Online Data Supplement

Toll-like receptor 2 (TLR2) Regulates Organic Dust-Induced Airway Inflammation

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Lung Macrophage Isolation Methodology

To isolate primary lung macrophages, WT and TLR2 KO mice were euthanized and the right ventricle was perfused with saline to remove blood cells from the pulmonary vasculature. Next, lungs were isolated, minced, and digested in a solution containing collagenase type I (324 U/mL; Fisher, Pittsburgh, PA), bovine DNase (75 U/mL; Sigma), porcine heparin (25 U/mL; Sigma) and phosphate buffered saline (PBS, pH 7.4) with Ca^{2+} and Mg^{2+}. After 1 hour rocking at room temperature, the lung solution was passed through nylon mesh (40 μM; Fisher) to remove large fragments. Each cell pellet was briefly resuspended in cold red blood cell lysing solution (ammonium chloride based buffer). After centrifugation, single cell suspensions were underlaid with lymphocyte separation solution (Fisher) and centrifuged at 400*g* for 20 minutes. Mononuclear cells were collected, washed, and suspended in complete RPMI media. For lung macrophage isolation, the mononuclear cells were incubated for 2 hours in culture medium. The cultures were enriched for lung macrophages by discarding the medium and washing the wells twice with PBS to remove non-adherent cells. Lung macrophage yield was > 92% as determined by Giemsa staining. Lung macrophages from TLR2-deficient and WT mice were then immediately stimulated with 1% DE in side-by-side experiments. After 24 hours, cell-free supernatants were collected and stored at -80°C for later cytokine/chemokine analysis. This optimal time (24 hour) and dose concentration of DE (1%) for eliciting cytokine/chemokine production was conducted prior to these studies (data not shown). Cell count and viability was assessed by trypan blue exclusion method after stimulation time period, and there was no evidence of cellular toxicity.

Organic Dust Extract (DE) Analysis

Previous analysis of this dust(1) revealed that prior to placing solution (extract form) and prior to sterile filtration, the presence of Gram-positive bacteria (98%; *Staphylococcus*, *Bacillus*, *Streptomycetes*, and *Enterococcus* species) and Gram-negative bacteria (2%) as identified by colony morphology. A semi-quantitative elemental analysis by inductively coupled plasma-mass spectrometry revealed the presence of trace metals (100% concentrate of dust, ng/mL: B, 1380; Mg, 144,600; Ti, 1166; Mn, 375.5; Fe, 4226; Co, 59.7; Ni, 371; Cu, 3295.5; Rb, 1076.5; Mo, 132; Zn, 8797.5; Research Triangle Park, Research Triangle, NC). The organic dust was also compared to house dust for chemical marker analysis by gas chromatography-tandem mass spectrometry analysis, and analysis revealed high contents of muramic acid (mean: 203.5 ng/mg [DE] vs. 25 ng/mg

[house dust]; muramic acid is a component of PGN) and slightly elevated 3-hydroxy fatty acid (0.0723 nmols/mg [DE] vs. 0.0494 nmols/mg [house dust]; 3-hydroxy fatty acid is a component of endotoxin), but no difference in ergosterol (3.53 ng/mg vs. 6.1 ng/mg).(1)

The total protein concentration in the dust extract (12.5% concentration) was approximately 160-320 μg/ml, by Bradford protein assay (Bio-Rad, Hercules, CA). The mean endotoxin concentration in a 12.5% concentration as determined by the Limulus amebocyte lysate gel clot assay (Cambrex, Walkersville, MD) was 0.384 μg/ml (range: 0.208-0.56 μg/ml).

Although it is not possible to scrub the dust extract of only TLR2-bearing components to the best of our knowledge, it is possible to inactivate biologic components and leave metals and particles intact by heat-inactivation (heating dust extract to 120°C for 24 hours). Since our previous *in vitro* studies found that heat-inactivated DE did not modify markers of antigen presenting cell function, phenotype or cytokine/chemokine secretion,(1) we investigated heat-inactivated DE-induced airway responses following a single exposure in our described murine model.

LPS challenge methodology and results

Although it has been previously demonstrated that these TLR2 deficient mice respond similarly as WT mice to lipopolysaccharide (LPS) challenge,(2) we sought to confirm here that the airway response observed in the TLR2-deficient mice was specific to the TLR2 receptor pathway and not secondary to an inherent hypo-responsive effect. WT and TLR2 KO mice were intranasally challenged once with LPS (100 μg per 30 μl, from *Escherichia coli* (O55:B5), Sigma) and euthanized 5hr post-exposure. BAL fluid was collected and total cell number and differential cell counts were determined. An LPS concentration of 100 μg is approximately 250X the equivalent concentration in 12.5% DE, and was utilized at this concentration to maximize airway inflammatory response to LPS.

There was no difference in total cellularity, macrophage, or neutrophil influx following a one-time challenge with LPS between WT and TLR2-deficient mice as shown in supplementary figure 1.

PGN challenge

To determine if a TLR2-agonist would induce similar airway inflammatory responses as that of DE, WT mice were intranasally challenged with *Staphylococcus aureus* peptidoglycan (PGN; 100 μg per 30 μl, Sigma), which also remains consistent with our prior work comparing DE-induced responses to PGN.(1, 3) A PGN concentration of 100 μg is approximately half the protein concentration in 12.5% DE. WT mice were challenged once (single exposure) or once daily for 2 weeks (repetitive exposure) to saline (vehicle control) or PGN and subsequently euthanized 5 hours after final exposure. BAL fluid was collected and total cell number, differential cell counts, and concentrations of TNF-α, IL-6, CXCL1, and CXCL2 levels were determined. In other experiments, dose-responsiveness to aerosolized methacholine (1.5-48.0 mg/ml) was obtained at three hours after challenge as described in the *Methods* section using the computerized small animal ventilator system (Finepointe,

Buxco, Wilmington, NC) and reported as total lung resistance (R_L). Lung sections from repetitively challenged mice were routinely processed and stained with hematoxylin and eosin as described in the *Methods* section.

Supplement References:

1. Poole JA, Alexis NE, Parks C, MacInnes AK, Gentry-Nielsen MJ, Fey PD, Larsson L, Allen-Gipson D, Von Essen SG, Romberger DJ. Repetitive organic dust exposure in vitro impairs macrophage differentiation and function. *J Allergy Clin Immunol* 2008;122:375-382, 382 e1-4.

2. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K and Akira S**.** Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999;11:443-51.

3. Poole JA, Wyatt TA, Von Essen SG, Hervert J, Parks C, Mathisen T, Romberger DJ. Repeat organic dust exposure-induced monocyte inflammation is associated with protein kinase C activity. *J Allergy Clin Immunol* 2007;120:366-73.