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

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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.

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

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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× or 8× 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% CO_2 to promote deep breathing, connected by polyethylene tubing (30 cm × 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× (1 μM ODN and 4 μM Pam2) or 8× 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×, 8× 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× O/P or PBS. Mice were euthanized after CO_2 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 TM Real-Time PCR System using Taqman® gene expression assays for IL-6 (cat: Mm.PT.51.12387735), IL-1β (cat: Mm.PT.51.17215823), TNFα (cat: Mm.PT.51.16622079), IFNγ (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 ΔΔCt method, where Ct is the threshold