Response of Human Pulmonary Epithelial Cells to Lipopolysaccharide Involves Toll-like Receptor 4 (TLR4)-dependent Signaling Pathways

EVIDENCE FOR AN INTRACELLULAR COMPARTMENTALIZATION OF TLR4*

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Pulmonary epithelial cells are continuously exposed to microbial challenges as a result of breathing. It is recognized that immune myeloid cells express Toll-like receptors (TLRs), which play a major role in detecting microbes and initiating innate immune responses. In contrast, little is known concerning the expression of TLR in pulmonary epithelial cells per se, their distribution within the cell, their function, and the signaling pathways involved. In this work, we demonstrated by reverse transcription-PCR and/or immunoblot that TLR4 and the accessory molecule MD-2 are constitutively expressed in distinct human alveolar and bronchial epithelial cells. We further characterized by flow cytometry, biotinylation/precipitation, and confocal microscopy the intracellular localization of TLR4 in these cells. Despite this intracellular compartmentalization of TLR4, pulmonary epithelial cells were responsive to the TLR4 activator lipopolysaccharide (LPS), a potent Gram-negative bacteria-associated molecular pattern. Using respiratory epithelial cells isolated from TLR4 knock-out and wild type mice, we demonstrated that TLR4 is the actual activating receptor for LPS in these cells. Furthermore we showed that this cell response to LPS involves a signaling complex including the kinases interleukin-1 receptor-associated kinase (IRAK), p38, Jnk, and ERK1/2. Moreover, using vectors expressing dominant-negative forms of MyD88 and TRAF6, we established that LPS-induced activation of respiratory epithelial cells is largely dependent on TLR4 signaling intermediates. Altogether these data demonstrate that TLR4 is a key element in the response of pulmonary epithelial cells to molecules derived from Gram-negative bacteria. The intracellular localization of TLR4 in lung epithelia is expected to play an important role in the prevention of the development of chronic inflammatory disease.

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The lung is constantly exposed to invading particles and potential pathogens. To cope with this pressure, the lung has evolved a sophisticated defense mechanism designed to clear offending agents while inducing a minimum amount of concomitant inflammation. At first, mechanical defenses constituted by the mucociliary escalator participate in the removal of material from the tracheobronchial tree (1). Then resident and recruited phagocytes in the lower respiratory tract and alveoli ingest particulate matter and pathogens that circumvent this first line of defense. Pulmonary epithelial cells also maintain mucosal integrity by modulating local immune responses. Thus, these cells respond to a range of stimuli by producing biologically active mediators, including cytokines and chemokines, that influence airway inflammation (2).

In professional immune cells, including monocytes, macrophages, T cells, and dendritic cells, many receptors participate in microbe detection. Toll-like receptors (TLRs) represent a conserved family of innate immune recognition receptors that play key roles in detecting microbes, initiating innate immune responses, and linking innate and adaptive immunity (3, 4). Among these stimuli, the cell wall of Gram-negative bacteria contains lipopolysaccharide (LPS), a potent proinflammatory pathogen-associated molecular pattern. The response to LPS is initiated upon its interaction with TLR4 in conjunction with the accessory molecules MD-2 and soluble or membrane-bound CD14 (5). The response is then transduced via the interleukin (IL)-1 receptor signaling complex, which includes two essential adaptor proteins, myeloid differentiation (MyD)88 and tumor necrosis factor receptor-associated factor (TRAF6) as well as the serine-threonine kinase known as IRAK. Other components involved in this signaling pathway include mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (Jnk), and p38 kinase (p38) (6, 7). This signal transduction pathway further coordinates the induction of multiple genes encoding inflammatory mediators and co-stimulatory molecules (8).

1 The abbreviations used are: TLR, Toll-like receptor; LPS, lipopolysaccharide; MyD, myeloid differentiation; TRAF, tumor necrosis factor receptor-associated factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; Jnk, c-Jun N-terminal kinase; p38, p38 kinase; IL interleukin; KC, keratinocyte-derived chemokine; FITC, fluorescein isothiocyanate; DN, dominant-negative; IRAK, IL-1 receptor-associated kinase; RT, reverse transcription; FCS, fetal calf serum; sLPS-NC-LC-biotin, sulfosuccinimidyl-6-biotinamido(hexanoate); TNF, tumor necrosis factor; PBS, phosphate-buffered saline; RANTES, regulated on activation normal T cell expressed and secreted.
Various studies have provided evidence that TLR4 plays a critical role in myeloid cells (9–11), but recent reports suggest that an LPS signaling system also exists in cells of epithelial origin. Thus, TLR4 is expressed in intestinal (12–15), renal (16), colonic, and gingival epithelial (17). However, the signaling pathways involved in the activation of these epithelia by LPS remain poorly defined. Furthermore little is known concerning the activation of pulmonary epithelial cells (18, 19), the distribution within the cell, its function, and the corresponding signaling pathways in pulmonary epithelial cells despite the fact that these cells are remarkable for their critical physiologic exposure to frequent airborne microbial challenges.

Here we demonstrate that TLR4 is constitutively expressed in distinct human alveolar and bronchial epithelial cells and describe the intracellular localization of this receptor. We also establish that LPS-induced stimulation of these cells is dependent on the activation of TLR4 signaling intermediates such as MyD88, IRAK, TRAF6, and MAPK.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—RPMI 1640, F-12K nutrient mixture (Kaighn’s modification), Dulbecco’s modified Eagle’s, and Ham’s F-12 cell culture media; antibiotics; glutamine; Hanks balanced salt solution; and trypsin-EDTA were from Invitrogen. Fetal calf serum (FCS) and human recombinant cytokines TNFα and IL-1β were from Immunogen Corp. (Los Angeles, CA).

**Cell Culture**—The human prononicytic cell line U937, the human alveolar epithelial cell line A549, the human tracheobronchial epithelial cell lines BEAS-2B and 16-HBE were obtained from the American Type Culture Collection (Manassas, VA). The human tracheal epithelial cells CFT-2 and NT-1 were obtained from the French Research Center for Human Use (INSERM U402, Paris, France). CFT-2 was derived from primary epithelial cell lines BEAS-2B and 16-HBE were obtained from the pulmonary epithelial cells despite the fact that these cells are remarkable for their critical physiologic exposure to frequent airborne microbial challenges.

**Response of Pulmonary Epithelium to LPS Involves TLR4**

**Isolation and Primary Culture of Murine Pulmonary Epithelial Cells**—Pulmonary epithelial cells from pathogen-free C57BL/6 mice were isolated according to a modified protocol of Corti et al. (21). In brief, lungs were perfused with 10 ml of RPMI 1640 medium (supplemented with 1% antibiotics and 2% L-glutamine) through the pulmonary artery until they were cleared of blood. Bronchoalveolar lavages were performed using 5 × 1 ml of PBS, 1 ml EDTA to remove alveolar leukocytes. One ml of 0.1% protease type XIV (Sigma) was instilled into the lungs through the trachea. Lungs were then removed and placed in a sterile tube containing 2 ml of RPMI 1640 medium (supplemented with 1% antibiotics and 2% L-glutamine) at 4 °C overnight for enzymatic digestion to occur. Lung tissues were further teased apart in RPMI 1640 medium, 10% FCS in a Petri dish. The cell isolate was passed through a 100-μm filter, and pulmonary epithelial cells were purified as follows. Collected cells were counted and incubated for 15 min at 4 °C at the appropriate ratios with magnetic activated cell sorting (MACS CD45 and CD90 microbeads, Miltenyi Biotech, Bergisch Gladbach, Germany), two antigens highly expressed by leukocytes and fibroblasts, respectively. Cells were then washed and diluted in PBS, 0.5% FCS, and CD45+CD90++cells were negatively selected by passing the antibody-coated cell suspension through a column on an AutoMACS magnetic cell separator (Miltenyi Biotech). Pulmonary epithelial cells thus purified and incubated in RPMI 1640 medium, 10% FCS. After 24 h, cells were stimulated for 6 h by 1 μg/ml LPS, and KC and IL-6 levels were measured in the resulting supernatants.

**RT-PCR**—Cells were grown on a cell culture flask (Techno Plastic Products, Trasadingen, Switzerland), and total RNA was extracted with TRIzol (Gibco BRL, Life Technologies). cDNA was obtained by incubating RNA with 1 ml of Superscript II RNase H minus (Promega) in a total volume of 50 μl in the manufacturer’s buffer for 1 h at 42 °C and 10 min at 70 °C. PCR was performed using specific primers (Gentest, Envy, France) for human TLR4 (sense, 5'-TGG ATG CTT CCT ATA AG-3'; antisense, 5'-GAA ATG GAG GCA CCC CTT C-3') and for human MD-2 (sense, 5'-GTT ACT GAT CCT CCT TGC ATT AGT-3'; antisense, 5'-TTG TAG CTA AAC TTA CAG CAC-3') (14, 22, 23). As an internal control, we used primers for the detection of human β-actin (sense, 5'-AAG GAG ACG CTT GAC GCC AAA C-3'; antisense, 5'-AGA CAG CAC GTG GTT GGC GTA CA-3'). Amplifications were performed in a Peltier thermal cycler apparatus (MJ Research, Watertown, MA) using Q-BioTag™ polymerase (Gibogene, Ilkirch, France). For the detection of TLR4 and MD-2, the thermocycling protocol was as follows: 95 °C for 3 min, 36 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min. For the detection of β-actin, only 30 cycles were applied, and the temperature of annealing was 62 °C. Amplification products were resolved on a 1.5% agarose gel containing 1× TAE buffer and stained with ethidium bromide. The gels were recorded after amplification with an Ultra-Lum system (Ultra-Lum, Claremont, CA).

**Immunoblotting**—Epithelial and monocyte cells were washed once with cold PBS and then lysed on ice in a lysis buffer (10 mM Tris, 150 mM NaCl, 3 mM EDTA) supplemented with protein inhibitors (100 μg/ml leupeptin, 10 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamide) and 1% (v/v) Triton X-100. Samples were further solubilized prior to electrophoresis by adding SDS (2%, v/v), and disulfide bonds were reduced with 5% (v/v) β-mercaptoethanol. An equal amount of protein (15 μg) was fractionated by SDS-PAGE on a 10% acrylamide gel, and proteins were further electrophoresed into a nitrocellulose membrane (Optitran BA-S 85, Genset, Evry, France) for human TLR4 (sense, 5'-TGG ATG CTT CCT ATA AG-3'; antisense, 5'-GAA ATG GAG GCA CCC CTT C-3') and for human MD-2 (sense, 5'-GTT ACT GAT CCT CCT TGC ATT AGT-3'; antisense, 5'-TTG TAG CTA AAC TTA CAG CAC-3'). Amplifications were performed in a Peltier thermal cycler apparatus (MJ Research, Watertown, MA) using Q-BioTag™ polymerase (Gibogene, Ilkirch, France). For the detection of TLR4 and MD-2, the thermocycling protocol was as follows: 95 °C for 3 min, 36 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min. For the detection of β-actin, only 30 cycles were applied, and the temperature of annealing was 62 °C. Amplification products were resolved on a 1.5% agarose gel containing 1× TAE buffer and stained with ethidium bromide. The gels were recorded after amplification with an Ultra-Lum system (Ultra-Lum, Claremont, CA).

**Immunoprecipitation and Kinase Assay**—In 24-well plates (4 × 10⁶ cells/well, BD Biosciences) in RPMI 1640 medium, 10% FCS. After 24 h, cells were stimulated for 6 h by 1 μg/ml LPS, and KC and IL-6 levels were measured in the resulting supernatants.

**Response of Pulmonary Epithelium to LPS Involves TLR4**
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**RESULTS**

Identification of Components of the LPS Receptor Complex in Pulmonary Epithelial Cells—TLR4 is the primary signal transducer involved in LPS signaling (5). RT-PCR was used to assess the expression of this receptor in distinct unstimulated human respiratory epithelial cells. Phorbol 12-myristate 13-acetate-differentiated U-937 cells (macrophage-like cells) have been shown previously to express TLR4 and were used as a positive control for this receptor (Fig. 1, lanes 1). As shown in Fig. 1, *left panel*, TLR4 is clearly expressed at the mRNA level in both human alveolar (A549) and tracheobronchial (BEAS-2B, 16-HBE, and NT-1) epithelial cell lines. A high level of expression is also observed in CPT-2, a human tracheal cell line with several phenotypic characteristics of cystic fibrosis respiratory epithelia (20). RT-PCR analysis of β-actin expression confirmed the quality of all mRNA preparations used for RT-PCR. Human TLR4 is also detectable in all five epithelial cell lines as an ~110-kDa protein (Fig. 1, *middle panel*). An immunoblot for β-actin demonstrated similar gel loading. The apparent discrepancy between mRNA and protein expression of TLR4 is consistent with previous studies using intestinal epithelial cells (28) and suggests that expression of this receptor at the mRNA level may not be directly correlated with its protein level.

Current research findings suggest that the activation of the innate immune system involves complex associations of receptors depending on cell types and bacterial stimuli. The LPS receptor complex of mononuclear phagocytes is composed of TLR4, MD-2, and CD14. To gain insight into the mechanism of LPS recognition by pulmonary epithelial cells, we first ana-
lyzed the expression of the accessory molecule MD-2 by the two cell lines A549 and BEAS-2B. Using RT-PCR, we observed that mRNA for MD-2 is expressed in these epithelial cells (Fig. 1, right panel). The presence of the MD-2 protein was not checked due to the lack of a specific antibody. Concerning the presence of CD14, this molecule was detected by flow cytometry at a low expression level at the surface of BEAS-2B cells (not illustrated). By contrast, CD14 protein staining could not be detected either on A549 cells (not shown) or in primary polarized bronchial epithelial cells (see Fig. 4, lower panels).

**Intracellular Localization of TLR4 in Pulmonary Epithelial Cells**—We first demonstrated by flow cytometry the absence of cell surface staining of TLR4 using A549 and BEAS-2B cells (Fig. 2, left panels). Positive staining of the monocytic cells U-937 confirmed the quality of the anti-TLR4 antibody and the flow cytometry protocol used (Fig. 2, inset). By contrast, CD14 protein staining could not be detected either on A549 cells (not shown) or in primary polarized bronchial epithelial cells (see Fig. 4, lower panels). As an independent measure of the intracellular presence of TLR4 in these cells, surface proteins were labeled by a membrane-impermeable form of biotin. Biotinylated proteins were then extracted with neutralizing agarose beads followed by an immunoblot with an anti-TLR4 antibody. Fig. 3A confirms that TLR4 is only detectable in the total or in the non-precipitated cytoplasmic fraction but not in the precipitated cell surface portion of respiratory epithelial cells. An immunoblot using horseradish peroxidase-conjugated streptavidin was also used to control the efficiency of the precipitation protocol (not shown). As an additional positive control of our biotinylation protocol, biotinylated proteins extracted from A549 and BEAS-2B cells were subjected to an immunoblot with an anti-CD87 antibody. Consistent with previous studies (24), we demonstrated that the CD87/plasminogen activator receptor is expressed both on the cell surface and the cytoplasmic compartments of pulmonary epithelial cells. Interestingly Fig. 3A also shows that TLR4 is clearly detectable both on the plasma membrane and intracellular fractions of U-937 cells. This observation is in good agreement with recent studies focusing on the distribution of TLR4 in human monocytes (29).

Furthermore immunolocalization by confocal microscopy confirmed the intracellular compartmentalization of TLR4. Staining in A549 and BEAS-2B cells exhibited a diffuse cytoplasmic pattern (Fig. 3B). This staining was not detected in samples in which the primary antibodies was omitted or in which a control irrelevant antibody was substituted for the primary antibody (Fig. 3B, right panels). More importantly, we demonstrated a similar intracellular, subapical pattern of TLR4 in human primary polarized bronchial epithelial cells (characterized by cilia on the apical cell surface visible using differential interference contrast microscopy (Fig. 4, upper panels)).

**LPS Induces the Secretion of IL-8 and IL-6 but Not RANTES from Pulmonary Epithelial Cells**—In view of the unexpected intracellular localization of TLR4 in respiratory epithelial cells, we considered whether these cells could nevertheless respond to LPS stimulation. Fig. 5, A and B, clearly shows that LPS purified from the respiratory pathogen *P. aeruginosa* strongly stimulated the release of IL-8 and IL-6 from BEAS-2B cells in a concentration-dependent manner in the presence of serum. This result is in agreement with previous studies (30). To rule out any stimulatory effect due to LPS contamination by other bacteria-derived components such as lipoproteins, experiments were also performed using LPS supplemented with 20 μg/ml polymyxin B, a well-characterized LPS inhibitor (31). Under these experimental conditions, IL-8 secretion by BEAS-2B cells
was completely abolished (not shown). Interestingly LPS does not induce the release of RANTES, whereas this chemokine is clearly secreted upon stimulation of BEAS-2B cells by an optimal concentration of TNFα or IL-1β (Fig. 5C). To check whether TLR4 is the actual activating receptor for LPS, activation was assessed in respiratory epithelial cells from TLR4 knock-out mice in comparison with cells from wild type animals. Fig. 5D clearly shows that LPS strongly up-regulates p38, Jnk, and ERK1/2 activity in murine pulmonary epithelial cells isolated from TLR4 knock-out (TLR4−/−) and wild type animals (TLR4+/+). Murine epithelial cells were stimulated for 6 h, and cytokine concentrations were determined by enzyme-linked immunosorbent assay. Results are representative of two distinct experiments performed in duplicate.

**Discussion**

Due to inhalation of particles containing bacteria and LPS from the commensal flora in the nasopharynx and the environment, the lung is constantly exposed to potentially inflammatory components. Along with alveolar macrophages, pulmonary epithelial cells are the first cells to be challenged by LPS, and...
Expression of epithelial TLR4 is not regulated by LPS. BEAS-2B cells were stimulated or not (NS) by 1 μg/ml P. aeruginosa LPS. A, expression of human TLR4 and β-actin mRNA was analyzed by RT-PCR. B, TLR4 protein expression was assessed by immunoblot as described in Fig. 1, and membranes were further reprobed with an anti-β-actin antibody (diluted 1:15,000) to confirm similar gel loading. Densitometric analysis was carried out using the NIH Image Version 1.62 software. Histogram bars show TLR4 normalized against β-actin expression and are representative of at least two distinct experiments.

Response of Pulmonary Epithelium to LPS Involves TLR4

Their response must be greatly regulated to prevent alteration of the mucosal barrier. The recent discovery of TLR4 as the receptor for LPS prompted us to investigate the contribution of this receptor to the molecular mechanisms underlying the activation of pulmonary epithelial cells by this key product of Gram-negative bacteria. Two previous studies reported the expression of TLR4 mRNA in these cells (18, 19). Here we confirm and extend these data by showing (i) that respiratory epithelial cells express a LPS receptor complex that includes TLR4 and MD-2, (ii) an unexpected intracellular compartmentalization of TLR4 that nevertheless allows LPS to strongly induce the secretion of proinflammatory mediators, (iii) that epithelial activation by LPS does not alter TLR4 expression at the mRNA or protein level or alter its intracellular localization, and (iv) that the LPS activation pathway shares elements with cells of the myeloid lineage in that TLR4-dependent signaling in epithelial cells involves intermediates such as MyD88, IRAK, TRAF6, and MAPK.

Secretion of inflammatory cytokines such as IL-8 and IL-6 by epithelial cells involves fairly elevated amounts (0.1–1 μg/ml) of LPS in comparison with phagocytic cells, which are stimulated by 1–10 ng/ml LPS (10, 11, 22). Thus, initiation and coupling to downstream signaling events appear to be less efficient in the pulmonary epithelium than in myeloid cells. We hypothesize that this different responsiveness to LPS may be related to a distinct compartmentalization of TLR4 in the two cell types. Although apparently discordant data have been reported, an intracellular localization of TLR4 has been observed in epithelial cells of intestinal origin. Thus, while Cario et al. (13) reported cell surface expression of TLR4, others observed the absence of this receptor in the same type of epithelium (34).

In the context of this discrepancy, a more recent study demonstrates the intracellular distribution of TLR4 in the Golgi apparatus where it co-localizes with internalized LPS and its absence on the surface of intestinal epithelial cells in contrast to its membrane expression on monocytes (15). Concerning pulmonary epithelial cells, by means of complementary techniques including flow cytometry, biotinylation/precipitation, and confocal microscopy using both cell lines and primary cells, we provide substantial evidence for an intracellular localization of TLR4 in these cells. Based on flow cytometry experiments, it is unlikely that LPS might recruit TLR4 to the cell surface upon cell activation. However, we cannot exclude the possibility that inflammatory mediators such as cytokines or bioactive lipids might be able to induce TLR4 relocalization. In agreement with the absence of TLR4 expression on the cell surface of pulmonary epithelial cells, it is also relevant to notice that addition of a blocking anti-TLR4 antibody (clone HTA125) in the extracellular medium had no effect on activation by LPS as assessed by the measurement of IL-8 secretion. By contrast, the same antibody reduced LPS-induced activation of U-937 cells by 68% (not shown). Thus, we speculate that the intracellular compartmentalization of TLR4 may prevent “inopportune” activation of pulmonary epithelial cells due to a regular
exposure to air containing trace amounts of LPS and as a consequence a chronic inflammatory state. In the context of this distinctive cell distribution, TLR4 signaling may therefore be triggered only upon exposure to a high amount of free or bacteria-associated LPS as occurs in occupational or infectious diseases (18, 35). Subsequently the pulmonary epithelium may then participate in the local innate response through the secretion of cytokines and antimicrobial peptides.

Interestingly, before the identification of TLR4 as an essential participant in LPS signaling, Wright and colleagues (36, 37) showed that LPS is rapidly delivered from the plasma membrane to an intracellular site and that agents that block vesicular transport alter cell responses to LPS. Moreover Vasselon et al. (38) demonstrated that monomeric LPS crosses the cell membrane and traffic within the cytoplasm independently of membrane CD14, while aggregates of LPS are internalized in association with CD14. In the present study, we attempted to immunolocalize CD14 in human primary polarized bronchial epithelial cells using confocal microscopy and a FITC-conjugated anti-CD14 antibody (clone MY4). In fact, no CD14 protein staining could be detected in lung epithelial samples. A similar result was observed using the pulmonary epithelial cell line A549 but was not seen with BEAS-2B cells, which express the distinctive cell distribution, TLR4 signaling may therefore be selectively activated in a time-dependent manner by LPS.

Regardless we may propose a hypothetical mechanism to construe how elevated concentrations of LPS activate human respiratory epithelial cells (see Fig. 7D). After a receptor-mediated active transfer or a passive delivery across the plasma membrane (36, 37, 41), LPS is internalized and rapidly encounters intracellular TLR4, and downstream signaling is triggered. Our present findings suggest that this process involves at least the signal-transducing molecules MyD88, IRAK, and TRAF6 and activation of the transcription factor NF-κB. Several authors proposed that these cells are CD14-negative (39, 40), while others demonstrated both CD14 mRNA and cell surface protein in human airway epithelial cells (2, 18, 19). In fact, these contradictory results may be explained by distinct basal activation or differentiation state of the epithelial cells used throughout these investigations.

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