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*J Immunol* 2002; 168:810-815; doi: 10.4049/jimmunol.168.2.810

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Toll-Like Receptor 4 Mediates Innate Immune Responses to \textit{Haemophilus influenzae} Infection in Mouse Lung\textsuperscript{1}

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Toll-like receptors (TLRs) have been implicated in the regulation of host responses to microbial Ags. This study characterizes the role of TLR4 in the innate immune response to intrapulmonary administration of \textit{Haemophilus influenzae} in the mouse. Two different strains of mice efficiently cleared aerosolized \textit{H. influenzae} concurrent with a brisk elaboration of IL-1\textbeta, IL-6, TNF-\alpha, macrophage-inflammatory protein (MIP)-1\textalpha, and MIP-2 in bronchoalveolar lavage and a corresponding mobilization of intrapulmonary neutrophils. Congenic strains of mice deficient in TLR4 demonstrated a substantial delay in clearance of \textit{H. influenzae} with diminished IL-1\textbeta, IL-6, TNF-\alpha, MIP-1\textalpha, and MIP-2 in bronchoalveolar lavage and a notable absence of intrapulmonary neutrophils. In TLR4-expressing animals, but not TLR4-deficient animals, TNF-\alpha and MIP-1\textalpha expression was up-regulated in epithelial cells of the conducting airway in response to \textit{H. influenzae} which was preceded by an apparent activation of the NF-\kappaB pathway in these cells based on the findings of decreased overall I\kappaB and an increase in its phosphorylated form. This study demonstrates a critical role of TLR4 in mediating an effective innate immune response to \textit{H. influenzae} in the lung. This suggests that the airway epithelia might contribute to sensing of \textit{H. influenzae} infection and signaling the innate immune response. The \textit{Journal of Immunology}, 2002, 168: 810–815.

The mammalian lung has evolved an elaborate network of defenses to maintain mucosal sterility despite ongoing inhalation of potential pathogens. Breaches in pulmonary host defense, such as in cystic fibrosis and in critically ill, ventilated patients, can lead to chronic and/or lethal pneumonia. Effector functions involved in pulmonary defense have been the subject of intensive investigation. The airway surface fluid contains a mixture of molecules which have direct antimicrobial activity such as lysozyme, lactoferrin, phospholipase A, anti-microbial peptides, and surfactant (1). The mechanical elimination of pathogens through mucociliary clearance also contributes to host defense. Cells resident to the noninfected lung, such as alveolar macrophages, secrete cytokines and chemokines in response to infection. Secretion of macromolecules rapidly mobilizes the influx of neutrophils and leads to a second wave of innate immunity. Finally, pathogen-derived macromolecules are processed by APCs to activate B and T cells as effectors of acquired immunity. Mechanisms for activating innate immunity and initiating the adaptive immune response are less well defined. A variety of cells express proteins called pattern recognition receptors that broadly recognize conserved structures in microorganisms called pathogen-associated molecular patterns (PAMPs)\textsuperscript{3} (2). Examples of pattern recognition receptors include CD14 and complement receptors (3, 4). The recent identification of mammalian homologs of the \textit{Drosophila} gene product d toll, called Toll-like receptors (TLRs), has revealed a step in the activation of innate immunity and its relationship to adaptive immunity (5).

A family of mammalian TLRs has been identified, each exhibiting substantial homology to type-I IL-1R (6–9). Binding of PAMPs to TLRs activates a number of signaling pathways (10, 11). TLR4 recognizes molecules of Gram-negative bacteria (e.g., LPS) (12, 13); TLR2 binds to components of Gram-positive bacteria (e.g., peptidoglycans and lipoteichoic acid) (14, 15), mycobacteria (16) and fungi (17); and TLR9 recognizes nucleic acid motifs (18). The net result of PAMP-TLR engagement is activation of intracellular signaling pathways such as NF-\kappaB and expression of genes involved in innate immunity (e.g., defensins and chemokines), and the initiation of adaptive immunity (e.g., CD86) (5).

Studies to date have focused on the role of TLRs in the initiation of host responses in the context of hematopoietic-derived cells such as macrophages (16, 17), dendritic cells (19, 20), and B and T lymphocytes (21). More recent studies have demonstrated expression of TLRs on endothelial cells (22, 23), cardiac myocytes (24, 25), and gut epithelial cells (26, 27), although the role TLRs play in these nonhematopoietic cells is unclear.

The current study focuses on the role of TLR4 in mediating the innate immune response in murine lung to \textit{Haemophilus influenzae} as a model pulmonary pathogen.

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\textsuperscript{*} Received for publication August 13, 2001. Accepted for publication November 6, 2001.

\textsuperscript{1} The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{2} This work was supported by the National Institutes of Health (National Institute of Diabetes and Digestive and Kidney Diseases Grant P30 DK47757 and National Heart, Lung, and Blood Institute Grant RO1 HL49040), the Cystic Fibrosis Foundation (to J.N.W.), and National Institute of Allergy and Infectious Diseases Grant AI44231 (to J.M.W.).

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Materials and Methods

Preparation of H. influenzae

A kanamycin-resistant encapsulated type-b strain (Eagan) H. influenzae H338 was grown to an OD of 0.4 in brain heart infusion broth supplemented with 2% (v/v) Fildes Enrichment (BD Biosciences, Cockeysville, MD), 2 µg/ml NAD (Sigma-Aldrich), St. Louis, MO), and 20 µg/ml kanamycin sulfate (Sigma-Aldrich) to mid-log phase, recovered by centrifugation, and resuspended in PBS (28).

Animal models

C3H/HeN and C57BL/10ScCr mice were purchased from the National Cancer Institute (Bethesda, MD). C3H/HeJ and C57BL/10ScSn mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Age-matched male mice were used for experiments. Anesthetized mice suspended vertically were administered H. influenzae intranasally at a dose of \(1 \times 10^3\) CFU in 50 µl of sterile PBS.

Preparation of BAL and lung homogenate

Mice were anesthetized and the trachea was exposed with a midline superficial incision. A 20-gauge catheter (BD Biosciences) attached to a 1-ml syringe (BD Biosciences) was used to cannulate the trachea, and the lungs were lavaged twice with 500 µl of PBS plus 5 mM EDTA plus 1% protease inhibitor (P8340; Sigma-Aldrich) chilled to 4 °C; lungs were then harvested and homogenized in 1 ml of PBS.

Bacterial counts

Bacteria were quantified by plating 10-µl serial dilutions of the bronchoalveolar lavage (BAL) or lung homogenates onto brain heart infusion agar plates (BD Biosciences) containing 1.5% Fildes Enrichment, 2 µg/ml NAD, and 20 µg/ml kanamycin sulfate. Plates were incubated overnight and colonies were counted. The selectable marker allowed for the elimination of contaminating flora.

Cell differential

Cells in the BAL were collected by centrifugation (2000 rpm for 4°C for 10 min), resuspended in PBS plus 4% BSA, transferred onto glass slides by using a Cytospin 3 centrifuge (Thermo Shandon, Pittsburgh, PA) for 15 min at 1500 rpm, and stained with Kwik Diff staining kit (Thermo Shandon). At least 100 cells were examined microscopically from each sample.

Cytokines

The BAL supernatants were stored at −70°C for the ELISA analysis of cytokines and chemokines. IL-1β, IL-4, IL-6, IL-10, TNF-α, and monocyte chemoattractant protein (MCP)-1 were analyzed by ELISA (BioSource International, Camarillo, CA) according to the manufacturer’s instructions. Macrophage-inflammatory protein (MIP)-1α and MIP-2 were also analyzed by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Immunocytochemistry

Abs for immunocytochemistry included anti-IgB-α, anti-IgM-α, anti-IL-1β, anti-IL-4, anti-IL-6, anti-IL-10, anti-TNF-α, anti-MIP-1α, anti-MCP-1, anti-MIP-2, anti-IP-10 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-monocyte/macrophage (MOMA)-2 (Serotec, Oxford, U.K.). All Abs were diluted according to the instructions of the manufacturer. Abs directed against phosphorylated IgB-α were developed in the mouse, anti-IgB-α Abs were developed in the rabbit, and anti-MOMA-2 Abs were developed in the rat; the other Abs were developed in the goat.

Serial cryostat sections (10 µm) were fixed for 5 min in acetone. Blocking was performed by a 1-h incubation with 10% goat serum or donkey serum, depending on the origin of the secondary Ab. Sections were incubated for 1 h with the primary Ab at room temperature followed by washes with PBS and an incubation with 1) 1/100 diluted rhodamine-labeled goat anti-mouse IgG (Sigma-Aldrich), 2) 1/100 diluted fluorescein- or rhodamine-labeled rabbit anti-goat IgG (Sigma-Aldrich), 3) 1/100 diluted fluorescein-labeled goat anti-rabbit IgG (Sigma-Aldrich), or 4) 1/100 diluted fluorescein-labeled sheep anti-rabbit (Serotec). Double immunostaining was also performed according to a modified protocol in which each segmented incubation was followed by extensive washing in PBS. Specimens were examined under a fluorescence microscope (Nikon Microphot-FXA; Nikon, Melville, NY). Negative controls consisted of preincubation with PBS, omission of the primary Ab, and substitution of the primary Ab by a non-immune control serum. The three types of controls mentioned above were performed for each experiment on the same day.

Morphometry

The number of cytokine-positive cells among MOMA-2-positive cells were assessed in TLR4−/− and TLR4+ mice. Cytokine-positive cells among MOMA-2-positive cells were counted in cross-sections of lung appropriately stained (double immunofluorescence), and the number of cytokine-positive cells was calculated as a percentage of at least 200 MOMA-2-positive cells from at least five randomly chosen different regions within a section of the lung by using an image analyzer (Phase Three Imaging Systems; Medicybernetics, Des Moines, IA). Each group consisted of two or more animals.

Statistical analyses

Data were analyzed for statistical significance using Student’s t test or Mann-Whitney rank sum test using SigmaStat 2.03 (SPSS, Chicago, IL). Differences were considered significant for p < 0.05.

Results

The model used to evaluate the initial response of the host to a bacterial pathogen is based on the administration of a kanamycin-resistant type-b strain (Eagan), H. influenzae, into lungs of mice. Analyses performed 6, 12, and 24 h after exposure to the pathogen focused on bacterial clearance and cytokines/chemokines and cells in BAL, as well as on immunohistochemistry for cytokine expression and regulatory proteins involved in the NF-κB pathway.

Experiments were performed in two strains of mice (i.e., C3H/HeN and C57BL/10 ScSn) and in congenic lines deficient in TLR4 (i.e., C3H/HeJ and C57BL/10 ScCr), which have previously been shown to be hyporesponsive to LPS. C3H/HeJ carries a missense mutation (P712H) in the TLR4 gene while a deletion in C57BL/10 ScCr ablates TLR4 protein and its mRNA (29). The mice will be referred to subsequently as HeN+, HeJ+, ScSn+, and ScCr−, with the superscript indicating the TLR4 status.

H. influenzae was administered via intranasal administration and lung was harvested at 12 and 24 h for recovery of the bacteria (Fig. 1). Pilot experiments indicated that >90% of the bacteria present in a total lung homogenate was found in BAL from both TLR4-expressing and TLR4-deficient animals; subsequent analyses of bacteria clearance were based on measurements from BAL. Studies in TLR4-expressing mouse lines revealed a decrease in recovered bacteria over 24 h with more effective clearance observed in TLR4-expressing mice compared to TLR4-deficient mice (Fig. 1). Statistical analyses indicated that bacteria clearance were based on measurements from BAL. Studies in TLR4-expressing mouse lines revealed a decrease in recovered bacteria over 24 h with more effective clearance observed in TLR4-expressing mice compared to TLR4-deficient mice (Fig. 1).

FIGURE 1. Clearance of H. influenzae. H. influenzae (1 × 10^7 CFU) was inoculated intranasally into age-matched male C57BL/10ScSn and C57BL/10ScCr, or C3H/HeN and C3H/HeJ mice. The mice were sacrificed after 12 or 24 h. Total CFU in BAL were determined. Each circle represents an individual animal. ○, TLR4-expressing animals; ●, TLR-deficient animals. This represents an aggregate of three independent experiments for C3H series and two independent experiments for the C57BL/10 series. Statistically significant differences in bacterial recovery were noted between TLR-expressing mice and the corresponding TLR-deficient mice except for the C57BL/10 series at the 12 h time point. **, p < 0.01; ***, p < 0.001.
the ScSn+ line when compared with HeN+. This finding contrasts with the findings in both TLR4-deficient lines, in which the presence of H. influenzae either did not decline as in ScCr– or actually increased 50-fold over input in HeJ+. Differences in recovered bacteria between HeN+ and HeJ– were statistically significant at both 12 h (p = 0.002) and 24 h (p < 0.001), while statistical significance was achieved in ScSn+ vs ScCr– at 24 h (p = 0.001).

To understand better the mechanism(s) involved in TLR-dependent bacterial clearance, BAL was analyzed for measures of inflammation including ELISA for the cytokines/chemokines IL-1β, IL-4, IL-6, IL-10, TNF-α, MCP-1, MIP-1α, and MIP-2, as well as cell counts and differentials. Time course studies revealed peak levels of cytokines 12 h after exposure to H. influenzae in all cases except for IL-1β, in which highest expression was noted at 24 h (Fig. 2). Analysis of BAL failed to reveal detectable levels of IL-4, IL-10, or MCP-1. The most striking finding is that cytokine/chemokine responses were consistently blunted in TLR-deficient strains as compared with the conegenic TLR-expressing strains (Fig. 2). The reduction in HeJ– as compared with HeN+ ranged from 2-fold for IL-6 (p = 0.044) to 9-fold for MIP-1α (p = 0.065); reductions observed in ScCr– relative to ScSn+ were more substantial, ranging from 12-fold for IL-6 (p = 0.009) to 40-fold for TNF-α (p = 0.004).

Total cells recovered in BAL were increased ~2-fold in TLR4-deficient strains as compared with the TLR4-expressing strains, with highest counts found at 24 h (data not shown). Distribution of cell types in BAL differed substantially as a function of TLR4 expression (Fig. 3). In each case, the majority of cells were identified as macrophages and neutrophils. A neutrophil-dominated cellular infiltrate was found in HeN+ and ScSn+, with 80 and 71% of the total cells being neutrophils, respectively. This finding contrasts with the findings in HeJ– and ScCr–, in which macrophages dominated, comprising 87 and 94% of the total cells, respectively. Differences between TLR-expressing lines and TLR-deficient lines were statistically significant (p < 0.001). PBS used to dilute bacteria for instillation does not itself induce any inflammation (data not shown).

Lung tissue was analyzed by immunohistochemical analysis to better identify the cellular source of cytokine/chemokines, which appeared in BAL. So as to characterize the initial response to infection, analyses were performed on tissues harvested at 6 and 12 h post-infection.

Cytokine/chemokine expression in macrophages was demonstrated by two-color immunofluorescence using MOMA-2+ as a marker for macrophages. An attempt was made to quantify expression by counting all MOMA-2+ cells with detectable cytokine/chemokine proteins, although this assay clearly was not quantitative (Table I). All cytokines/chemokines detected in BAL were indeed observed in macrophages. In some cases, expression was diminished in TLR4-expressing cells as compared with TLR4-deficient cells (Table I). Analysis of MIP-2 expression in representative macrophages is shown in Fig. 4. In both TLR-expressing strains, MIP-2 expression was found in more macrophages than that which was observed in TLR-deficient cells. Immunohistochemical analysis of lung tissues also demonstrated expression of TNF-α and MIP-1α in epithelia of the conducting airway of HeN+ and ScSn+ mice 12 h after exposure to H. influenzae; however, the peak expression was diminished in the corresponding TLR-deficient strains HeJ– and ScCr– (Fig. 5A).

To assess further the role of the airway in sensing and modulating the infectious process, lung tissue was evaluated for IκB-α, which is critical to the control of the NF-κB pathway. This analysis

![Figure 2](Image 50x167 to 278x319)

**FIGURE 2.** Cytokine response in the BAL. Levels of IL-1β, IL-4, IL-6, IL-10, TNF-α, MCP-1, MIP-1α, and MIP-2 were analyzed by ELISA from BAL of C3H/HeN, C3H/HeJ, C57BL/10ScSn, and C57BL/10ScCr. In uninfected mice, levels were undetectable. IL-1β, IL-6, TNF-α, MIP-1α, and MIP-2 increased following infection, whereas IL-4, IL-10, and MCP-1 remained undetectable. Data for those cytokines detected in BAL are presented. All represent 12 h time points except for IL-1β, which was harvested after 24 h (mean ± SD for five to seven independent animals). Open bars, TLR4-expressing animals; filled bars, TLR4-deficient animals. Statistically significant differences were noted for all comparisons between TLR-expressing mice and the corresponding TLR-deficient mice except for the C57BL/10 series for MIP-1α, as follows: *, p < 0.05; **, p ≤ 0.01; and ***, p < 0.001.

<table>
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<td>29 ± 3</td>
<td>16 ± 2</td>
<td>38 ± 4</td>
<td>12 ± 2</td>
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*Details of the analysis are provided in Materials and Methods and Fig. 4. Analyses were performed on tissue harvested 12 h after exposure to bacteria. Data represent the mean ± SD for two animals in three independent experiments.*
Discussion

The goal of this study was to evaluate the role of TLRs in mounting an immune response following exposure to a bacterial pathogen associated with lower respiratory tract disease. The specific model developed for this study was intrapulmonary administration of the Gram-negative bacterium *H. influenzae* to mice deficient in TLR4. *H. influenzae* colonizes the mucosal surface of the human nasopharynx. It is also a common cause of diseases such as otitis media and pneumonia when host factors allow it access to normally sterile surfaces in the upper and lower respiratory tract, respectively. This organism appears to contribute to early pathogenesis of pulmonary disease in cystic fibrosis where it colonizes the airway. *H. influenzae* can be recovered from the respiratory tract of 20% of cystic fibrosis infants before 2 years of age (30). A family of 10 and 9 genes expressing TLR has been described in humans and in mice, respectively, although specific function has been assigned to only a few, of which TLR4 has served as a prototype (31). In vitro studies clearly demonstrate TLR4 mediated activation of NF-κB pathway in response to LPS, which is an important structural component of Gram-negative bacteria (12, 13). Importantly, two strains of mice deficient in TLR4, along with congenic normals, are available for in vivo studies (29).

Our studies demonstrate a critical role of TLR4 in sensing and affecting the initial innate immune response to an intrapulmonary challenge with *H. influenzae*. Production of proinflammatory cytokines in the lungs of normal mice following *H. influenzae* is substantially diminished in TLR4-deficient mice, and the neutrophilic response is blunted. These TLR4-dependent abnormalities in innate immunity correlated with delays in bacterial clearance. Importantly, these findings in host response were demonstrated in two distinct TLR4-deficient lines of mice, arguing against genetic differences other than TLR4 deficiency as the basis for defective innate immunity within the congenic series. Direct comparison between the different genetic backgrounds studied (i.e. C3H vs C57BL/10) is difficult because they differ at many genetic loci. Previous studies regarding in vivo function of TLR4 have characterized host responses to purified bacterial components such as LPS or to bacterial challenge outside the lung (32). Kurt-Jones et al. (33) did show delayed clearance of the viral respiratory pathogen respiratory syncytial virus from TLR4-deficient mice.

In further characterizing the role of TLR4 in the lung, we attempted to evaluate the relative contribution of the airway epithelium vs resident immune effector cells such as macrophages. Previous studies have focused on the role of TLRs in dendritic cells (19, 20), macrophages (16, 17), and lymphocytes (21) as sensors and modulators of the immune response. While TLRs have been expressed in nonhematopoietic cells such as cardiac muscle (24, 25), endothelial cells (22, 23), and gut epithelial cells (26, 27), the role they play at these sites has not been defined.

Our hypothesis was that the airway epithelium would indeed serve as an important sensor and transducer of the innate immune response to *H. influenzae* challenge. Previous studies have demonstrated induction of the NF-κB pathway in cultured airway epithelial cells in response to bacteria and proinflammatory cytokines (34, 35). More recently, we showed expression of TLR2 and TLR4 in human airway epithelial cells and have linked the activation of TLR2 cells by PAMPs of Gram-positive bacteria to signaling of NF-κB and activation of an inducible defensin gene (our unpublished observation). In this study, immunohistochemical studies localized the production of proinflammatory cytokines to both the

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**FIGURE 4.** Expression of MIP-2 in alveolar macrophages. Cryostat sections (10 µm) of lungs from C3H mice (wild-type (HeN⁺)) and TLR4-deficient mice (HeJ⁻), C57BL10 mice (wild-type (ScSn⁺)) and TLR4-deficient mice (ScCr⁻) harvested 12 h after exposure to *H. influenzae* were double-immunostained for MOMA-2 (left panels) or MIP-2 (middle panels) and examined under a triple filter with nuclei stained by 4',6'-diamidino-2-phenylindole (right panels). In the absence of primary Ab or when the primary Ab was substituted by a non-immune serum, no signal was observed (data not shown). The results shown are representative of three different and reproducible experiments; the pictures were taken in the same conditions (brightness, contrast, magnification) on the same day.

- **A** and **B**: HeN⁺ lungs treated with MOMA-2 and MIP-2 antibodies, respectively.
- **C** and **D**: Same as **A** and **B** but with HeJ⁻ lungs.
- **E** and **F**: Same as **A** and **B** but with ScSn⁺ lungs.
- **G** and **H**: Same as **A** and **B** but with ScCr⁻ lungs.
- **I** and **L**: Same as **A** and **B** but with a triple filter.
resistant macrophages and epithelia of the conducting airway from the bronchi to terminal bronchioles. Furthermore, *H. influenzae* challenge resulted in a brisk activation of the NF-κB pathway in the airway that was TLR4 dependent.

Our studies demonstrate a critical role of TLR4 in the early host response to *H. influenzae* in the lung and implicate both the alveolar macrophages and conducting airway epithelia in sensing and modulating the initial response. The slight differences in the expression of cytokines/chemokines in the alveolar macrophage between wild-type and mutant mice emphasize the role of the airway epithelium in modulating the early host response to *H. influenzae* infection. We have not assessed quantitatively the contribution of each cell type to the innate immune response, although both appear to be TLR4 dependent. Recently, TLR2 was implicated directly in the effector response of macrophages to *H. influenzae* and immunostained for TNF-α (upper panels) and MIP-1α (lower panels). Immunostaining for TNF-α was observed in the airway epithelia of the wild-type strains. In the TLR4-deficient mice the intensity of the signal was much lower compared with the wild-type mice. The intensity of the immunostaining of the apical part of the airway epithelial cells for MIP-1α was diminished in the TLR4-deficient mice compared with the wild-type mice. In absence of primary Ab (control) or when the primary Ab was substituted by a nonimmune serum (data not shown), no signal was observed. The results shown are representative of three different and reproducible experiments.

**References**


