MD-2–Dependent Pulmonary Immune Responses to Inhaled Lipooligosaccharides: Effect of Acylation State

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Methods

Experimental Design

The experimental design for the dose-response study and the time course study is shown in Figure E1.

Production of Lipooligosaccharide

Metabolically ^{14}C-labeled LOS was isolated from an acetate auxotroph of wild type *N. meningitidis* serogroup B (NMBACE1) and from a *msbB* mutant derived from NMB as previously described (E1, E2, E3). In brief, LOS was purified after growth of the bacteria in Morse's minimal medium supplemented with ^{14}C acetate (2 mM; 0.1 μCi/ml) to yield ^{14}C-LOS of sufficient specific radioactivity (300 cpm/μg) to facilitate accurate quantification of LOS. Both LOS^{wt} and LOS^{msbB} were extracted by hot phenol-water, precipitated by ethanol and after resuspension and sonicated in water, further purified by ultracentrifugation or gel filtration chromatography as previously described (E1). LOS stocks were stored at 4º C.

Measurement of Endotoxin Concentration

Concentrations of LOS (endotoxin activity (units); EU) were measured using the kinetic chromogenic *Limulus* Amebocyte Lysate assay (E4) and indicated an activity of LOS^{wt} of 18,000,000 EU/ml (1200 μg/ml) and for LOS^{msbB} 33,800,000

(2250 µg/ml). LOS solutions were diluted using sterile, pyrogen-free (pf) water containing 0.05% Tween-20 and assayed at multiple dilutions. Two-fold serial dilutions of endotoxin standards were prepared using sterile, pf water with Tween-20 in borosilicate glass tubes that were depyrogenated by heating for 4 h at 200^{o}C. A twelve-point calibration curve from 0.049 to 100 EU of standard *E. coli* 0111:B4 endotoxin was measured. The absorbance in each microplate well was measured at 405 nm every 30 s for 90 min. Endotoxin determinations were based upon the maximum slope of the absorbance versus time plot for each well. Four assay reagent blank wells serve as reference and control for the pf status of the reagent water, centrifuge tubes, pipette tips and microplates. The endotoxin value for each solution was calculated from the arithmetic mean of those dilutions that fell in the middle two-thirds of the standard curve.

Bronchoalveolar Lavage

At the appropriate time points mice were euthanized and BAL fluid was collected as previously described (E4). Tubes of BAL fluid were centrifuged and the supernatants were stored in aliquots at -80ºC for cytokine analysis. The residual cell pellet was resuspended with Hank's Balanced Salt Solution (LifeTechnologies, Grand Island, NY) and total cells were counted by hemocytometer. For the differential enumeration of cells, Hank's Balanced Salt Solution was added to further dilute the suspension and cells were centrifuged onto slides. Slides were dried and stained with Diff-Quik (Dade Behring, Newark,

NY) and differential cell counts were performed in duplicate counting 200 cells each time.

References

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E2. Gioannini TL, Teghanemt A, Zhang D, Coussens NP, Dockstader W, Ramaswamy S, Weiss JP. Isolation of an endotoxin–MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *PNAS* 2004; 101: 4186–4191.

E3. Teghanemt A, Zhang D, Levis EN, Weiss JP, Gioannini TL. Molecular basis of reduced potency of underacylated endotoxins. *J Immunol* 2005; 175:4669-4676.

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Figure E1. Experimental protocol for the dose-response and time course study.