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The contribution of toll-like receptor activation to lung damage after donor brain death

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Abstract

Introduction—Donor brain death is the first injurious event which can produce inflammatory dysfunction after pulmonary transplantation. This study was designed to determine whether stimulation of the toll-like receptor system contributes to the changes produced by brain death.

Materials and Methods—Rats were repeatedly treated with specific agonists for toll-like receptor 4 or toll-like receptor 2/6 to desensitize these receptors. Brain death was then induced by inflation of a balloon catheter within the extradural space. Mean arterial pressure changes and inflammatory markers were measured serially by protein and mRNA analysis.

Results—Both desensitizing pre-treatments prevented the neurogenic hypotension ($p < 0.001$) and metabolic acidosis ($p < 0.001$) observed in control animals after brain death. These treatments also reduced the levels of TNF α and CXCL1 in serum and bronchoalveolar lavage fluid, although desensitization of toll-like receptor 4 produced a greater inhibition than desensitization of toll-like receptor 2. Desensitization of toll-like receptor 4 also reduced ($p < 0.05$) expression of the adhesive integrin CD11b on blood neutrophils after brain death. Examination of mRNA levels in lung tissue 5 hours after brain death showed that desensitization of TLR4 limited the expression of IFN γ , IFN β and CXCL10, whilst desensitization of TLR2/6 only reduced the expression of IFN γ .

Conclusion—These results indicate that activation of toll-like receptor signalling pathways can contribute to the lung damage produced by brain death; this may increase subsequent graft injury following transplantation.

Keywords

brain death; primary graft dysfunction; inflammation; toll-like receptors

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Introduction

Primary lung graft dysfunction (PGD) is a form of acute lung injury that develops in recipients early after pulmonary transplantation (1). The incidence of the most severe form of PGD is 30% (2); this is the leading cause of 30 day mortality, accounting for 29% of early deaths (3). It has been thought that PGD is principally a manifestation of ischaemia-reperfusion injury (4). However, it is now accepted that PGD is the result of a series of insults that start in the donor during the events that led to brain death and culminates following reperfusion of the organ in the recipient (5).

Toll-like Receptors (TLRs) are key sensors of the innate immune system and are pattern recognition receptors that sense pathogen associated molecular patterns (PAMPs) (6) through leucine-rich repeats in their extracellular domain. Even in the absence of infection, injured cells can release damage associated molecular patterns (DAMPs), which activate TLRs (7). The intracellular portion of TLRs contains a TLR/IL1R receptor (TIR) interaction domain required for forming complexes with adaptor molecules, such as myeloid differentiation primary response protein (MyD88) and TIR domain-containing adaptor inducing IFN β (TRIF). Stimulation of all TLRs except TLR3 causes activation of the MyD88-dependent pathway, resulting in the activation of transcription factors including AP-1 and NF κ B and subsequent synthesis of pro-inflammatory cytokines. In addition, stimulation of TLR3 or TLR4 induces the production of type I interferons and CXCL10 following activation of the TRIF-dependent (MyD88-independent) signalling pathway which results in phosphorylation of the interferon regulatory factor, IRF3 (8).

The central role played by TLRs in ischaemia reperfusion injury (IRI) has been demonstrated in animal models of IRI in the heart (9), lung (10), liver (11) and kidney (12). Both MyD88-dependent (13) and TRIF-dependent signal transduction pathways have been demonstrated to be important (14), with immune and non-immune cells having the potential to respond to TLR stimulation. For this reason, PGD can be considered a response, at least in part, to injury caused by TLR ligands (15, 16); some of these ligands may have an endogenous source (17).

In a rodent model of hepatic IRI, prior treatment with the TLR4 agonist lipopolysaccharide (LPS) improved survival and maintained liver function (18). It is known that primary stimulation of a TLR can reduce the effect of subsequent stimulation by induction of a process termed endotoxin tolerance which can dramatically reduce the release of cytokines such as TNF α (19). This tolerance can persist for up to 3 weeks after primary treatment and is thought to involve the induction of specific regulatory proteins including SOCS-1, IRAK-M and SHIP (20), which can inhibit both MyD88-dependent and TRIF-dependent pathways.

Animal models of IRI are canonical and the contributing factors that compromise allograft function are rather more complicated (21). Ischaemia surrounding the transplant event can be divided into three distinct phases (22). The first involves vasoconstriction and hypotension associated with brain death and donor organ retrieval. The second phase is the cold ischaemic interval associated with preservation and storage, and a third can occur during graft revascularization. The initial modifiable insult to the donor thus occurs following brain death.

It is well established that brain death influences the outcome of lung transplantation (23, 24), and that the magnitude of the pulmonary inflammatory response in the donor predicts PGD in the recipient (25). However, the role played in this process by TLRs and their downstream signalling pathways is poorly understood. In this study rats were pre-treated daily for 5 days with specific TLR agonists to desensitize TLR4 or TLR2/6. Groups of agonist pre-treated

and matched control animals were then used to investigate changes in the systemic and pulmonary inflammatory response following brain death.

Materials and Methods

Animals and the induction of receptor tolerance

Twenty nine outbred male Wistar rats were used (mean mass: 372 g, 95% CI: 359 – 384 g) for the study. The procedures were performed according to the regulations of the Animals (Scientific Procedures) Act 1986 (project license reference no: PPL 60/3456).

Endotoxin tolerance was induced by intraperitoneal injection of highly purified lipopolysaccharide (LPS from *Escherichia coli* O111: B4; a selective TLR4 agonist) or a synthetic diacylated lipoprotein derived from *Mycoplasma salivarium* (FSL-1; fibroblast stimulating lipopeptide 1; a selective TLR2/6 agonist (26)) diluted in 0.5 ml of apyrogenic 0.9% saline; both agonists were from Invivogen, San Diego, CA. On the first day of tolerance induction (day 0), rats received an intraperitoneal injection of 20 µg/kg of LPS (n=10) or FSL-1 (n=9), followed by 100 µg/kg on days 1, 2, 3 and 4. This regimen was adapted from established rodent models in which endotoxin tolerance was produced using these doses of LPS (27) and FSL-1 (28). The mass-based equipotency of these agents has also been demonstrated in rats by the induction of both febrile and cytokine responses (29). Control animals (n=10) received equivalent volumes of sterile saline. The rats were weighed prior to each injection and were used in the brain death model 3 days after the final injection.

Brain death model

All animals were anaesthetized in a chamber with 5% isoflurane, intubated with a 14G cannula and ventilated with an Inspira Advanced Safety Volume Control ventilator (Harvard Apparatus) with tidal volume 8 ml/kg, frequency 50 cycles/min, inspiration/expiration ratio = 1/2 and inspired oxygen fraction 1.0. The rats were maintained with inhalation of 2.5% isoflurane. A tail vein was cannulated with a 22G cannula for the administration of fluid. The left groin was dissected and the left femoral artery was cannulated with a 24G cannula for continuous blood pressure monitoring and blood sampling. All rats received intravenous infusion of 5 ml/kg/h gelatin solution (Gelofusine, B.Braun Melsungen AG, Germany).

In three animals from each group, a sternotomy was performed immediately after line insertion, the left lung was excised and snap frozen in liquid nitrogen and stored at –80°C. In the remaining animals (LPS pre-treated, n=7; FSL-1 pre-treated, n=6; control, n=7), the scalp was incised and the skull vault was exposed. A burr hole was drilled to expose the dura. A 4F Fogarty balloon catheter (Edwards Lifesciences) was inserted in the extradural space pointing caudally. The balloon was inflated with 0.5 ml of water over 30 s to induce brain death. Brain death was confirmed immediately by pupil dilatation, apnoea, and disappearance of corneal reflexes. This method of brain death induction is 100% effective as determined by the disappearance of brain stem auditory evoked potentials (30).

Sampling

The animals were monitored for 5 h after brain death. Arterial blood pressure was continuously measured and the trace was digitally stored using Spike3 software (version 3.17, Cambridge Electronic Designs, UK). Blood gases were measured using arterial blood samples collected immediately after insertion of the arterial line, and 1, 3 and 5 h after brain death. Blood for measurement of serum cytokines was collected immediately after insertion of the arterial line, and 1 and 5 h after balloon inflation. Blood was also collected for measurement of CD11b/CD18 expression by neutrophils immediately after insertion of the

arterial line, and 3 and 5 h after balloon insertion/brain death. At the end of the monitoring period animals underwent laparotomy and sternotomy. The rats were exsanguinated by severing the inferior vena cava below the diaphragm and the lungs were excised *en bloc* with the heart. After clamping the left main bronchus, bronchoalveolar lavage (BAL) of the right lung was performed three times with 1 ml of normal saline each time. The apical segment of the left upper lobe was used to estimate wet/dry mass ratio. The remainder of the left lung was snap-frozen in liquid nitrogen and stored at -80°C .

BAL fluid processing

The BAL fluid was centrifuged at 800xg for 10 min. The supernatant was then aliquoted and frozen at -80°C .

Flow cytometric determination of blood neutrophil CD11b/CD18 expression

Immediately after collection, whole blood was incubated with the following fluorochrome-conjugated antibodies: CD45-FITC, CD45-FITC/CD11b-RPE and CD45-FITC/CD18-RPE (Serotec, Oxford, UK) for 15 min in the dark. The red cells were then lysed (Lysing Solution; Becton Dickinson, Oxford, UK). Flow cytometric analysis was performed using a Becton Dickinson FACScan and the data were analyzed using the CellQuest software program (Becton Dickinson). The increase in expression was expressed as median fluorescence relative to baseline expression.

Measurement of cytokine concentration

The cytokines TNF α , CXCL1 and IFN γ were measured in serum and BAL fluid using commercially available ELISA kits (R&D Systems, Abingdon, UK). Assays were performed in duplicate; the coefficient of variation was less than 10% in all cases. All samples were assayed at several dilutions to eliminate the hook effect.

RNA isolation and quantitative PCR

Total RNA was isolated from rat lung using TRI REAGENT^{TM} (Sigma, St Louis, USA) according to the manufacturer's protocol. Following ethanol precipitation the RNA was cleaned using RT² qPCR-Grade RNA Isolation kit (SuperArray, Frederick, MD, USA). RNA concentration and purity was determined by UV spectrophotometry. cDNA synthesis was performed from 1 μg total RNA using the RT² First Strand Kit (SuperArray, Frederick, MD, USA) according to the supplier's protocol. Amplification and detection were performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the TLR signalling pathway RT² Profiler^{TM} PCR 84 gene assay (PARN-018A-12; SuperArray, Frederick, MD, USA). Samples from 3 animals in each group were analysed after tolerance induction and the remaining animals (LPS pre-treated, n=7; FSL-1 pre-treated, n=6; control, n=7) were analysed 5 h after the induction of brain death; each assay was performed in duplicate. The samples were verified for reverse transcription and polymerase chain efficiency and the absence of genomic DNA contamination using the RT² RNA QC PCR assay (SuperArray, Frederick, MD, USA). In brain dead control animals, results were expressed as $2^{-\Delta\Delta\text{CT}}$ (CT threshold cycle) and normalized against control animals prior to brain death. In TLR agonist pre-treated groups, the results were normalized against control animals following brain death.

Statistical analysis

Statistical analysis of the data was performed using the Statistical Package for Social Sciences (SPSS v11; Chicago, IL, USA). Comparisons between two independent groups of observations with normal distribution were performed using the Student's t-test. Paired observations within a group were compared with the paired t-test (normal distribution) or the

Wilcoxon signed-rank test (non-parametric distribution). Comparisons between three or more independent groups of observations with normal distribution were performed with one-way analysis of variance, or the Welch test, if the variances of the groups were unequal. Subsequently, each group was compared with the Brain-death group using contrast tests. For three or more groups of observations with non-parametric distribution, the Kruskal-Wallis test was used and then each group was compared with the Brain-death group using the Mann-Whitney U-test. Comparisons between groups for a categorical variable were performed with the Fisher's exact test. Data are reported as mean $\pm$ SEM. Differences were considered significant at the level $p < 0.05$.

Results

Change in body mass

Following the initial injection of either LPS or FSL-1, the animals showed a significant reduction in body mass ($p < 0.05$). The reduction in body mass following injection of LPS was much more pronounced compared to FSL-1-treated animals ($p < 0.01$). FSL-1-treated animals started to gain body mass following the third injection, whilst LPS-treated animals did not start to gain mass until after the fourth injection (Figure 1).

Haemodynamic changes

There was no difference overall in baseline mean arterial pressure (MAP) (overall mean: 79.2 ± 2.09 mmHg) between the groups before brain death (Figure 2). Balloon inflation caused immediate brain death in all animals which was accompanied by a hypertensive response. MAP increased significantly and acutely, and peaked at 151.8 ± 1.72 mmHg (mean difference from baseline: 72.55 mmHg; 95% CI: 76.78, 68.31 $p < 0.001$). Prior treatment with either LPS or FSL-1 did not alter the magnitude of the hypertensive crisis ($p = 0.28$).

After 3-4 minutes, the hypertensive crisis was replaced by neurogenic hypotension. In saline-treated control animals the MAP continued to deteriorate over the duration of the 5h observation period to a final value of 23.0 ± 0.34 mmHg. However, in the LPS and FSL-1-treated groups there was no such deterioration and the final MAP (overall mean: 44.5 ± 3.05 mmHg) was similar to that immediately after the hypertensive crisis (overall mean: 48.3 ± 1.93 mmHg; $p = 0.14$). After only one hour of observation, the MAP of both treated groups was significantly different from the control group ($p < 0.001$).

Arterial blood gases and pulmonary oedema

There was no significant difference in oxygenation between the saline-treated control group and groups of rats pre-treated with either LPS or FSL-1. Rats in the saline-treated control group developed a progressive metabolic acidosis following the induction of brain death. At 5h following brain death base excess was significantly different from baseline (mean: -6.6 ; 95% CI: $-3.5, -9.7$; $p < 0.001$). Prior treatment with LPS or FSL-1 prevented the development of a metabolic acidosis, maintaining base excess within normal limits. The wet/dry mass ratio of the lungs was similar in all experimental groups.

Serum cytokine levels

No TNF α was detected in serum before the induction of brain death in all of the experimental groups. In saline-treated control animals there was a significant increase in serum TNF α within 1 h when compared to baseline ($p < 0.001$). Prior treatment with FSL-1 or LPS significantly reduced the amount of serum TNF α released in response to brain death ($p = 0.002$) (Figure 3a). Furthermore, the level of TNF α in serum 1 h after brain death was significantly lower in the LPS group compared to the FSL-1-treated group ($p = 0.02$). The

level of serum TNF α in the control group decreased significantly by 5 h ($p=0.04$) but remained significantly higher than the level observed in the LPS-treated animals ($p=0.001$) and FSL-1-treated animals ($p=0.005$). The level of serum TNF α at 5 h remained significantly lower in the LPS-treated group than the FSL-1-treated group ($p=0.04$).

As with TNF α , no CXCL1 was detected in serum prior to brain death. After balloon inflation in the control group there was a rapid and progressive increase in serum CXCL1. At 1 h, the level in controls was significantly greater than that measured in FSL-1 ($p=0.02$) or LPS ($p=0.003$) treated animals (Figure 3b). The differences between treated groups and controls were more marked 5 h following brain death ($p<0.001$).

IFN γ was not detected in the serum of animals in any experimental groups until the 5h time-point. The level of IFN γ was significantly greater in brain dead controls than in animals pre-treated with either LPS or FSL-1 at this time-point ($p<0.001$). In addition, the level of IFN γ was significantly greater in FSL-1-treated animals than those treated with LPS ($p=0.007$; data not shown).

BAL cytokine levels

TNF α levels in BAL fluid were significantly higher in brain dead controls when compared to LPS-treated ($p<0.001$) or FSL-1-treated ($p=0.002$) animals (Figure 4a). The levels of CXCL1 were also higher in brain dead control animals than either of the treated groups ($p<0.05$; figure 4b). Levels of IFN γ in BAL also tended to be higher in controls than in either treated group but this did not reach statistical significance ($p=0.08$; data not shown).

Expression of CD11b/CD18 by neutrophils

The baseline expression of CD11b and CD18 was similar in all experimental groups (CD11b: $p=0.61$ and CD18: $p=0.80$). Following the induction of brain death, the expression of CD11b increased progressively with time in all groups (Figure 5a). However, by 3 h the expression of CD11b was significantly lower in LPS-treated animals than either saline-treated controls ($p=0.004$) or FSL-1 preconditioned animals ($p=0.03$; Figure 5b). These differences were more marked at 5 h. The expression of CD11b by FSL-1-treated animals did not differ significantly from controls at any point (Figure 5b).

The expression of CD18 increased progressively with time in brain dead control animals. However, CD18 expression by LPS and FSL-1-treated animals remained relatively stable and was significantly lower than control in both treated groups of animals at the 3 h ($p<0.05$) and 5 h ($p<0.001$) time-points (Figure 5c).

TLR pathway gene expression in lung tissue

The 84 genes examined can be divided into those encoding TLRs, genes for adaptor or TLR interacting proteins, and downstream pathway genes. The expression of only one gene was significantly reduced 5 h after brain death; this gene encodes sterile α and TIR motif containing 1 (SARM1). However, 39 of the 84 genes showed a significant (<0.05) and >3 fold increase in expression 5 h after brain death (Table 1). Particularly noteworthy amongst the genes induced by brain death at this time point were cytokines (IL6, IL1 β , TNF α , IL1 α , IL10 and IFN γ), chemokines (CXCL10, CCL2) and colony stimulating factors (Csf2 and Csf3). There was no significant alteration in gene expression from normal animals following desensitization with LPS before brain death. Following FSL-1 pre-treatment there was only a change in gene expression for IL1 α prior to brain death; the expression of this gene was reduced three fold (data not shown).

Following the induction of brain death, the LPS-treated group showed a significant, >3 fold downregulation in the expression of type I and type II interferons (IFN β : $p=0.03$ and IFN γ : $p=0.02$) and the chemokine CXCL10 ($p=0.007$) when compared to the group of saline-treated control animals (Figure 6). The greatest significant increase in gene expression (2.6 fold) in this group of animals was observed for SARM1 (data not shown). Of the 3 cytokine genes regulated by LPS pre-treatment, the FSL-1-treated group only produced a significant downregulation of IFN γ compared to control animals ($p=0.008$) (Figure 6).

Discussion

Toll-like receptors recognise conserved pathogen-associated molecular patterns such as lipopolysaccharide, lipopeptides and nucleic acids. In humans there are ten TLRs that have been characterized on the basis of their specificity for ligands derived from micro-organisms (31). However, there is increasing evidence that damaged tissues can also release endogenous ligands termed alarmins, including heparan sulphate (32), hyaluronan (33) and high mobility group box 1 (HMGB1) (34) that can activate TLRs in the absence of infection. There is a clear potential for these endogenous ligands to be generated during the process of brain death in organ donors (35, 36). Indeed, a recent report demonstrated increased pre-implantation expression of HMGB1 by renal tubules in kidneys from deceased donors compared with those from living donors (37). This might increase innate inflammation and PGD after organ transplantation from brain dead donors.

Exogenous or endogenous ligands activate specific TLR dimers. Most TLRs, including TLR4, form homodimers whilst TLR2 forms a heterodimer with either TLR1 or TLR6. When activated, all TLRs except TLR3 recruit the adapter molecules MyD88 and TIRAP leading to activation of transcription factors including NF κ B and AP1 and the release of pro-inflammatory factors, including TNF α , IL6 and IFN γ . Toll-like receptors 3 and 4 also recruit the adapter molecules TRIF and TRAM leading to the activation of IRF3 and the release of type I interferons and CXCL10 (31), with a later release of TNF α (38).

Whilst TLR2 and TLR4 have been shown to play a role during IRI (10, 13), most of the studies of downstream signalling have focused on the MyD88-dependent pathway (10, 13). However, a recent study indicated a contribution of the TRIF-dependent pathway by showing that animals deficient in the IRF3 component of this pathway were relatively resistant to IRI (14). The current study was performed to assess the potential of separate desensitization of the TLR4 and TLR2/6 receptors by repeated pre-treatment with the specific ligands LPS and FSL-1 respectively to modulate the inflammatory response following brain death.

The development of TLR desensitization during serial administration of LPS or FSL-1 was demonstrated by measurement of an initial reduction in body mass followed by a sustained increase following repeated injections of either agonist (39). However, seventy two hours after the final injection of either agonist, no detectable difference was observed between treated and untreated groups of animals using a targeted, 84-gene quantitative RT-PCR assay. This finding is consistent with the failure of a comprehensive hybridization micro-array analysis to detect changes in gene expression in leukocytes more than 24 hours after stimulation with endotoxin (40), despite the induction of tolerance by this protocol.

The induction of brain death led to a transient hypertensive crisis which was rapidly replaced by hypotension. This hypertensive crisis is mediated largely by catecholamines, with the autonomic storm causing stress failure of pulmonary capillaries, neurogenic pulmonary oedema and deterioration in oxygenation (36). This was not prevented by prior treatment with either TLR ligand. However, previous research demonstrates that this form of

lung injury is potentially treatable and improves with optimal hemodynamic management (41). Prior treatment with the TLR agonists reduced the sustained decrease in mean arterial pressure following the hypertensive crisis. The hypotension observed after brain death has previously been attributed to catecholamine-induced myocardial ischemia (42), tachyphylaxis of adrenoreceptors (43, 44) and the altered neurohormonal environment of brain death (45). The observation that the deterioration in hemodynamic status is more marked in control animals than those preconditioned with TLR ligands suggests that pro-inflammatory cytokines such as TNF α also contribute to the hemodynamic collapse that follows brain death. The hemodynamic consequence of circulating TNF α may also explain the beneficial effect of glucocorticoid pre-treatment in a model of brain death (46).

Within 5 h of the induction of brain death, the transcription of IFN γ and the TRIF-dependent mediators IFN β and CXCL10 were downregulated in the lungs from LPS pre-treated animals. The increased expression of SARM1 in these animals might also be relevant as the product of this gene is known to reduce TRIF-dependent TLR signalling (47). In FSL-1 pre-treated animals, the transcription of TRIF-dependent inflammatory mediators in lungs following brain death was not different from levels observed in saline-treated controls. This is an important finding as full activation of TLR/type 1 IFN signalling is necessary for deleterious hyperinflammatory reactions (48). Interestingly, hepatic IRI in rodents is also a TLR4 mediated event, dependent on the induction of type I but not type II IFNs (49), and CXCL10 (14).

Whilst the concentrations of TNF α and CXCL1 in serum and bronchoalveolar lavage fluid were dramatically reduced after brain death in groups of animals pre-treated with either LPS or FSL-1, the level of both these cytokines was significantly lower in the LPS-treated group. It is clear that functional TLR2 and TLR4 are expressed by leukocytes and pneumocytes (50, 51), allowing both cell types to respond to appropriate agonist stimulation. The results from analysis of RNA extracted from unfractionated lung tissue will certainly reflect the response to brain death of multiple cell types. The failure of pre-treatment with either LPS or FSL-1 to reduce the level of mRNA encoding TNF α or CXCL1 in lung tissue after brain death is consistent with previous reports that TLR tolerance can also be mediated by post-transcriptional mechanisms (52).

The induction of CD11b and CD18 expression by neutrophils is a sensitive marker of cell activation by pro-inflammatory chemokines (53), and TLR agonists such as LPS (54). The current study demonstrated that the expression of CD11b is dramatically lower following brain death in animals pre-treated with LPS when compared with either FSL-1-treated animals or saline-treated controls. However, the expression of CD18 was reduced after brain death in groups of animals pre-treated with either LPS or FSL-1. The recruitment of neutrophils is central to the development of acute lung injury (25). Integrins on leukocytes mediate the firm adhesion to endothelial cells by interacting with intercellular adhesion molecules. In models of acute lung injury using LPS, the integrin Mac-1 (CD11b/CD18) appears to play a dominant role as antibodies to Mac-1, and not to lymphocyte function associated antigen-1 (LFA-1; CD11a/CD18), can inhibit neutrophil migration to the lung (55).

In conclusion, this study suggests that stimulation of both TLR4 and TLR2/6 can contribute to the inflammatory sequelae of brain death which may prime the lung for subsequent IRI and PGD. The results also show that inflammatory lung injury, which was thought to be triggered by the hypertensive crisis (24, 36), can be uncoupled from the hemodynamic response to brain death by specific desensitization of TLR signalling. Hemodynamic injury and the subsequent inflammatory response that accompanies brain death in the donor may therefore be linked by the generation of endogenous TLR ligands. Further research will

identify the TLR ligands released during brain death, potentially allowing beneficial modulation of their downstream effects and mitigation of lung dysfunction after transplantation.

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Figure 1.

Changes in body mass during and after treatment of animals with TLR ligands. Animals in the control group (n=10; -●-) show a steady gain in mass. The group treated with purified LPS (n=10; -■-) shows an initial decrease in mass followed by an increase in mass after the fourth injection. The group treated with FSL-1 (n=9; -▲-) also shows an initial decrease followed by an increase in mass after the third injection. Error bars show SEM.

Figure 2.

Mean arterial blood pressure (MAP) prior to brain death (-15 minutes), during the induction of brain death (0 minutes) and after the induction of brain death (5 minutes – 300 minutes). Results are for control (saline pre-treated) animals (n=7; -●-), LPS pre-treated animals (n=7; -■-) and FSL-1 pre-treated animals (n=6; -▲-); error bars show SEM, these bars are too small to view for the control group.

Figure 3.

The change in serum cytokine levels after brain death. In all cases the open bars indicate the control group (n=7), the grey shaded bars indicate the LPS-treated group (n=7) and the black bars indicate the FSL-1-treated group (n=6). (a) Serum TNFα in all experimental groups 1 and 5 hr after balloon inflation. (b) Serum CXCL1 in all experimental groups 1 and 5 hr after balloon inflation. Error bars show SEM; * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001 in comparison with control animals.

Figure 4.

The change in cytokine levels in bronchoalveolar lavage fluid after brain death. In all cases the open bars indicate the control group (n=7), the grey shaded bars indicate the LPS-treated group (n=6) and the black bars indicate the FSL-1-treated group (n=6). (a) TNFα levels in donor bronchoalveolar lavage fluid five hours following the induction of brain death in all experimental groups. (b) CXCL1 levels in donor bronchoalveolar lavage fluid five hours following the induction of brain death in all experimental groups. Error bars show SEM; * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ in comparison with control animals.

Figure 5.

The change in integrin expression by neutrophils after brain death. (a) Flow cytometric histograms demonstrating the change in expression of CD11b on whole blood neutrophils compared to control (CD45) 5 hours following the induction of brain death. (b) The change in expression CD11b following the induction of brain death in control animals (open bars; n=7), animals pre-treated with LPS (grey shaded bars; n=7) and those pre-treated with FSL-1 (black bars; n=6). (c) The change in expression CD18 following the induction of brain death in control animals, animals pre-treated with LPS and those pre-treated with FSL-1. The dotted line represents baseline antigen expression in normal animals; error bars show SEM; * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ in comparison with control animals.

Figure 6.

The relative change in the expression of mRNA encoding IFN γ , IFN β and CXCL10 in lung tissue sampled 5 h after the induction of brain death in animals pre-treated with purified LPS (n=7) or FSL-1 (n=6) compared to saline-treated controls (n=7). The black bars indicate IFN γ , the open bars indicate IFN β and the grey shaded bars indicate CXCL10. Error bars show SEM; * indicates $p<0.05$, ** indicates $p<0.005$ in comparison with control animals.

Table 1

Genes in lung tissue showing a significant, >3-fold increase in mRNA expression 5 h after the induction of brain death. Samples were assayed in duplicate for each of 3 control animals sampled before brain death and 7 control animals sampled after brain death

Gene of Interest	Fold Increase in Gene Expression 5 hours after Brain Death ($2^{-\Delta\Delta CT}$ brain death sample/ $2^{-\Delta\Delta CT}$ control sample)	p value
IL6	4370	p<0.0001
Csf3	2856	p<0.0001
CXCL10	1552	p=0.0003
CCL2	279	p=0.0001
IL1 β	109	p=0.0002
TNF α	87	p=0.0003
IL1 α	72	p=0.0005
COX2	55	p=0.001
IL10	44	p=0.0004
IFN γ	32	p=0.01
Csf2	25	p<0.0001
IRF1	24	p=0.003
TLR2	20	p=0.0007
Peptidoglycan recognition protein 1	18	p=0.0001
Macrophage inducible C-type lectin	18	p=0.0002
CD14	17	p=0.0008
NF κ B 2	15	P=0.0002
NF κ B inhibitor, α	14	p=0.0002
Interferon inducible dsRNA dependent protein kinase	11	p=0.007
Receptor (TNFRSF)-interacting serine-threonine kinase 2	10	p=0.0004
IFN β	10	p=0.01
IL12	9	p=0.0038
NF κ B 1	9	P=0.0007
TNF β	9	p=0.0277
TLR1	9	p=0.0032
Rel	7	p=0.0027
CD80	7	p=0.0052
Mitogen activated protein kinase 3	6	p=0.003
TNF Receptor	6	p=0.0021
TLR6	6	p=0.0021
TLR5	5	p=0.0012
CCAAT/enhancer binding protein	5	p=0.0004
TNFAIP3 interacting protein 2	4	p=0.0097

Gene of Interest	Fold Increase in Gene Expression 5 hours after Brain Death ($2^{-\Delta\Delta CT}$ brain death sample/ $2^{-\Delta\Delta CT}$ control sample)	p value
CD86	4	p=0.0129
NFκB 3	4	p=0.0051
MyD88	3	p=0.0023
NFκB inhibitor, β	3	p=0.0023
IL1 Receptor	3	p=0.0024
Caspase 8	3	p=0.0289