treated allografts would eventually have become totally occluded. An important finding of our study was that simvastatin inhibited the development of OAD without any background immunosuppression. This is a highly relevant issue considering the strong
alloimmune response which develops in the heterotopic allograft model in fully-mismatched animals (Hertz et al. 1993). While simvastatin treatment alone is not sufficient to prevent OAD, our results suggest that lung transplant recipients may benefit from the pleiotropic effects of statin treatment. As we already have some evidence of the beneficial effect of statins after lung transplantation (Johnson et al. 2003) further clinical studies with statins should be planned. Furthermore, a decreased innate response in the lung transplant could prevent initial graft injury and thereby adaptive immune activation. Earlier, pravastatin was shown to increase early survival after orthotopic lung transplantation (Li et al. 2006). In addition, anti-inflammatory effects of statins could have an impact on the decrease of acute rejection episodes in the follow-up.

In clinical use, simvastatin and other statins have less severe adverse effects than traditional immunosuppressive drugs. According to our results and other evidence mentioned earlier, it may be possible that simultaneous use of statin after transplantation could also reduce the need of immunosuppression in humans. For these reasons, the role of statin therapy in lung transplant patients should be re-evaluated. Furthermore, preoperative donor treatment with statins was shown to decrease IRI, adaptive immune activation, and cardiac allograft vasculopathy in experimental heart transplantation (Tuuminen et al. 2011). This effect should be investigated in the context of lung transplantation using an orthotopic lung transplant model well-suited for IRI-studies. This would enable studying different statins and determining which statin has the best therapeutic profile or whether there is a class effect. Given the well-known safety profile and inexpensive cost of statins, translational clinical trials are also highly feasible. In order to assess whether donor statin therapy reduces IRI after transplantation, our program has already initiated a prospective randomized study of donor statin therapy in heart, lung, liver, and kidney transplantation with the title “The impact of donor statin therapy on the development of infections and chronic rejection” (Donor Simvastatin Treatment in Organ Transplantation (SIMVA), ClinicalTrials.gov Identifier: NCT01160978).

5. **HIF-1 overexpression in recipient myelomonocytic cells increases tolerogenic T-cell activity**

In our study, we show that constant activity of HIFs in myelomonocytic cells of the recipients reduced inflammation in the allografts in otherwise non-immunosuppressed recipients. This result was somewhat surprising as prior reports have implicated a central role for HIF-1 in promoting inflammation (Cramer et al. 2003, Anand et al. 2007, Nizet and Johnson 2009). One possible mechanism of action by which HIF-1 overexpression reduced inflammation might have been due to increased tolerogenic T-cell activity. In our study, mVHL−/− allografts showed signs of increased FoxP3 activity implying tolerogenic Treg activation. Increased FoxP3 expression was also observed in recipients’ spleens, suggesting increased Treg activity in the secondary lymphoid organs. We also observed reduced mRNA expression of IL-6 in mVHL−/− allograft recipients. IL-6 is a potent inhibitor of TGF-β driven induction of CD4+ FoxP3+ regulatory T cells (Bettelli et al. 2006). Interestingly, we also noticed reduced mRNA expression of IL-6 and simultaneously increased mRNA expression of FoxP3 in mVHL−/− allografts in our study. Furthermore, reduced mRNA expression of MCP-1 and reduced allograft infiltration of inflammatory cells into the allograft was seen. Altogether these results suggest a decreased adaptive immune response in mVHL−/− and T regulatory pathway activation in these allografts at 10 days. Ben-Shoshan et al.
recently reported that hypoxia controls Treg homeostasis via HIF-1 (Ben-Shoshan et al. 2008). The number as well as the potency of Tregs are also enhanced in hypoxia (Ben-Shoshan et al. 2008). In our study, VHL-deficiency in the myeloid cells may have resulted in increased Treg activity leading to reduced inflammatory and immune responses. Therefore it seems that the final effect of HIF-1 in any one setting is likely dependent on the prevailing microenvironment. The potential protective role of HIF-1 is tempting as the HIFs are major regulators of the cellular response to hypoxia. A targeted control of protective properties of HIFs could reduce ischemic injuries of the organs, especially during a transplant operation.

6. Hypoxia-inducible factor-1 in recipient myelomonocytic cells delays obliterative airway disease in mouse tracheal allografts

In addition to increased tolerogenic activity, there is another mechanism through which HIF-1 may have reduced inflammation and thereby the development of OAD in our study. The difference between mVHL−/− and mHIF−/− animals was still evident when tacrolimus was administered suggesting that tacrolimus treatment did not abrogate the beneficial effects of myelomonocytic overexpression of HIF-1. HIF-1 overexpression was associated with a lesser degree of epithelial loss at 10 days in the allografts. As the rate of epithelial loss was similar in all allografts early after transplantation, it is unlikely that early epithelial injury due to ischemia was affected by myeloid cell HIF-1 expression. It is more likely that the reduced inflammatory response allowed better epithelial regeneration in tracheal allografts. Allograft overexpression of HIF-1α is reported to promote repair of airway microvasculature through the induced expression on pro-angiogenic factors in mice (Jiang et al. 2011). Reduced epithelial injury could explain the delay in OAD development as we know the importance of a proper epithelium during this process (Neuringer et al. 2002).

It should be noticed, that mVHL−/− leads to stabilization of both HIF isoforms, HIF-1α and HIF-2α (Krieg et al. 2000). However, we did not dissect which one of the isoforms is responsible for the beneficial effect. It has been shown that both macrophages and neutrophils are able to express both HIF-1α and HIF-2α (Poitz et al. 2014; Thompson et al. 2014). However, as the OAD development was significantly accelerated in mHIF-1α−/− recipients one may assume that HIF-1 activity was the major contributor to the decreased OAD development in mVHL−/− recipients. In addition, it has been reported that HIF-2α regulates mainly key neutrophil functions (Thompson et al. 2014), but we did not detect any significant difference in the number of MPO+ neutrophils between the groups at 3 or 10 days in our study.

Again, as in our simvastatin study, without any co-administered immunosuppressant it is only possible to slow down the development of OAD by influencing HIF-1α stability. Our results support a role for HIF-1 in alleviating early allograft injury and inflammation and a possible novel therapeutic target in preventing OAD.
STUDY LIMITATIONS

Both tracheal allograft models used in our study have several limitations that should be considered when interpreting the results. The transplantation was performed over a full MHC mismatch and no immunosuppression was used making this a very robust model and the ensuing loss of epithelium and OAD development were much more accelerated compared to the clinical setting.

Additionally, in this study we used knockout animals with HIF-1α or VHL deficiency in myelomonocytic cells. However, one should keep in mind, that while the majority of the myelomonocytic cells has a targeted deletion, the deletion efficiency of HIF-1α or VHL is not perfect (Cramer et al. 2003). Finally, we focused on the RT-PCR analysis of mRNA levels in tissue samples in all three studies. However, the mRNA analysis gives insight only to current cellular activity and does not necessarily reflect protein expression. The mRNA analysis also gives little information regarding the already synthesized intracellular proteins, their deposition and release. RT-PCR analysis was chosen, because it is the most standardized method in analysis of mRNA levels in our laboratory and it also offers the broadest analysis repertory of different factors in our hands.
CONCLUSIONS OF THE STUDY

Firstly, simvastatin treatment enhanced early epithelial recovery after transplantation and inhibited allograft infiltration of inflammatory cells and expression of lymphocyte chemokine and proinflammatory cytokine mRNA. Importantly, simvastatin inhibited the development of OAD (II).

Secondly, continuous HIF-1 expression in myeloid cells improved epithelial recovery, reduced inflammatory cell accumulation, and increased regulatory FoxP3 mRNA expression in mouse tracheal allografts. Importantly, these effects led to better preservation of tracheal epithelium and a decrease in the development of OAD. All these beneficial effects were observed without any immunosuppression. Our results suggest that HIF-1 has a protective role in preventing OAD in an experimental model for lung transplantation (III).

Finally, our findings here show that the activation of innate immune responses by early ischemia takes place in both syngrafts and allografts in the rat heterotopic allograft model. However, in the presence of alloantigens, early ischemic injury induced both innate and adaptive immune responses which were followed by Th17 activation and afterwards by a sustained Th1 immune response. This was accompanied by infiltration of the allograft with proinflammatory effector cells that target the tracheal epithelium leading to progressive fibroproliferation and total tracheal occlusion if left untreated. The increased knowledge on early mechanisms of immune activation enables future studies that aim at interrupting the molecular pathways leading to alloimmune activation and OAD (I).
Keuhkonsiirto on loppuvaiheen keuhkosairauksissa, kuten keuhkoaltaumataudissa, ainoa käytettävissä oleva parantava hoitomuoto. Vaikka keuhkonsiirto-ototilaen hyhytaikaisennuste on jatkuvasti parantunut uusien lääkkeiden, kehittyneen kirurgisen tekniikan ja tehohoidon myötä, pitkäaikaisennustetta heikentää keuhkosirrannäisen krooninen toimintahäiriö. Sen alamuodoista bronchiolitis obliterans-syndrooma eli BOS on parhaiten tunnettava muoto.

Kudostasolla pienissä keuhkoputkissa on jatkuva tulehdustila, jonka seurauksena pienet hengitystiet lopulta ahtautuvat lisääntyynen sidekudoskasvuun seurauksena ja potilaan suorituskyky heikenee merkittävästi. BOS diagnooidaan kliinisin löydyksen. Diagnosti voidaan tehdä, jos keuhkonsiirteen toiminta selvästi heikenee ilman muuta selittävää syytä. BOS on merkittävän syy keuhkonsiirteiden tuhoamiseen ja potilaan krooniseen toimintahäiriöön. Se on parhaiten tunnettu muoto.

Keuhkonsiirto-ototilaen jälkeen pienissä keuhkoputkissa on jatkuva tulehdustila, jonka seurauksena pienet hengitystiet lopulta ahtautuvat lisääntynen sidekudoskasvuun seurauksena ja potilaan suorituskyky heikenee merkittävästi. BOS diagnooidaan klinisin löydyksen. Diagnosti voidaan tehdä, jos keuhkonsiirteen toiminta selvästi heikenee ilman muuta selittävää syytä. BOS on merkittävän syy keuhkonsiirteiden tuhoamiseen ja potilaan krooniseen toimintahäiriöön. Se on parhaiten tunnettu muoto.
Toiminta voi tilanteesta riippuen olla luonteeltaan tulehdusta kiihdyttävä tai rauhoittavaa. Tutkimuksemme kolmannessa vaiheessa meillä oli käytössämme geenimuunneltuja hiiriä, joilla oli myeloisissa soluissa jatkuva HIF-1 aktivaatio. Samaa koe-eläinmallia käyttäen teimme näille geenimuunnelluille hiirille henkitorvisiirtoja. Jatkuva HIF-1 aktivaatio hidasti sidekudoskasvua siirteissä kontrollisiirteisiin verrattuna ja sillä oli siis siirteitä suojaava vaikutus tässä mallissa.

Tutkimuksemme tulokset viittaavat siihen, että keuhkonsiirteen alkuvaiheen hapenpuutteesta johtuva vaurio ja sitä seuraava synnynnäisen immuniteetin aktivaatio ovat merkittäviä syitä myöhemmin ilmaantuvan keuhkopathian ahtauttavan sidekudoskasvuun kehittymisessä. Statiinilääkityksen mahdollinen hyöty keuhkonsiirtopotiilaita hoidettaessa tulisi myös arvioida uudelleen. Lisäksi näissä tutkimuksissa käytämämme keuhkonsiirtomalli koe-eläimillä vaikuttaa olevan edelleen hyvinkin käyttökelpoinen siihen asti, kunnes merkittävästi parempi keino hyljinnän tutkimiseen kehitetään.
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