DR. CLAIRE H. MASTERSON (Orcid ID : 0000-0002-9863-5324)

DR. NEIL J ROWAN (Orcid ID : 0000-0003-1228-3733)

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TITLE

Purified β-glucans from the Shiitake mushroom ameliorates antibiotic-resistant *Klebsiella pneumoniae*-induced pulmonary sepsis

AUTHORS

C. H. Masterson[1]*, E. J. Murphy[2], H. Gonzalez[1], I. Major[3], S. D. McCarthy[1], D. O'Toole[1], J. G. Laffey[1,4], N.J. Rowan[5]

*Corresponding author

AFFILIATIONS

[1]Lung Biology Group, National University of Ireland, Galway, Galway, Ireland, [2] Bioscience Research Institute, Athlone Institute of Technology, Athlone, Ireland, [3]Materials Research Institute, Athlone Institute of Technology, Athlone, Ireland, [4]Anesthesia and Intensive Care Medicine, University Hospital Galway, Galway, Ireland, [5]Centre for Disinfection, Sterilisation and Biosecurity, Athlone Institute of Technology, Athlone, Ireland

ABBREVIATED RUNNING HEADLINE

β-Glucans for Pulmonary Sepsis

DETAILS OF CORRESPONDING AUTHOR

Dr Claire Masterson, Lung Biology Group, Biosciences Research Building, National University of Ireland, Galway, Ireland. Email: claire.masterson@nuigalway.ie

Significance and Impact of the Study: Bacterial infection remains the main cause of Acute Respiratory Distress Syndrome (ARDS) and the need for an effective therapeutic intervention is evident. This study has demonstrated for the first time that purified β-glucans from the Shiitake mushroom *Lentinus edodes* can be used to attenuate the injury resulting from antibiotic-resistant *Klebsiella pneumoniae* pulmonary infection. Intravenous administration of β-glucan shows potential for treating sepsis-induced lung injury as it effectively reduces bacterial load, inflammatory white cell influx, protein leakage to the lungs and improves lung physiological parameters. Use of Lentinan shows promise as potential therapeutic intervention to combat bacterial pulmonary sepsis.

Abstract: Bacterial infection remains the main cause of Acute Respiratory Distress Syndrome (ARDS) and is a leading cause of death and disability in critically ill patients. Here we report on the use of purified β-glucan (Lentinan) extracts from *Lentinus edodes* (Shiitake) mushroom that can reduce infection by a multidrug-resistant clinical isolate of *K. pneumoniae* in a rodent pneumonia model, likely through immunomodulation. Adult male Sprague-Dawley rats were subjected to intra-tracheal administration of *K. pneumoniae* to induce pulmonary sepsis and randomised to three groups; Vehicle control (Vehicle, n=12), Commercial Lentinan (CL, n=8), or in-house extracted Lentinan (IHL, n=8) was administered intravenously 1h post infection. Physiological parameters and blood gas analysis were measured, bacterial counts from bronchoalveolar-lavage (BAL) were determined, along with differential staining of white cells and measurement of protein concentration in BAL 48h after pneumonia induction. Use of IHL extract significantly decreased bronchoalveolar-lavage (BAL) CFU counts. Both CL and IHL extractions reduced protein concentration in BAL. Use of IHL resulted in an improvement in physiological parameters compared to controls and CL. In conclusion administration of Lentinan to treat sepsis-induced lung injury appears safe and effective and may exert its effects in an immunomodulatory manner.

Key words: beta glucans; medicinal mushrooms; *Klebsiella pneumoniae*; lung injury; sepsis

Introduction

Acute respiratory distress syndrome (ARDS) is a leading cause of death and disability in critically ill patients (Rubenfeld et al., 2005) resulting from a loss of respiratory system compliance and hypoxia due to alveolar-capillary barrier disruption (Ferguson et al., 2005) which can lead to multiple organ failure and death (Ware and Matthay, 2000). Although ARDS can arise from many causes, sepsis-associated ARDS is the most common type (Bellani et al., 2016) with approximately 40% of patients with severe sepsis, and 23% of mechanically ventilated patients developing ARDS (Bellani et al., 2016, McNicholas et al., 2018). The inflammatory response associated with the presence of a microbial infection involves the recruitment of inflammatory white cells and an over-production of inflammatory cytokines leading to alveolar-capillary barrier damage and pulmonary oedema instigating further infiltration, inflammation, and widespread damage (Grommes and Soehnlein, 2011). Interventions to combat the progression of ARDS to date have been mostly unsuccessful (Laffey and Kavanagh, 2018, Lewis et al., 2019). Thus far the most promising therapy for the treatment of ARDS has been the implementation of supportive therapies such as fluid management, ventilatory strategies and prone positioning to improve oxygenation (Kon et al., 2015). The collective failure of pharmacologic therapies shows a clear need for the introduction of novel treatments for sepsis and associated ARDS. Targeting the immune response as a method of controlling the inflammatory response would serve as an attractive option in combating the initial infection and detrimental outcome of an overzealous inflammatory milieu in sepsis induced ARDS.

β-glucans are polysaccharides naturally found in the cell walls of plants, bacteria, fungi, yeast and algae (Sullivan et al., 2006). They are comprised of chains of D-glucose rings connected commonly through a 1-3 glycosidic bond. Depending on source, β-glucans vary in chain length and level of side branching. Mushroom β-Glucans, including Lentinan extracts from *Lentinus edodes* (Shiitake), consist of a 1,3 linked back bone with 1,6 linked side branches (Wan-Mohtar et al., 2016). The molecular structure of different β-glucans confers different properties including solubility and receptor binding which is of interest in relation to leukocyte activation. For example, β-Glucans with poor solubility confer direct leukocyte activation due to clustering of the receptor binding site (Sahasrabudhe et al., 2016), whereas modified soluble forms of β-Glucan enhanced leukocyte activity without complete activation (Poutsiaka et al., 1993). Dectin-1 is a β-Glucan specific receptor (Herre et al., 2004) found predominantly on macrophages and neutrophils as well as dendritic cells and T-Lymphocytes (Brown et al., 2003, Taylor et al., 2002) and functions as a receptor specific for β-Glucans with 1,3 and/or 1,6 linkages (Willment et al., 2003). It has been known for quite some time that β-glucans are the effective components in fungal products, and

mushroom extracts have been used as medicine for thousands of years (Chang, 2002, Sullivan et al., 2006).

Recently, there has been an increasing interest in the use of β-glucans from natural sources for treating infection in animals and humans (Carballo et al., 2019), including use of Shiitake mushrooms (Zhang et al., 2019). Carballo and co-workers (Carballo et al., 2019) exploited use of β-glucans from yeast in feed to modulate immune responses to control *Vibrio* genus-related infection in farmed fish. McCarty et al. recently described the potential role of β-glucan as natural nutraceutical for boosting type 1 interferon response to RNA viruses such as influenza and coronavirus (McCarty and DiNicolantonio, 2020). Sepsis, influenza and coronavirus cause an inflammatory storm in the lungs and it is this inflammatory storm that leads to respiratory distress, organ failure and death (Shi et al., 2020). There is emerging evidence to suggest that certain nutraceuticals, such as β-glucans, may help reduce inflammation in the lungs with RNA viruses and other problematic bacterial pathogens (Vetvicka and Vetvickova, 2015). β-glucans may also help boost type 1 interferon response to these pathogens, which is the body's primary means of help create antimicrobial antibodies to fight off these infections (McCarty and DiNicolantonio, 2020). Here we aimed to investigate the potential for our in-house purified lentinan β-glucan preparation (compared to commercially produced lentinan β-glucan) in combating antibiotic-resistant *Klebsiella pneumoniae* pulmonary infection.

Results & Discussion

The characteristics of β-Glucan compounds varies depending on preparation

Immunomodulatory polysaccharides, such as β-glucans, are non-toxic and do not result in side effects commonly seen with the use of bacterial or synthetic compounds (Rice et al., 2004) making them an attractive adjunct therapeutic strategy in critically ill or high-risk patients. Megazyme analysis of CL and IHL samples revealed differences in both β and α-glucan % w/w content. CL was shown to have a significantly higher α glucan content at 16±1.5% compared with only 2±0.5% for IHL extract. However, IHL had a significantly higher β-glucan content at 76± 3.5% compared with 48±3.0 for CL samples (Figure S1). Further studies by our group using the same samples revealed differences between specimens including variances in particle size and particle dispersity, elemental content, but also that each sample contained the same β-glucan compound (Murphy et al., 2020). This corroborates findings of Zhang et al., (Zhang et al.) where similar medicinal fungi may exhibit significant differences in the molecular mass range profile and chain conformation of the beta-glucan samples. A study conducted by Vetvicka et al., (Vetvicka and Vetvickova, 2018) suggested that the use of a highly purified yeast-derived β-Glucan was superior to crudely isolated, less active β-glucans from various sources. In rodent models of septic shock and as we have shown here, the IHL which contains a purer composition of β-glucan out-performs the commercially sourced CL. β-glucans at concentrations used in this study (20mg.kg^{-1}) were based on the maximum dose of CL that could be administered due to the viscosity of the solution, and did not affect viability or proliferation of *K. pneumoniae* bacteria (data not shown). This suggests an indirect effect of the compounds as described previously (Vetvicka and Vetvickova, 2015) whereby β-glucans act upon the immune system to instigate clearance of infection and are not directly antibacterial themselves. Recent linked in vitro studies using same β-glucan extracts revealed that both IHL and CL demonstrated low to minimal toxicity in pulmonary cell lines. We observed that IHL and CL had both inflammatory and anti-inflammatory properties, with IHL showing a greater immune-modulating profile compared to CL supporting the immunomodulation potential of this novel β-glucan extract (Murphy et al., 2020).

B-Glucan can effectively attenuate bacterial counts and white cell infiltrates to the lungs during pneumonia

Whilst direct antibacterial properties of CL and IHL could not be demonstrated, here we have shown that the numbers of viable bacteria present in the BAL fluid of treated animals was significantly reduced. The administration of 20mg.kg^{-1} β-glucan was significantly effective at lowering BAL WCC compared to vehicle (Figure 1, Panel B, $P<0.05$), however only the IHL was shown to significantly reduce the numbers of viable

K. pneumoniae in the BAL fluid (Figure 1, Panel A, P<0.05). Differential counts of the total white cells in the BAL fluid revealed that both CL and IHL could reduce the numbers of inflammatory PMNs infiltrating to the lung (Figure1, Panel C, P<0.01) but only IHL increased the numbers of monocytes/macrophages in the BAL fluid (Figure1, panel D, P<0.05). It has been shown in several studies that β-glucans exert a potent effect on cells of the innate immune system which would explain the reduction in bacterial numbers and the differences in innate immune cell populations as shown here. B-Glucans are not produced by humans or animals and are therefore classed as 'non-self' upon introduction to the body activating innate and adaptive immune responses (Brown and Gordon, 2005). B-Glucans can bind to and activate circulating monocytes increasing their cytotoxic activity, phagocytic activity, reactive oxygen species and nitric oxide production, and cytokine secretion (Chan et al., 2009). In mouse models of influenza yeast β-glucan has been shown to increase dendritic cell activation conferring protection from infection (Camilli et al., 2018, Vetvicka and Vetvickova). In humans, administration of β-glucan has been previously shown to increase blood monocyte proliferation and maturation of dendritic cells (Chan et al., 2007), here we have demonstrated an increase in monocyte numbers in the BAL fluid of treated animals whilst also demonstrating a decrease in the inflammatory PMN fraction.

B-Glucans improve lung function and levels of circulating metabolites

Administration of IHL significantly improved arterial oxygenation (P<0.05) during pneumonia compared to vehicle controls. CL also improved arterial oxygen levels, albeit not significantly (Figure 2, Panel A). Static lung compliance measurements revealed that lung structural indices were also improved. Blood gas analyses of circulating metabolites revealed a significant decrease in plasma lactate levels after treatment with IHL β-glucans (Figure 2, Panel B, P<0.05). The administration of IHL β-Glucan also returned blood glucose levels back to normal compared to vehicle and sham (Figure 2, Panel C).

While both CL and IHL increased compliance toward normal values, only IHL reached significance (Figure 3, Panel A, P<0.05). The wet:dry ratio, an indicator of lung leak and tissue edema, was positively decreased by IHL compared to vehicle control (Figure 3, Panel B, P<0.001) and arterial-alveolar gradient values trended toward a decrease in the IHL treated group (Figure 3, Panel C).

B-Glucans enhance the inflammatory response to infection

Cytokine measurements in the BAL of animal models revealed that the treatment of *K. pneumonia* using β-glucans seemed to enhance the inflammatory response to infection by significantly decreasing the levels of anti-inflammatory IL-10 (P<0.01) and modestly increasing levels of TNF-α (P=0.108) compared to vehicle control (Figure 4, Panels A & B). Measurement of BAL protein levels indicated that treatment with

β-glucan significantly decreased the levels of protein representing a decrease in alveolar-capillary leakage (Figure 4, Panel C, $P<0.05$) and supports the finding that IHL could improve lung structural indices as shown by measurements in static lung compliance and wet:dry ratio (Figure 3, Panels A & B). A study has demonstrated that the prophylactic administration of β-glucan can confer a protective effect, lowering TNF-α production and enhancing endotoxin clearance (Vereschagin et al., 1998). Here however we have demonstrated a very modest increase in inflammatory TNF-α accompanied by a significant decrease in anti-inflammatory IL-10. This may be explained by the prophylactic administration used in previous studies which may have resulted in a state of 'trained immunity' (Quintin et al., 2012). In contrast, β-glucan in the present study was administered 1h post bacterial inoculation.

There is strong potential to produce commercially scalable production of β-glucans from Shiitake mushroom mycelium using controlled bioreactor studies for global research and innovation (Wan-Mohtar et al., 2016). With more research such as the present study and others (Vetvicka and Vetvickova, 2018) showing that the production process can significantly impact the activity and efficacy of the extracted β-glucan it is imperative that it is given due attention. Here we have shown that the isolation of a pure β-glucan product from the Shiitake mushroom is effective in attenuating the injury parameters associated with bacterial pneumonia and propose that the use of naturally derived immunomodulators to be a novel strategy in combating ARDS.

Materials and Methods

Klebsiella pneumoniae-Induced Lung Injury: All work was approved by the Animal Care Research Ethics Committee of the National University of Ireland, Galway and conducted under license from the Health Products Regulatory Authority, Ireland (Licence number AE19125/P053, Number of animals approved on licence - 246; Animals enrolled in this series - 28). Specific-pathogen-free adult male Sprague Dawley (CD) rats (Charles River Laboratories, Kent, UK) weighing between 300–450g were used in all experiments. These animals were chosen based on the successful establishment of bacterial pneumonia using intratracheal *E. coli* by our research group which has been used in several studies (O'Croinin et al., 2005, Chonghaile et al., 2008, Devaney et al., 2015, Masterson et al., 2018). The use of rats allows for adequate sample sizes of arterial blood, bronchoalveolar lavage (BAL) fluid, and tissues for analysis.

In all groups, animals were anaesthetised using vaporised isoflurane (Iso-Vet, Chanelle, Co. Galway, Ireland). The animals were orotracheally intubated under direct vision using a guide wire and a 14G catheter (BD Insyte®; BD Biosciences). A bolus of $1x10^9$ CFU of *K. pneumoniae* in a 300µL PBS suspension was instilled followed by a bolus of air. The animals were allowed to recover from anaesthesia as previously described (Curley et al., 2017, Devaney et al., 2015, Devaney et al., 2013, O'Croinin et al., 2005, O'Croinin et al., 2008) before proceeding to treatment. Piloting results for the *K. pneumoniae* pneumonia model are included in the supplementary data (Figure S2).

Clinical Isolate

K. pneumoniae clinical isolate was provided by Tullamore General Hospital (Health Service Executive, Co. Offaly, Ireland) as a stab culture. Using a clinical and laboratory standards institute (CLSI)-compliant Vitek 2 automated analyser to obtain antibiotic sensitivities, this organism was determined to be resistant to gentamicin and aztreonam and therefore categorized as a multidrug resistant extended-spectrum beta-lactamase (ESBL) as per European Antimicrobial Resistance Surveillance Network guidelines 2017 (Giske, 2017). Of note, the patient from whom the isolate was derived was successfully treated using meropenem following the failure of broad-spectrum antibiotics. The sample was isolated from blood but originated from the urine via a urinary tract infection complication.

K. pneumoniae cultures were propagated using tryptone soya broth (TSB; Fisher Scientific Ireland) and colonies were identified using UTI Brilliance Clarity Agar (LIP Fannin, Galway, Ireland). Colony forming units were quantified using a combination of serial dilution cultures and optical density readings at 600nm. Cultures were prepared for animal models of pneumonia at $1x10^9$CFU/300µL in sterile PBS (Sigma Aldrich, Ireland).

Beta Glucan isolation and preparation

Commercial lentinan (CL) was sourced from Carbosynth (FL33321, Berkshire, UK) and resuspended in sterile H_2O (Sigma Aldrich, Ireland). In-house produced Lentinan (IHL) was extracted from the fruiting bodies of *Lentinus edodes* which were purchased from Fancy Fungi Ltd (Co. Wexford, Ireland). Lentinan extracts were analyzed for (1,3)-(1,6)-beta-glucan content using the Megazyme yeast and mushroom kit (K-YBGL, Megazyme Ltd., Co. Wicklow, Ireland). Assays were carried out according to manufacturer's instructions. Briefly, samples were milled, and placed in 12M H_2SO_4 at -4^{o}C for 2 hours to solubilize the glucans. The samples were then hydrolyzed in 2M H_2SO_4 at 100^{o}C for 2 hours. After incubation any remaining glucan fragments were quantitatively hydrolyzed to glucose using a mixture of *exo*- 1,3-β-glucanase and β-glucosidase which gives a measurement of total glucan. The alpha glucan and sucrose content of the sample is determined by hydrolyzing specifically to D-glucose and D-fructose. Glucose was measured with amyloglucosidase and invertase using reagent. β-glucan was determined by the difference in both measurements.

In-house vs commercial Lentinan Beta Glucans in bacterial pneumoniae: One hour following intra-tracheal instillation of *K. pneumoniae* bacteria, animals were randomized to receive: (i) Vehicle (PBS, 300µL, n=12); (ii) 20mg.kg^{-1} IHL β-Glucan (n=8); (iii) 20mg.kg^{-1} CL β-Glucan (n=8) and the degree of injury assessed at 48 hours post pneumonia induction.

In Vivo Assessment: Animals were anaesthetized with subcutaneous ketamine 75mg.kg^{-1} (Ketalar; Pfizer, Cork, Ireland) and medetomidine 0.5mg.kg^{-1} (Domitor; Vétoquinol, Dublin, Ireland), intravenous and intra-arterial access was secured, and a tracheostomy tube was inserted. Anaesthesia was maintained with 2mg.kg^{-1} Alfaxan® (Jurox PLC, uk) and paralysis with 0.5mg.kg^{-1} atracurium besylate (Tracrium, GlaxoSmithKline, Dublin, Ireland), and mechanical ventilation commenced using 7mL.kg^{-1} tidal volume. Arterial blood pressure, airway pressure, lung static compliance and arterial blood gas analyses were performed as previously described (Curley et al., 2012, Devaney et al., 2015).

Ex Vivo Analyses: Following exsanguination under anaesthesia, bronchoalveolar lavage (BAL) was performed, and BAL fluid differential leukocyte counts, and lung bacterial colony counts were completed. BAL concentrations of TNF-α and IL-10 cytokines were determined using ELISA (R&D Systems, Abingdon, UK) and total BAL protein measured (Micro BCA; Pierce, Rockford, IL). All *ex vivo* analyses were performed by blinded investigators.

Statistical Analysis: A sample size of 8-12 animals per group was determined for a 3 group design based on previously published experimental data from these models by our group (Chonghaile et al., 2008,

O'Croinin et al., 2005, Devaney et al., 2015). Data are reported as means (±SD) or as medians [interquartile range]. Data was analysed using Sigma Stat (SYSTAT® software, Richmond, CA). The distribution of all data was tested for normality using Kolmogorov-Smirnov tests. Data were analysed by student's unpaired T-Tests or one-way ANOVA as appropriate, with post hoc testing using Student-Newman-Keuls between group comparisons as appropriate. Underlying model assumptions were deemed appropriate based on suitable residual plots. A two-tailed P value of <0.05 was considered significant.

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Conflict of Interest

No conflict of interest declared.

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Figure 1

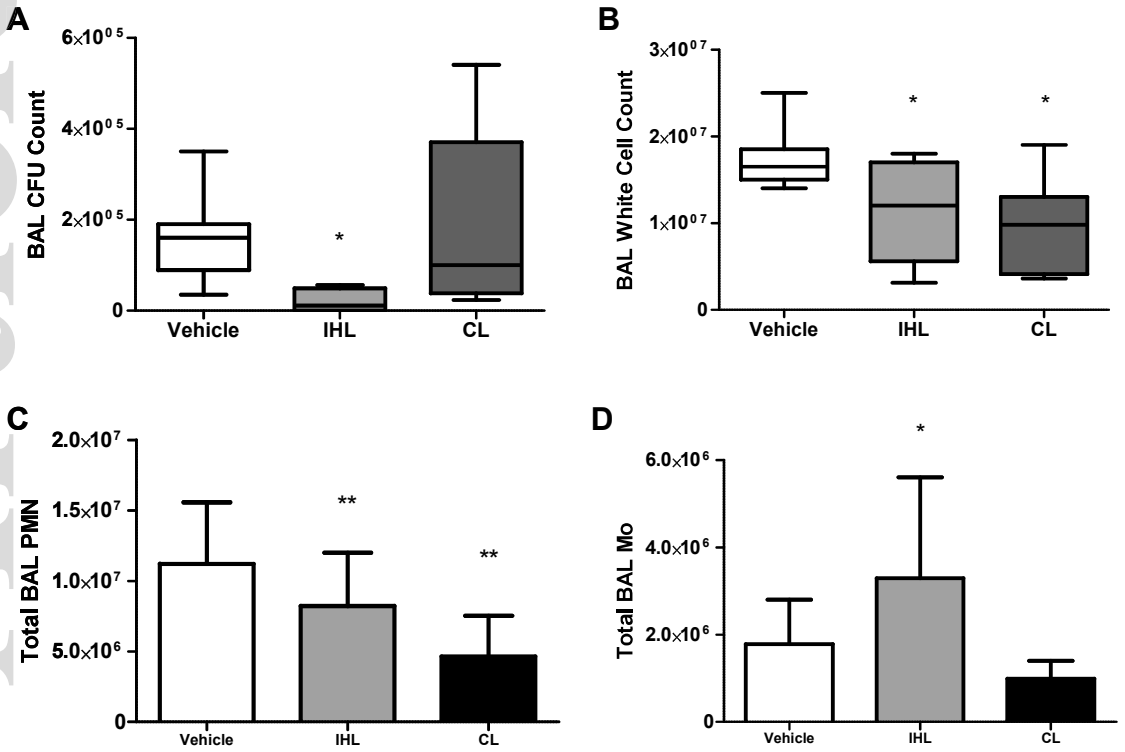

Figure 2

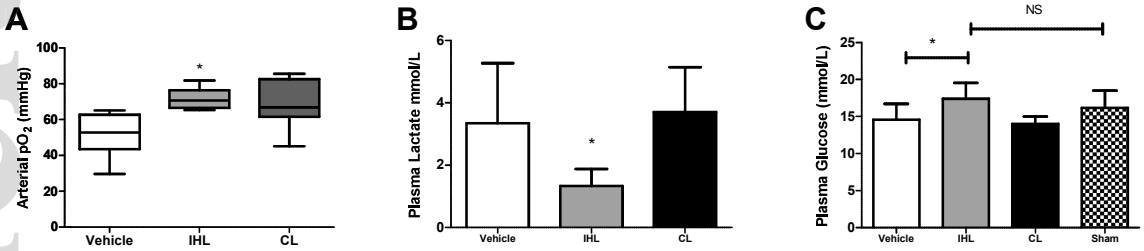

Figure 3

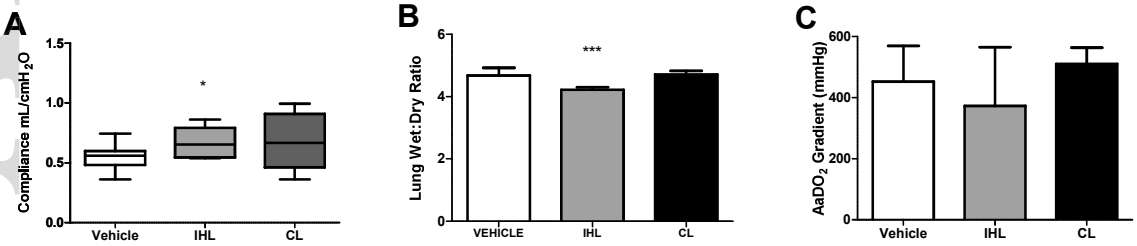

Figure 4

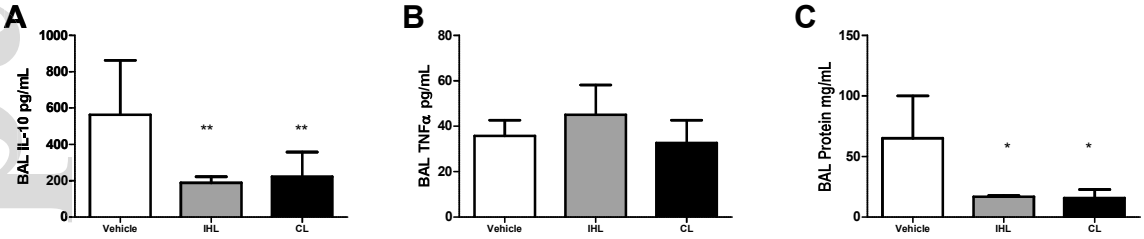

Figure Legends

Figure 1: The effects of IHL and CL on the bacterial counts and white cell infiltrates to the lungs during pneumonia. Rodent models of *K. pneumoniae* infection were treated intravenously with 20mg.kg^{-1} of IHL or CL β-glucan. Bacterial and white cell counts were performed on the BAL fluid (Panels A & B). Differential staining of the BAL white cells allowed numeration of the PMN (Panel C) and Mo fractions (Panel D).

Data are expressed as mean ± SD (n= 6-10/group). *=p<0.05, **=p<0.01 (1-way Anova with Newman Keuls Multiple Comparison Test) wrt Vehicle group. IHL = In-house Lentinan; CL=Commercial Lentinan; CFU = colony forming unit; BAL=bronchoalveolar lavage; PMN = Polymorphonuclear neutrophils; Mo = Monocytes

Figure 2: The effects of IHL and CL on blood gas incides during pneumonia. Rodent models of *K. pneumoniae* infection were treated intravenously with 20mg.kg^{-1} of IHL or CL β-glucan. Samples of arterial blood were analyzed using a blood gas analyzer during mechanical ventilation. Arterial oxygenation (Panel A), and plasma lactate and glucose readings were recorded (Panels B & C).

Data are expressed as mean ± SD (n= 6-10/group). *=p<0.05, NS = Not significant (1-way Anova with Newman Keuls Multiple Comparison Test) wrt Vehicle group and/or Sham group. IHL = In-house Lentinan; CL=Commercial Lentinan; pO_2 = partial pressure of oxygen.

Figure 3: The effects of IHL and CL on physiological parameters during pneumonia. Rodent models of *K. pneumoniae* infection were treated intravenously with 20mg.kg^{-1} of IHL or CL β-glucan. Static compliance was measured under anaesthesia (Panel A), and samples of lung tissue were dried post mortem for analysis of wet:dry ratios (Panel B). Samples of arterial blood were analyzed using a blood gas analyzer during mechanical ventilation at FiO_2 1.0 and $AaDO_2$ calculated (Panel C).

Data are expressed as mean ± SD (n= 6-10/group). *=p<0.05, ***=p<0.001, (1-way Anova with Newman Keuls Multiple Comparison Test) wrt Vehicle group. IHL = In-house Lentinan; CL=Commercial Lentinan; $AaDO_2$ = Arterial alveolar Oxygen Gradient.

Figure 4: The effects of IHL and CL on cytokine profiles during pneumonia. Rodent models of *K. pneumoniae* infection were treated intravenously with 20mg.kg^{-1} of IHL or CL β-glucan. Post mortem BAL

samples were analyzed for anti-inflammatory IL-10 (Panel A), inflammatory TNF-α (Panel B) and protein concentration was assessed using a protein assay of BAL fluid (Panel C).

Data are expressed as mean ± SD (n= 6-10/group). *=p<0.05, **=p<0.01, (1-way Anova with Newman Keuls Multiple Comparison Test) wrt Vehicle group. IHL = In-house Lentinan; CL=Commercial Lentinan; BAL = Bronchoalveolar lavage; IL-10 = Interleukin 10; TNF-α = Tumor necrosis factor alpha.