

Activation of lung toll-like receptors does not exacerbate sickness responses to lipopolysaccharide in mice



Adam K. Walker^{a,*}, Jennifer Hsieh^a, Katherine V. Luu^a, Aiat A. Radwan^a, Gabriella R. Valverde^b, Burton F. Dickey^b, Michael J. Tuvim^b, Robert Dantzer^a

^a Department of Symptom Research, Laboratory of Neuroimmunology of Cancer-Related Symptoms (NICRS), Division of Internal Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

^b Department of Pulmonary Medicine, Division of Internal Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

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ABSTRACT

Pneumonia represents a leading cause of death. Recently, a novel treatment strategy for pneumonia has involved enhancing the host pulmonary innate immune response by pre-exposure to aerosolized toll-like receptor (TLR)9 and TLR2/6 agonists, known as O/P. O/P inhalation in mice has been demonstrated to stimulate innate lung immunity, and thus increase survival against subsequent pneumonia infection while producing barely detectable increases in systemic cytokines. Here, we examined the safety of O/P treatment when used in mice that are inflamed systemically. Swiss-Webster mice were treated with two doses of aerosolized O/P (1× or 8×) vs phosphate buffered saline (PBS) either immediately before intraperitoneal injection of 0.1 mg/kg lipopolysaccharide (LPS) or PBS (equivolume) or 2 h after. Sickness responses (reduced body weight, food intake, activity and social interaction) were examined at 2 and 5.5 h post-treatment. Immediately following behavioral testing, mice were euthanized, perfused with PBS, and brains, spleens, livers and lungs snap frozen for assessment of pro-inflammatory cytokine mRNAs. While O/P treatment alone increased lung IL-1β, IFNγ and TNF-α, no such effects were observed in the brain, spleen or liver. Furthermore, there was no evidence that O/P treatment administered before or after LPS had any synergizing effect to potentiate the cytokine response to LPS in any compartment measured. Supportive of these findings were the measures of sickness behaviors that did not show any increased sickness response in O/P-treated mice exposed to LPS, suggestive that the cytokine signal produced in the lungs from O/P inhalation did not propagate to the brain and synergize with LPS-induced neuroinflammation. These findings support the safety of the use of O/P inhalation as a preventative measure against pneumonia and demonstrate a unique ability of the lungs to compartmentalize pulmonary inflammation and limit propagation of the cytokine signal to the brain.

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1. Introduction

Pneumonia is one of the leading causes of death worldwide from infection (File, 2003; Liu et al., 2010). Recent efforts to develop novel therapeutic strategies have demonstrated that exploitation of the lung's innate defense mechanisms can enhance resistance to pulmonary infections in preclinical murine models. Specifically, inhalation of synthetic toll-like receptor (TLR)9 and TLR2/6 agonists combined, known as oligodeoxynucleotide and

Pam2 (O/P), can significantly accelerate pathogen killing in the lungs and reduce mortality rates to pneumonia (Duggan et al., 2011). While this O/P treatment can induce robust pulmonary inflammation, it appears that the pro-inflammatory cytokines induced by inhalation of innate immune ligands are primarily contained within the lungs (Clement et al., 2008; Tuvim et al., 2009; Evans et al., 2010). Indeed, preliminary efforts to explore the safety of this O/P treatment determined almost no adverse events from O/P inhalation even when administered well above the anticipated clinical dose. Specifically, a 1× O/P dose was found to be maximally effective in inducing resistance to lung infection. However, even at an 8× dosage, minimal, if any, adverse physiological or behavioral responses could be observed (Alfaro et al., 2014).

While these findings are promising, pneumonia often develops on a background of systemic inflammation where individuals are

* Corresponding author. Address: Department of Symptom Research Laboratory of Neuroimmunology of Cancer-Related Symptoms at the Institute of Biosciences and Technology, Texas A&M Health Sciences Center, 2121 W Holcombe Boulevard, Room 1025, Houston, TX 77030, USA. Tel.: +1 713 794 4854; fax: +1 713 745 3475.

E-mail address: akwalker@mdanderson.org (A.K. Walker).

already immunocompromised or inflamed, such as with cancer patients (Joos and Tamm, 2005; Fekrazad et al., 2010). This can, in turn, exacerbate the symptoms of pneumonia and increase mortality rates. Therefore, while minimal to no adverse events or sickness responses have been observed with the O/P treatment alone in healthy animals, it is necessary to confirm that activation of toll-like receptors (TLRs) and the induction of pro-inflammatory cytokines in the lungs does not synergize with pre-existing or subsequent systemic inflammation and further potentiate sickness and cytokine responses. Therefore, we examined the extent of adverse outcomes from O/P treatment in a more clinically relevant model, in which mice were treated with either a $1\times$ or $8\times$ O/P aerosol dose and with an intraperitoneal injection of lipopolysaccharide (LPS) to mimic systemic inflammation. Sickness responses including body weight loss, reductions in food consumption, locomotor activity and social interaction were assessed alongside pro-inflammatory cytokine responses in several compartments of the body.

2. Methods

2.1. Animals and treatments

Swiss Webster (6–8 weeks old, $n = 42$) (Charles River) females were used in these experiments. Mice were housed individually in standard shoebox cages in a temperature (23 °C) and humidity (45–55%) controlled environment with a 12/12-h modified dark-light cycle (lights on at 2200 h). Food and water were available *ad libitum*. Mice were handled and habituated to the aerosol chamber daily for 1 week prior to experimentation.

For O/P treatment mice were placed in a 10 L polyethylene exposure chamber downstream of an Aerotech II nebulizer (Biodex Medical Systems) driven by 10 L/min of air supplemented with 5% CO₂ to promote deep breathing, connected by polyethylene tubing (30 cm \times 22 mm). The exposure chamber was vented to a biosafety hood by another segment of polyethylene tubing. Mice were exposed to aerosolized synthetic TLR agonists (2,3-bis(palmitoyloxy)-2-propyl-Cys-Ser-Lys-Lys-Lys-Lys-OH (Pam2CSK4 or Pam2) as the trifluoroacetic acid salt was purchased from Bachem, and 5' TCG TCG TCG TTC GAA CGA CGT TGA T 3' oligodeoxynucleotide as the sodium salt on a nuclease-resistant phosphorothioate backbone (ODN M362 or ODN) from TriLink BioTechnologies) dissolved in 6 mL of endotoxin-free sterile water at $1\times$ (1 μ M ODN and 4 μ M Pam2) or $8\times$ concentrations for 20 min, with ~ 4 mL of solution typically delivered. Controls were exposed to aerosolized phosphate buffered saline (PBS) for 20 min.

LPS treatment involved an intraperitoneal injection of freshly made solutions of 0.1 mg/kg LPS (L-020M4062, serotype 0127:B8; Sigma, St. Louis, MO) prepared with sterile endotoxin-free isotonic PBS or PBS (equivolume). This dose of LPS was chosen to induce mild sickness behaviors and cytokine activation at the time point of measurement, while still providing room for the potential of elevated responses for the combined effect of the TLR agonist treatment with the LPS.

For Experiment 1 mice were treated with aerosolized $1\times$, $8\times$ or PBS and then injected with LPS or PBS immediately after. For Experiment 2 mice were pretreated with LPS or PBS and then 2 h later treated with aerosolized $8\times$ O/P or PBS. Mice were euthanized after CO₂ anesthesia 6 h post-LPS. This time interval was chosen to allow us to carry out behavioral observation of the experimental animals during the sickness response to LPS and still be able to measure activation of the inflammatory response at the periphery and in the brain (Dantzer et al., 2008). The brain, lung, liver and spleen were removed after perfusion with sterile PBS. All biochemical assays on brain tissue were conducted on entire half brains cut

down the midline to include a representation of all brain regions. Fig. 1 outlines the experimental design and timing of behavioral experiments.

All protocols in this study were approved by the Institutional Animal Care and Use Committees (IACUC) of Texas A&M University or The University of Texas MD Anderson Cancer Center.

2.2. Behavior

To assess whether localized pulmonary inflammation caused by O/P inhalation was capable of potentiating the inflammatory sickness and cytokine response caused by a systemic inflammatory challenge, we examined body weight loss, food disappearance and sickness behaviors following aerosolized treatment with TLR 9 and TLR 2/6 agonists and intraperitoneal injection of LPS. All behavioral experiments were performed during the first 6 h of the dark phase of the light cycle. Changes in locomotor activity and social exploration were assessed 2 and 5.5 h after drug administration. We selected behavioral endpoints that we had already demonstrated to be very sensitive to systemic inflammation-induced sickness (Kent and Bluthe, 1992).

2.2.1. Locomotor activity

Locomotor activity was assessed as previously described (Walker et al., 2013). Briefly, mice were individually placed in clean, novel cages identical to the home cage but without bedding or litter. The cage was divided into four virtual quadrants and activity was measured by counting the number of quadrant entries, operationalized as when all four paws entered the quadrant, over a five-min period. Rearing, including wall climbing was also counted. Counting was conducted by a well-trained observer who was blind to the treatments. A new cage was used for each mouse.

2.2.2. Social exploration

Immediately following testing for locomotor activity, a naïve, untreated mouse of the same sex and age was placed into the novel cage. Time spent interacting with the novel mouse was scored over a 5 min period. Interaction time was counted only if the experimental mouse initiated contact with the novel conspecific, which included nose to nose interaction, climbing over or under, and chasing the novel mouse.

2.3. Biochemistry

2.3.1. RNA extraction and reverse transcription

Total RNA from liver, lung, spleen and whole brain samples were extracted in TRIzol[®] reagent (Life Technologies Corporation, Carlsbad, CA). All reverse transcription reactions were performed in a Bio-Rad C1000 Touch[™] Thermal Cycler, using an Ambion[®] PureLink[®] RNA Mini reverse transcriptase kit (Life Technologies Corporation, Carlsbad, CA; cat # 12183018A) according to manufacturer's instructions, with random decamer primers for each reaction.

2.3.2. Real-time reverse transcription polymerase chain reaction (RT-PCR)

Real-time RT-PCR was carried out on an Applied Biosystems ViiA7[™] Real-Time PCR System using Taqman[®] gene expression assays for IL-6 (cat: Mm.PT.51.12387735), IL-1 β (cat: Mm.PT.51.17215823), TNF α (cat: Mm.PT.51.16622079), IFN γ (cat: Mm.PT.45.13380517), IL-10 (Mm.PT.56a.13531087) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cat: Mm.PT.39.1) purchased from Integrated DNA Technologies as previously described (O'Connor et al., 2009). Reactions were performed in duplicate. Relative quantitative measurement of target gene levels was performed using the $\Delta\Delta$ Ct method, where Ct is the threshold

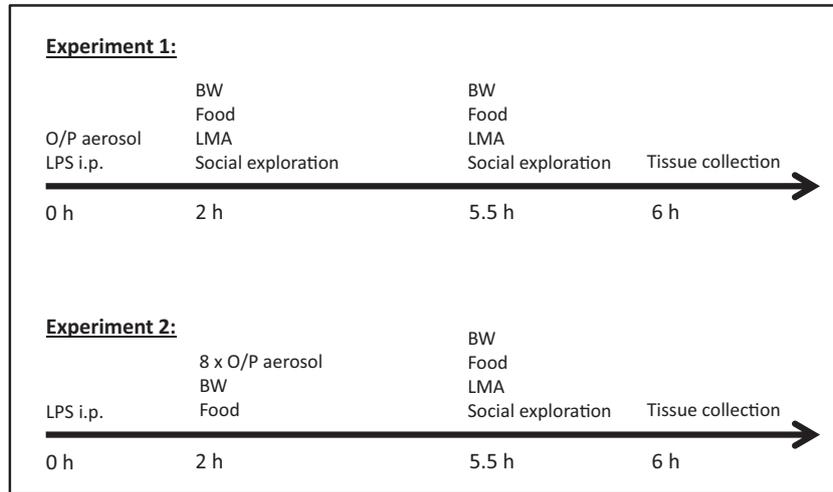


Fig. 1. Experimental timeline. Mice were treated with aerosolized oligodeoxynucleotide and Pam2 (O/P) and lipopolysaccharide LPS (ip) and assessed for measures of sickness such as body weight loss (BW), food disappearance, locomotor activity (LMA) and social exploration.

concentration (O'Connor et al., 2009). GAPDH was used as the endogenous housekeeping control gene.

2.4. Bronchoalveolar lavage (BAL) fluid preparation and cell analysis

For Experiment 2 cell counts of BAL fluid were assessed. Bronchoalveolar lavage (BAL) fluid was obtained by instilling and collecting two aliquots of 1 mL each of PBS through a luer stub adapter cannula (BD Biosciences) inserted through rings of the exposed trachea at the indicated time points. Total leukocyte count

was determined with a hemocytometer (Bright-Line, Hauser Scientific) and slides for differential cell count were prepared by cytopsin of 200 µl of BAL fluid at 300×g for 5 min, followed by Wright-Giemsa staining.

2.5. Statistical analysis

Two-way analyses of variances (ANOVAs) (LPS vs PBS × O/P (0, 1 or 8×)) were performed for all measures of biochemistry for tissue collected at 6 h. Three-way repeated measures ANOVAs (LPS vs

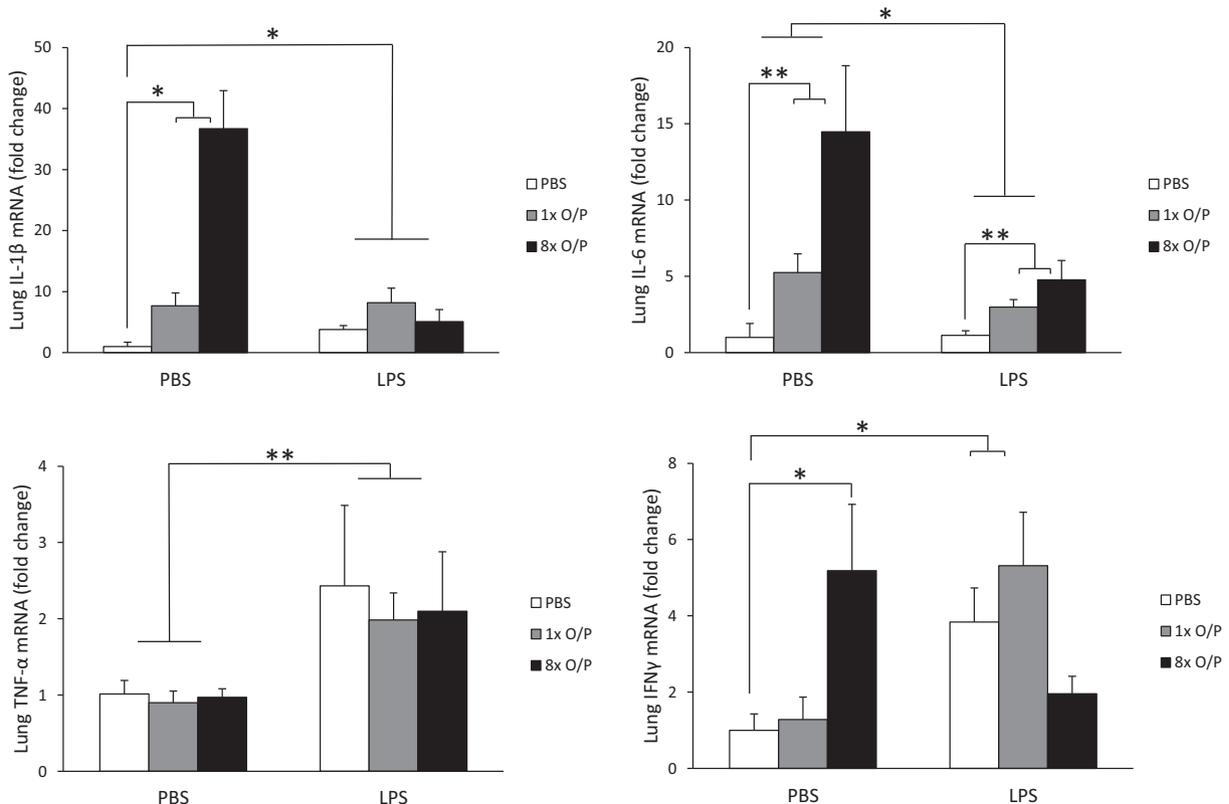


Fig. 2. Oligodeoxynucleotide and Pam2 (O/P) inhalation and systemic lipopolysaccharide (LPS) increases pro-inflammatory cytokines in the lung. Mean IL-1β, IL-6, TNF-α and IFNγ mRNA fold differences (± SEM) of mice treated with aerosolized O/P or PBS immediately before LPS or PBS (ip). *p < 0.05; **p < 0.01; ***p < 0.0001.

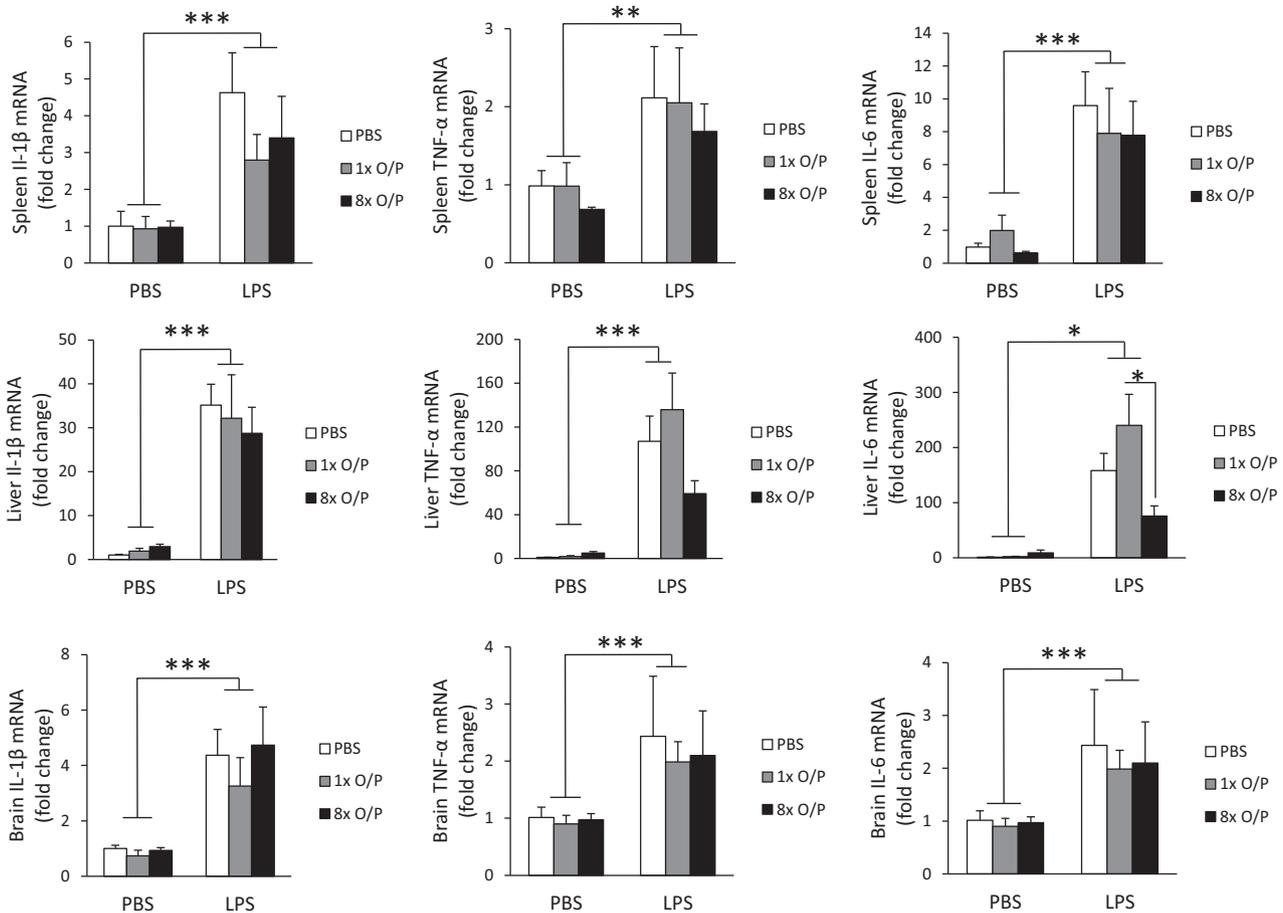


Fig. 3. Oligodeoxynucleotide and Pam2 (O/P) inhalation immediately before intraperitoneal lipopolysaccharide (LPS) administration does not potentiate spleen, liver and brain cytokine responses to LPS. Mean IL-1 β , IL-6 and TNF- α mRNA fold differences (\pm SEM) of mice treated with aerosolized O/P or PBS and LPS or PBS (ip). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

PBS \times O/P (0, 1 or 8 \times) vs PBS \times time as a repeated factor) were performed for measures of sickness behaviors, body weight loss and food consumption. Where applicable, planned comparisons were always performed against PBS/PBS negative control to determine changes in behavior and elevations in cytokine concentrations from baseline levels. Planned comparisons were determined using Fisher's PLSD adjusted for the number of comparisons to control for family wise error. Only relevant significant comparisons between groups are indicated on figures.

3. Results

3.1. Aerosolized TLR treatment activates lung pro-inflammatory cytokines (Fig. 2)

LPS increased lung IL-6 ($F_{(1,29)} = 4.29$, $p < 0.05$) and TNF- α ($F_{(1,32)} = 7.44$, $p = 0.01$) mRNA concentrations. O/P treatment also increased lung IL-6 mRNA ($F_{(1,29)} = 6.06$, $p < 0.01$). A significant interaction between LPS and O/P treatment was observed for lung IL-1 β mRNA ($F_{(2,28)} = 17.65$, $p < 0.0001$). LPS treatment significantly increased IL-1 β compared to PBS/PBS controls and O/P treatment alone dose-dependently increased IL-1 β mRNA compared to PBS/PBS controls ($p < 0.05$ for all). A significant interaction between LPS and O/P treatment was also observed for lung IFN γ mRNA ($F_{(2,28)} = 5.59$, $p < 0.01$). O/P treatment alone significantly increased lung IFN γ mRNA at the 8 \times dosage ($p < 0.05$). LPS treatment increased lung IFN γ mRNA, which reached significance for

LPS-treated mice independently of O/P treatment ($p < 0.05$ for all). Interestingly, 8 \times O/P significantly decreased the response to LPS for all cytokines except TNF- α ($p < 0.05$ for all).

3.2. Aerosolized TLR treatment does not potentiate spleen, liver and brain pro-inflammatory cytokine responses to systemic LPS (Fig. 3)

LPS significantly increased spleen IL-1 β ($F_{(1,33)} = 19.64$, $p < 0.0001$), IL-6 ($F_{(1,32)} = 28.28$, $p < 0.0001$) and TNF- α ($F_{(1,33)} = 8.85$, $p = 0.005$) mRNA. O/P treatment had no effect on spleen cytokine mRNA levels either alone or in interaction with LPS.

LPS also significantly increased brain IL-1 β ($F_{(1,32)} = 24.30$, $p < 0.0001$), IL-6 ($F_{(1,32)} = 7.44$, $p = 0.01$) and TNF- α ($F_{(1,32)} = 7.44$, $p = 0.01$) mRNA. O/P treatment had no effect on brain cytokine mRNA levels either alone or in interaction with LPS.

LPS significantly increased liver IL-1 β ($F_{(1,32)} = 50.30$, $p < 0.0001$) and TNF- α ($F_{(1,33)} = 50.11$, $p < 0.0001$) mRNA with no effect of O/P treatment. A significant interaction between LPS and O/P treatment was observed for liver IL-6 mRNA ($F_{(2,33)} = 5.23$, $p = 0.01$). Post hoc comparisons revealed that all LPS-treated groups had significantly higher hepatic IL-6 mRNA than their respective controls, and IL-6 mRNA was significantly higher for mice treated with 1 \times O/P and LPS compared to those treated with 8 \times O/P and LPS ($p < 0.05$ for all), suggestive of a suppression of the response to LPS by 8 \times O/P. O/P controls did not significantly differ from PBS/PBS controls.

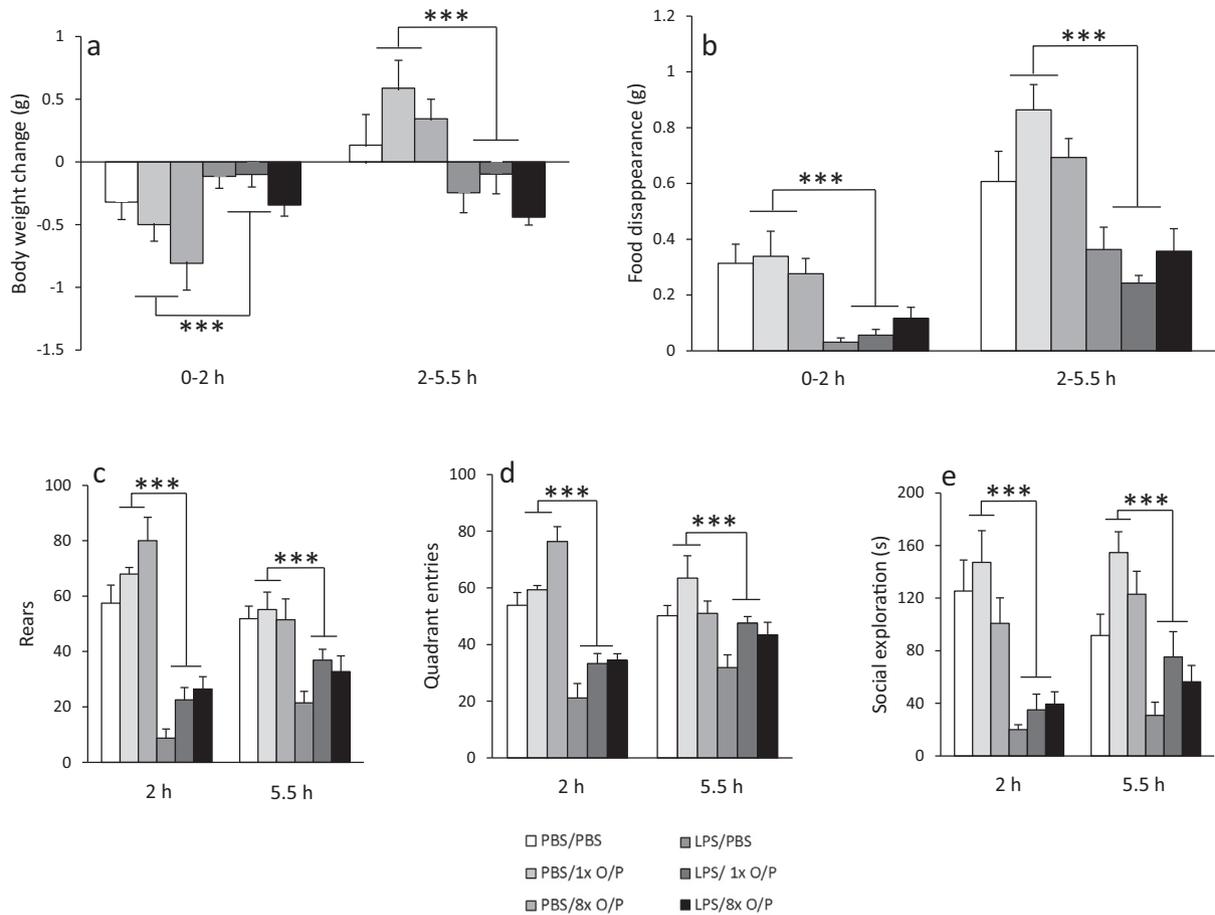


Fig. 4. Oligodeoxynucleotide and Pam2 (O/P) inhalation immediately before intraperitoneal lipopolysaccharide (LPS) administration does not synergize with LPS-induced sickness. (a, b) Mean body weight change (g) and food disappearance (g) (+ SEM) for mice treated with aerosolized oligodeoxynucleotide and Pam2 (O/P) or PBS and LPS or PBS (ip) between 0–2 and 2–5.5 h. (c–e) Mean number of rears, quadrant entries, and time engaged in social exploration (\pm SEM) for mice treated with aerosolized O/P or PBS and LPS or PBS (ip) at 2 and 5.5 h. *** $p < 0.0001$.

3.3. Aerosolized TLR treatment does not induce IL-10 in distal compartments to attenuate the response to systemic LPS

Given that there are some instances where O/P inhalation appeared to reduce the pro-inflammatory response to LPS, we examined whether O/P treatment may actually induce an anti-inflammatory response. LPS significantly increased IL-10 mRNA in the spleen ($F_{(1,34)} = 8.33$, $p < 0.01$), brain ($F_{(1,33)} = 3.69$, $p < 0.05$) and liver ($F_{(1,33)} = 19.22$, $p = 0.0001$). However, O/P treatment had no effect on IL-10 either alone or in interaction with LPS in any of these organs. No effect of LPS or O/P treatment was observed for IL-10 mRNA in the lung.

3.4. Aerosolized TLR treatment does not potentiate sickness responses to systemic LPS

The effects of O/P treatment with or without LPS administration are presented in Fig. 4.

3.4.1. Body weight and food consumption

LPS reduced body weight between 0–2 h and 2–5.5 h ($F_{(1,36)} = 24.88$, $p < 0.0001$). A main effect of O/P treatment was observed whereby mice treated with 8 \times O/P lost more weight than 1 \times O/P-treated mice ($F_{(2,36)} = 3.91$, $p < 0.05$). LPS reduced food consumption ($F_{(1,36)} = 68.01$, $p < 0.0001$). Aerosolized O/P treatment,

irrespective of dose, did not alter LPS-induced reductions in food consumption.

3.4.2. Locomotor activity

Main effects of LPS and O/P treatment were observed for locomotor activity across time, although no interaction was found. LPS significantly reduced quadrant entries and rears at 2 and 5.5 h ($F_{(1,35)} = 20.34$, $p < 0.0001$ and $F_{(1,35)} = 30.51$, $p < 0.0001$, respectively). A significant O/P treatment \times time interaction was observed for quadrant entries ($F_{(2,35)} = 5.41$, $p = 0.009$). Planned comparisons revealed that 8 \times O/P treatment and 1 \times O/P treatment actually increased quadrant entries at 2 and 5.5 h respectively ($p < 0.05$ for both). A main effect of O/P treatment was observed for rears such that both doses of O/P treatment significantly increased rearing compared to controls ($F_{(2,35)} = 5.04$, $p = 0.01$).

3.4.3. Social exploration

LPS significantly reduced social exploration time ($F_{(1,36)} = 57.83$, $p < 0.0001$), whereas O/P treatment significantly increased social exploration compared to controls ($F_{(2,36)} = 3.95$, $p = 0.03$).

3.5. Pre-treatment with systemic LPS does not potentiate aerosolized TLR-induced lung inflammation

In the previous experiments we demonstrated that treatment with O/P immediately before systemic LPS treatment did not exac-

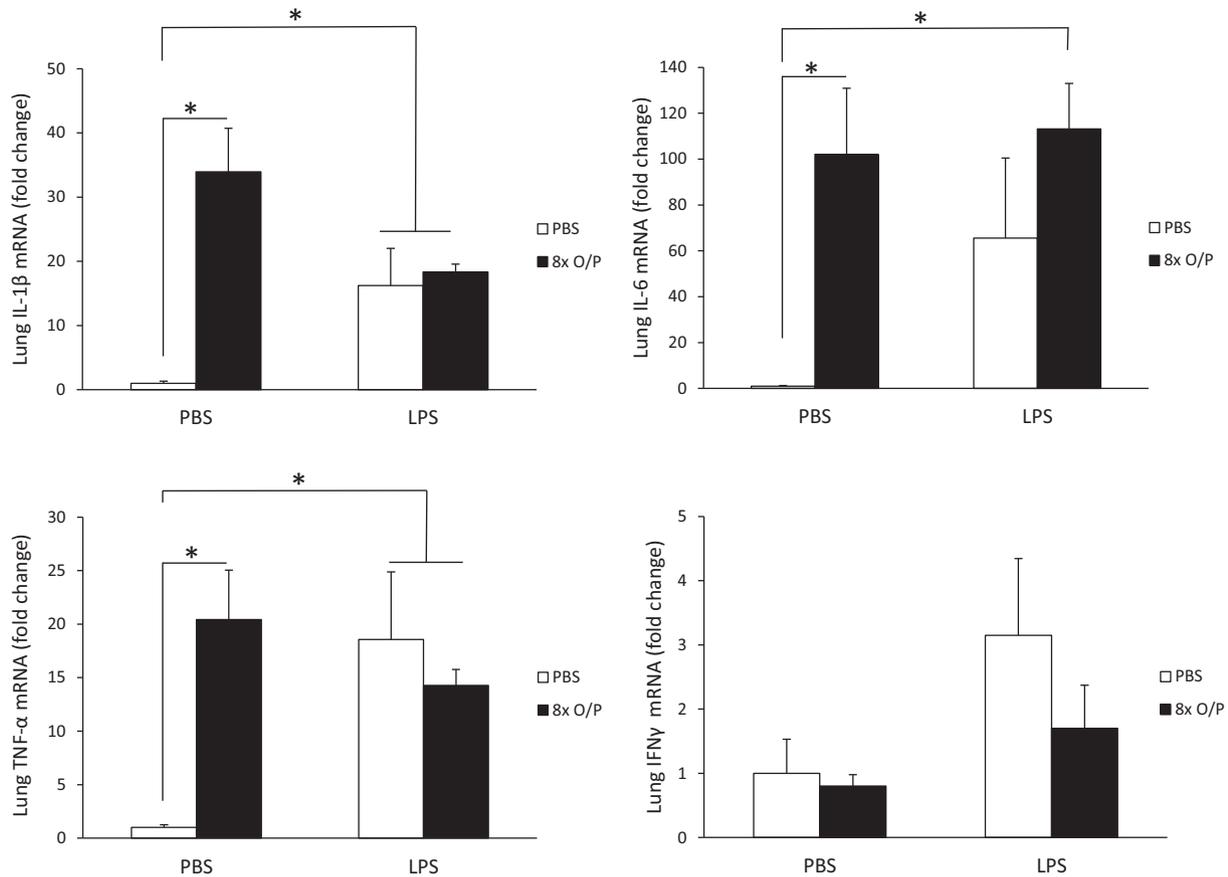


Fig. 5. Oligodeoxynucleotide and Pam2 (O/P) inhalation does not potentiate pre-existing systemic lipopolysaccharide (LPS)-induced increases in pro-inflammatory cytokines in the lung. Mean IL-1 β , IL-6, TNF- α and IFN γ mRNA fold differences (\pm SEM) of mice treated with aerosolized O/P or PBS 2 h after LPS or PBS (ip). * $p < 0.05$.

erbate symptoms of LPS-induced sickness or inflammatory responses. Here we examined a more clinically relevant question as to whether the maximal 8 \times O/P dose potentiates sickness when given after LPS-induced systemic inflammation has already reached its peak.

3.5.1. Changes in lung leukocyte levels

We examined changes in lung leukocyte levels in the same mice by measuring total and differential cell counts in BAL fluid. A significant interaction between LPS and O/P treatment was observed for the total number of cells in BAL fluid ($F_{(1,15)} = 5.03$, $p < 0.05$), whereby O/P treatment significantly increased neutrophil levels which was attenuated in the presence of LPS ($p < 0.05$). Of the cells induced by 8 \times O/P almost 90% were neutrophils with almost no neutrophils observed in the control groups. Only approximately 40% of total cells were neutrophils however in the mice pre-treated with LPS. The remaining percentage of total cells in each case were macrophages. Minimal to no lymphocytes or eosinophils were observed in BAL fluid.

3.5.2. Lung cytokine levels

Fig. 5 depicts the pro-inflammatory cytokine mRNA levels observed in the lungs of mice treated with 8 \times O/P 2 h after LPS treatment. Significant O/P \times LPS interactions were observed for lung IL-1 β ($F_{(1,14)} = 11.84$, $p < 0.01$) and TNF- α ($F_{(1,14)} = 7.96$, $p < 0.05$) mRNA whereby both O/P and LPS alone or in combination increased mRNA levels above that of PBS-treated controls ($p < 0.05$ for all). The same trend was observed for IL-6 mRNA but only O/P

treatment reached significance ($F_{(1,14)} = 8.15$, $p < 0.05$). No significant effects for IL-10 and IFN γ mRNA were observed.

3.6. Aerosolized TLR treatment does not potentiate spleen, liver and brain cytokine responses to pre-treatment with LPS (Fig. 6)

LPS increased spleen IL-1 β ($F_{(1,15)} = 10.73$, $p < 0.05$), TNF- α ($F_{(1,15)} = 4.89$, $p < 0.05$) and IL-6 ($F_{(1,14)} = 4.90$, $p < 0.05$) mRNA with no effect of O/P treatment. No significant differences were observed for IL-10.

LPS increased brain IL-1 β ($F_{(1,13)} = 15.37$, $p < 0.01$), TNF- α ($F_{(1,12)} = 12.24$, $p < 0.01$) and IL-6 ($F_{(1,12)} = 4.81$, $p < 0.05$) mRNA with no effect of O/P treatment. No significant differences were observed for IL-10.

LPS increased liver IL-6 ($F_{(1,13)} = 5.33$, $p < 0.05$) and TNF- α ($F_{(1,14)} = 6.74$, $p < 0.05$) mRNA with no effect of O/P treatment. Trends also indicated LPS-induced increases in liver IL-1 β but did not reach significance.

3.7. Aerosolized TLR treatment does not potentiate sickness responses to pre-existing inflammation caused by systemic LPS (Fig. 7)

3.7.1. Body weight and food consumption

LPS reduced body weight between from 2–5.5 h ($F_{(1,28)} = 50.27$, $p < 0.0001$). LPS reduced food consumption between 2 and 5.5 h ($F_{(1,28)} = 22.54$, $p < 0.0001$) with no effect of pre-treatment with 8 \times O/P.

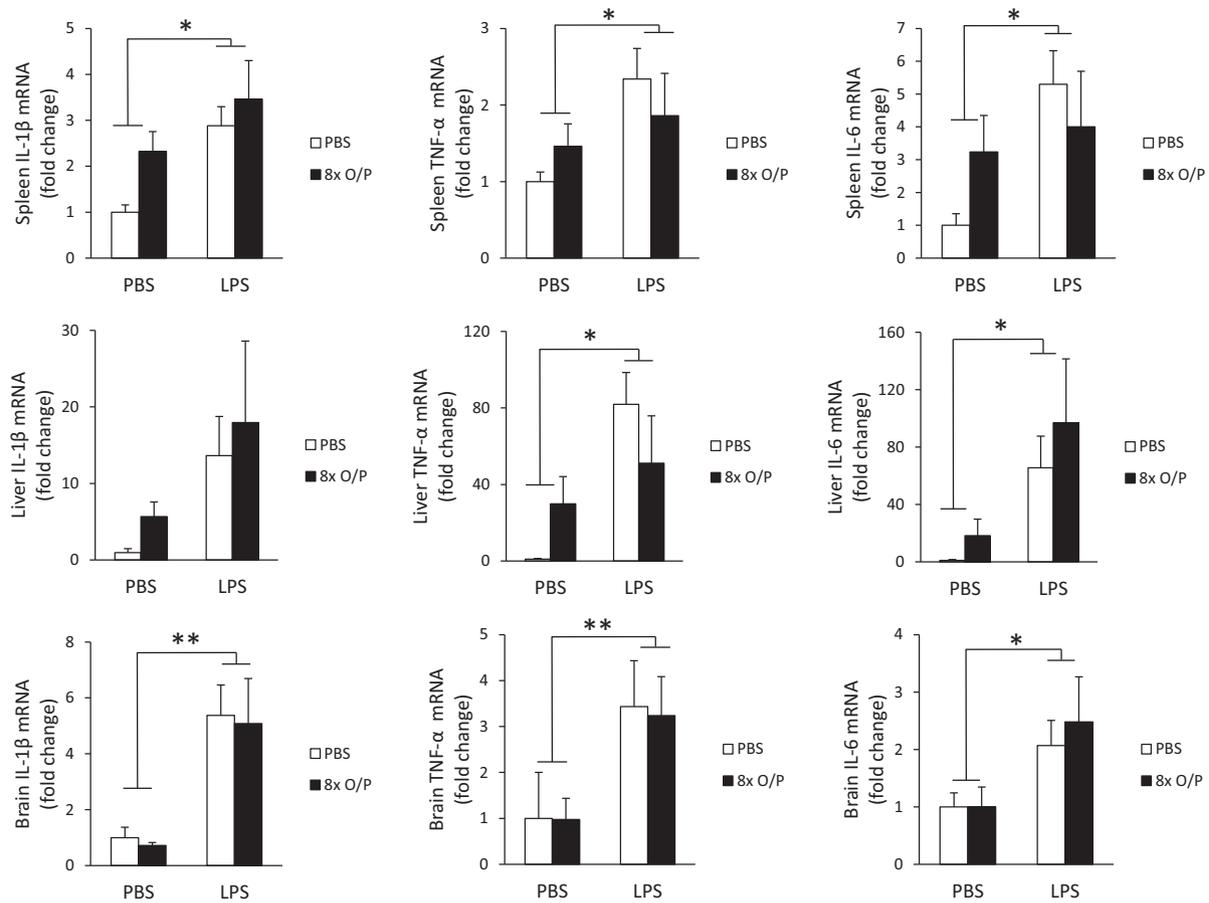


Fig. 6. Oligodeoxynucleotide and Pam2 (O/P) inhalation does not potentiate pre-existing lipopolysaccharide (LPS)-induced spleen, liver and brain cytokine responses. Mean IL-1 β , IL-6 and TNF- α mRNA fold differences (\pm SEM) of mice treated with aerosolized O/P or PBS 2 h after LPS or PBS (ip). * p < 0.05; ** p < 0.01.

3.7.2. Locomotor activity

LPS significantly reduced both quadrant entries ($F_{(1,27)} = 4.51$, $p < 0.05$) and rears ($F_{(1,27)} = 14.44$, $p < 0.001$). There was no effect of O/P treatment either alone or in interaction with LPS.

3.7.3. Social exploration

LPS significantly reduced social exploration time ($F_{(1,28)} = 5.96$, $p < 0.05$). No effect of O/P treatment either alone or in interaction with LPS was observed.

4. Discussion

Activation of the lungs' innate immune response by aerosolized exposure to TLR9 and TLR2/6 ligands (O/P) can provide protection against lung infections in mice (Duggan et al., 2011). Here we show that while O/P treatment induces a rather robust inflammatory response in the lungs, it appears to be largely contained there. Indeed, there is little evidence that the cytokine signal generated in the lungs from O/P inhalation propagates to the brain to induce significant sickness responses. While these effects of O/P treatment have been previously reported (Clement et al., 2008; Tuvim et al., 2009; Evans et al., 2010; Alfaro et al., 2014), no attempt has yet been made to examine the safety of using O/P inhalation in a clinically relevant population of individuals who are at greater risk of developing pneumonia and respiratory complications from infection as they are already immunocompromised or inflamed. Therefore, the present experiments were carried out to test for a possible propagation of inflammation induced by stimulation of TLR9 and

TLR2/6 in the lung to the rest of the body and the brain by using a LPS challenge, and cytokine expression and sickness as read-outs. The results we obtained provide no evidence in favor of such a propagation. Instead, they indicate that the activation of the innate immune system that is triggered by the aerosolized TLR ligands remains localized in the lung. This compartmentalization is interesting and in contrast with the concept that peripheral inflammation, whatever its origin, is able to propagate to the brain and ultimately influence behavior (Dantzer et al., 2008).

Several key pieces of evidence point toward this compartmentalization. Firstly, there was evidence of O/P-induced inflammation in the lungs as shown by increases in IL-1 β , IL-6, TNF- α and IFN γ mRNA with concomitant increases in these cytokines also being observed in response to LPS across the two experiments. This was also evidenced by increased neutrophils in lung lavage fluid in response to 8 \times O/P treatment in Experiment 2. While systemic LPS administered either immediately after O/P aerosolization or 2 h before O/P aerosolization elevated the pro-inflammatory cytokine concentrations of other tissues including the liver, spleen and brain as expected, there was no substantial evidence of O/P treatment either alone or in conjunction with LPS to increase cytokines in these tissues. Secondly, examination of specific symptoms of sickness was consistent with the biochemical analyses. LPS significantly reduced food consumption, locomotor activity and social exploration which was unaffected by O/P inhalation irrespective of whether it was administered immediately prior to or 2 h after LPS. LPS also reduced body weight however, PBS-treated controls actually lost significantly more weight than mice treated with LPS between 0 and 2 h, but completely recovered and gained

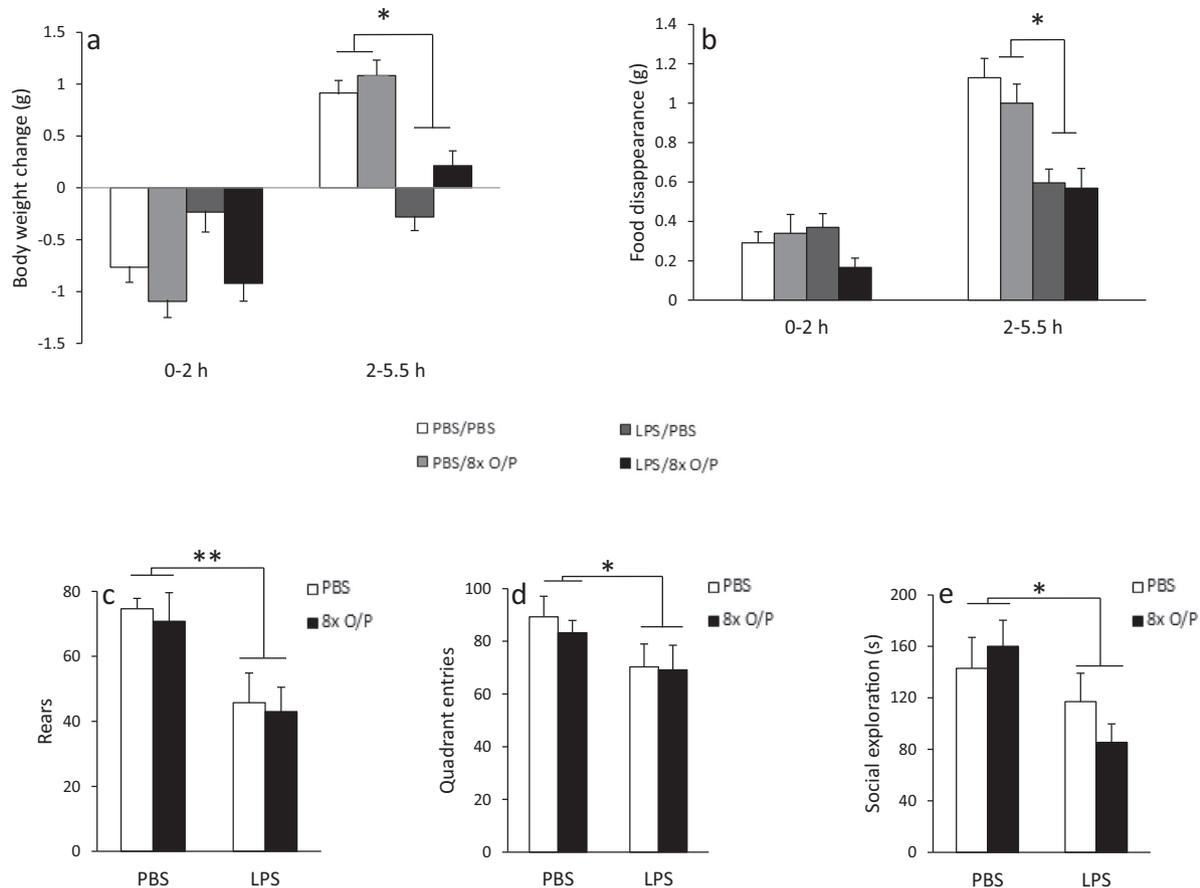


Fig. 7. Oligodeoxynucleotide and Pam2 (O/P) inhalation 2 h after intraperitoneal lipopolysaccharide (LPS) administration does not synergize with LPS-induced sickness. (a, b) Mean body weight change (g) and food disappearance (g) (\pm SEM) for mice treated with aerosolized oligodeoxynucleotide and Pam2 (O/P) or PBS 2 h after LPS or PBS (ip) between 0–2 and 2–5.5 h. (c–e) Mean number of rears, quadrant entries, and time engaged in social exploration (\pm SEM) for mice treated with aerosolized O/P or PBS 2 h after LPS or PBS (ip) at 2 and 5.5 h. * $p < 0.05$; ** $p < 0.01$.

weight between 2 and 5.5 h. This body weight loss between 0–2 h in the mice receiving aerosols is likely accounted for by the fact that the mice huddle in a corner shortly after aerosol administration, and are not observed to drink or eat during this time.

Curiously, the data from Experiment 1 and Experiment 2 not only dispute the notion that activation of pulmonary TLR9 and TLR2/6 potentiates LPS-induced sickness and systemic inflammation, but even indicates a dose-dependent attenuation by aerosolized O/P of the response to LPS in some instances. For instance, there are consistent trends showing an increase in activity following O/P treatment in Experiment 1. While this does not negate the fact that O/P inhalation does not appear to induce sickness, it is possible that O/P inhalation influences brain activity outside of inflammatory pathways. Also, 8 \times O/P when administered immediately prior to LPS particularly shows a reduced response in the presence of systemic LPS for IFN γ in the lung and for TNF- α and IL-6 in the liver. Again, 8 \times O/P when administered 2 h after systemic LPS shows a reduced response for IL-1 β in the lung as well as a decrease in the number of neutrophils in BAL fluid compared to when 8 \times O/P was administered without LPS. To determine whether these effects were due to O/P inhalation actually inducing an increase in anti-inflammatory cytokines we measured IL-10 mRNA in each compartment. No significant relationship between IL-10 and O/P treatment was observed in the lungs, liver, spleen or brain and cannot explain the O/P-induced attenuation of the response to LPS. Nor is it likely that a tolerance effect can explain this phenomenon as the cytokine response to LPS in the lungs was not lower than in other organs. It is possible that the activation of lung

TLRs by the high dose of O/P activated the cholinergic anti-inflammatory reflex that then attenuates the response to LPS, however this requires confirmation. Similarly, the reduction in neutrophils in BAL fluid in Experiment 2 when mice were treated with 8 \times O/P and LPS can be accounted for by the known increase in neutrophil and monocyte adhesion to endothelial cells in response to LPS. LPS has been demonstrated to increase numerous adhesion molecules and a rapid reduction in the number of circulating neutrophils (Jersmann et al., 2001; Bradfield et al., 2007; Hickey and Kubers, 2009). This has been well demonstrated in the lungs, and it has been suggested that activated neutrophils may become lodged in the narrow lung capillaries making it difficult for migration (Hickey and Kubers, 2009). This is likely to account for the reduced number of total cells observed in the BAL fluid of mice treated with 8 \times O/P and LPS compared to the BAL fluid of mice treated with 8 \times O/P alone and may not actually represent an attenuated response.

Containment of O/P-induced inflammation to the lungs indicates that the lung microenvironment is capable of regulating its inflammatory response somewhat independently of the rest of the body. This idea is supported by recent findings showing that alveolar macrophages are not influenced by systemic events and respond differently than circulating monocytes. For instance, Philippiart et al. (2012) demonstrated that alveolar macrophages do not develop endotoxin tolerance unlike monocytes and peritoneal macrophages after injection with LPS in mice. This suggests localized mechanisms specific to the lung microenvironment. The mechanism responsible for containment within the lungs in the

present study most likely reflects the difference between the molecular responses to TLR 9 and TLR 2/6 activation by O/P treatment compared to conditions of pulmonary infection such as influenza. That is, both the TLR agonists and influenza, for example, act at the level of the epithelial cells. However, unlike with the TLR agonist inhalation, invading pathogens will cause epithelial cell death and activate 'danger' signals. This in turn, will lead to a high degree of macrophage recruitment and hence systemic spill over. Therefore, lung endothelial inflammation in the absence of cell death, as is the case with exposure to aerosolized TLR 9 and TLR 2/6 agonists, can be considered safe from propagating the cytokine signal to other compartments and inducing sickness.

5. Conclusion

The evidence provided here supports the safety of using TLR 9 and TLR 2/6 agonists as a protective therapy against pneumonia in both healthy at-risk individuals and already immunocompromised or inflamed patients. We report no evidence of O/P-induced exacerbation of the LPS response either behaviorally or in regards to cytokine activation in distal peripheral and central compartments of the body. Furthermore, we demonstrate the ability of the lung microenvironment to compartmentalize itself from the rest of the body and contain inflammation to the lungs without propagation to the brain under conditions that lack endothelial cell death and macrophage recruitment. This compartmentalization challenges the existing beliefs that peripheral inflammation, whatever its origin, is able to propagate to the brain and ultimately influence behavior.

Conflicts of interest

Robert Dantzer works as a consultant for Ironwoods Pharmaceuticals. Michael J. Tuvim and Burton F. Dickey have received Grant support from the US National Institutes of Health for studies on inducible innate resistance; they are inventors of technologies related to inducible resistance for which patent applications have been made; and they have ownership interests in Pulmotect, Inc., which has licensed technologies from their employer, MD Anderson Cancer Center, for stimulating innate immunity to prevent respiratory infections and has sponsored research in their laboratories. All other authors declare no competing interests.

Authors' contributions

A.K.W. participated in the study conception, design, coordination, data collection, statistical analysis and drafting of the manuscript. J.H., K.V.L. and A.R. participated in the behavioral testing and qRT-PCR. G.R.V. was responsible for the O/P treatment preparation and administration, and lung lavage and cell counts of BAL fluid. B.F.D., M.J.T. and R.D. participated in the study conception, design, coordination, and drafting of the manuscript. All authors read and approved the final manuscript.

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