TLR-4 pathway mediates the inflammatory response but not bacterial elimination in *E. coli* pneumonia

Janet S. Lee,1 Charles W. Frevert,1 Gustavo Matute-Bello,1 Mark M. Wurfel,1 Venus A. Wong,1 Shu-Min Lin,2 John Ruzinski,1 Steve Mongovin,1 Richard B. Goodman,1 and Thomas R. Martin1

1Veterans Affairs Puget Sound Health Care System and the Division of Pulmonary & Critical Care Medicine, University of Washington, Seattle, Washington; 2Division of Pulmonary, Allergy, & Critical Care Medicine, Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania; and 3Department of Thoracic Medicine II, Chang Gung Memorial Hospital, Taipei, Taiwan

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TLR-4 pathway mediates the inflammatory response but not bacterial elimination in *E. coli* pneumonia. *Am J Physiol Lung Cell Mol Physiol* 289: L731–L738, 2005. First published July 15, 2005; doi:10.1152/ajplung.00196.2005.—We examined the role of Toll-like receptor (TLR-4) in modifying the lung inflammatory response and its effects on the bacterial recovery from the lungs following inhaled *Escherichia coli* in two different strains of TLR-4 mutant mice that are hyporesponsive to LPS. The C57BL/10ScCr(*tlr4*-*del*) mutant mice containing a deletion mutation in the TLR-4 gene showed lower proinflammatory cytokine levels, lower lung MPO activity, and lower neutrophil accumulation in the air spaces but showed no differences in the inflammatory cytokine levels, lower lung MPO activity, and lower neutrophil accumulation. The C3H/HeJ(*tlr4-*del*) and the C57BL/10ScCr(*tlr4*-*del*) mice do not show differences in bacterial clearance or inflammatory responses between TLR-4 mutant and wild-type mice. The C3H/HeJ mice is the result of a single amino acid substitution (P712H) in the cytoplasmic domain of TLR-4. The C57BL/10ScCr(*tlr4*-*del*) mice were reported to contain a second mutation in the IL-12Rβ2 gene, whereas the C57BL/10ScCr(*tlr4*-*del*) mice do not. Several animal studies have used either the C3H or the C57BL/10 TLR-4 mutants to address the role of TLR-4 in mediating the clearance of gram-negative bacteria from the lungs (3, 13, 16, 29), and two studies have investigated the responses of both strains of TLR-4 mutants to a specific pathogen (16, 29). Although TLR-4 mutants of the C3H and C57BL/10 strains have impaired bacterial clearance following intranasal *Klebsiella pneumoniae* (29) and *Bordetella bronchiseptica* (16), all studies have shown that TLR-4 is essential for adequate pulmonary host defenses. The C3H/HeJ mice showed no difference in bacterial clearance or inflammatory responses in the lungs following intranasal inoculation of *Legionella pneumophila* when compared with a related sub-strain wild type for TLR-4 (13). It has been suggested that the low endotoxic potential of *L. pneumophila* may account for apparent lack of differences in bacterial clearance and inflammatory responses between TLR-4 mutant and wild-type mice (3, 13). Alternatively, TLR-4 may not be necessary for lung host defense against all gram-negative bacteria.

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Lee, Janet S., Charles W. Frevert, Gustavo Matute-Bello, Mark M. Wurfel, Venus A. Wong, Shu-Min Lin, John Ruzinski, Steve Mongovin, Richard B. Goodman, and Thomas R. Martin. 2005. TLR-4 pathway mediates the inflammatory response but not bacterial elimination in *E. coli* pneumonia. *Am J Physiol Lung Cell Mol Physiol* 289: L731–L738, 2005. First published July 15, 2005; doi:10.1152/ajplung.00196.2005.—We examined the role of Toll-like receptor (TLR-4) in modifying the lung inflammatory response and its effects on the bacterial recovery from the lungs following inhaled *Escherichia coli* in two different strains of TLR-4 mutant mice that are hyporesponsive to LPS. The C57BL/10ScCr(*tlr4*-*del*) mutant mice containing a deletion mutation in the TLR-4 gene showed lower proinflammatory cytokine levels, lower lung MPO activity, and less parenchymal and peribronchial inflammation compared with the C57BL/10ScSn mice, a related TLR-4 wild-type substrain. However, the C57BL/10ScCr(*tlr4*-*del*) mice do not show differences in bacterial clearance or inflammatory responses between TLR-4 mutant and wild-type mice. The C3H/HeJ(*tlr4-*del*) and the C57BL/10ScCr(*tlr4*-*del*) mice do not show differences in bacterial clearance or inflammatory responses between TLR-4 mutant and wild-type mice. The C3H/HeJ mice is the result of a single amino acid substitution (P712H) in the cytoplasmic domain of TLR-4. The C57BL/10ScCr(*tlr4*-*del*) mice were reported to contain a second mutation in the IL-12Rβ2 gene, whereas the C57BL/10ScCr(*tlr4*-*del*) mice do not. Several animal studies have used either the C3H or the C57BL/10 TLR-4 mutants to address the role of TLR-4 in mediating the clearance of gram-negative bacteria from the lungs (3, 13, 16, 29), and two studies have investigated the responses of both strains of TLR-4 mutants to a specific pathogen (16, 29). Although TLR-4 mutants of the C3H and C57BL/10 strains have impaired bacterial clearance following intranasal *Klebsiella pneumoniae* (29) and *Bordetella bronchiseptica* (16), all studies have shown that TLR-4 is essential for adequate pulmonary host defenses. The C3H/HeJ mice showed no difference in bacterial clearance or inflammatory responses in the lungs following intranasal inoculation of *Legionella pneumophila* when compared with a related sub-strain wild type for TLR-4 (13). It has been suggested that the low endotoxic potential of *L. pneumophila* may account for apparent lack of differences in bacterial clearance and inflammatory responses between TLR-4 mutant and wild-type mice (3, 13). Alternatively, TLR-4 may not be necessary for lung host defense against all gram-negative bacteria.

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Address for reprint requests and other correspondence: T. R. Martin, Div. of Pulmonary and Critical Care, Dept. of Medicine, Univ. of Washington, 1660 S. Columbian Way, GMR 151-L, Seattle, WA 98108 (e-mail: trmartin@u.washington.edu).
Our objective in this study was to determine whether E. coli, a respiratory pathogen and strong inducer of proinflammatory cytokine production by alveolar macrophages in vitro, elicits similar innate inflammatory responses and bacterial proliferation in the lungs of TLR-4 mutant mice of two different strain backgrounds. We compared two different TLR-4 mutants, the C57BL/10ScN(tlr4\textsuperscript{del}) and C3H/HeJ(tlr4\textsuperscript{del}) mice, with their related TLR-4 wild-type strains. The TLR-4 mutants will be referred to as the ScN(tlr4\textsuperscript{del}) and the HeJ(tlr4\textsuperscript{del}) mice, with the information in parenthesis to indicate their mutant status.

Our findings indicate that both TLR-4 mutants showed reduced inflammatory responses when compared with their respective wild-type mice, as measured by lung cytokine levels, lung MPO activity, and net neutrophil counts in the bronchoalveolar lavage (BAL) fluid. However, the inflammatory responses were not related to their ability to eliminate E. coli from the lungs. On the basis of these findings, we conclude that functional TLR-4 is not a generalizable requirement for adequate pulmonary host defenses against gram-negative bacteria.

MATERIALS AND METHODS

Animals.

C57BL/10ScN(tlr4\textsuperscript{del}) littermate breeders were originally obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in the vivarium of the Veterans Affairs (VA) Puget Sound Health Care Systems Research facility. Random animals were genotyped by PCR with published primers that spanned the deletion site observed in the C57BL/10ScN(tlr4\textsuperscript{del}) mice (22). Others have reported a point mutation in the IL-12R gene that underlies the IL-12 unresponsiveness of LPS-defective C57BL/10ScCt(tlr4\textsuperscript{del}) mice (21). We sequenced the C57BL/10ScN(tlr4\textsuperscript{del}) mice at the IL-12R gene and found that our C57BL/10ScN(tlr4\textsuperscript{del}) strain did not contain this second mutation. For the in vitro E. coli studies, the C57BL/10ScSnj mice with intact TLR-4 signaling were used as the respective control strain for the C57BL/10ScN(tlr4\textsuperscript{del}) mutant mice. For the in vivo E. coli studies, the C57BL/10ScSn mice with intact TLR-4 signaling were used as the respective control strain for the C57BL/10ScN(tlr4\textsuperscript{del}) mutant mice. The C3H/HeJ(tlr4\textsuperscript{del}) mutant and C3H/HeSnj TLR-4 wild-type mice were also obtained from Jackson Laboratory. Age- and sex-matched animals were used in all experiments. Animals were maintained in specific pathogen-free cages. Experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the VA Puget Sound Health Care Systems and the University of Washington.

Bacteria. E. coli, serotype K-1, was a clinical isolate obtained from the blood of a patient with biliary sepsis. The pathogenicity of this bacteria in mice was previously described (18). Methods used to pass and store the bacteria have been detailed elsewhere (7). An aliquot of frozen bacteria was thawed and inoculated into 50 ml of Luria broth (GIBCO-BRL Laboratories, Gaithersburg, MD), which was incubated for 6 h at 37°C and shaken at 225 rpm. For the aerosol experiments, this culture was inoculated into 1 liter of Luria broth and incubated for another 14 h under the same conditions. The broth was centrifuged at 9,000 rpm, and the bacterial pellet was washed twice with sterile 0.9% NaCl. The washed bacterial pellet was resuspended in 22 ml of 0.9% NaCl and divided equally into two neublizers. Quantitative cultures were performed on the bacterial slurry.

Pneumonia studies. The inhalation method of delivering live bacteria to mice has been previously described (17, 18). After bacterial delivery, the mice were either killed immediately to establish the initial bacterial inoculum or killed at 6 or 24 h for measurements of bacterial burden. At the specified times, mice were killed with 120 mg/kg pentobarbital and exsanguinated by direct cardiac puncture. The thoracic cavity was opened by midline incision. The trachea was exposed and cannulated with a 20-gauge catheter, which was secured with a 2-0 silk suture. The left main stem bronchus was identified and divided at the hilum, and the entire left lung was placed in 1 ml of sterile H2O2 for subsequent homogenization. BAL was performed on the right lung with 0.9% NaCl containing 0.6 mM EDTA instilled in one aliquot of 0.6 ml, followed by three aliquots of 0.5 ml. The right lung was subsequently fixed with 4% paraformaldehyde at an inflation pressure of 15 cmH2O.

Alveolar macrophage cytokine production. Alveolar macrophages were obtained from BAL performed on whole lungs by the method described in Pneumonia studies. Cell counts and cytospins were performed to determine the number of alveolar macrophages obtained per animal. The alveolar macrophages at a concentration of 1 × 10\textsuperscript{5}/ml were immediately exposed to heat-killed E. coli at 10\textsuperscript{5} colony-forming units (cfu)/ml for 2 and 6 h. Supernatants were then collected and stored at −70°C.

Phagocytosis assay. Alveolar macrophages were obtained from BAL performed on whole lungs by the method described in Pneumonia studies. This assay was based upon a modification of a protocol by Schif and colleagues (24). E. coli (K-12 strain) BioParticles (BODIPY FL conjugate; Molecular Probes, Eugene, OR) were opsonized with heat-inactivated mouse serum before exposure to alveolar macrophages at a ratio of 10:1 (cell: bacterium). After a 2-h incubation in the dark at 37°C, the reaction was terminated by being placed on ice. The suspension was centrifuged, and 0.1% Trypan blue was briefly added to the cell pellet to quench external fluorescence. The cells were washed carefully in assay buffer twice, and linear mean fluorescence intensity of the alveolar macrophages was measured by flow cytometry (FACScan; Becton Dickinson, San Jose, CA).

Sample processing. Total cell counts in the BAL fluid were performed with a Neubauer chamber. We performed cell differentials by counting 200 consecutive cells from cytospin preparations stained with Diff Quik. The remainder of the BAL fluid was spun at 200 g, and supernatants were stored at −70°C. The left lung homogenates were separated into three aliquots: the 1st for quantitative cultures, the 2nd for cytokine measurements, and the 3rd for MPO determinations. For the cytokine measurements, the lung homogenate was added to a buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl\textsubscript{2}, and 1 mM MgCl\textsubscript{2}. The mixture was sonicated and spun at 12,000 g for 30 min, and the supernatants were stored at −70°C.

Bacterial recovery measurements. Quantitative cultures were performed on the lung homogenates obtained from each animal at the various times. For E. coli, we performed quantitative cultures by spreading serial 10-fold dilutions of the lung homogenates in warm LB agar by the pour plate method.

MPO activity assay. The Amplex red peroxidase assay kit (Molecular Probes) allows for the detection of H2O2 or peroxidase activity in tissue. This assay was used to determine MPO activity in the lung homogenates. Lung tissue homogenates were prepared as above and then mixed with reaction buffer, H2O2, and Amplex red reagent according to the manufacturer’s instructions. The Amplex red reagent reacts with H2O2 in the presence of peroxidase to produce the fluorescent molecule resorufin. The fluorescence of each sample was measured with a cytofluorometer set at excitation wavelength of 530 nm and emission wavelength of 590 nm (CytoFluor II; PerSeptive Biosystems, Foster City, CA). We obtained the MPO activity by calculating resorufin units for each unknown lung homogenate sample using a resorufin standard curve.

Cytokine measurements. KC, macrophage inflammatory protein (MIP)-2, TNF-α, and IFN-γ concentrations in the lung homogenates...
and BAL fluid were measured with commercially available ELISA kits (R&D Systems, Minneapolis, MN). The sensitivities were 7.8 pg/ml for KC and 23.4 pg/ml for both MIP-2 and TNF-α. A direct comparison between the ELISA method and a bead-based cytometric immunoassay system (Luminex, Austin, TX) was made in selected samples. KC, MIP-2, and TNF-α concentrations obtained by the ELISA and Luminex methods correlated well with each other. Measurements of IL-1β and IL-10 in the lung homogenates were made with the Luminex assay. The sensitivities were 23.4 pg/ml for IL-10 and 19 pg/ml for IL-1β.

Quantitative morphometry of airways. The right lungs were fixed with 4% paraformaldehyde at an inflation pressure of 15 cm H2O as described in Pneumonia studies. Lungs were cut in 4- to 6-μm-thick sagittal sections and stained with hematoxylin and eosin. Midsagittal sections were used for quantitative morphometry. Methods for quantitative morphometry were obtained and modified from George et al. (8). In brief, conducting airways were identified in section at >20, and images were captured using Q-imaging micropublisher (QiMaging, Burnaby, BC, Canada). The analysis was performed with Image ProPlus software (Media Cybernetics, Silver Spring, MD). Airways were divided into three groups based on diameter: 1) small airways 90 μm or less in diameter, 2) medium airways 90–129 μm in diameter, and 3) large airways >129 μm in diameter. Obliquely cut airways showing a length to width ratio >2.5 were excluded from analysis. We calculated airway epithelial area and subepithelial area by defining the internal perimeter, external perimeter, and basement perimeter on the digital images. The entire section of each lung tissue slide was examined under >20 by a reviewer who was unaware of the identity of the slides, and the conducting airways meeting the criteria above were scanned and analyzed.

Statistical analysis. Nonparametric analysis (Mann-Whitney U-test) was used to make statistical comparisons between animal groups with the StataSE 8 software package (Stata Corp LP, College Station, TX).

RESULTS

Lung cytokine responses of the ScN(tlr4lp-Δ) mutant and TLR-4 wild-type mice following inhalation of E. coli. We examined the in vivo cytokine responses in the lungs following exposure to E. coli serotype K-1 by inhalational challenge. The ScN(tlr4lp-Δ) mice showed significantly lower concentrations of the proinflammatory cytokines KC, MIP-2, and TNF-α in the lungs 6 h following exposure to live bacteria compared with the TLR-4 wild-type mice (P < 0.03) (Fig. 1). There were no significant differences in the concentrations of IL-1β and IL-10.

Neutrophilic responses of the ScN(tlr4lp-Δ) mutant and TLR-4 wild-type mice following inhalation of E. coli. We measured lung homogenates’ MPO activity as a marker of the total neutrophil content in the lungs. The ScN(tlr4lp-Δ) mutant mice showed lower lung MPO activity at both 6 and 24 h (P < 0.05) compared with the TLR-4 wild-type mice (Fig. 2A). At 6 h, BAL polymorphonuclear neutrophil (PMN) counts in the ScN(tlr4lp-Δ) mutant mice exceeded the PMN counts of the wild-type mice (P < 0.01) (Fig. 2B). By 24 h, however, the BAL PMN counts of the ScN(tlr4lp-Δ) mutant mice had reached a plateau and were significantly less than in the wild-type mice (P < 0.001). Thus the overall neutrophil response was lower in the ScN(tlr4lp-Δ) mutant mice despite an initial rapid recruitment of neutrophils into the air spaces.

Bacterial recovery in the lungs of the ScN(tlr4lp-Δ) mutant and TLR-4 wild-type mice following inhalation of E. coli. Despite the lower inflammatory responses observed in the ScN(tlr4lp-Δ) mice, these mice had lower bacterial recovery from the lungs 6 h after exposure to inhaled E. coli compared with the wild-type mice (P < 0.001) (Fig. 3). The differences at 6 h were seen at two different inhaled doses of E. coli, and the differences in bacterial recovery from the lungs of the ScN(tlr4lp-Δ) and wild-type mice were still detectable at 24 h. The difference in bacterial burden correlated with the clinical appearance of the mice, as the ScN(tlr4lp-Δ) mice showed less respiratory difficulty and less hunching and ruffling of fur during the experiment compared with the wild-type mice. Thus, in the ScN(tlr4lp-Δ) mice, reduced inflammation was associated with enhanced bacterial elimination from the lungs following aerosol challenge with E. coli.

TLR-4 mutation reduces alveolar macrophage cytokine responses to E. coli in vitro. We determined whether the alveolar macrophages from the ScN(tlr4lp-Δ) mutant mice have an earlier and more intense proinflammatory response in vitro than those of the TLR-4 wild-type mice that could enhance bacterial elimination in vivo. We incubated alveolar macrophages from ScN and wild-type mice with heat-killed E. coli and then measured KC, MIP-2, and TNF-α in the supernatants at 2 and 6 h. The alveolar macrophages of the ScN(tlr4lp-Δ) mutant mice showed consistently less proinflammatory cytokine production in response to E. coli in vitro than the TLR-4 wild-type mice (Table 1). Thus differences in the alveolar macrophage proinflammatory responses do not explain the enhanced bacterial elimination in the ScN(tlr4lp-Δ) mice in vivo.

Phagocytosis of E. coli by alveolar macrophages derived from ScN(tlr4lp-Δ) and TLR-4 wild-type mice. To determine whether differences in phagocytic activity of alveolar macrophages contribute to differences in bacterial elimination, we incubated alveolar macrophages from ScN and wild-type mice with BODIPY-labeled E. coli in vitro. The phagocytic activity of the ScN(tlr4lp-Δ) macrophages was similar to that of the TLR-4 wild-type mice (Fig. 4). Thus the phagocytic activity of
the ScN(tlr4Lps-del) alveolar macrophages does not explain the enhanced elimination of inhaled *E. coli* from the lungs.

**Histopathological changes in the ScN(tlr4Lps-del) mutant and TLR-4 wild-type mice following inhalation of *E. coli*.** When lung tissue was analyzed by light microscopy 6 h after bacterial exposure, the lungs of the ScN(tlr4Lps-del) mice showed less neutrophilic infiltrates, hemorrhage, protein deposition, and peribronchial infiltrates compared with the wild-type mice (Fig. 5, A–D). Interestingly, the TLR-4 wild-type mice showed prominent inflammatory cell infiltration in the peribronchial regions as well as in the parenchyma immediately surrounding the airways (Fig. 5C). It appeared that the influx of inflammatory cells and edema into the submucosal region of the airways contributed to the peribronchial thickening. We measured the airway submucosal area as described by George and colleagues (8) and found that the TLR-4 wild-type mice but not the ScN(tlr4Lps-del) mice showed significant increases in airway thickening of the small and large airways from 0 to 6 h (*P* = 0.005 for small airways, *P* = 0.006 for large airways) (Fig. 5E). There was also an increase in airway thickening of the medium airways of the TLR-4 wild-type mice compared with the ScN(tlr4Lps-del) mice, but this was not statistically significant (*P* = 0.08 for medium airways) (Fig. 5E). Thus the lungs of the TLR-4 wild-type mice showed airway thickening and inflammation following inhaled *E. coli* that was associated with impaired bacterial elimination.

**Lung cytokine responses of the HeJ(tlr4Lps-d) mutant and respective TLR-4 wild-type mice following inhalation of *E. coli*.** To determine the lung inflammatory responses and bacterial recovery in TLR-4 mutants of a different strain background, we examined cytokine responses to an additional strain of *E. coli* that was associated with impaired bacterial elimination.

### Table 1. Cytokine concentrations from alveolar macrophage supernatants following stimulation with *Escherichia coli* in vitro

<table>
<thead>
<tr>
<th>Time, h</th>
<th>TLR-4 wild type</th>
<th>ScN(tlr4Lps-del)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1,045±215</td>
<td>6,082±3,062</td>
</tr>
<tr>
<td>6</td>
<td>80±17*</td>
<td>892±135*</td>
</tr>
<tr>
<td>KC, pg/ml</td>
<td>7,234±1,059</td>
<td>61,799±11,681</td>
</tr>
<tr>
<td>MIP-2, pg/ml</td>
<td>4,138±742</td>
<td>15,285±2,133</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>5,55±5,100</td>
<td>9,683±653†</td>
</tr>
</tbody>
</table>

Cytokine concentrations depicted are means ± SE. Statistical comparisons were made between ScN(tlr4Lps-del) mutant and Toll-like receptor (TLR)-4 wild-type mice for cytokine concentrations at 2 and 6 h by Mann-Whitney rank-sum *U*-test (*n* = 4 animals in each group). *P* < 0.03; †*P* < 0.05, MIP, macrophage inflammatory protein.
TLR-4 IN E. coli PNEUMONIA

Fig. 4. Alveolar macrophages (AM) obtained from TLR-4 wild-type and ScN(tlr4lps-d) mice were incubated with heat-inactivated serum opsonized E. coli BioParticles at a ratio of 10:1 (10 bacteria to 1 cell). AM phagocytosis is expressed as mean fluorescence intensity (MFI) of the AM populations by flow cytometry. Results indicate that there is no difference in the in vitro phagocytic activity of AM derived from ScN(tlr4lps-d) and TLR-4 wild-type mice.

DISCUSSION

TLR-4 is a key pattern recognition receptor that is essential for recognition of bacterial LPS, but its role in the response to live bacteria is less clear. Although several studies have suggested that intact TLR-4 is required for adequate pulmonary host defenses against the gram-negative bacteria H. influenzae (29) and B. bronchiseptica (16), it is not clear whether this applies to all gram-negative bacteria. The main goal of this study was to examine tissue inflammatory responses and bacterial proliferation in TLR-4 mutants of different strain backgrounds. We used E. coli, a respiratory pathogen and a strong inducer of proinflammatory cytokine production from alveolar macrophages in vitro. The main finding is that while the lung inflammatory responses to inhaled E. coli are TLR-4 dependent, control of bacterial proliferation in the lungs is a more ground and their related wild-type mice, the HeJ(tlr4lps-d) and HeSnJ mice were exposed to inhaled E. coli. Like the findings in the ScN(tlr4lps-d) mutant mice, the HeJ(tlr4lps-d) mice showed significantly lower concentrations of the proinflammatory cytokines KC, MIP-2, and TNF-α in the lungs 6 h following exposure to live bacteria compared with the appropriate wild-type mice (P < 0.01) (Fig. 6).

Neutrophilic responses of the HeJ(tlr4lps-d) mutant and respective TLR-4 wild-type mice following inhalation of E. coli. The lung MPO activity was reduced in the HeJ(tlr4lps-d) mutant mice at 6 h (0.56 ± 0.015) compared with TLR-4 wild type (1.0391 ± 0.086, P < 0.001; data not shown). The total neutrophil content in the lungs at 6 h, measured by MPO activity, paralleled the BAL neutrophil counts, as the HeJ(tlr4lps-d) mutant mice had lower BAL PMN counts than the wild-type mice at 6 h (P < 0.01) (Fig. 7). Thus the overall neutrophil response was consistently lower in the HeJ(tlr4lps-d) mutant mice compared with the TLR-4 wild-type mice, and this was similar to the response seen in the ScN(tlr4lps-d) mutant strain.

Bacterial recovery in the lungs of the HeJ(tlr4lps-d) mutant and respective TLR-4 wild-type mice following inhalation of E. coli. Despite the lower proinflammatory cytokine and neutrophil responses in the HeJ(tlr4lps-d) mice, the inhaled E. coli were cleared from the lungs at similar rates in HeJ(tlr4lps-d) and TLR-4 wild-type mice over the first 6 h after bacterial aerosol exposure (Fig. 8).
complex response that does not appear to depend on TLR-4 as assessed in the inhalation experiments reported here.

We found a dissociation between tissue inflammatory responses following inhalational challenge with *E. coli* and bacterial recovery in the lungs of the ScN(*tlr4*del) mutant and the ScSn TLR-4 wild-type mice. The ScN(*tlr4*del) mutant mice had less inflammation in the lungs, reflected by lower MPO activity, lower cytokine responses, and less peribronchial and surrounding parenchymal inflammation at 6 and 24 h. Despite the lower lung inflammatory responses, the ScN(*tlr4*del) mutant mice had more rapid bacterial elimination 6 and 24 h after exposure to the same inhaled dose of *E. coli* as the related TLR-4 wild-type mice.

Using an intraperitoneal challenge model, Haziot et al. (10) found that mouse strains lacking functional CD14 or TLR-4 had less bacterial dissemination from the peritoneal cavity, suggesting more effective bacterial containment and/or elimination at the site of bacterial entry [CD14−/− and ScN(*tlr4*del) mice]. This was associated with early and intense neutrophil recruitment into the peritoneal cavity that was independent of CD14 and TLR-4 signaling.

Our findings in the ScN(*tlr4*del) mutant mice support the findings of Haziot and colleagues, although there are some differences. As in the Haziot study, we found an early increase in BAL PMN in the ScN(*tlr4*del) mutant mice at 6 h (Fig. 2B), even though the whole lung MPO activity was decreased at 6 and 24 h (Fig. 2A). This suggests that, although the ScN(*tlr4*del) mutant mice had fewer total neutrophils in the lungs by 6 and 24 h (based on MPO measurements), a greater percentage of the neutrophils in the lung microvasculature migrated into the air spaces within the first 6 h after infection. Unlike the study of Haziot and colleagues, this early neutrophil recruitment was only transient, because the BAL PMN counts were significantly lower at 24 h than in the ScSn mice (Fig. 2B). We also found that the overall tissue inflammatory response of the ScN(*tlr4*del) mutant mice was significantly lower compared with the control substrain, as measured by cytokine concentrations and MPO activity in the lungs, as well as histopathology. Thus, in the lung compartment, impaired TLR-4-dependent signaling in the ScN(*tlr4*del) mutant mice is associated with more rapid PMN recruitment to the site of infection and more rapid bacterial inactivation but an overall reduction in tissue proinflammatory activity.

We entertained the possibility that initial proinflammatory events are increased in the lungs of the ScN(*tlr4*del) mutant mice as an explanation for the disconnection between the lower inflammatory responses and more effective bacterial elimination in these mice at 6 and 24 h. However, proinflammatory signaling in alveolar macrophages from the ScN(*tlr4*del) mutant mice was consistently less than in the TLR-4 wild-type
macrophages at 2 and 6 h in response to E. coli (Table 1). This finding is inconsistent with the hypothesis that the early inflammatory response in the lungs of the ScSn(tlr4Lps-del) mutant mice is greater than in the TLR-4 wild-type mice.

We also examined phagocytic activity of alveolar macrophages to determine whether macrophages from ScSn(tlr4Lps-del) mutant and TLR-4 wild-type mice have differences in phagocytic activity that would explain the differences in the elimination of inhaled E. coli from the lungs. However, alveolar macrophages from ScSn(tlr4Lps-del) and TLR-4 wild-type mice had similar phagocytic activity for E. coli in vitro (Fig. 4).

One potential explanation for the differences in bacterial elimination of E. coli between ScSn(tlr4Lps-del) mutant mice and ScSn TLR-4 wild-type mice may have to do with differences in the responses in the airways. The histopathological studies showed that the TLR4 wild-type mice had inflammation in and around small and medium airways and significant airway mucosal thickening and edema, whereas the airways of the ScSn(tlr4Lps-del) mice were virtually normal, consistent with the reduced inflammatory responses that we detected in the lungs. Consistent with this difference, Lorenz and colleagues (14) have shown that the ScSn wild-type mice have increased airway resistance following inhaled LPS compared with other wild-type mice. Thus it is possible that airway wall inflammation and edema, and possibly bronchoconstriction, all combine to limit the cross-sectional area of the airways in the wild-type mice. This could have two effects that would delay bacterial elimination: first, these factors would favor greater bacterial deposition in the airways, as opposed to the alveolar spaces, and bacterial clearance is dependent upon the phagocytic activity of macrophages located in the latter region (9, 12). Second, these factors are likely to impair mucociliary clearance, which would further impair the clearance of inhaled bacteria from the lungs. Ironically, the reduced inflammatory response in the airways in the TLR4-deficient mice would favor more peripheral deposition of bacteria, where bacterial inactivation is more rapid. This would increase bacterial clearance in the mutant mice relative to the wild-type mice, in which airway inflammation and narrowing favor more proximal deposition of bacteria in the airways.

When we examined the tissue inflammatory responses and bacterial recovery in the lungs of the HeJ(tlr4Lps-d) mutant and HeSnJ wild-type mice following inhaled E. coli, the HeJ(tlr4Lps-d) mice had lower lung concentrations of inflammatory cytokines, lower neutrophil content in the lungs reflected by MPO in lung homogenates, and lower BAL fluid neutrophil counts. However, the HeJ(tlr4Lps-d) mice and the HeSnJ mice had similar bacterial recovery in the lungs despite significant differences in tissue inflammatory response at 6 h. Although our findings contrast with a study by Cross and colleagues (5), which showed that the HeJ(tlr4Lps-d) mice were more susceptible to the lethal effects of intraperitoneal E. coli than the wild-type strain, this difference may be explained by differences in the primary sites of infection (peritoneum vs. the lungs) and suggests that host responses to bacterial challenge in the lungs are more complex than at other tissue sites.

Lettinga and colleagues (13) have shown that TLR-4 does not appear to play a critical role in a murine model of L. pneumophila, another respiratory pathogen in humans. Bacterial clearance in the HeJ(tlr4Lps-d) mice was identical to the wild-type strain following intranasal challenge with L. pneumophila. In contrast to our studies, the tissue inflammatory responses were also similar in both the TLR-4 mutant and wild-type mice. Lettinga and colleagues proposed that the low endotoxic potential of L. pneumophila explains the failure to see a difference between TLR-4 mutant and wild-type mice. Low endotoxic potential does not explain the findings in our E. coli pneumonia model, as our data show that E. coli is a strong inducer of TLR-4-dependent proinflammatory cytokine production both by alveolar macrophages in vitro and in the lungs of mice in vivo.

In summary, we show that, whereas mice with two different types of TLR-4 mutations have markedly reduced intrapulmonary inflammatory responses to inhaled E. coli, reduced inflammatory responses are not necessarily associated with impaired elimination of E. coli from the lungs. Therefore, the absence of functional TLR-4 does not necessarily promote proliferation of E. coli in the lungs, as previously shown for gram-negative bacteria such as H. influenzae (29) and the mouse pathogen B. bronchiseptica (16). Our findings do not imply that cytokines and recruited inflammatory cells are unimportant in the containment of E. coli in the lungs. Rather, the data suggest that other gene(s) or factors in addition to TLR-4, such as mucociliary clearance, contribute to overall bacterial elimination from the lungs. We conclude that 1) strain backgrounds of TLR-4 mutants and wild-type mice are important considerations when interpreting studies of host defense mechanisms in the lungs and 2) intact TLR-4 signaling is required for adequate tissue inflammatory and cytokine responses but is not an essential requirement for the control of proliferation of all gram-negative bacteria from murine lungs.

REFERENCES


