Lipid A fraction of LPS induces a discrete MAPK activation in acute lung injury

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tionally, we have studied the relative potency of Kdo₂-lipid A compared with crude Escherichia coli LPS. Lastly, we explored whether there is a TLR4-independent mechanism for Kdo₂-lipid A-induced inflammation.

MATERIALS AND METHODS

Mice. Male C57BL/6J and C3H/HeJ mice 6–8 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in specific pathogen-free conditions and provided with food and water ad libitum. All experiments were conducted in accordance with Institutional Animal Care and Use Committee-approved protocols.

Materials. Isoflurane was obtained from Abbott (Chicago, IL). E. coli 0111:B4 endotoxin and PD-98059 were purchased from Sigma Chemical (St. Louis, MO). An aliquot of Kdo₂-lipid A was a generous gift from Dr. Robert Murphy and thereafter was purchased from Avanti Polar Lipids (Alabaster, AL). Antibodies to JNK, p38, and ERK1/2 and to their phosphorylated forms were purchased from Cell Signaling Technologies (Beverly, MA). Control cell lysates (293 control cell extracts with or without UV treatment) were from Cell Signaling (Danvers, MA). Dulbecco’s modified Eagle’s medium (DMEM) and Hanks’ balanced salt solution (HBSS) were obtained from Invitrogen (Carlsbad, CA). RPMI 1640, 25 mM HEPES, L-glutamine, and fetal bovine serum (FBS) was obtained from Media-tech (Herdon, VA). SDS-PAGE and gel transfer were performed with the Invitrogen NuPAGE system. The lysis buffer solution contained 0.1% Triton X-100, 0.1 mM DTT, 50 mM HEPES, and 10% glycerol in distilled water (dH₂O). Complete protease inhibitor cocktail tablets were obtained from Roche Applied Science (Indianapolis, IN).

In vivo ALI model. ALI was induced by intratracheal administration of O4:B111 E. coli LPS or Kdo₂-lipid A in doses ranging from 10 to 300 μg per mouse. In this procedure, ablended-20-gauge needle (Hamilton, Reno, NV) was passed through the mouth into the trachea of an anesthetized mouse suspended by the incisors, and the LPS (in 50 μl of sterile PBS) or Kdo₂-lipid A solution (in 50 μl of sterile vehicle) was deposited. Mice were humanely euthanized, and lungs were perfused with ic saline and harvested at specific time points after administration. Lungs were snap-frozen for future analysis. Preliminary studies determined that this method was associated with 95% survival at 24 h.

Primary peritoneal macrophage studies. To elicit the accumulation of macrophages in the peritoneal cavity, mice (C57BL/6J) were injected intraperitoneally with 1 ml of 3% thioglycollate broth (Sigma-Aldrich) as previously described (40). Three days after thioglycollate injection, macrophages were recovered by peritoneal lavage method reported for MPO assay; we have validated a method that is commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to manufacturer’s instructions. Western blot analysis. SDS-PAGE and immunoblotting was performed to evaluate levels of phosphorylated and total p38, ERK, and JNK in both whole lung homogenates and peritoneal macrophages, essentially as previously described (47). After removing supernatants from cell culture plates for cytokine detection, macrophages were lysed in ice-cold lysis buffer containing complete protease inhibitor cocktail (Roche Applied Science) and then resuspended, and debris from the lysed cell was pelleted by centrifugation at 14,000 g for 20 min. The supernatant was then removed and stored at −86°C. For in vivo animal experiments, total lungs were removed and homogenized on ice with a PowerGen homogenizer (Fisher Scientific; Pittsburgh PA) at maximum speed in complete protease inhibitor cocktail. Homogenates were centrifuged at 14,000 g for 20 min, and supernatants were collected. All samples were analyzed for protein content using a Bio-Rad DC Protein Assay kit with BSA as standards. For the Western blots, equal amount of protein (20 or 50 μg for each lung homogenate, 20 μg for each macrophage cell lysate) were loaded and run on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) with a molecular weight marker. Protein was then electrotransferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA) and blocked with 5% nonfat dry milk, 20 mM TBS, with 0.1% Tween. After blocking, the membrane was incubated overnight at 4°C with a rabbit polyclonal specific primary antibody to phosphorylated JNK, ERK, or p38 using a dilution of 1:1,000 in 5% BSA followed by anti-rabbit or anti-rat immunoglobulin horseradish peroxidase-coupled secondary antibody at a dilution of 1:3,000 in 5% nonfat dry milk. After washing five times, bands were detected using Western blotting detection reagents (ECL or ECL Plus; PerkinElmer Life Sciences, Boston, MA). The membranes were then stripped using stripping buffer (63 mM Tris·HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol from Bio-Rad) and reprobed with antibodies specific for total JNK, ERK, or p38. Band density was measured using a chemiluminescence system running Quantity One software (Bio-Rad).

Statistical analysis. For each experimental condition, the entire group of animals was prepared and studied at the same time. Data are presented as means ± SE for each experimental group. SigmaStat v3.11 was used for one-way analysis of variance, the Tukey-Kramer
multiple comparisons test for multiple groups, and Student’s t-test for comparisons between two groups. $P < 0.05$ was considered significant.

RESULTS

Intratracheal instillation of lipid A as well as LPS induces ALI. At 6 and 24 h, intratracheal administration of Kdo2-lipid A resulted in ALI comparable in magnitude and distribution to that induced by LPS (Fig. 1A). Since LPS-induced ALI has been extensively characterized (22, 47), we sought to compare the severity of lung injury induced by lipid A or LPS. Our histological studies demonstrated similar accumulation of neutrophils and mononuclear cells into the pulmonary interstitium and interstitial pulmonary edema. The semiquantitative lung injury score (Fig. 1B) was comparably and significantly increased compared with vehicle ($P < 0.001$) in lipid A and LPS groups at 6 and 24 h (6-h control vs. lipid A vs. LPS: $1.5 \pm 0.37, 9.6 \pm 1.20, 9.2 \pm 1.13$; 24-h control vs. lipid A vs. LPS: $1 \pm 0.21, 9.8 \pm 0.66, 10.3 \pm 0.67$).

Increased lung MPO at 6 h after lipid A instillation. In dose-ranging studies, we determined the optimal dose of lipid A and LPS to be 30 µg per mouse based on comparable submaximal lung injury (Fig. 1A) and time course of MPO activity (Fig. 2A). MPO activity was comparable 24 h after 30 or 100 µg of lipid A or LPS in wild-type mice ($n = 6$ each group; $P = 0.094$ and 0.153, respectively) and was minimally different with the 10-µg dose [difference = 0.17 optical density units (OD)$\cdot$min$^{-1}$$\cdot$mg$^{-1}$ lung tissue; $P = 0.034$]. Unless otherwise specified, all subsequent experiments were conducted with the 30 µg per mouse dose of LPS or Kdo2-lipid A to minimize saturation of the MPO response signal.

Change in MPO activity was comparable at 2 and 24 h between lipid A 30 µg- and LPS 30 µg-treated animals (Fig. 2B) By contrast, MPO activity was increased 2.4-fold in lipid

Fig. 1. A: lung histology from LPS- and lipid A-treated mice (30 µg per mouse). Lungs were harvested, and inflation-fixed sections were stained with hematoxylin and eosin. Representative images of lungs 6 and 24 h after LPS, lipid A, and vehicle control exposure are shown at $\times400$ magnifications. Two additional experiments provided similar results. B: semiquantitative acute lung injury (ALI) score was comparably and significantly increased compared with vehicle ($P < 0.001$) in lipid A and LPS groups at 6 and 24 h (6-h control vs. lipid A vs. LPS: $1.5 \pm 0.37, 9.6 \pm 1.20, 9.2 \pm 1.13$; 24-h control vs. lipid A vs. LPS: $1 \pm 0.21, 9.8 \pm 0.66, 10.3 \pm 0.67$; $P < 0.001$ for LPS and lipid A vs. vehicle at 6 and 24 h).
A- compared with LPS- and vehicle-treated mice at 6 h after instillation (lipid A, 0.88 ± 0.25 OD·min⁻¹·mg⁻¹, n = 6; LPS, 0.37 ± 0.21 OD·min⁻¹·mg⁻¹, n = 12; vehicle, 0.205 ± 0.02 OD·min⁻¹·mg⁻¹, n = 20; P < 0.05; Fig. 2B). MPO was also comparably low in vehicle-treated mice or C3H/HeJ mice treated with LPS or lipid A at 6 and 24 h. There were no significant differences among C3H/HeJ mice groups treated with vehicle, lipid A, or LPS (Fig. 2C).

We next wanted to exclude a possible active vehicle effect by the Kdo2-lipid A carrier (10% ethanol, 40% propylene glycol, 50% dH2O) on the magnitude of ALI as measured by MPO. Therefore, we also instilled PBS in some mice. There was no significant difference in MPO activity between PBS and ethanol/propylene glycol-treated mice or compared with mice without any treatment at 24 h.

Fig. 2. A: lung myeloperoxidase (MPO) assays. MPO activity was comparable 24 h after 30- or 100-μg lipid A or LPS in wild-type mice (n = 6 each group; P = 0.094 and 0.153, respectively) but not after 10-μg dose (**P = 0.034). Whole lung MPO activity is presented as means ± SE (n = 6 per group). Time course of lung MPO in wild-type (B and C) or C3H/HeJ (C) mice treated with intratracheal instillation of LPS (30 μg per mouse in 50 μl of sterile PBS), lipid A (30 μg per mouse, 50-μl sterile vehicle), vehicle (50 μl; 10% ethanol, 40% propylene glycol, 50% distilled water), or PBS (50 μl). Lungs were harvested from nonmanipulated mice (no treatment) or mice with different treatments at various time points (n = 4–20). *P < 0.001 (ANOVA, Bonferroni correction) compared with no treatment (n = 4). LPS 2 h (n = 4), lipid A 2 h (n = 6), vehicle 6 h (n = 20), PBS 6 h (n = 4), LPS 6 h (n = 4), vehicle 24 h (n = 10), PBS 24 h (n = 4), and all C3H/HeJ groups (no Tx, n = 2; vehicle 24 h, n = 6; lipid A 6 h, n = 10; lipid A 24 h, n = 16; LPS 24 h, n = 6); **P < 0.05 lipid A 6 h vs. LPS 6 h. OD, optical density units.

Fig. 3. A: immunoblots for phosphorylated (p ERK) and total ERK (t ERK) expression in mouse lungs receiving different treatment after 2 and 6 h. V, vehicle; L30, LPS 30 μg per mouse; A30, lipid A 30 μg per mouse; L100, LPS 100 μg per mouse; A100, lipid A 100 μg per mouse. Gels were loaded with 20 μg per well total protein. Four additional experiments for each condition provided similar results. B: increased expression of phosphorylated ERK in lipid A-treated lungs (30 μg per mouse). Total ERK staining is shown as a control for protein loading in the immunoblots. Gels were loaded with 50 μg per well total protein. Representative experiments from separate mice (C57BL/6J) are shown. Four additional experiments for each condition provided similar results. C: densitometry results from 5 independent experiments are presented. *P < 0.001; †P < 0.05.
Discrete MAPK activation between lipid A- and LPS-treated lungs. Phospho-ERK activation was readily detected early (2 h post-treatment) in both lipid A- and LPS-treated lungs with subsequent decrease at 6 and 24 h (Fig. 3A). High dose (100 µg) more than intermediate dose (30 µg) LPS or lipid A treatment induced maximal and comparable phospho-ERK activation at 2 h. Pronounced whole lung phospho-ERK activation (normalized for total ERK) was appreciated at 6 and 24 h after lipid A but not LPS or vehicle treatment (6 h, 0.88 ± 0.03 vs. control, 0.33 ± 0.04, P < 0.001; 24 h, 0.269 ± 0.02 vs. control, P < 0.001; Fig. 3B). Phospho-ERK activation was further enhanced at 24 h compared with 6 h (1.73 ± 0.08 vs. 0.88 ± 0.03; P < 0.01). There were no statistical differences among vehicle, LPS 6-h, and LPS 24-h groups (Fig. 3C).

Because whole lung phospho-JNK and phospho-p38 activation were not detected at 2 and 6 h even with high-dose (100 µg per mouse) LPS or lipid A treatment in gels loaded with 20 µg of total protein, gels were loaded with 50 µg per well total protein, and immunoblots were incubated with ECL substrate (ECL Plus) and exposed to radiographic film for up to 5 min (Figs. 3B and 4).

Phospho-p38 activation (normalized for total p38) was comparable between groups at 6 h (Fig. 4, A and B). No obvious phospho-JNK activation was detected after 6 or 24 h in either LPS or lipid A lungs in our model. To further confirm these results regarding activation of phospho-JNK, we performed the immunoblots along with positive and negative JNK control lysates. The phospho-JNK activation relative to total JNK in the lung homogenates was much lower than that of positive control cell lysates.

Similar cytokine secretion pattern in macrophage cells stimulated with lipid A or LPS. To compare the efficiency of lipid A vs. LPS and ascertain the potential contribution of macrophages to the differences in MPO and MAPK signaling at 6 h, we conducted studies with primary peritoneal macrophages ex vivo. Based on previous work evaluating the effects of lipid A stimulation of macrophages (35), we used 100 ng/ml Kdo₂ lipid A to maximally stimulate thioglycollate-elicited macrophage cells. As shown in Fig. 5A, MIP-2 release was maximal at 2 h (1,700.4 ± 12.6 pg/ml for LPS). Lipid A and LPS induced comparable cytokine concentrations at 4 h of incubation. KC secretion was also comparable in lipid A- and LPS-stimulated macrophages (Fig. 5B).

Blockade of phospho-ERK activation diminishes the cytokine release from macrophage cells stimulated with lipid A or LPS. We next determined the requirement for ERK-dependent signaling by lipid A compared with LPS. The ERK inhibitor (PD-98059) was used to confirm the role of phospho-ERK activation on proinflammatory effects in vitro. One hour before stimulation by lipid A or LPS, we incubated the...
thioglycollate-elicited macrophage cells with the ERK inhibitor. PD-98059 (100 μM) blocked LPS- and lipid A-mediated increase in phospho-ERK activation (Fig. 6, A and B) in macrophages to a comparable level. Reduction in supernatant cytokine concentrations between lipid A and LPS was also comparable (Fig. 6, B and C), suggesting that phospho-ERK activation was sufficient to account for the lipid A- or LPS-induced proinflammatory cytokine secretion in vitro.

**DISCUSSION**

We have demonstrated for the first time that purified lipid A induces ALI in mice in a magnitude comparable to LPS and that this is associated with whole lung ERK (p44/42) activation. Furthermore, we have demonstrated that the lipid A-induced ALI is TLR4-dependent by comparing our observations in TLR4 mutant (C3H/HeJ) mice that have previously been demonstrated to be resistant to high-dose LPS-mediated lung neutrophil influx and ALI (16, 17).

Intratracheal LPS induces acute lung inflammation characterized by a pronounced biphasic leukocyte infiltration: initial massive neutrophil accumulation followed by a late mononuclear cell influx (15). A significant limitation of previous LPS ALI studies in animal models is the use of crude preparations of E. coli-derived LPS. These preparations contain multimeric molecules with a wide range of molecular weights owing to microheterogeneity in the length and composition of the terminal glycan chains. This precludes a direct comparison of molar concentrations of LPS with lipid A. As a result, our experiments have used direct weight-based comparisons. The possibility, however, exists that since lipid A (MW 2238.75) is in molar excess to LPS at equal weight (average MW 109) (30) that the differences in ERK activation are due exclusively to TLR4 receptor saturation. Although this is not excluded in the current studies, the equivalent doses of lipid A and LPS resulted in a comparable histological magnitude of ALI at 6 and 24 h. Even when comparing MPO and ERK activation responses across a 2 log difference in doses (100 μg LPS vs. 10 μg lipid A), this appears to be an unlikely explanation for the differential response. Since the histological assessment of neutrophil infiltration (as a component of total cellular inflammation) is relatively insensitive as a single parameter, it is possible that the composite lung injury score underestimates the difference in lung neutrophils at 6 h as indicated by the significant difference in the more sensitive MPO activity. This analysis clearly has some limitations but is consistent with previous studies concerning this compound (35).

LPS is not an effective chemoattractant for neutrophils but can trigger an inflammatory cascade via the synthesis of cytokines, eicosanoids, and other proinflammatory mediators by resident alveolar macrophages and other cells (31). TLR4 activation of mononuclear cells via the MAPK cascade is thought to be an essential step for subsequent chemokine release (10). A number of lines of evidence support an important role for MPO in the microbicidal activity of neutrophils (24) as evidenced by neutrophil MPO constituting greater than 5% of the dry weight of the cells (42). The primary outcome measure for our study was thus whole lung MPO activity as a marker of neutrophil accumulation in the injured lung. Notably, the time course of increasing MPO activity differed between lipid A and LPS at a submaximal dose (30 μg per mouse) at 6 h but not 24 h. Furthermore, MPO activity (Fig. 2A) was comparable between LPS- and lipid A-treated animals at 24 h with the high dose (100 μg per mouse) but not with the low dose (10 μg per mouse) possibly suggesting a threshold effect for TLR4-specific receptor activation.

**Fig. 6.** A: ERK inhibitor (100 μM PD-98059; PD) blocks phosphorylated ERK activation in thioglycollate-elicited macrophage cells stimulated with V (PBS), L (100 ng/ml LPS), and A (100 ng/ml Kdo2-lipid A). After 4 h of incubation, macrophages were lysed in ice-cold lysis buffer, and cell extracts were analyzed. Total ERK staining is shown as a control for protein application to the Western blots. Densitometry results (B) are similar to the expression pattern of cytokines released in the supernatants (C).
C3H/HeJ mice harbor a point mutation at codon 712 of the Tlr4 gene resulting in a substitution of a highly conserved proline by histidine within the cytoplasmic domain of TLR4 and resulting in a defect in signal transduction (33). Jayaseelan and coworkers (16) have previously demonstrated that TLR4 mutant (C3H/HeJ) mice do not develop ALI after challenge with aerosolized LPS-induced ALI. Using C3H/HeJ mice, we confirmed that functional TLR4 was required for neutrophilic lung inflammation in response to maximal doses of both LPS and lipid A.

For these studies, LPS was formulated in sterile saline (PBS) for intratracheal instillation as previously described (47). Since Kdo2-lipid A is not soluble in saline, we used an organic vehicle (10% ethanol, 40% propylene glycol, 50% dH2O; Ref. 46) to solubilize Kdo2-lipid A and avoid lipid micelle formation that might occur in aqueous solutions. Importantly, we confirmed that there was no active vehicle effect in wild-type mice (Fig. 1).

Interestingly, we noted that the MPO activity in lungs from lipid A-injured mice was much greater than in LPS-treated animals at 6 h. We therefore reasoned that divergent MPO activity at 6 h might result from differences in TLR4-dependent signal transduction in response to lipid A compared with LPS.

Each of the three major MAPK pathways (ERK p44/42, Ref. 11; p38, Ref. 19; and JNK, Ref. 26) have been mechanistically implicated in the initiation and evolution of LPS-mediated neutrophil accumulation in the lung and consequent ALI (2). One explanation for such disparate observations is that investigators use different crude LPS extracts and different routes of administration to induce ALI thus resulting in variable patterns of MAPK activation. We therefore characterized MAPK expression patterns in the whole lung homogenates of lipid A-induced ALI compared with LPS. The key observation from these experiments was that a submaximal dose of lipid A (30 μg per mouse) induced significantly more whole lung phospho-ERK activation at 2, 6, and 24 h than an equivalent dose of LPS. By contrast, whole lung phospho-p38 activation was comparable between LPS- and lipid A-treated animals. Although chemical MAPK inhibition abrogates LPS-induced ALI (19, 26), the present data do not prove that ERK activation is either necessary or sufficient for lipid A-induced ALI. However, we speculate that there may be a component of TLR4-independent repression by whole LPS in vivo or, alternatively, that lipid A may have greater receptor potency. Specific ERK inhibition in lipid A- compared with LPS-treated animals may provide a clearer understanding of how MAPK-mediated lung inflammation is affected by these compounds.

Furthermore, since monocyte-derived chemokines such as KC are thought to contribute significantly to neutrophil migration into the injured lung (28), we conducted in vitro experiments and confirmed the relationship between phospho-ERK activation and cytokine expression in murine macrophages stimulated with either LPS or Kdo2-lipid A. However, these responses were comparable in magnitude between lipid A and LPS unlike the observations in whole lung for phospho-ERK and MPO activation. It is possible therefore that whereas macrophage-derived ERK activation is important in both lipid A- and LPS-induced ALI, cells other than macrophages or neutrophils (13), such as lung structural cells including pulmonary microvascular endothelial cells and alveolar pneumocytes (1) that do express TLR4 and respond to LPS, account for the differential ERK activation in our system. We are actively exploring this possibility.

In contrast to several previous reports (26), we were unable to detect measurable phospho-JNK activation in our murine ALI model. Several possible reasons exist for this. First, most studies of JNK-mediated LPS activation have been conducted using isolated peripheral blood leukocytes (25) or other noninflammatory cell lines such as epithelial cells (12) but not whole lung. Additionally, there is some inconsistency between the measured activation of ERK, JNK, or p38 (5, 44, 45) in these studies. Second, interspecies and strain variation may account for our observations. LPS-induced ALI in rats was associated with significant ERK and JNK phospho-activation at 24 h. NF-κB-mediated cytokine production was significantly reduced by chemical JNK inhibition in that model (26).

Our observations led to the conclusion that structural differences between whole LPS and the lipid A moiety may account for the striking differences in MAPK activation patterns in whole lung but not isolated murine macrophages. TLR4-mediated activation requires engagement of the LPS-LPS binding protein (LBP)-CD14 complex (14). The crystal structure of CD14 and the physicochemical implications for LPS binding have recently been elucidated (21). The LPS-binding pocket, located on the side of the horseshoe near the NH2 terminus is entirely hydrophobic except for the rim and provides a highly conserved lipophilic domain for binding of the lipid portion of LPS. The long carbohydrate chain of LPS is hydrophilic and negatively charged and appears to have its own binding site. This is suggested by the observation that LPS retains some affinity for CD14 even after enzymatic delipidation (23). Additionally, Ding and coworkers (7) have reported preserved but attenuated NF-κB activation in LPS-stimulated murine peritoneal macrophages from C3H/HeJ mice, suggesting a partial TLR4-independent mechanism for whole LPS activation. Therefore, our findings of differential activation of MAPK in ALI between lipid A and LPS may in part be explained by differential molecular interactions with CD14 and consequent engagement with the TLR4-MD-2 complex on the cell membrane or potentially a TLR4-independent mechanism.

MPO and cytokine production by heterogeneous resident lung and inflammatory cell populations could potentially confound these results. Polymorphonuclear leukocytes and macrophages are the most important regulators of LPS-induced ALI (15, 28). To investigate the contribution of inflammatory cell signaling, we chose to study the in vitro effects of LPS and lipid A in thioglycollate-elicited peritoneal macrophages. These cells have previously been demonstrated to be predominantly of bone marrow-derived origin (32). While recognizing that these cells might be phenotypically and functionally different from pulmonary monocyte/macrophages, it has recently been described that lung migratory macrophages in LPS-induced ALI are also bone-marrow derived (personal communication, William J. Janssen, National Jewish Research and Medical Center, Denver, CO). Further specific characterization of the differences between nonresident pulmonary and peritoneal macrophages, although beyond the scope of the present study, is an active area of investigation.
Taken together, we have demonstrated that the lipid A fraction of LPS induces ALI in mice in association with whole lung ERK activation and significantly greater MPO activity than LPS 6 h after submaximal dosing. This may in part be accounted for by differential TLR4 signaling in lung parenchymal cells but not macrophages.

Clinical studies with the lipid A-specific monoclonal antibody HA-1A failed to conclusively provide clinical benefit in terms of mortality or improvement of pulmonary organ failure/ALI in patients with bacterial sepsis and shock (3, 29). Our data suggests that LPS activation of inflammation in ALI may be more complex than simply lipid A-mediated TLR4 activation and perhaps account for these disappointing clinical outcomes. Future studies of LPS-mediated ALI should address both lipid A-dependent and -independent activation of the inflammatory cascade.

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