

MD-2–Dependent Pulmonary Immune Responses to Inhaled Lipooligosaccharides

Effect of Acylation State

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Endotoxins represent one of the most potent classes of microbial immunoreactive components that can cause pulmonary inflammation. The aim of this study was to compare the inflammatory potency of two types of *Neisseria meningitidis* endotoxins (lipooligosaccharides) in lungs: wild type (hexaacylated, LOS^{wt}) and mutant type (pentaacylated, LOS^{msbB}), and to determine the importance of MD-2 in endotoxin responses in lungs *in vivo*. Endotoxin-normoresponsive mice (BALB/c) were exposed to selected doses of penta- and hexaacylated lipooligosaccharides (LOS) by nasal aspiration. Cellular and cytokine/chemokine inflammatory responses in bronchoalveolar lavage were measured at 1-, 4-, 8-, 16-, 24-, and 48-hour time points. MD-2–null mice were exposed to one dose of hexaacylated LOS and inflammatory responses were measured after 4 and 24 hours. Inhalation of hexaacylated LOS resulted in strong inflammatory responses, while pentaacylated LOS was much less potent in inducing increases of neutrophils, TNF- α , macrophage inflammatory protein-1 α , IL-6, granulocyte colony-stimulating factor, and IL-1 β concentration in bronchoalveolar lavage. Similar kinetics of inflammatory responses in lungs were found in both types of endotoxin exposures. Inhalation of hexaacylated LOS in MD-2–null mice resulted in significantly lower numbers of neutrophils in bronchoalveolar lavage than in normoresponsive mice. Markedly lower inflammatory potency of pentaacylated LOS was observed compared with hexaacylated LOS. Hyporesponsiveness in MD-2–null mice after nasal aspiration of wild-type LOS indicate its essential role in airway responsiveness to endotoxin.

Keywords: endotoxin; lipooligosaccharide; inhalation; pulmonary inflammation

Innate immunity represents the first line of defense against infection. When bacteria and bacterial components invade the host, they stimulate a variety of specialized host cells, including epithelial cells, monocytes, macrophages, neutrophils, and endothelial cells, as part of the acute inflammatory response (1). These responses are crucial for host defense but, in excess, can be very harmful causing local and multi-system derangements, including pulmonary inflammation (2), shock, and occasionally death (3).

Lipopolysaccharides (LPS) or endotoxins represent one of the most potent classes of microbial immunoreactive components.

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CLINICAL RELEVANCE

Alteration of fatty acyl chains within lipid A markedly affects inflammatory potency *in vivo* by impairing Toll-like receptor 4 activation by endotoxin:MD-2 complexes. Blunted airway responses to inhaled endotoxin in MD-2–null mice demonstrates the essential role of MD-2.

Endotoxins are composed of a lipid A region, responsible for proinflammatory activity, covalently linked to an oligo- or polysaccharide chain. Lipopolysaccharides lacking the distal O-specific carbohydrate are termed lipooligosaccharides (LOS) (4), and are characteristic of the endotoxins produced by many gram-negative bacterial inhabitants of the oropharyngeal flora. One such LOS derived from *Neisseria meningitidis* has been studied as a model compound for immune recognition (5–9).

Endotoxins are highly water insoluble, existing either as integral components of the gram-negative bacterial outer membrane or, after extraction, as aggregates shielding the lipid A region from water (4). Maximum host cell responsiveness to endotoxin requires at least four extracellular and cell surface host proteins (LPS binding protein [LBP], cluster determinant 14 [CD-14], lymphocyte antigen 96 [MD-2], and Toll-like receptor [TLR]4). These proteins engage endotoxin in a series of sequential protein–endotoxin and protein–protein interactions (8). LBP catalyzes the transfer of LPS from bacterial membranes (10) or aggregates of purified LPS to CD-14 either in soluble form or membrane-bound via a glycosyl-phosphatidylinositol (GPI)-linked membrane anchor (11). Monomeric endotoxin:CD-14 complexes that are released are the preferred substrate form of endotoxin for MD-2, presented either in extracellular form (6, 7) or membrane bound via association with TLR4 (9). Simultaneous engagement of MD-2 with endotoxin and TLR4 results in receptor and cell activation, initiating a signal transduction cascade leading to the release of proinflammatory mediators (1, 12).

The chemistry and geometry of the isolated endotoxin molecule and of supramolecular assemblies of endotoxin can greatly affect the proinflammatory activity of endotoxin (13–15). Among the most important structural determinants of endotoxin activity are the number, chain length, and location of fatty acyl chains within lipid A (13–17). In general, the most potent endotoxin species are hexaacylated, whereas either less or more highly acylated endotoxins are substantially less potent inducers of host proinflammatory responses (15, 18). Differences in the potency of hexa-, tetra-, and pentaacylated endotoxins reflect differences in the ability of endotoxin-bound MD-2 to induce activation of TLR4 (17, 19). On the basis of these observations, it has been suggested that the elaboration of underacylated endotoxins by *Pseudomonas aeruginosa* early in the evolution of pulmonary infection in cystic fibrosis (15, 20,

21) and by *Yersinia pestis* in pneumonic plague (22) contributes greatly to the virulence of these airway bacterial pathogens by blunting early host inflammatory responses needed for efficient mobilization of host defenses. However, the ability of the mammalian airway to mount graded responses to administered endotoxin, depending on endotoxin lipid A properties, and the role of MD-2 in airway responses to endotoxin have not yet been directly tested.

The first aim of this study was to determine the ability of *Neisseria meningitidis* serogroup B (NMB) LOS to induce airway inflammation *in vivo* and to compare the inflammatory potency of wild-type hexaacylated NMB LOS (LOS^{wt}) with that of pentaacylated LOS (LOS^{msbB}) derived from the *msbB* mutant strain of NMB. The second aim was to determine the role of MD-2 in airway responsiveness to LOS. Some of the results have been previously reported in the form of abstracts (23–25).

MATERIALS AND METHODS

Lipooligosaccharide

Metabolically ¹⁴C-labeled LOS was isolated from an acetate auxotroph of wild-type NMB and from a *msbB* mutant derived from NMB as previously described (5, 6, 17) (see the online supplement). Concentrations of LOS (endotoxin activity [units]; EU) were measured using the kinetic chromogenic *Limulus* Amebocyte Lysate assay as previously described (26) and indicated an activity of LOS^{wt} of 18,000,000 EU/ml (1,200 µg/ml) and for LOS^{msbB} 33,800,000 (2,250 µg/ml) (see the online supplement). Stocks of LOS were diluted fresh before use with sterile, pyrogen-free saline.

Animals

Six-week-old male BALB/c mice from Jackson Laboratories (Bar Harbor, ME) were used. After a 10-day quarantine and before exposures, two mice were necropsied and evaluated for inflammation to ensure the health of the shipment. MD-2-null mice on C57BL/6 background were bred and genotyped in our vivarium. Exposed and control BALB/c and MD-2-null mice were housed separately and supplied with HEPA-filtered air, filter-sterilized water, and ovalbumin-free mouse food (sterile Teklad 5% stock diet; Harlan, Madison, WI) *ad libitum*. Experimental protocols were approved by the Institutional Animal Care and Use Committee and adhered to NIH Guidelines.

Experimental Protocol

Dose–response and time course studies were performed using LOS^{wt} and LOS^{msbB} isolated from the wild-type and mutant type of NMB serogroup B. In the dose–response study, groups of 6- to 10-week-old endotoxin-normoresponsive (BALB/c) mice were exposed to one of five LOS^{wt} doses (5, 10, 30, 300, and 3,000 EU/mouse) or one of three LOS^{msbB} doses (30, 300, and 3,000 EU) as shown in Figure E1 in the online supplement. Control groups inhaled sterile, pyrogen-free saline. LOS was inhaled by nasal aspiration under short-term Halothane anesthesia. Four hours after exposure mice were killed by injection with Nembutal (150 mg/kg, intraperitoneally) and processed for necropsy, bronchoalveolar lavage (BAL), and histopathology. In the time-course studies, groups of six to nine mice were exposed to sterile, pyrogen-free saline (controls), LOS^{wt}, or LOS^{msbB} (30 and 300 EU/mouse). At 1, 4, 8, 16, 24, and 48 hours after exposure to LOS^{wt} and 4 and 24 hours after exposure to LOS^{msbB}, BAL fluid and lungs were processed. MD-2-null mice were exposed to 300 EU LOS^{wt} (*n* = 17) and necropsied 4 or 24 hours after exposure.

BAL and Processing of Lungs

At the appropriate time points mice were killed and BAL fluid was collected as previously described (26) (see the online supplement). After lavage, lungs were perfused through the heart with sterile saline, cannulated, and fixed in zinc formalin. Cassettes with the lungs were processed and lungs were embedded in paraffin and cut in 5-µm

sections. Slides were stained with hematoxylin and eosin and evaluated for inflammation.

Cytokine/Chemokine Assays

Supernatants from BAL fluid were assayed to determine cytokine/chemokine concentrations using a multiplexed fluorescent bead-based immunoassay (BioRad Laboratories, Inc., Hercules, CA). The BioPlex mouse cytokine system was used to measure concentrations of 18 cytokines/chemokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 [p40], IL-12 [p70], IL-17, granulocyte colony-stimulating factor [G-CSF], granulocyte macrophage colony-stimulating factor [GM-CSF], murine CXCL1 (KC), TNF-α, macrophage inflammatory protein [MIP]-1α, IL-6, IFN-γ, and regulated on activation, normal T cell expressed and secreted [RANTES]) or, for some groups, 6 cytokines (TNF-α, MIP-1α, G-CSF, IL-6, IL-1β, and IL-17). Cytokine concentrations below the valid range of the standard curve were assigned the limit of detection divided by $\sqrt{2}$ (27).

Statistical Analysis

Log transformation of BAL cells and cytokine concentrations yielded normally distributed data. Data were represented by their geometric mean and SEM. Statistical analyses were performed comparing LOS^{wt}- and LOS^{msbB}-exposed mice to controls using two-sided Dunnett *t* for multiple comparisons (Ver. 15; SPSS, Inc., Chicago, IL). Comparisons of LOS treatment versus control across time points were performed using ANOVA. Comparisons of dose–response curves between treatments used generalized linear models. *P* values ≤ 0.05 were considered significant and ≤ 0.01 highly significant.

RESULTS

Comparison of Dose-Dependent Effects of Nasally Instilled LOS^{wt} and LOS^{msbB}

Endotoxin-responsive BALB/c mice were used to establish dose–response, time-course, and histopathologic changes in the lungs induced by exposure to purified LOS aggregates. Early inflammatory responses in the lungs were manifest in BAL fluid primarily by recruitment of neutrophils and increased concentrations of inflammatory cytokines and chemokines. Experiments showed that increasing LOS^{wt} doses from 0 (controls) to 5, 10, 30, 300, and 3,000 EU/mouse induced

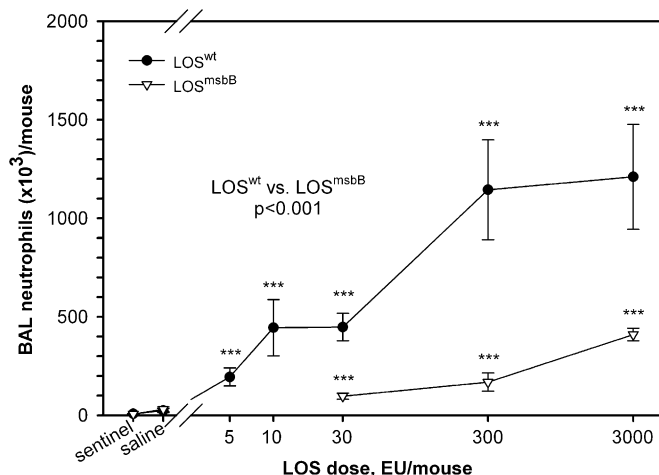


Figure 1. Dose–response data showing the number of neutrophils in bronchoalveolar lavage (BAL) after nasal aspiration of LOS^{wt} and LOS^{msbB} in comparison with controls ($***P < 0.001$). There was a significantly higher neutrophilic response in LOS^{wt}- than in LOS^{msbB}-exposed mice 4 hours after exposure ($P < 0.001$).

significant increases of total cell counts (not shown) and neutrophils (Figure 1) in the BAL fluid compared with controls ($P < 0.01$). Exposure to LOS^{msbB} also induced a significant increase in neutrophils recovered in the BAL fluid in comparison with controls ($P = 0.05$), but required 10- to 100-fold higher concentrations of LOS to induce a response comparable to that induced by LOS^{wt} (Figure 1). Both LOS^{wt} and LOS^{msbB} induced accumulation of TNF- α , MIP-1 α , IL-6, G-CSF, and IL-1 β in BAL fluid; however, LOS^{wt} was approximately 100 times more potent (Figure 2). In mice exposed to LOS^{wt} , little change was observed in BAL fluid concentrations of IL-1 α , IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-17, GM-CSF, KC, IFN- γ , and RANTES (data not shown).

Kinetics of Airway Inflammation Induced by Nasally Instilled LOS^{wt} and LOS^{msbB}

To determine if markedly reduced airway inflammatory responses to LOS^{msbB} versus LOS^{wt} manifest at 4 hours were due to an altered magnitude of host responses and/or altered kinetics of the inflammatory responses, two doses of LOS^{wt} and LOS^{msbB} (30 and 300 EU/mouse) were selected for a time-course study. Pulmonary inflammatory cell responses to inhaled LOS^{wt} at 1, 4, 8, 16, 24, and 48 hours are shown in Figure 3. A similar time course of total cell and neutrophilic response was found in both LOS^{wt} dose-exposure groups of mice. Total cell counts and neutrophils were greatly increased within 4 hours of

exposure and remained very high for 24 hours. Total and neutrophil concentrations in BAL were still above baseline levels at 48 hours. In mice treated with 30 or 300 EU LOS^{wt} /mouse (Figure 3, middle and top panels, respectively), we observed a 14- to 32-fold increase in neutrophils at the 4-hour time point compared with controls (Figure 3, bottom panel). Neutrophils continued to increase for the first 24 hours after initial exposure. Nearly all the increase in total BAL cells was attributable to neutrophils. Intra-nasal instillation of LOS^{msbB} induced much less accumulation of neutrophils in BAL at all time points examined (compare Figures 3 and 4), indicating that the magnitude but not the kinetics of airway inflammatory responses is affected by the dose or potency of the LOS administered. A similar pattern was observed for the cytokines/chemokines (TNF- α , MIP-1 α , IL-6, G-CSF, and IL-1 β) induced by instillation of LOS^{wt} , except that the levels of these proteins in BAL returned close to baseline within 16 to 48 hours (Figure 5). At the doses tested, LOS^{msbB} induced only very modest increases in BAL of the same cytokines and chemokines induced more robustly by LOS^{wt} (see Figure 2) with apparently similar kinetics (data not shown).

Dose- and Time-Dependent Histopathologic Changes in the Lungs of Mice Exposed to Nasally Instilled LOS^{wt} and LOS^{msbB}

In addition to lung lavage cellular and cytokine responses, we also examined the lungs for histopathologic changes. Four hours

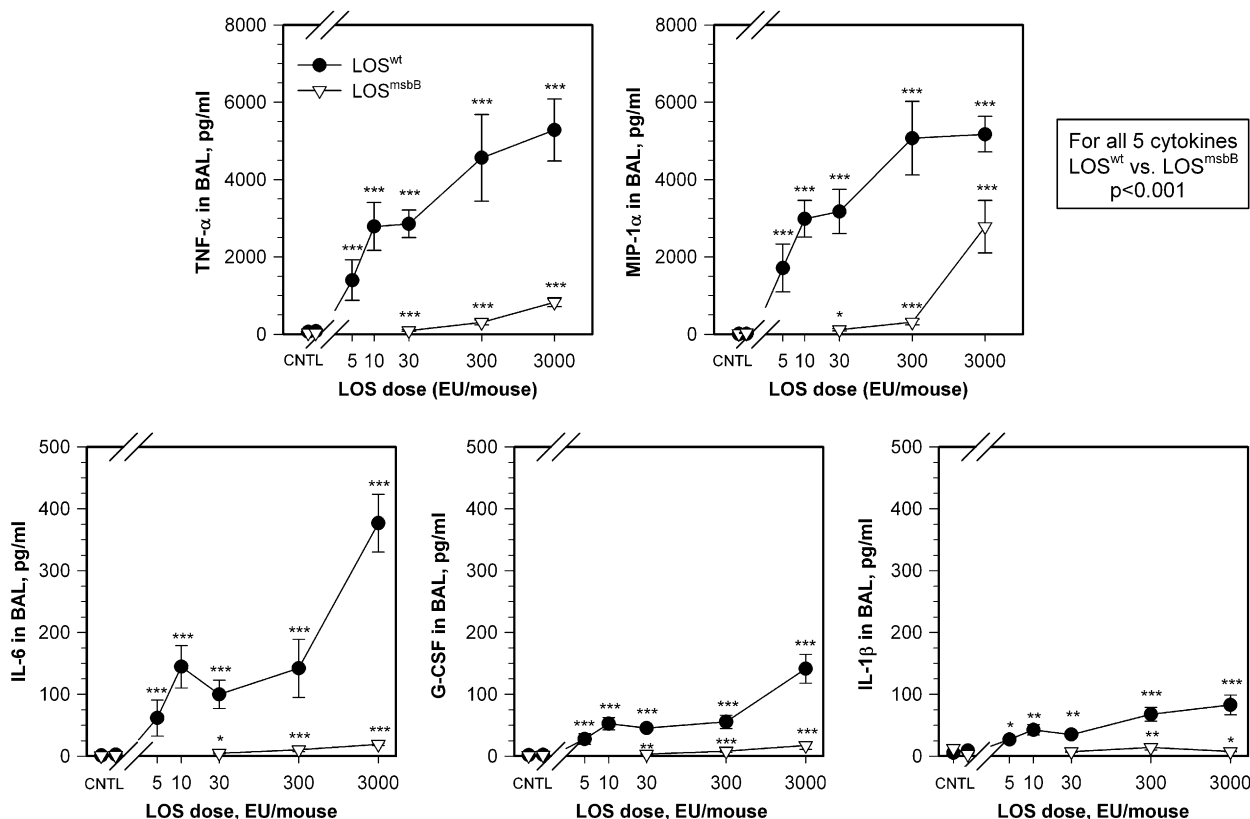


Figure 2. Cytokine dose–response curves for TNF- α , macrophage inflammatory protein (MIP)-1 α , IL-6, granulocyte colony-stimulating factor (G-CSF), and IL-1 β in BAL fluid of LOS^{wt} - in comparison with LOS^{msbB} -exposed mice. Data show a dose–response relationship where increasing doses of endotoxin produced significant increases of TNF- α , MIP-1 α , G-CSF, IL-6, and IL-1 β in BAL fluid of LOS^{wt} -exposed mice in comparison with those exposed to LOS^{msbB} ($P < 0.001$ for each cytokine). Each data point compared with respective control: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

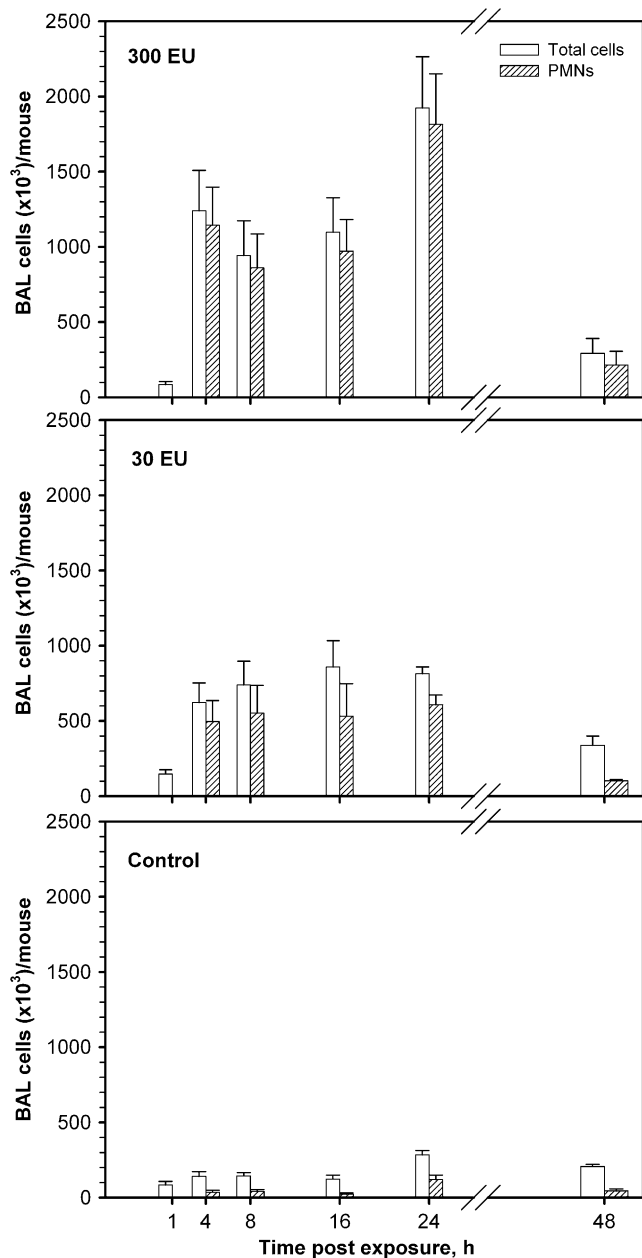


Figure 3. Time-course for pulmonary inflammatory responses (total cells and neutrophils) in BAL after the nasal aspiration of LOS^{wt} in the dose of (top panel) 300 EU/mouse and (middle panel) 30 EU/mouse in comparison with (bottom panel) saline (control). The higher endotoxin dose showed maximal total cells and neutrophils in BAL at 24 hours, with a small elevation of neutrophil counts persisting at 48 hours.

after exposure to LOS^{wt} or LOS^{msbB} at 30, 300, or 3,000 EU/mouse, the tissues exhibited peribronchiolar neutrophilia that was not evident in sentinels or saline and increased with increasing dose. The abundance of neutrophils was far greater in the lungs of the LOS^{wt} exposure group compared with the LOS^{msbB} group of mice (Figure 6A), with increasing numbers of neutrophils in the peribronchiolar and perivascular tissue up to 24 hours. The accumulation of neutrophils seen microscopically corresponded closely to the dose- and time-dependent accumulation of these cells in BAL fluid in response to LOS exposure (Figure 6B).

Airway Responses to Nasally Instilled LOS Is MD-2 Dependent

Previous studies in mice have demonstrated the prominent roles of LBP, CD14, and TLR4 in airway/lung responses to inhaled endotoxin (28–30), but the role of MD-2 has not yet been directly examined. Previous *in vitro* studies have demonstrated the critical role of MD-2 in determining the different potencies of hexaacetylated versus underacetylated endotoxins, including that of LOS^{wt} versus LOS^{msbB}. The findings above demonstrating the marked differences in proinflammatory potency of nasally instilled LOS^{wt} versus LOS^{msbB} are thus consistent with an important role of MD-2 in determining airway responses to instilled LOS. To test the role of MD-2 more directly, we bred and back-crossed MD-2-null mice (31) onto the C57BL/6 background for more than 7 generations and then compared the responsiveness of wild-type and MD-2-null mice to nasally instilled LOS^{wt}. In contrast to the wild-type mice, the MD-2-null mice did not display appreciable cellular airway inflammatory changes (Figure 7) in response to instilled LOS^{wt} 4 or 24 hours after exposure. Lavage cellularity in exposed MD-2-null mice were no different than in saline-exposed controls. These findings show clearly the importance of MD-2 in sensitive airway inflammatory responses to nasally instilled endotoxin.

DISCUSSION

The ability of the immune system to recognize endotoxin (lipopolysaccharides and lipooligosaccharides) is crucial in host defense against many gram-negative bacteria but can also lead to inflammation-driven host pathology. Accordingly, the LPS response system is highly complex and controlled, providing many levels of regulation. Factors regulating the expression and function of LPS-binding protein, CD-14, MD-2, and TLR-4 likely affect the host response to environmental endotoxin exposure and constitute an important gene-environment interaction affecting the incidence and severity of diseases such as asthma, asthma-like syndrome, organic dust toxic syndrome, and some forms of bioaerosols-induced alveolitis.

Inflammatory responses to inhaled endotoxin have been studied in both humans (2, 32–34) and mice (35–38). In general, inhalation or nasal aspiration of endotoxin by mice induces similar host responses and changes (35, 39), and these reflect human airway responsiveness (33).

The data presented in this study demonstrate that the ability of mice to mount rapid and robust airway inflammatory responses to nanogram amounts of nasally instilled endotoxin depends on the acylation state but not, apparently, specific features of the oligo(poly)saccharide chain of endotoxin. The magnitude and kinetics of airway inflammatory responses to meningococcal LOS observed in this study closely resembled those previously described for other administered purified hexaacetylated LPS species (33, 37, 40). Thus, meningococcal LOS or *Escherichia coli* or *P. aeruginosa* LPS each induced neutrophil recruitment to the airway that was manifest within 4 hours and persisted for at least 48 hours. The kinetics of cytokine responses induced by airway exposure to purified LOS aggregates or by purified *P. aeruginosa* LPS (40) was also similar and resembled that investigated by exposure of humans to inhaled grain dust containing LPS (33). Taken together, these findings suggest a similar capacity of the airways/lungs of mice and humans to respond to a broad array of inhaled or instilled endotoxin species of varied bacterial origin.

In contrast, our studies revealed substantial differences in the magnitude of airway responses to endotoxin species that

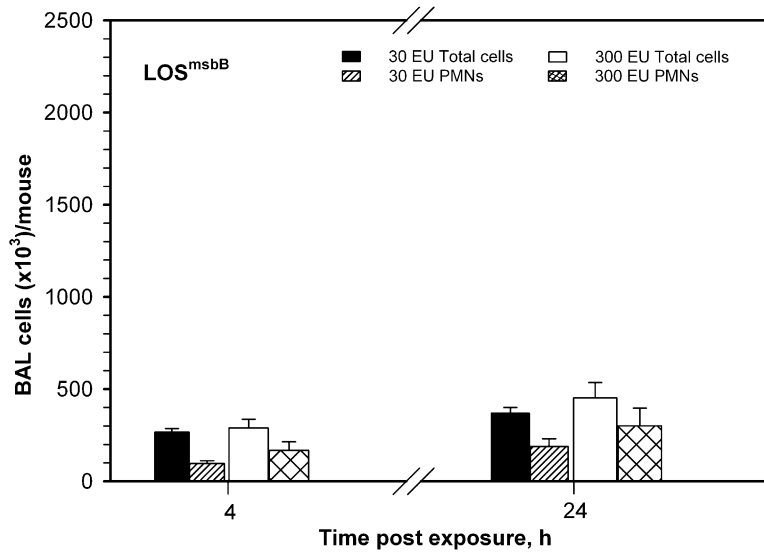


Figure 4. Pulmonary inflammatory responses after nasal aspiration of LOS^{msbB} in the dose of 30 EU and 300 EU/mouse at 4- and 24-hour time points shown on the same scale as in Figure 3. Responses at both doses and time points were much lower than observed in LOS^{wt} -exposed mice.

differ in the lipid A region by only one fatty acid/endotoxin molecule. Approximately 100-fold higher amounts of pentaacylated LOS^{msbB} , in comparison to hexaacylated LOS^{wt} , were needed to induce comparable airway inflammatory responses. The potency of tetraacylated endotoxin species is likely to be even lower (18). If such differences are also manifest during infections of the airways and lungs, this would result in delayed mobilization of airway host defenses, giving invading bacteria more time to further multiply, adapt, and disseminate. Our findings thus support earlier speculations that the elaboration of

pentaacylated LPS by *P. aeruginosa* initially colonizing individuals with cystic fibrosis and tetracylated LPS by *Y. pestis* greatly increases host susceptibility to these airway pathogens.

The differences in potency we observed between LOS^{wt} and LOS^{msbB} in inducing airway inflammatory responses *in vivo* were strikingly similar to differences previously observed in LBP-dependent *in vitro* activation by these LOS species of cells expressing mCD14, MD-2, and TLR4 (17). Our findings thus strongly suggest that the molecular requirements for airway responsiveness to endotoxin correspond closely to the require-

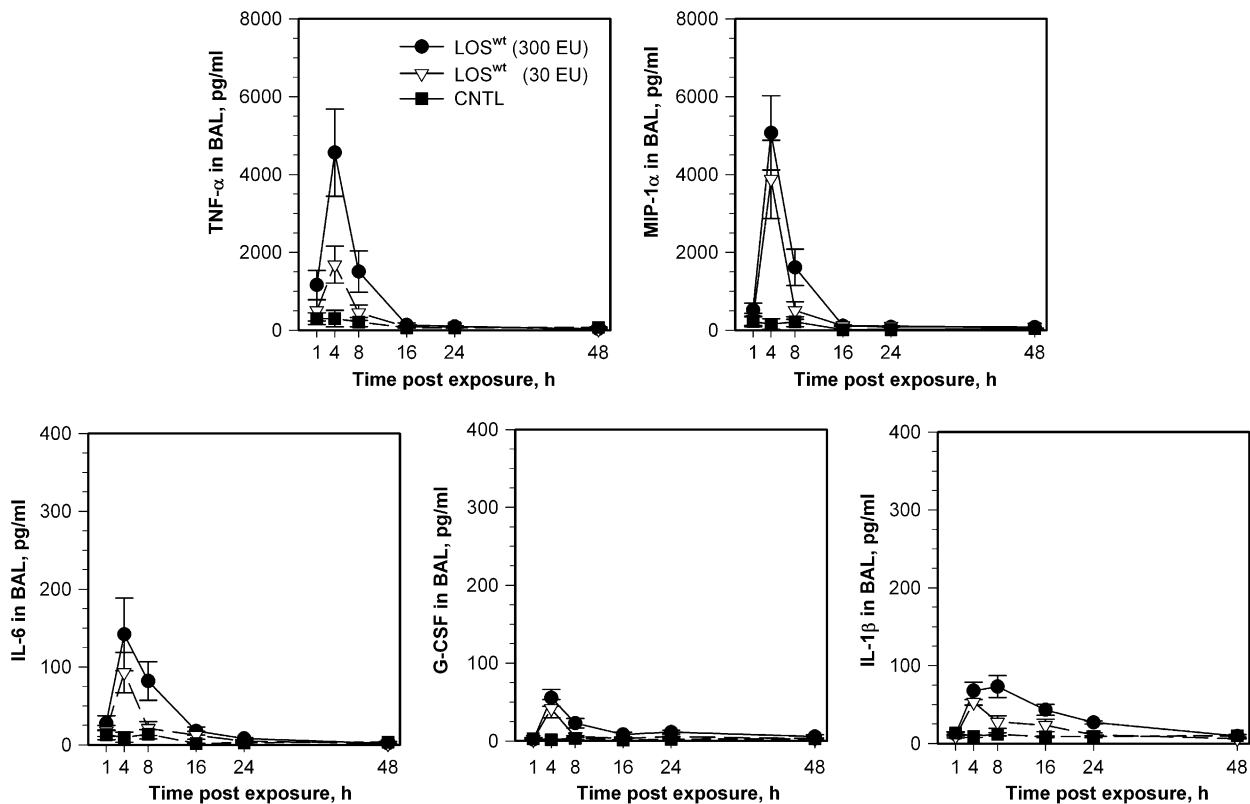


Figure 5. Time course for the concentration of $TNF-\alpha$, $MIP-1\alpha$, $IL-6$, $G-CSF$, and $IL-1\beta$ in lavage after inhalation of LOS^{wt} in the dose of 300 and 30 EU/mouse in comparison with controls.

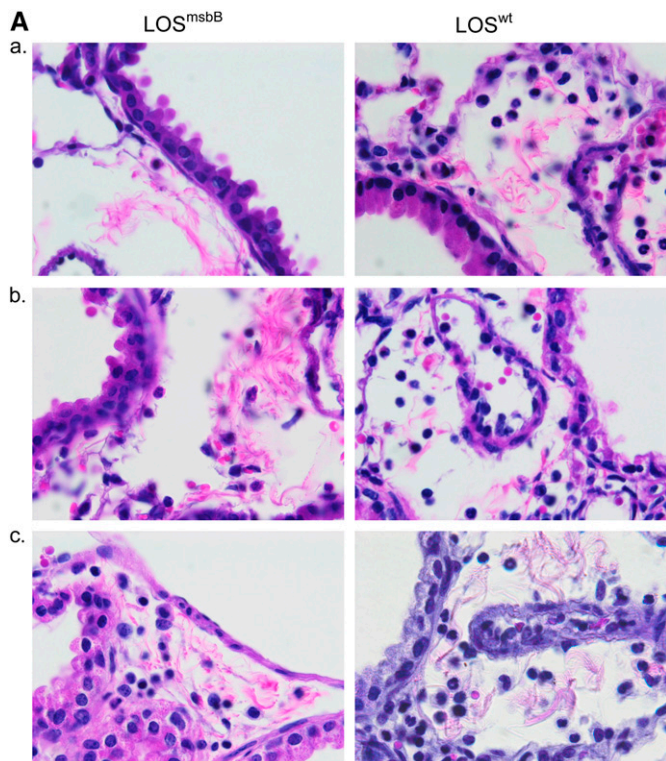


Figure 6. Representative photomicrographs of lungs stained with hematoxylin and eosin. (A) Hexa- and pentaacylated LOS (LOS^{wt} and LOS^{msbB})-induced accumulation of neutrophils in the peribronchiolar region 4 hours after nasal aspiration of (a) 30 EU, (b) 300, and (c) 3,000 EU/mouse. Magnification: $\times 100$. (B) Neutrophil recruitment in the peribronchiolar region 1, 4, 8, 16, 24, and 48 hours after exposure of 300 EU of LOS^{wt} . Magnifications: $\times 40$ and $\times 100$.

ments defined *in vitro* for LBP/CD-14/MD-2/TLR4-dependent cell activation by endotoxin. Previous studies in mice have demonstrated the prominent role of LBP, CD14, and TLR4 in airway/lung responses to endotoxin (28–30) and our findings in this study now definitively establish an essential role for MD-2 as well (Figure 7). MD-2-null mice do not mobilize airway inflammatory responses to nasally instilled LOS aggregates but are responsive to purified monomeric LOS:MD-2 (unpublished observation), confirming that the loss of responsiveness of MD-2-null mice to endotoxin is due to the absence of MD-2. *In vitro*, differences in potency of hexaacylated versus underacylated endotoxin species in inducing TLR4-dependent cell activation reflect differences in the functional properties of endotoxin bound to MD-2 (17, 19). Thus, the differences we observed in this study in the ability of LOS^{wt} versus LOS^{msbB} to induce airway inflammation most likely reflect differences in the functional properties of hexacylated versus pentacylated LOS bound to MD-2 in the airway.

In vivo studies in mice have suggested that TLR4-dependent responses to endotoxin in the resting airway are mediated mainly by bone marrow-derived cells (e.g., alveolar macrophages), with airway epithelia less directly involved (29). We have shown that primary cultures of well-differentiated human and murine airway epithelia are hyporesponsive to endotoxin, despite expression of CD14 and TLR4, because of limited expression of MD-2 (7) (unpublished observations). The demonstration in this study that MD-2 is required for airway responses to nasally instilled endotoxin is consistent with the

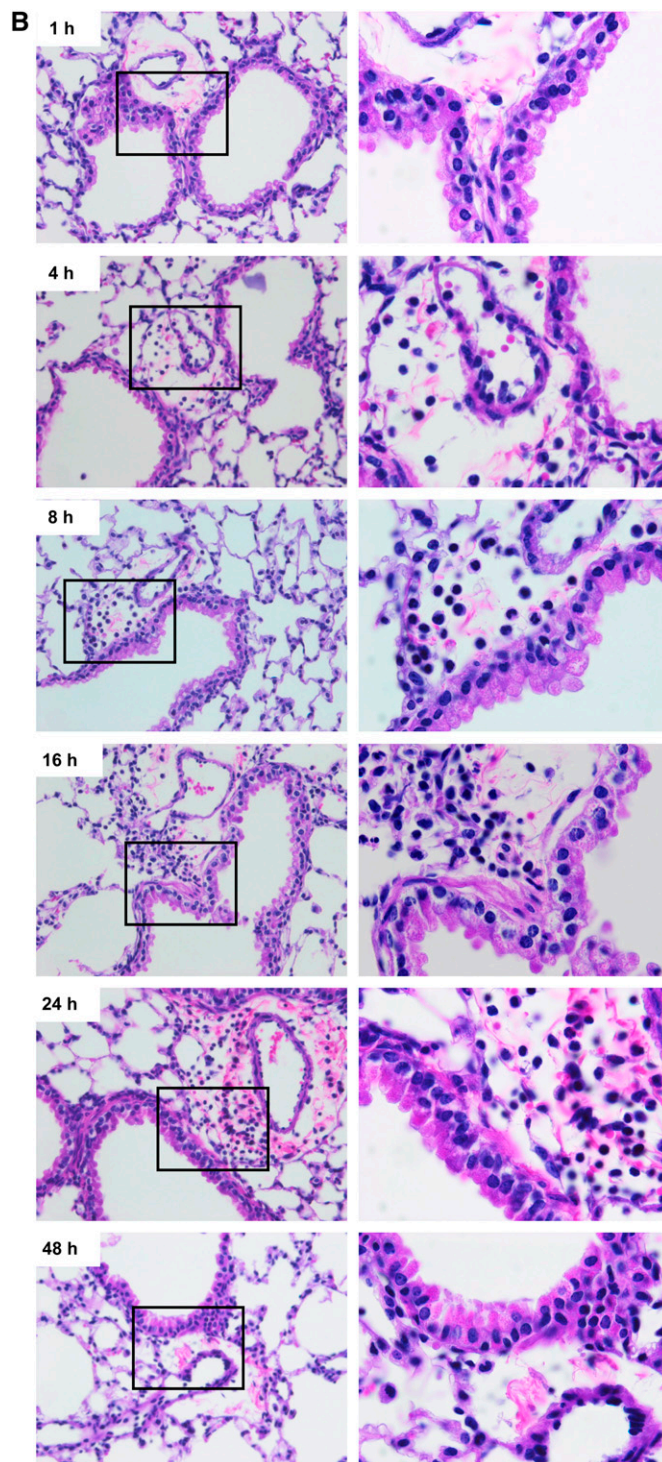


Figure 6. (Continued).

speculation that it is the absence of MD-2 expression by airway epithelia in the resting airway that normally limits their direct responses to inhaled or aspirated endotoxin. The production of MD-2 by alveolar macrophages but not by airway epithelia may help to promote host responses to deeply invading bacteria while minimizing inflammatory reactions to more incidental exposure to endotoxin-bearing environmental pollutants.

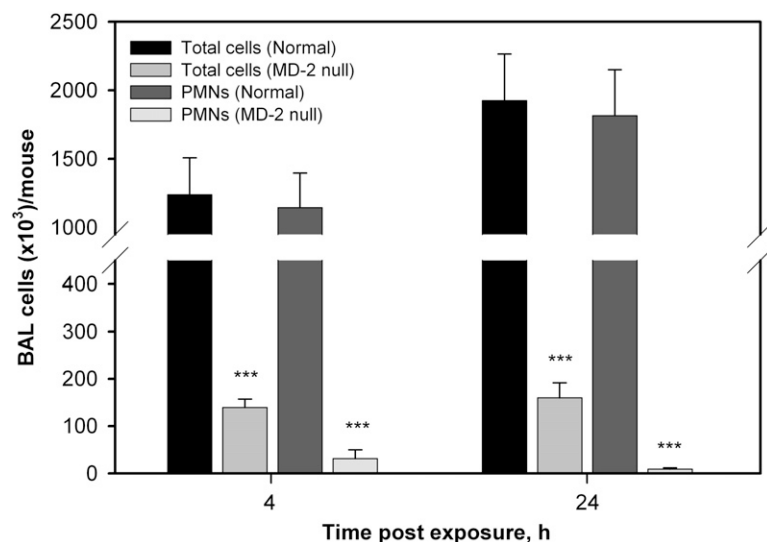


Figure 7. Number of total cells and neutrophils in bronchoalveolar lavage fluid from normoresponsive mice in comparison with MD-2^{-/-} mice at 4 and 24 hours after exposure (****P* < 0.001).

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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