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Decreased efferocytosis and mannose binding lectin in the airway in bronchiolitis syndrome

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Short running title: Decreased efferocytosis and MBL in BOS

Key words: Lung transplant, BOS, efferocytosis, macrophage, mannose binding lectin, complement, BAL

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Background: Mannose binding lectin (MBL) is a key mediator of both innate immunity and efferocytosis (phagocytosis of apoptotic cells) in the airway. Defective efferocytosis results in a net increase in apoptotic material that can undergo secondary necrosis, leading to tissue damage and chronic inflammation. We have shown reduced MBL and efferocytosis in other chronic inflammatory lung diseases; we therefore hypothesized that reduced MBL and efferocytosis in the airways may be a determinant of bronchiolitis obliterans syndrome (BOS) following lung transplantation. **Methods:** We investigated MBL (ELISA), MBL-mediated complement deposition (UC4, ELISA) and efferocytosis of apoptotic bronchial epithelial cells (flow cytometry) in bronchoalveolar lavage (BAL) and peripheral blood from 75 lung transplant recipients (16 with stable graft function, 34 stable with proven infection, 25 with BOS) and 14 healthy controls.

Results: In plasma, MBL levels were highly variable (0-17.538 μg/mL), but increased in infected subjects vs control (p=0.09) or stable groups (p=0.003). There was a similar increase in UC4 in infected patients and a significant correlation between MBL and UC4. There was no correlation between MBL and time post-transplant. In BAL, MBL levels were less variable (0-73.3 ng/mL) and significantly reduced in patients with BOS vs controls and stable groups. Efferocytosis was significantly reduced in the BOS group vs control and stable groups (Mean [SEM] Control: 20% [1.3%], Stable 20.5% [2.5%], Infected 17.3% [2.8%], BOS 11.3% [1.5%, p=.04]).

Conclusions: Low levels of MBL in the airway may play a role in reduced efferocytosis, subsequent tissue damage and BOS following lung transplantation.

Lung transplantation is now recognized as an effective treatment option for a variety of end-stage lung diseases and is associated with improvements in life expectancy and quality of life. However the five-year survival is currently only around 60%, the worst of any solid organ transplant, with death beyond the acute phase being largely due to progressive and treatment refractory airway remodeling (obliterative bronchiolitis, OB) manifest clinically as bronchiolitis obliterans syndrome (BOS). BOS is thought to follow persistent alloreactive, infective, and non-specific epithelial injury with dysregulated epithelial repair[1]. Although the predominant histopathologic finding in patients with OB is of fibro-proliferative small airway obliteration, large airways are also affected with the eventual development of bronchiectasis, leading directly to patient morbidity and mortality.

The well-ordered process of apoptosis is important for regulation and maintenance of normal tissue homeostasis. During resolution of airway inflammation, apoptotic epithelial cells are removed by phagocytosis by alveolar macrophages (a process termed 'efferocytosis'). The pathological concept of failed efferocytosis has been described in regard to a number of lung diseases including pulmonary fibrosis[2], cystic fibrosis[3] chronic granulomatous disease [4] smokers and patients chronic obstructive pulmonary disease (COPD) [5-8].

It is considered that the failed efferocytosis results in a net increase in apoptotic material that may lead to secondary necrosis of this material resulting in increased tissue damage and chronic inflammation. This was clearly shown in our studies of COPD where we found evidence of secondary necrosis (increased lactate dehydrogenase in BAL) and that the increased numbers of apoptotic bronchial epithelial cells negatively correlated with efferocytosis[6,9]. In lung transplant recipients we have also described an accumulation of apoptotic material associated with an increase in the cytotoxic mediator granzyme b in the airways [10,11].

Mannose binding lectin (MBL) is a key mediator of both innate immunity and efferocytosis and is thus likely to be important in protecting against lung tissue damage. In other inflammatory chronic lung diseases including COPD, we have shown reduced levels of MBL that correlated with a defect in the

ability of alveolar macrophages to phagocytose apoptotic bronchial epithelial cells (efferocytosis)[8]. We further showed that treatment of smoking mice (which replicate the inflammation and macrophage dysfunction found in our human subjects) with plasma-derived MBL improved efferocytosis by both alveolar and lung tissue macrophages *in vivo*, providing further evidence of the importance of this pathway[8].

We therefore hypothesized there would be decreased efferocytosis associated with low levels of MBL in the airways of patients with BOS. It is probable that these deficiencies would significantly contribute to increased numbers of uncleared apoptotic cells and secondary necrosis with tissue damage and an abnormal inflammatory response leading to in OB following lung transplantation. Importantly, this pathway may provide new treatment options for BOS, an area of major unmet clinical need. Our lung transplant patients undergo frequent bronchoscopic evaluation, either in the context of surveillance to detect pre-clinical histological signs of rejection or in the context of acute deteriorations where samples are required for a specific diagnosis. These have provided an ideal opportunity to evaluate the role of efferocytosis and key mediators in both stable subjects and those with infection, acute rejection or BOS.

Materials and Methods

Subjects

Subjects were recruited from the South Australian Lung Transplant Service. Rejection status was categorized both clinically and histologically on transbronchial biopsies according to standard criteria. Pathologists were blinded to the results of flow cytometric testing. Patients were maintained on standard immunosuppressive therapy (Cyclosporin A or Tacrolimus, mycophenolate or azathioprine and prednisolone). Bronchoalveolar lavage (BAL) and peripheral blood (collected into lithium heparin) was obtained from lung transplant recipients with (a) stable graft function (blood from n=16; BAL from n=10), (b) BOS (blood n=25; BAL n=11) (c) stable function with infection (blood n=34; BAL n=10) and d) normal controls (never-smokers with no history of respiratory or allergic disease and normal spirometry) (blood and BAL, n=14). All groups were age-matched as closely as possible. Patient demographics are presented in Table 1.

Flexible bronchoscopy

Bronchoscopy was performed according to expert consensus recommendations for the performance of bronchoscopy for investigative purposes (American Thoracic Society) and as previously reported by us[5-8]. Briefly, BAL was collected after wedging the bronchoscope tip into a subsegmental airway. A 50mL aliquot of sterile normal saline (at room temperature) was instilled with a syringe then aspirated using low suction into a 50mL plastic suction trap. Two further aliquots of saline were instilled and aspirated in the same way into two further traps. The first aliquot was not used for analysis due to bronchial contamination but processed for microbiological testing. Aspirated BAL was immediately transferred to 50mL polypropylene tubes (to avoid attachment of cells to the polystyrene plastic trap) and kept on ice.

Preparation of BAL

BAL was prepared and alveolar macrophages were isolated from BAL by adhering to plastic as previously reported[5]. BAL was frozen at -80^{0}C.

Measurement of soluble MBL

MBL levels were determined in batches of undiluted frozen BAL and plasma samples using commercial ELISA kits (HyCult Biotechnology, Uden, The Netherlands) following instructions supplied by the manufacturer. The lower limit of detection was 0.41 ng/mL.

Complement activation assay

C4 deposition was measured using ELISA as previously described[13] which arbitrarily quantifies C4 deposited onto a solid phase mannan surface. After MBL binding to mannan, subsequent C4 deposition was measured using a biotinylated anti-C4 antibody (Sigma-Aldrich, Castle Hill, NSW, Australia). 1mL of Statens Serum Institue Standard (Statens, Denmark) was arbitrarily assigned 1000U C4 deposition. MBL-mediated C4 deposition was measured in UC4/µl. samples.

Efferocytosis of apoptotic bronchial epithelial cells by alveolar macrophages

A flow-cytometric efferocytosis assay was performed using alveolar macrophages from BAL as previously reported[5,14]. Briefly immortalized normal bronchial epithelial cells (16HBE) were induced to undergo apoptosis using UV radiation for 20 min then stained with mitotracker red (MTR) (50µL, 25µg/mL) and used as phagocytic targets. One mL aliquots of alveolar macrophages ($4x10^5$/mL) in culture medium were adhered to 24 well culture plates for 2h, then fluid removed and 1mL apoptotic cells ($4x10^6$/mL) added for 1.5h.

Statistical analysis

We used the Shapiro-Wilk test for normality, ANOVA plus post hoc t-test or Kruskal Wallis plus post hoc Mann Whitney as relevant. Correlations were performed using Pearson Correlation Coefficients, significance $p<0.05$

Results

Measurement of MBL

In plasma, MBL levels were highly variable (0-17.538 μg/mL). No significant differences were noted among the transplant groups vs non-transplant controls, although there was a trend (p=0.099) for increased MBL in the infection group vs controls (Figure 1A). There was a significant (p=0.003) increase in MBL in the infection group vs stable transplant patients (Figure 1A). Notably, in plasma, 34% transplant patients with stable graft function (7/16) or BOS (7/25) met the accepted definition of MBL deficiency (less than 0.1μg/mL)[12], whereas only 1/34 with infection and two of the 14 control subjects were MBL deficient.

In BAL, MBL levels were lower than in plasma but less variable (range 0-74 ng/mL) and significantly reduced in patients with BOS vs healthy controls and transplant patients with stable graft function. There were no significant differences in MBL in BAL from any other transplant group (MBL ng/mL: (mean ± SEM) Controls: 6.2 ± 1; stable 8.6 ± 3.8; stable infected 10.7 ± 2.7; BOS 2.2 ± 1.7) (Figure 2). There was no significant correlation between plasma and BAL levels of MBL (Pearson correlation 0.168, p=0.263).

Complement activation assay

In BAL, values were below the lower limits of detection for the assay. In plasma, values ranged from 0.013 to 0.634 UC4/uL, and no significant differences were noted between non-transplant controls and transplant groups (Figure 1B) There was a significant (p=0.003) increase in MBL mediated C4 deposition in the infection groups vs stable transplant patients (p=0.037) and a trend for an increase in the BOS vs stable groups (p=0.096) (Figure 1B).

There was a significant correlation between MBL levels in plasma and UC4/uL (Pearson correlation 0.805; p=<0.001) (Figure 1C).

Efferocytosis of apoptotic bronchial epithelial cells by alveolar macrophages

Alveolar macrophages from patients with BOS had significantly reduced ability to efferocytose apoptotic airway epithelial cells compared to patients with stable graft function. Investigations of the further group of stable patients with infection showed alveolar macrophage phagocytic ability that was not significantly decreased vs the 'stable' group (Figure 3).

Correlations between MBL, efferocytosis and time post-transplant

Consistent with our previous findings of a significant correlation between MBL and efferocytosis in patients with COPD[8], the present study noted a trend for correlation between alveolar macrophage efferocytosis ability and MBL levels in BAL (Pearson correlation 0.399, p=0.090, n=25). There was no significant correlations between MBL and time post-transplant or pre-transplant diagnosis (Pearson correlation -0.204; p=0.115).

Discussion

Current diagnosis of OB is difficult; histopathology assessment is notoriously unreliable[15] and physiologic techniques are insensitive to early changes in resistance in the small airway, so that by the time a clinical diagnosis of BOS is made, a substantial proportion of airways may have been irretrievably obliterated. Therapeutic intervention is likely to be more effective if applied earlier; therefore, a more complete understanding of the pathogenesis of BOS is urgently required to enable and identify new treatment targets.

The processes leading up to BOS are incompletely understood but are thought to include persistent alloreactive, infective, and non-specific epithelial injury with dysregulated epithelial repair[1]. These processes culminate in remodeling and fibrotic obstruction of the small airways. The initiators of these changes are most likely multi-factorial, and we have shown that T-cells from patients with BOS have increased levels of pro-inflammatory cytokines and granzymes associated with graft rejection, epithelial cell death and the process of epithelial mesenchymal transition[11,16,17]. We have further shown increased numbers of apoptotic bronchial epithelial cells in the airways of lung transplant recipients and suggested that secondary necrosis of the uncleared apoptotic material could contribute to the perpetuation of chronic inflammation and tissue damage that precedes BOS[10].

The present study extended these observations to show that the increased number of apoptotic cells in the airway in BOS may result from a defective ability of alveolar macrophages to phagocytose these cells (efferocytosis), associated with low levels of MBL (required for effective efferocytosis). The changes

were not related to time post-transplant. The relevance of uncleared apoptotic cells in subsequent development of fibrosis has been highlighted by several studies including a comprehensive review by PM Henson in 2003[2]. One potential consequence of these accumulated cells may be high levels of the pro-fibrotic cytokine TGF-β (produced by macrophages ingesting the apoptotic cells) that may contribute to the pulmonary fibrosis in BOS[2].

Although traditionally recognized as a mediator of host defense by its ability to facilitate macrophage phagocytosis of pathogens, MBL is now recognized to regulate the clearance of apoptotic cells[18,19], by a process that involves recognition of mannose containing pathogen associated molecular patterns (PAMPs) and 'altered self' involved in the macrophage-target cell interaction[18]. As such, the reduced levels of MBL noted in BOS may provide an environment which promotes the significant defect in efferocytosis of apoptotic cells and progression to airway obliteration in OB. Low plasma levels of MBL are associated with increased susceptibility to infection, and poorer prognosis in cystic fibrosis[20,21]. The association of low MBL in plasma from both donor and recipient with susceptibility to infection has also been well-described in a variety of transplant settings including liver, renal, kidney-pancreas and heart[22-24]. The low MBL levels were, in most cases, associated with MBL gene polymorphisms[25-27].

Despite these numerous studies of MBL in plasma, there have been no studies investigating the role of MBL in the airway or with regard to efferocytosis of apoptotic cells and the link to tissue damage, inflammation and progression to BOS in lung transplant patients. The present study found differences in MBL levels in plasma and airway. Significantly reduced levels of MBL were noted in the airway of patients with BOS vs non-transplant controls or stable lung transplant patients whereas, in the blood, levels were highly variable and no difference was found between the transplant groups. This is consistent with previous studies by our group and others that have shown that plasma levels of MBL are highly variable, thought to be due to the genetic polymorphisms complicating correlation with disease processes[12,26].

Although we found no evidence from peripheral blood for a difference in the ability of transplant patients and controls to activate complement in response to MBL binding to solid phase mannose moieties, we were not able to assess complement activation in the airway, as levels were below the limits of detection in BAL. Complement binds directly and specifically to surface blebs of apoptotic cells including vascular endothelial cells[27-29]; it is therefore probable that the uncleared apoptotic cells that remain as a result of the defective efferocytosis in OB have the potential to directly activate complement and contribute to complement mediated tissue injury. It is also possible that even low levels of MBL in BAL from patients with BOS are still sufficient to activate complement and mediate an effect on C4 deposition in the airway, but not sufficient to exert the essential pro-efferocytosis effects on alveolar macrophages. This is supported by a previous report that showed that increased complement activation despite low levels of plasma MBL contributed to graft rejection following heart transplantation[22]. It is also likely that MBL may exert its effects differentially in the peripheral and lung compartments; a 'double edged sword effect'. This may be due to the unique ability of alveolar macrophages to retain very high concentrations of certain mediators (eg, macrolide antibiotics) when compared to plasma concentrations[30].

These findings have direct implication for potential adjunct treatment for BOS and provide a rationale for investigating MBL as a therapy in this disease. MBL has been successfully administered by an intra-nasal route for treatment of murine model of invasive pulmonary aspergillosis[31]. In humans, plasma-derived MBL has been applied in a phase I human study in MBL-deficient healthy male subjects, establishing the safety of the approach[32]. A second generation nonofiltered plasma-derived MBL has been developed by Statens Serum Institute, Denmark, and has now successfully undergone phase II human studies[33,34]. Consistent with this evidence we have previously demonstrated that we could improve efferocytosis and reduce inflammation by treating mice that had a smoke-induced MBL and efferocytosis deficiency with aerosolized plasma derived MBL[11].

Data from this study strongly implicate the MBL pathway in the defective macrophage efferocytosis function in OB, and suggest that modulation of efferocytosis in the airway using MBL therapy may be a

useful adjunct therapeutic strategy to prevent the progression of BOS following lung transplantation. Intra-nasal or aerosolized administration of MBL would be an attractive treatment option for these patients.

	Control	Stable	Stable + Infection	BOS
n	14	16	34	25
Age	48.5 ± 3	48.1 ± 5	45.1 ± 2	43.6 ± 5
Months post-transplant		9.9 ± 1	14.6 ± 2	28.0 ± 7
FEV_1 [%Pred]	98.1 ± 3	82.6 ± 5	72.1 ± 4	47.7 ± 6

Table 1. Demographic details of study subjects. Data presented as Mean ± SEM. Tx: Transplant, FEV_1: forced expiratory volume in 1 second.
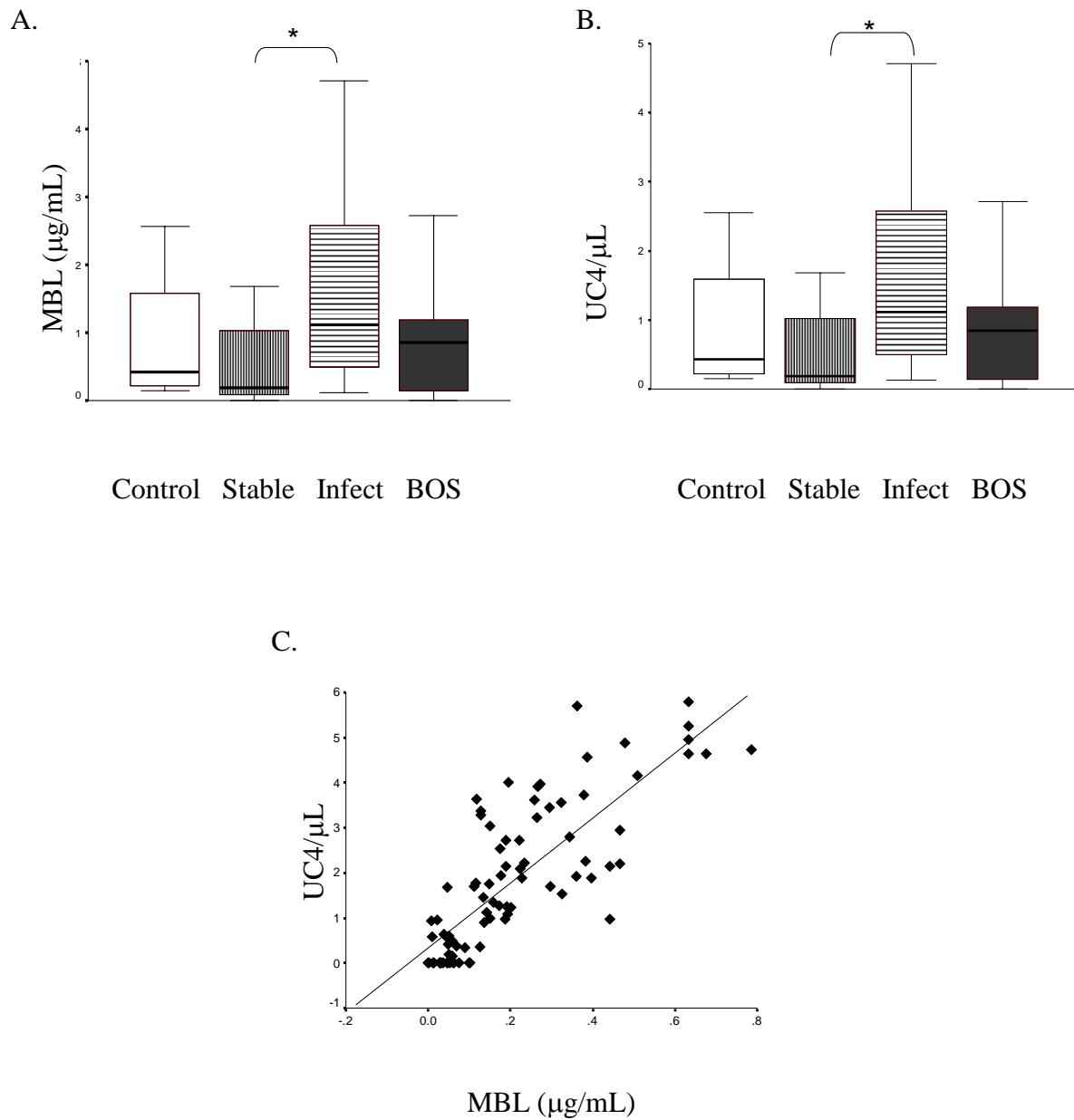
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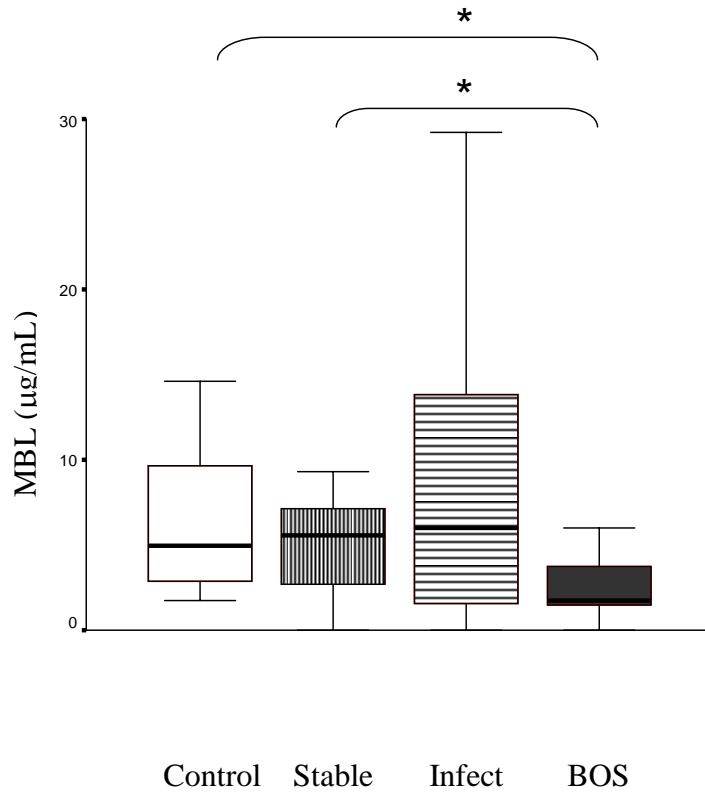
Figure 2. MBL was determined by ELISA in BAL from healthy controls, lung transplant patients with stable graft function (Stable), patients with stable graft function but proven infection (Infect) and those with bronchiolitis obliterans syndrome (BOS). *significant (p<0.05) decrease 'BOS' vs 'Controls' or 'Stable' groups.
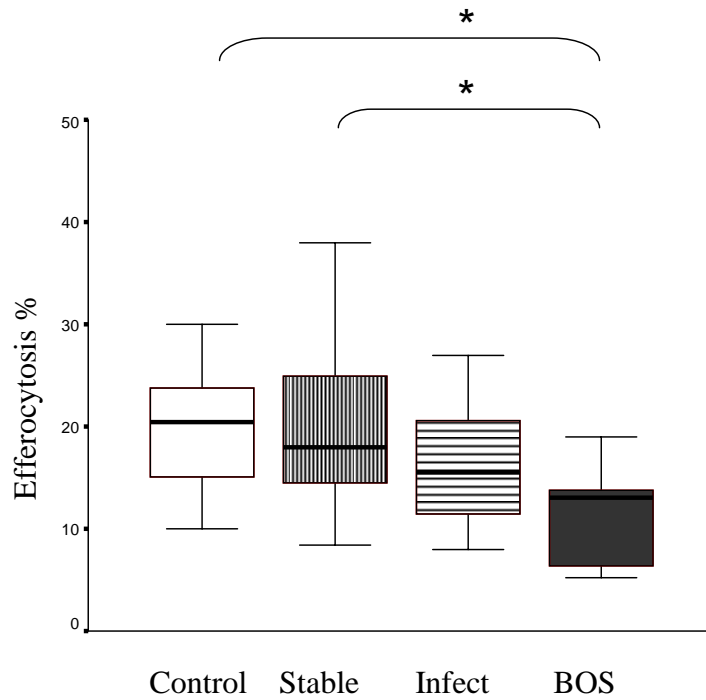
Figure 3. Efferocytosis of apoptotic bronchial epithelial cells by alveolar macrophages obtained from bronchoalveolar lavage from never-smoker controls, lung transplant patients with stable graft function (Stable), patients undergoing an acute rejection episode (Acute rejection), patients with stable graft function but proven infection (Infection) and those with bronchiolitis obliterans syndrome (BOS). *significant (p<0.05) decrease 'BOS' vs 'Controls', 'Stable' or 'Infection' groups.

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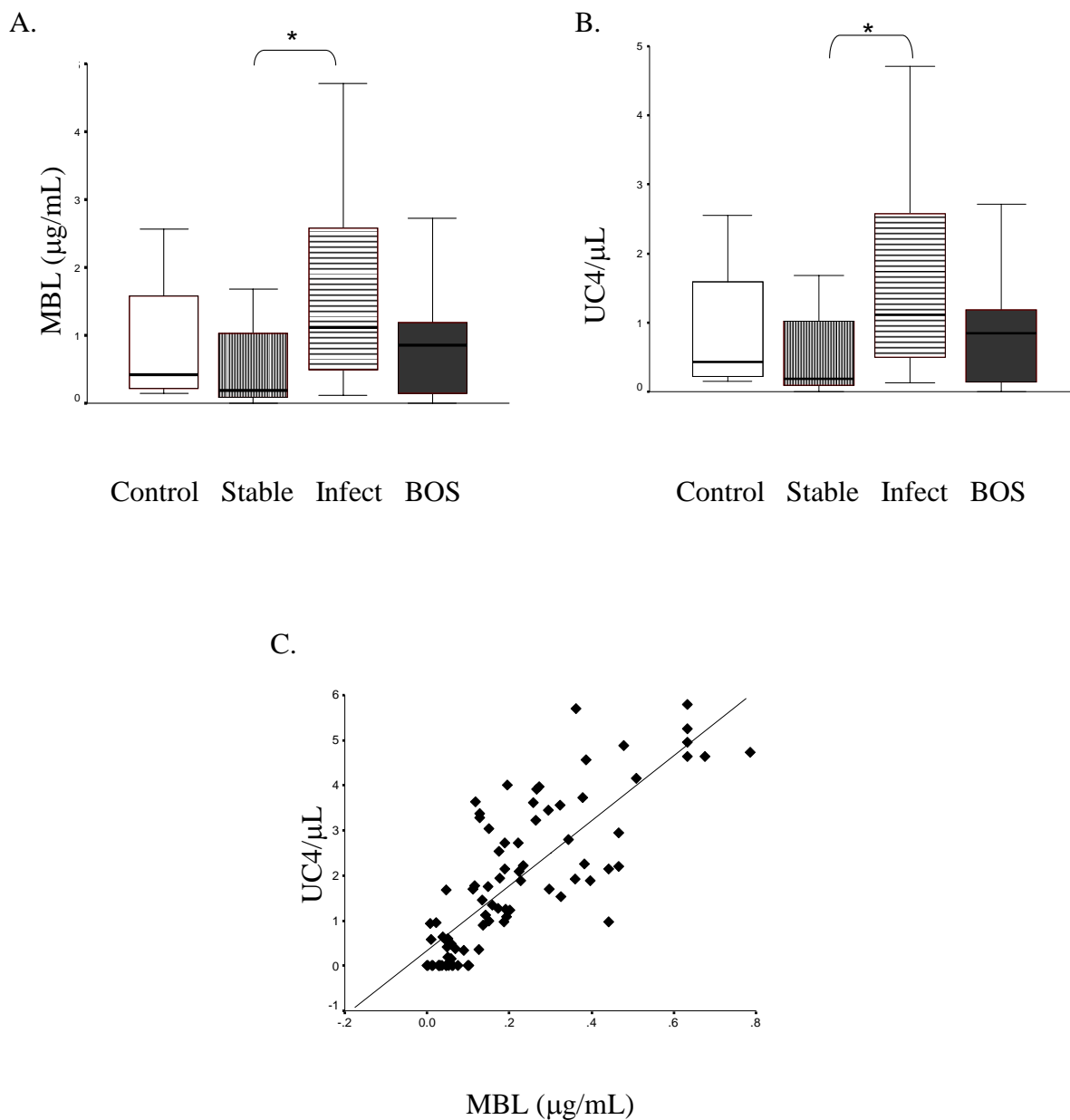
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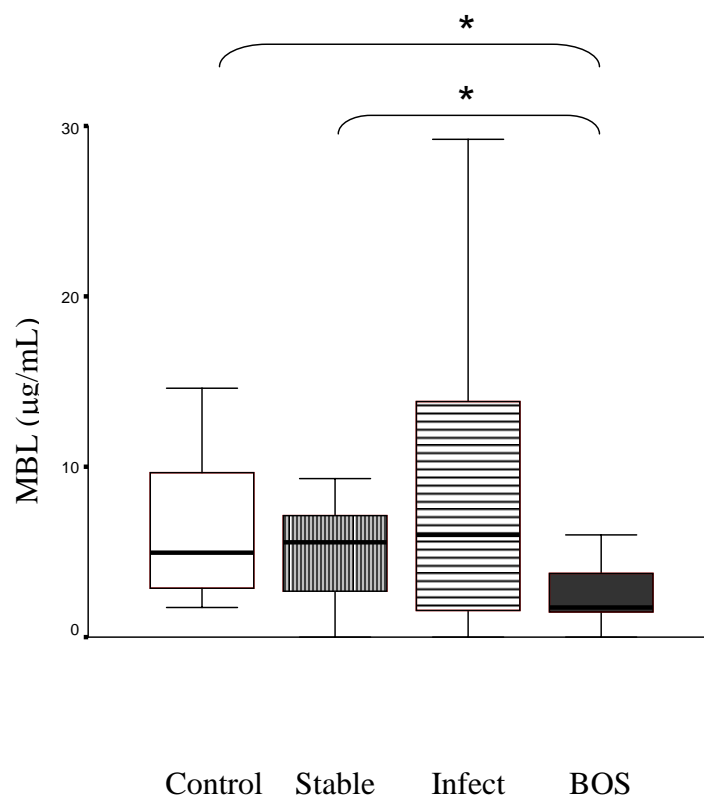
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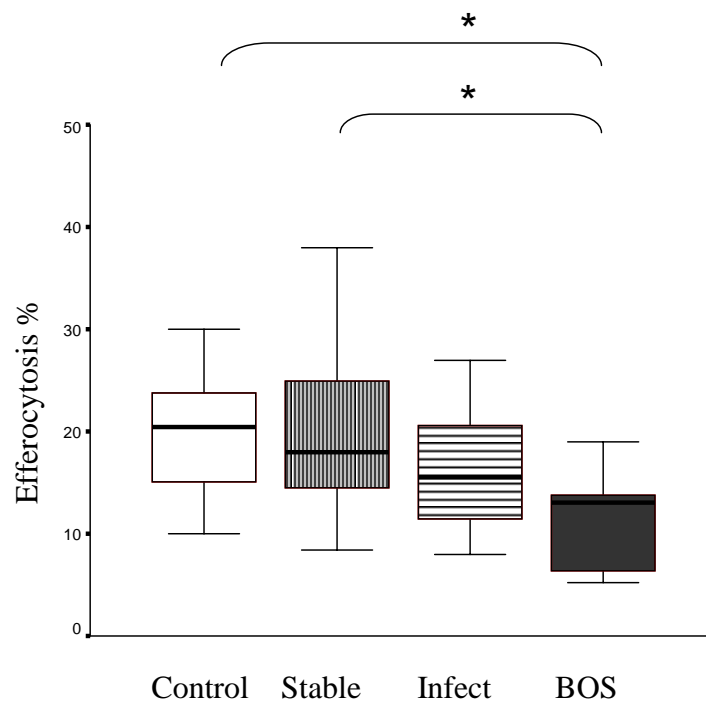'Controls' or 'Stable' groups.

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COVER LETTER:

The manuscript " Decreased efferocytosis and mannose binding lectin in the airway in bronchiolitis syndrome" has not been published previously and it is not under consideration for publication elsewhere.

All authors are in agreement with the content of the manuscript.

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Sandra Hodge

On behalf of all authors

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