Pulmonary Antimicrobial Peptide Expression in Lung Transplant Recipients with Bronchiolitis Obliterans Syndrome (BOS)

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Running head: Antimicrobial peptides and BOS

Wordcount: 3756
Abstract
Mechanisms other than classical alloimmunity are increasingly implicated in the pathogenesis of Bronchiolitis Obliterans Syndrome (BOS). We hypothesised that expression of antimicrobial peptides (AMPs), key elements of innate immunity, may be abnormal in the transplanted lung and contribute to BOS pathogenesis. Pulmonary expression of the neutrophil-derived AMPs, hCAP-18/LL-37 and α-defensins (HNP1-3), and the epithelial-cell derived AMPs human β-defensin-2 (hBD-2), elafin and secretory leukoprotease inhibitor (SLPI) were measured by ELISA in bronchoalveolar lavage fluid (BAL) from stable lung transplant recipients (n=30) and those with BOS (n=14). The relationship between airway pathogens and AMP levels was investigated. The presence of airway pathogens in stable recipients was associated with increased levels of neutrophil-derived AMPs and SLPI. When only recipients without airway pathogens were analysed, those with BOS (n=7) had elevated hCAP-18/LL-37 (p=<0.001) and HNP1-3 (p=0.004) compared to stable recipients (n=23). In contrast expression of the proteinase inhibitor SLPI was significantly lower in BOS compared to stable recipients (p=<0.001).

Even in the absence of airway pathogens, BOS is associated not only with increased airway levels of AMPs from degranulated neutrophils but also with reduced expression of SLPI. This pattern of AMP expression may favour non-alloimmune injury to the airway by reducing anti-protease defence and increasing inflammation and fibrosis.

Word count: 204

Keywords: Defensins, SLPI, elafin, cathelicidin
**Introduction**

Lung transplantation provides a realistic therapy for selected patients with end stage lung disease. However, long term survival after lung transplantation is limited to 49% at 5 years. The main cause of death in lung transplant recipients surviving more than one year is obliterative bronchiolitis (1, 2) the clinical correlate of which is BOS. This is manifest histologically by chronic inflammation and fibroproliferative repair in the small and medium sized airways eventually leading to luminal obliteration. Frequent acute rejection episodes are a recognised risk factor for BOS (3-5) and to date it has been attributed to a manifestation of chronic rejection occurring via alloimmune mechanisms. However, BOS is associated with a neutrophilia in both bronchoalveolar lavage (BAL) fluid and on endobronchial biopsy (6-9) rather than a pure lymphocytic infiltrate. Despite the advent of more effective immunosuppression, reductions in the frequency of acute rejection have not been mirrored by reductions in the prevalence of BOS (10). This raises doubts as to whether alloimmunity represents the only, or even the predominant mechanism in BOS pathogenesis.

The transplanted lung is exposed to the environment and has an innate immune system which is highly developed to protect against micro-organisms. The innate immune response may induce injury by directly attracting inflammatory cells, but also by orchestrating subsequent adaptive immune responses by acting as ‘danger signals’ which could induce alloimmunity (11). The antimicrobial peptides (AMPs) are a group of cationic peptides highly conserved throughout evolution, which form essential elements of the innate immune system. The AMPs found in the human airways include the cathelicidin hCAP-18/LL-37, alpha and beta defensins and the proteinase inhibitors,
secretory leukoprotease inhibitor (SLPI) and elafin [(12, 13). Although SLPI is considered to be an antimicrobial protein, it was originally discovered based on its elastase inhibitory activity. Subsequently it has also been shown to inhibit inflammatory gene expression eg in macrophages and to be involved in epithelial proliferation (14). AMP release is stimulated by the presence of inflammatory cytokines and microbial products and they act directly as endogenous antibiotics against a range of microorganisms (15-17). The AMPs modulate inflammation and immunity as well as influencing repair processes. They have a wide range of immunomodulatory properties including chemotactic activity for neutrophils, eosinophils, monocytes, dendritic cells and T cells. In addition, they affect the maturation and up-regulate the endocytic capacity and co-stimulatory molecule expression of dendritic cells and promote the repair of epithelial surfaces by cell proliferation and inhibition of apoptosis. (18-22). Therefore these molecules play a key role in orchestration of the subsequent adaptive cellular or humoral response to injury favouring a repair response.

We hypothesised that expression of the antimicrobial peptides in the airway of lung transplant recipients would be increased in the presence of airway pathogens and in the presence of BOS. This would contribute to the recruitment of immune cells, release of proinflammatory chemokines and creation of an environment favouring airway damage and subsequent epithelial injury or repair. We aimed to investigate levels of the AMPs, derived from both neutrophils and epithelial cells, in the airways of stable lung transplant recipients and those with BOS and determine the relationship between airway pathogens and AMP levels.
Materials and methods

Study population

Lung transplant recipients under regular follow up at the Cardiopulmonary Transplant Unit at Freeman Hospital, Newcastle Upon Tyne were recruited into the study. BOS was diagnosed according to standard International Society for Heart and Lung Transplantation (ISHLT) criteria (23). Patients undergoing either surveillance or investigative bronchoscopies were eligible for inclusion in the study.

The routine patient immunosuppressive regime was with prednisolone, cyclosporine and azathioprine. Medication dose was adjusted according to trough levels. Tacrolimus and/or mycophenolate mofetil were substituted when clinically indicated.

Microbiological prophylaxis was prescribed in accordance with local protocols. Flucloxacillin, metronidazole and nebulised colomycin was used in recipients for up to 1 week post-transplant based on pre-transplant and donor microbiology. Nebulised colomycin (2 mega units, b.i.d) was used for patients colonised with Pseudomonas aeruginosa pre-transplant or with BAL positivity for Pseudomonas. Cotrimoxazole 960 mg three times per week was used for Pneumocystis carinii prophylaxis after 1 week, oral gancyclovir for patients with cytomegalovirus (D+/R–) mismatch was continued for 3 months post-transplant. Fungal prophylaxis comprised of voriconazole in patients with Aspergillus fumigatus (either pre-transplant or in BAL post-transplant) and fluconazole if Candida was present in either donor or recipient BAL.

Ethical approval for this study was granted by the Local Research Ethics Committee and informed consent was received from all study patients.
Sampling

Bronchoscopy (Olympus FB 45.5, Olympus, Tokyo, Japan) was performed in patients premedicated with intravenous midazolam. Bronchoalveolar lavage (BAL) was performed using normal saline (NaCl 0.9%) instilled in three aliquots of 60mls into the right middle lobe or the lingula of the transplanted lung. Fluid was recovered by gentle suction and pooled. A portion was evaluated for the presence of bacteria, viruses and fungi by standardised microbiological culture methods including the use of selective agars and extended culture times. Any bacterial, fungal or viral growth was considered positive. 6-8 transbronchial biopsies were taken to exclude acute vascular rejection based on standard ISHLT criteria.

BAL fluid was gauze-filtered, centrifuged, and the acellular portion stored in aliquots at -80 degrees until later analysis by ELISA and Western blot. A total and differential cell count was performed using a Neubauer Haemocytometer, and Giemsa stained cytospin preparations. A minimum of 500 cells were counted.

ELISA

Established sandwich ELISAs were used to determine AMP levels in the cell-free BAL supernatant. Commercially available kits were used for Human neutrophil alpha defensins (Human Neutrophil Peptides [HNP] (1-3)) and Elafin from Hycult Biotechnology (Uden, Netherlands), and IL-8 from Biosource International (Nivelle, Belgium). ELISAs were performed according to manufacturer’s instructions.

hBD-2 ELISA was performed using a well defined methodology (24). SLPI, hCAP18/LL-37, and human neutrophil elastase ELISAs were performed as developed in house at Leiden University Medical Centre, Leiden, Netherlands (25-27).
**Gel electrophoresis and Western blotting for hCAP-18/LL-37 and SLPI**

Electrophoresis and Western blotting were performed on selected representative samples, (8 stable and 8 BOS). This was used to determine the presence of intact hCAP-18 and its cleavage to LL-37, and the presence of SLPI and its degradation products. Samples were subjected to sodium dodecyl sulphate (SDS) poly acrylamide gel electrophoresis (PAGE). This was performed on a 16.5% Tris/Tricine gel and separated proteins were transferred to a polyvinylidene-difluoride (PVDF) membrane (Biorad). Non-specific binding sites were blocked overnight and then the membrane was incubated for LL-37 for 1 hour with mouse monoclonal anti-LL-37 antibody 1.1C12 (28), and for SLPI with polyclonal rabbit anti-SLPI IgG (26). Secondary antibodies were horse radish peroxidase-conjugated goat anti-mouse for LL-37 and swine anti-rabbit for SLPI (DAKO, Denmark). The enhanced chemoluminescent (ECL) Western blotting detection system was used to reveal immunoreactivity.

**Statistical Analysis**

Statistical analysis was performed using SPSS version 10.0 (SPSS Inc Chicago). Data were initially assessed for normality. None of the data on AMP levels was normally distributed so non-parametric tests were used for their analysis. Non-parametric data are presented as a median and range, while normally distributed data are expressed as mean +/- SD. Differences between groups were assessed using Student’s t-tests and Kruskal-Wallis test. Where there were significant differences between groups on the Kruskal-Wallis test further comparisons were tested with the Mann-Whitney U test. A Bonferroni-Holm correction was used to correct for multiple comparisons between
groups. Correlations were assessed using Spearman rank correlation. Differences with a two sided p-value of 0.05 were considered to be statistically significant.

Results
Sixty-two lung transplant recipients, 34 female and 28 male underwent bronchoscopic evaluation and were included in this study. Of the 62 patients investigated 14 had BOS diagnosed according to standard ISHLT criteria. The BOS stages were BOS 0-p n=2, BOS 1 n=2, BOS 2 n=6 and BOS 3 n=4. 18 recipients had acute rejection ISHLT grade 2 or higher on transbronchial biopsy and the remaining 30 had stable lung function without evidence of acute rejection or BOS. The 18 patients with acute rejection were excluded from further analysis in this study. The study group therefore consisted of 14 recipients with BOS and 30 stable recipients. The patient demographics and pre-transplant diagnoses are shown in Table 1. There were no gender or age differences between the two groups. Immunsuppression at the time of BAL sample is shown in Table 2. As would be expected, the BOS group were significantly further from time of transplant than the stable group.

Neutrophils, IL-8 and neutrophil elastase
The degree of airway neutrophilia, BAL IL-8 and human neutrophil elastase (HNE) concentrations together with the antimicrobial peptide levels in BAL fluid were compared between stable patients and patients with BOS (Table 3). Transplant recipients with BOS had a significantly higher percentage of neutrophils present in their BAL fluid together with increased levels of HNE. There were higher IL-8 levels in BOS recipients (p=0.044), but this did not remain statistically significant after the Bonferroni-Holm
correction for multiple comparisons. As would be expected levels of the neutrophil derived AMPs hCAP-18/LL-37 and HNP 1-3 correlated positively with the total neutrophil count. In addition, levels of hCAP-18/LL-37 and HNP 1-3 were significantly increased in BOS even in the absence of a significantly higher total neutrophil count. There was, however, no significant difference in the epithelial-derived AMPs elafin and hBD-2 between BOS and stable recipients. In contrast, SLPI, an epithelial-derived AMP with potent serine proteinase inhibitory activity, was present in significantly lower levels in BAL fluid of recipients with BOS than stable recipients.

Since the presence of pathogens is likely to have a major confounding effect on neutrophil influx and AMP expression in the airway, the data was re-examined in both stable and BOS recipients with and without positive cultures of BAL fluid (Table 4). Samples where BAL fluid microbiological culture was negative will be referred to as samples with no pathogens through the remainder of this paper. In stable recipients the presence of pathogens was associated with a significant increase in BAL neutrophilia and elevation of both IL-8 and HNE. However, in recipients with BOS, the presence of pathogens was not associated with a further increase in total neutrophil count or neutrophil percentage or with any additional increase in levels of IL-8 or HNE.

**Neutrophil derived antimicrobial peptides and epithelial cell derived antimicrobial peptide hBD-2**

The AMPs derived predominantly from neutrophils, LL-37 and the alpha defensins HNP 1-3, were both significantly higher in stable transplant recipients who had pathogens cultured from their BAL compared with stable recipients who were culture negative. Interestingly however, in recipients with BOS there was no additional innate response to
the presence of pathogens. Importantly the data showed that even in the absence of pathogens there was a significantly higher level of both LL-37 and HNP 1-3 in recipients with BOS compared to stable recipients (Figure 1).

Neutrophils store the precursor cathelicidin hCAP-18 in their specific granules and proteolytic processing to the active peptide LL-37 occurs after neutrophil stimulation (29). The hCAP-18/LL-37 ELISA used is based on antibodies raised against LL-37 and therefore detects both the precursor hCAP-18 and processed forms of hCAP-18 containing the LL-37 sequence. We therefore used Western blot analysis to differentiate intact hCAP-18 from processed hCAP-18 containing LL-37 in the BAL samples. The results showed that the ratio between hCAP-18 and LL-37 differs amongst transplant recipients. Some recipients have a much higher ratio of active peptide to hCAP-18 than others (Figure 2).

Human beta defensin-2 is mainly derived from epithelial cells and is not produced by neutrophils. There was no significant difference in levels of hBD-2 between stable and BOS recipients either in the presence or absence of pathogens (Figure 3).

**Proteinase inhibitors**

The two AMPs with anti-proteinase activity, elafin and SLPI, are both produced predominantly by epithelial cells. There were no significant differences in the levels of elafin found for any of the groups in our study. In contrast there were markedly different responses in SLPI expression in stable and BOS recipients. In stable recipients SLPI was elevated in the presence of pathogens, which was not observed in recipients with BOS. In those recipients without detectable pathogens SLPI levels were significantly lower in recipients with BOS compared to those with stable lung function (Figure 4).
Discussion

We have shown that lung transplant recipients with BOS show a different pattern of antimicrobial peptide expression than stable recipients. BOS is characterised by significantly elevated levels of LL-37 and the alpha defensins HNP 1-3, significantly reduced levels of SLPI and no difference in levels of hBD-2 and elafin. The main findings of this study are summarised in Figure 5.

These results add to previous findings by Nelsestuen et al who showed in a proteomic survey of BAL protein content that elevated levels of the alpha defensins were associated with subsequent development of BOS (30). Both LL-37 and HNP1-3 are predominantly derived from neutrophils, although other cellular sources have been identified, most notably epithelial cells may contribute to LL-37 levels in airways (16). hCAP18/LL-37 is stored in the specific granules of neutrophils and (29) is processed to the active molecule LL-37 extracellularly by proteinase 3, although other proteinases may also contribute to processing (28, 31). Western blot analysis for LL-37 shows that the ratio between the precursor hCAP-18 and LL-37 differs among transplant recipients irrespective of the presence of BOS. Therefore, Western blotting appears to provide additional information to that obtained by ELISA. However, because BAL was not collected in a mixture of proteinase inhibitors, we cannot exclude the possibility that post-collection processing may have affected our results. The alpha defensins are stored in the azurophilic granules of neutrophils at levels of 5000µg per 10⁹ cells (32-34). Both HNP1-3 and hCAP-18/LL-37 are released from neutrophils during degranulation. Our results suggest that the neutrophils present in the airways of transplant recipients with BOS are activated and
undergoing degranulation with release of granule contents into the airway contributing to ongoing airway injury. Palmer and colleagues have demonstrated an association between a Toll-like receptor 4 (TLR-4) polymorphism, which makes the host hyporesponsive to bacterial LPS, and a reduced incidence of acute rejection and a trend to reduction in BOS stage 2 or 3 compared to those without the polymorphism (35). This leads us to speculate that key elements of the pulmonary innate response may play an aetiological role in BOS development. Indeed Ross et al have shown elevation of human beta defensin 2 (hBD-2) in transplant recipients with BOS compared with recipients with stable lung function (36).

We found no elevation of hBD-2 levels in patients with BOS in comparison with stable recipients. hBD-2 is produced mainly by epithelial cells and its expression is induced by the presence of microbial products (37, 38). Indeed, we did observe a biologically plausible elevation in hBD-2 level in stable recipients with infection in comparison to stable recipients without infection although this did not reach statistical significance. Our results contrast with the previous findings of Ross et al that hBD-2 was increased in the presence of BOS (36). In that study 6 out of the 8 samples from BOS recipients were culture positive for pathogens. This high level of culture positivity may account for differences between our findings, although we did not find any increase in hBD-2 levels in recipients with BOS who had pathogens present. Details of drug treatment were not given in the Ross paper whilst in our study 43% of the BOS transplant recipients were treated with azithromycin. Azithromycin is known to reduce epithelial cell IL-8 production in vitro (39) and BAL neutrophilia (40), so although there is currently no evidence for an effect of azithromycin on hBD-2, it is possible that there is an effect.
which accounts for the difference between our findings and those of Ross et al. We have done a subgroup analysis comparing the levels of the AMPs in recipients with BOS with and without azithromycin treatment. We found no significant differences between the two groups, however since the sub groups are small (comparable with the group sizes in the Ross study) these results should be interpreted with caution.

Our results demonstrated that the presence of pathogens in recipients with BOS was not associated with a significant additional increase in levels of any of the AMPs measured in contrast to the noticeable, but statistically non-significant response to pathogens seen in stable recipients. We speculate that the neutrophil derived AMPs are already abundantly present due to neutrophil degranulation and that no additional response is possible in the presence of pathogens. However, the lack of response from the epithelial derived AMPs SLPI, elafin and hBD-2 suggests a blunted epithelial antimicrobial response to pathogens in recipients with BOS.

The significant reduction in SLPI seen in BOS patients is of interest, particularly in the absence of a similar reduction in the other antiproteinase measured, elafin. There are several possible explanations for this finding: the results may reflect reduced production, an increase in cell association or retention of SLPI leading to reduced recovery in BAL or increased degradation. Since both elafin and hBD-2 are also derived from airway epithelial cells, it is unlikely that decreased SLPI levels simply reflect extensive epithelial damage. Jaumann et al have previously shown in vitro that TGF-β1 is an potent inhibitor of SLPI production (41). There is evidence that the airway environment of transplant recipients with BOS is rich in TGF-β1 (42). We can therefore speculate that the TGF-β1 levels seen in BOS may be high enough to significantly reduce SLPI production. There is
also evidence that in the presence of increased amounts of neutrophil elastase SLPI is not released from epithelial cells but remains cell associated (43). In this case it may not be efficiently recovered by BAL and the low levels we observed may not necessarily reflect a reduction in antiprotease defence, but a reduction in fluid-phase SLPI. It is also possible that the intraluminal polymyxoid fibrinous deposits seen in BOS act as a sink for SLPI based on charge interaction. This is unlikely, because only the SLPI level was reduced and not the other (sometimes more cationic) peptides. Hirsch et al have also demonstrated reduced levels of SLPI in lung transplant recipients with BOS. This study demonstrated that the reduction in SLPI was inversely correlated with the presence of oxidised methionine in BAL fluid. The authors speculated that oxidative stress in BOS leads to increased consumption of SLPI by proteolytic and oxidative inactivation (44). We considered the possibility that the lower SLPI levels in BOS patients are explained by degradation of SLPI, as previously shown in cystic fibrosis (45). We therefore investigated SLPI degradation products by Western blot analysis of our samples. Despite the fact that we used a polyclonal antibody that detects multiple epitopes on SLPI, we found no evidence of SLPI degradation in any of the samples assayed. Therefore, the lower SLPI levels in BOS patients are not likely explained by SLPI degradation.

We considered the possibility that the observed differences in AMP levels reflected differing immunosuppressive regimes of patients with BOS and stable patients. The overwhelming majority of recipients commenced cyclosporine, azathioprine and prednisolone at the time of transplantation. The most common alterations over time were removal of azathioprine, due to neutropenia, and a switch from cyclosporine to
tacrolimus due to side effects of cyclosporine or recurrent acute rejection episodes. At
the time BAL fluid was obtained most recipients with BOS were on a regime containing
a calcineurin inhibitor and prednisolone, and most stable recipients were on a regime
containing a calcineurin inhibitor, azathioprine and prednisolone. This difference
between groups seemed to reflect the fact that BOS recipients were significantly longer
after transplant than stable recipients with the main reason for the discontinuation of
azathioprine being a previous neutropenic episode. Little is known about the effects of
immunosuppressant drugs on AMP levels, but it is unlikely that the higher incidence of
azathioprine treatment in the stable group would explain the differential pattern of AMP
expression we find between stable and BOS groups. A limitation of this study is that it is
cross-sectional and so does not examine the changes in AMP levels over time. There was
a statistically significant difference between the two study groups in the length of time
after transplantation before BAL was collected. We are currently conducting a
longitudinal study to further clarify this issue. Also the mean age of BOS recipients in
this study was lower than in the stable recipients by 10 years, but this difference was not
statistically different. Previous work has suggested a change in levels of SLPI over time,
with decreasing amounts found in saliva with increasing age (46). However, in our study
there was no correlation between age and any of the antimicrobial peptide levels
measured.
Our findings support our hypothesis that, with or without the presence of pathogens, in
BOS there are elevated levels of the neutrophil derived antimicrobial peptides. The
presence of high levels of LL-37 and the alpha defensins would be expected to lead to
release of potent chemokines and changes in the maturation and function of dendritic
cells (18, 22). In addition, these antimicrobial peptides have been shown to exert 
chemotactic activity for both dendritic cells and T cells. Therefore, increased local levels 
of LL-37 and neutrophil α-defensins could increase the initiation and propagation of an 
adaptive immune response that may contribute to rejection. Both neutrophil alpha 
defensins and LL-37 have been shown to affect epithelial wound repair. Specifically 
neutrophil alpha defensins are mitogenic for fibroblasts (47) and increase collagen type I 
and III expression in both dermal and corneal fibroblasts (48, 49), suggesting that 
increased levels of alpha defensins may contribute to tissue fibrosis. The higher levels of 
alpha defensins we found in transplant recipients with BOS may therefore be associated 
with an exaggerated repair response to airway damage. Our results also imply that there 
is a protease/antiprotease imbalance in the airway in BOS with excess free neutrophil 
elastase present which is not counteracted by SLPI. This situation would be expected to 
contribute to further airway damage and inflammation. If the level of SLPI in the airway 
is truly reduced then the consequences of this would be decreased antimicrobial defence 
with risk of further infections perpetuating airway inflammation, and decreased 
protection against neutrophil-derived proteinase inhibitors with decreased epithelial 
repair.

In summary, the antimicrobial peptide responses demonstrated in BOS will contribute to 
a proinflammatory microenvironment and may promote alloimmune injury and an 
exaggerated repair response which could drive the airway remodelling which is 
characteristic of BOS. Treatment approaches which address abnormalities in innate 
immune responses in lung transplant recipients may be an important adjunct to classical 
immunosuppressive therapies in protecting the graft and require more evaluation.
Acknowledgements: The authors would like to acknowledge valuable technical assistance from Gail Johnson, Lynda Archer, Dennis Ninaber, Bram van der Linden and Renate Verhoosel.
References


Table 1: Patient demographics

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<th>BOS n=14</th>
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<tr>
<td>Sex</td>
<td>13M:17F</td>
<td>8M:6F</td>
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<td>Age (years)</td>
<td>43 (SD 11)</td>
<td>33 (SD 18)</td>
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<td>Days after Transplant</td>
<td>270 (SD 272)</td>
<td>744 (SD 579)</td>
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<td>CF</td>
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<td>Stenotrophomonas maltophilia</td>
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CF cystic fibrosis, PPH primary pulmonary hypertension
Table 2: Immunosuppressive regime at the time of BAL sample

<table>
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<tr>
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<th>Stable</th>
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<td>Cyclosporine, azathioprine, prednisolone</td>
<td>3 (21%)</td>
<td>5 (17%)</td>
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<tr>
<td>Tacrolimus, azathioprine, prednisolone</td>
<td>1 (7%)</td>
<td>10 (33%)</td>
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<td>Tacrolimus, MMF, prednisolone</td>
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<td>Cyclosporine, prednisolone</td>
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<td>1 (3%)</td>
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<td>6 (43%)</td>
<td>3 (10%)</td>
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<td>Tacrolimus, azathioprine</td>
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<td>3 (10%)</td>
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<tr>
<td>Cyclosporine, azathioprine</td>
<td></td>
<td>5 (17%)</td>
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<tr>
<td>Tacrolimus</td>
<td>1 (7%)</td>
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Table 3: Comparison between bronchoalveolar lavage fluid in stable transplant recipients (n=30) and recipients with BOS (n=14).

<table>
<thead>
<tr>
<th></th>
<th>Stable n=30 Median (range)</th>
<th>BOS n=14 Median (range)</th>
<th>P value (Kruskal Wallis)</th>
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<td>Total neutrophils x10⁴</td>
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<td>Percentage neutrophils</td>
<td>2.1 (0-72.4)</td>
<td>19.7 (0-100)</td>
<td>0.008</td>
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<td>IL-8 pg/ml</td>
<td>42 (0-8759)</td>
<td>590 (16.5-15008)</td>
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<td>Human neutrophil elastase ng/ml</td>
<td>7 (1-2886)</td>
<td>75 (2-454)</td>
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<td>hCAP-18/LL-37 ng/ml</td>
<td>0.4 (0-21)</td>
<td>10 (1-38)</td>
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<tr>
<td>HNP1-3 ng/ml</td>
<td>4 (0-3212)</td>
<td>510 (0-9512)</td>
<td>0.008</td>
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<td>hBD-2 pg/ml</td>
<td>137.5 (0-2450)</td>
<td>73 (0-3490)</td>
<td>0.959</td>
</tr>
<tr>
<td>SLPI ng/ml</td>
<td>156 (56-1926)</td>
<td>44 (0-525)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Elafin pg/ml</td>
<td>607 (0-15541)</td>
<td>0 (0-4827)</td>
<td>0.749</td>
</tr>
</tbody>
</table>
Table 4: Patient characteristics and BAL parameters in stable recipients and recipients with BOS grouped according to the presence of cultured pathogens

<table>
<thead>
<tr>
<th></th>
<th>Stable patients n=30</th>
<th>BOS n=14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No pathogens (n=23)</td>
<td>Pathogens (n=7)</td>
</tr>
<tr>
<td>Gender</td>
<td>10M:13F</td>
<td>3M:4F</td>
</tr>
<tr>
<td>Age (years)</td>
<td>42 (22-62)</td>
<td>38 (22-55)</td>
</tr>
<tr>
<td>Days after transplant</td>
<td>186 (10-1533)</td>
<td>313 (34-368)</td>
</tr>
<tr>
<td>Total neutrophils x10^4</td>
<td>0.2 (0-58.8)</td>
<td>8.2 (0.1-63.4)</td>
</tr>
<tr>
<td>% neutrophils</td>
<td>1.4 (0-26.6)</td>
<td>46.6 (1.6-72.4)</td>
</tr>
<tr>
<td>IL-8 pg/ml</td>
<td>25 (0-2320)</td>
<td>1664 (45-8759)</td>
</tr>
<tr>
<td>HNE ng/ml</td>
<td>6 (1-733)</td>
<td>1374 (13-2886)</td>
</tr>
</tbody>
</table>
Figure legends

**Figure 1**: Levels of the neutrophil derived antimicrobial peptides LL-37 and HNP 1-3 in BAL fluid. Comparison is made between four separate groups of transplant recipients, those with stable lung function and no pathogens (n=23), those with stable lung function and pathogens cultured from BAL fluid (n=7), recipients with BOS and no pathogens cultured from BAL (n=7) and recipients with BOS and pathogens present on culture of BAL (n=7).

**Figure 2**: Western blot analysis of BAL samples using monoclonal anti-LL-37 antibody. BAL samples were separated by SDS-PAGE, analysed by Western blot using monoclonal anti-LL-37, and immunoreactive bands were visualized using enhanced chemiluminescence. Lanes 1-4: recipients with BOS; lanes 5-8: stable recipients. Large arrows indicate intact hCAP-18 and LL-37, small arrows indicate intermediate processing product.

**Figure 3**: Levels of human beta-defensin-2 in the four recipient groups.

**Figure 4**: Levels of the proteinase inhibitors SLPI and elafin in each of the four recipient groups.

**Figure 5**: Summary of antimicrobial peptide levels in the different study groups
Figure 1

- **LL-37**
  - Stable no pathogens
  - Stable with pathogens
  - BOS no pathogens
  - BOS with pathogens
  - p = 0.002
  - p = 0.001

- **HNP 1-3**
  - Stable no pathogens
  - Stable with pathogens
  - BOS no pathogens
  - BOS with pathogens
  - p = 0.007
  - p < 0.001
Figure 2

hCAP-18

LL-37

lane 1 2 3 4 5 6 7 8
Figure 3

hBD-2

0 500 1000 1500 2000 2500 3000 3500 hBD-2 pg/mL

Stable no pathogens  Stable with pathogens  BOS no pathogens  BOS with pathogens
Figure 4

SLPI

Elafin

Stable no pathogens
Stable with pathogens
BOS no pathogens
BOS with pathogens

SLPI ng/ml

Elafin pg/ml

p=0.001
p=0.006
p<0.001

0
500
1000
1500
2000

0
2500
5000
7500
10000
12500
15000
17500
### Figure 5

#### Pro-inflammatory Antimicrobial
- α-defensins
- LL-37
- HNE

#### Anti-inflammatory Antimicrobial
- SLPI
- Elafin

<table>
<thead>
<tr>
<th>State</th>
<th>Pro-inflammatory</th>
<th>Anti-inflammatory</th>
<th>Equilibrium between damage and repair</th>
<th>Transient increase in innate immune response to deal with pathogens</th>
<th>Chronic pro-inflammatory state promoting airway damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable with pathogens</td>
<td>♦α-defensins</td>
<td>♦LL-37</td>
<td></td>
<td>♦SLPI</td>
<td></td>
</tr>
<tr>
<td>BOS</td>
<td>♦α-defensins</td>
<td>♦LL-37</td>
<td></td>
<td>♦SLPI</td>
<td></td>
</tr>
</tbody>
</table>