CD14 is an essential mediator of LPS-induced airway disease

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Brass DM, Hollingsworth JW, McElvania-Tekippe E, Garantziotis S, Hossain I, Schwartz DA. CD14 is an essential mediator of LPS-induced airway disease. Am J Physiol Lung Cell Mol Physiol 293: L77–L83, 2007. First published March 23, 2007; doi:10.1152/ajplung.00282.2006.—Chronic lipopolysaccharide (LPS) inhalation in rodents recapitulates many classic features of chronic obstructive pulmonary disease seen in humans, including airways hyperresponsiveness, neutrophilic inflammation, cytokine production, and small airways remodeling. CD14-deficient mice demonstrate no discernable physiological or inflammatory response to a single LPS inhalation challenge. However, the physiological (airways hyperresponsiveness) and inflammatory (presence of neutrophils and TNF-α in whole lung lavage fluid) responsiveness to inhaled LPS in C57BL/6CD14−/− mice was restored by instilling soluble CD14 intratracheally. Intratracheal instillation of wild-type macrophages into C57BL/6CD14−/− mice restored neutrophilic inflammation only and failed to restore airways hyperresponsiveness or TNF-α protein in whole lung lavage. These findings demonstrate that CD14 is critical to LPS-induced airway disease and that macrophage CD14 is sufficient to initiate neutrophil recruitment into the airways but that CD14 may need to interact with other cell types as well for the development of airways hyperresponsiveness and for cytokine production.

chronic obstructive pulmonary disease (COPD) has emerged as the fourth leading cause of death in the United States (1). In this disease, neutrophils are recruited to the airway in response to both cigarette smoke and bacterial products. Inhalation of bacterial endotoxin or lipopolysaccharide (LPS) causes acute and chronic airway disease in mice (5, 12, 23), and chronic LPS inhalation serves as a model of human environmental airway disease and COPD. Additionally, environmental LPS can contribute to exacerbations of allergic asthma (29, 30) increasing the likelihood of uncontrolled disease and increased health care costs. Understanding the acute response to inhaled LPS will provide important insight into the management of chronic inflammatory diseases such as asthma and COPD.

The response to inhaled LPS absolutely requires Toll-like receptor 4 (TLR4) (21) and at low LPS doses is dependent on LPS-binding protein (LBP) for a complete acute response to inhaled LPS (6). TLR4 and LBP are also required for the development of airway remodeling and concomitant persistent airways hyperresponsiveness (AHR) to inhaled methacholine (MCh) consequent to chronic LPS exposure. However, one must ask whether other components that recognize LPS and initiate TLR4 activation are essential to the airway response to LPS.

One such component of the LPS response, CD14, has been shown to be important in the in vitro response to LPS and exists as a glycosylphosphatidylinositol-linked surface receptor found on myeloid cells (15, 45) or in soluble form (2). In vitro, LPS-responsive cells lacking membrane-bound CD14 (mCD14) become sensitive to low concentrations of LPS in the presence of soluble CD14 (sCD14) (11). sCD14 appears to derive from monocytes (3) as well as from the liver (20, 40) and is found in normal serum at microgram concentrations (2). In mice, CD14 deficiency imparts resistance to LPS administered intratracheally (10). It has also recently been shown that the response to low-dose LPS instilled intratracheally is completely dependent on CD14, whereas at higher doses the response is also dependent on CD11b (24). Additionally, it has also been demonstrated that intravenous injection of an anti-CD14 antibody attenuates LPS-induced lung injury (41). We therefore investigated the role of sCD14 and mCD14 on macrophages in the development of inflammation and AHR in response to inhaled LPS to better understand the biology of chronic LPS inhalation as a model of environmental airways disease and COPD.

MATERIALS AND METHODS

Animals

Five or six male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) and five or six male CD14−/− mice at 8 wk of age were used for each group in this study. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Duke University Medical Center and were carried out in accordance with the standards established by the United States Animal Welfare Acts.

LPS Preparation and Aerosol Exposures

LPS was purchased as purified lyophilized powder [lot 110K4060; 25 mg; 30,000,000 endotoxin units (EU)/mg] prepared by phenol extraction from Escherichia coli serotype 0111:B4 from Sigma. LPS was reconstituted with 10 ml of sterile PBS. Immediately before use, LPS stock was diluted in 200 ml of PBS for aerosolization.

sCD14 Instillation

sCD14 was purchased from R&D Systems, Minneapolis, MN (cat. no. 982-CD-100) and resuspended in endotoxin-free PBS. A total of
either 15 or 8 μg per mouse was delivered in 40 μl of endotoxin-free PBS via oropharyngeal aspiration (9).

**LPS Exposure**

Mice were placed in individual compartments of stainless steel wire cage exposure racks in 135-l Hinner-style exposure chambers. Animals were exposed for 2.5 h, and LPS solution was aerosolized with a constant output atomizer (model 3076; TSI, Minneapolis, MN) with all output directed to the exposure chamber. Filtered, dehumidified air was supplied to the nebulizer at 30 psi gauge pressure. The exposure chamber was vented at a flow rate of 35.0 l/min.

**LPS Assay**

The airborne concentration of LPS was assessed by sampling 80 l of air drawn from the exposure chamber through 25-mm binder-free glass fiber filters (Gelman Sciences, Ann Arbor, MI) held within a 25-mm polypropylene inline air-sampling filter holder (Gelman Sciences) and tested for LPS concentration by using a chromogenic Limulus amebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD) according to the manufacturer’s instructions. LPS aerosol concentrations were 4–6 μg/m³ of air.

**Airway Physiology**

**Whole body plethysmography.** Airway responsiveness to inhaled MCh challenge was assessed in unrestrained, unanesthetized mice by using whole body plethysmography (Buxco Electronics, Troy, NY). Lung function was evaluated at 0, 5, 10, and 20 mg/ml of inhaled MCh.

**Airway Pressure Time Index**

Mice were anesthetized with pentobarbital sodium (60 mg/kg) intraperitoneally. Airway pressure (43) was measured by a differential pressure transducer connected to the side port of a surgically inserted tracheostomy cannula used to ventilate the animals with 6–8 ml/kg total volume at 125 breaths per minute. Neuromuscular blockade was accomplished with doxacurium chloride (0.25 mg/kg) and assessed by spontaneous respiratory efforts. Intravenous MCh (25, 100, and 250 μg/kg) was administered into the jugular vein.

**Adoptive Transfer**

Adoptive transfer of alveolar macrophages was achieved by harvesting whole lung lavage from five unexposed donor animals per recipient and pooling cells. A total of 2 × 10⁷ cells, which comprised of more than 98% alveolar macrophages, were then intratracheally instilled into each recipient animal anesthetized with isoflurane. Five adoptive transfer recipient animals per group were then phenotyped for their response to inhaled LPS.

**Whole Lung Lavage**

Mice were euthanized by CO₂ inhalation, and lungs were lavaged through PE-90 tubing with 6.0 ml of sterile saline, 1 ml at a time, at a pressure of 20 cmH₂O. The lavage fluid was centrifuged for 5 min at 200 g, the supernatant was decanted and stored at −70°C for further use, and the cell pellet was resuspended with Hanks’ balanced salt solution (without Ca or Mg). A small aliquot of this resuspension was used to count total lavaged cells per animal with a hemocytometer. One hundred microliters of the cell suspension were spun onto a slide with a cytocentrifuge (Shandon, Southern Sewickley, PA). Cells were stained with Hema-3 (Biochemical Sciences, Swedesboro, NJ) stain for differentials, air-dried, and covered with a coverslip with Cytoseal (Stephens Scientific, Kalamazoo, MI).

**Cytokine Analysis in Whole Lung Lavage Fluid**

ELISA for TNF-α was performed on aliquots of lung lavage fluid from each animal in duplicate according to the manufacturer’s instructions (R&D Systems). The lower limit of detection was 5.1 pg/ml.

**Statistical Analyses**

All data are expressed as means ± SE. For all groups, the total and differential cell counts from lung lavage fluid, the airway responsiveness to MCh challenge, and TNF-α protein expression were compared...
between C57BL/6 and C57BL/6CD14−/− mice. The differences between variables in each comparison were analyzed by Mann-Whitney U test. Probability values of \( P < 0.05 \) (2-tailed) were considered statistically significant.

RESULTS

C57BL/6CD14−/− Mice are Unresponsive to Inhaled LPS

We first asked whether C57BL/6CD14−/− mice are responsive to inhaled LPS. To address this, we exposed C57BL/6 and C57BL/6CD14−/− mice to LPS by inhalation for 2.5 h. In unexposed C57BL/6 and C57BL/6CD14−/− mice, there was no difference between groups in total cells or percent neutrophils in whole lung lavage cells (Fig. 1A). Of the mice from the LPS-exposed groups, only the C57BL/6 mice had an increase in total cells in whole lung lavage or in the percentage of neutrophils in the differential counts (Fig. 1A).

We next asked whether C57BL/6CD14−/− mice develop AHR to inhaled MCh challenge after a single acute LPS exposure. Before LPS inhalation, there was no difference between C57BL/6 and C57BL/6CD14−/− mice in their responsiveness to inhaled MCh. After LPS inhalation, the C57BL/6 mice had AHR as measured by whole body plethysmography at all concentrations of MCh. C57BL/6CD14−/− mice had an elevated response to inhaled MCh only at the 20 mg/ml concentration (Fig. 1B).

Acute LPS inhalation causes an increase in cytokine protein levels in whole lung lavage fluid (44). We asked whether C57BL/6CD14−/− mice respond to inhaled LPS with increases in TNF-α in the lavage fluid. There was no detectable TNF-α protein in whole lung lavage fluid in either C57BL/6 or C57BL/6CD14−/− mice exposed to air alone (Table 1). Immediately after a single acute LPS inhalation challenge, only

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**Table 1. TNF-α in whole lung lavage fluid measured by ELISA**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>TNF-α, pg/ml</th>
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<tbody>
<tr>
<td>C57BL/6</td>
<td>Air</td>
<td>ND</td>
</tr>
<tr>
<td>CD14−/−</td>
<td>Air</td>
<td>ND</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>LPS</td>
<td>729.51 ± 125.47</td>
</tr>
<tr>
<td>CD14−/−</td>
<td>LPS</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, none detected.
C57BL/6 mice had detectable TNF-α protein in the lavage fluid (Table 1).

**sCD14 Restores Inflammation and AHR to C57BL/6CD14−/− Mice in Response to Inhaled LPS**

We asked whether sCD14 instilled into the lungs of C57BL/6CD14−/− mice via oropharyngeal aspiration would restore all of the features of the response [polymorphonuclear leukocytes (PMNs), cytokines, and AHR] to inhaled LPS observed in wild-type C57BL/6 mice. To answer this question, we instilled 15 µg of sCD14 into the lungs of C57BL/6CD14−/− mice and exposed them to either inhaled LPS or filtered air for 2.5 h. There was no difference in the concentration of inflammatory cells in the lavage fluid between air-exposed C57BL/6 mice or C57BL/6CD14−/− mice receiving sCD14 or LPS-exposed C57BL/6CD14−/− mice receiving vehicle alone (Fig. 2A). However, C57BL/6CD14−/− mice receiving intratracheally instilled sCD14 had significantly elevated total cells in whole lung lavage fluid, and the majority of these cells were neutrophils (Fig. 2A). Oropharyngeally administered sCD14 restored the ability of C57BL/6CD14−/− mice to produce TNF-α protein in their lungs in response to inhaled LPS (Table 2).

We asked whether sCD14 instilled via oropharyngeal aspiration would restore MCh responsiveness to C57BL/6CD14−/− mice exposed to inhaled LPS. C57BL/6CD14−/− mice that received vehicle alone and were exposed to inhaled LPS were not different from unexposed mice in their responsiveness to inhaled MCh challenge (Fig. 2B). C57BL/6 mice and C57BL/6CD14−/− mice receiving sCD14 had similar responses to inhaled MCh challenge and were significantly elevated compared with control mice (Fig. 2B). This result was confirmed by airway pressure time index (Fig. 2C).

We asked whether sCD14 instilled into the lungs of C57BL/6CD14−/− mice would restore their ability to produce TNF-α protein in whole lung lavage fluid after acute LPS inhalation challenge. Air-exposed C57BL/6CD14−/− mice receiving sCD14 had no measurable TNF-α in whole lung lavage fluid, and LPS-exposed C57BL/6CD14−/− mice receiving vehicle alone had no TNF-α in whole lung lavage fluid either (Table 2). Oropharyngeally administered sCD14 restored the ability of C57BL/6CD14−/− mice to produce TNF-α protein in their lungs in response to inhaled LPS (Table 2).

**sCD14 Enhances the Response to Inhaled LPS in C57BL/6 Mice**

Because the response to bacterial endotoxin is so important in the response to gram-negative infection in mammals, we asked whether exogenously added sCD14 would enhance or attenuate the airway inflammatory response to inhaled LPS in C57BL/6 mice. To address this question, we instilled 5, 15, or 25 µg of sCD14 into the lungs of C57BL/6 mice via oropharyngeal aspiration before LPS inhalation challenge. LPS-exposed C57BL/6 mice receiving 25 µg of sCD14 had significantly enhanced pulmonary inflammation compared with LPS-exposed C57BL/6 mice receiving vehicle alone (Fig. 3A, left). The profile of cells in whole lung lavage remained unchanged, however, with the majority being PMNs (Fig. 3A, right).

We also asked whether sCD14 instilled into the lungs of C57BL/6 mice would affect TNF-α protein production in whole lung lavage fluid after acute LPS inhalation challenge. Air-exposed C57BL/6 mice receiving sCD14 had no measurable TNF-α in whole lung lavage fluid. Exogenously added sCD14 had no effect on TNF-α protein concentration in the lung despite the increased cellularity at the 25 µg dose of sCD14 (Table 3).

**Oropharyngeal Instillation of Alveolar Macrophages from C57BL/6 Mice into C57BL/6CD14−/− Mice Restores Neutrophilic Inflammation but Not Cytokine Production or AHR in Response to Inhaled LPS**

We asked whether CD14 expression on macrophages alone would restore the ability of C57BL/6CD14−/− mice to respond to inhaled LPS. Therefore, we adoptively transferred naïve wild-type alveolar macrophages into the lungs of C57BL/6CD14−/− mice via oropharyngeal aspiration. Air-exposed C57BL/6CD14−/− mice receiving wild-type macrophages had pulmonary cellularity that was not different from naïve C57BL/6 mice (Figs. 4A and 1A). LPS-exposed C57BL/6CD14−/− mice receiving vehicle alone had pulmonary inflammation that was significantly elevated over unexposed mice.

### Table 2. TNF-α in whole lung lavage fluid measured by ELISA

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>TNF-α, pg/ml</th>
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<tbody>
<tr>
<td>C57BL/6</td>
<td>Air</td>
<td>ND</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>LPS</td>
<td>1,441.38±213.78</td>
</tr>
<tr>
<td>CD14−/−</td>
<td>Air</td>
<td>ND</td>
</tr>
<tr>
<td>CD14−/− + sCD14</td>
<td>LPS</td>
<td>605.25±94.86</td>
</tr>
<tr>
<td>CD14−/− + Vehicle</td>
<td>LPS</td>
<td>ND</td>
</tr>
<tr>
<td>sCD14, soluble CD14</td>
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sCD14, soluble CD14.

### Table 3. TNF-α in whole lung lavage fluid measured by ELISA

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>TNF-α, pg/ml</th>
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<tbody>
<tr>
<td>C57BL/6 + Vehicle</td>
<td>LPS</td>
<td>2,254.51±824.15</td>
</tr>
<tr>
<td>C57BL/6 + 5 µg sCD14</td>
<td>LPS</td>
<td>2,116.57±322.78</td>
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<tr>
<td>C57BL/6 + 15 µg sCD14</td>
<td>LPS</td>
<td>1,828.53±406.00</td>
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<tr>
<td>C57BL/6 + 25 µg sCD14</td>
<td>LPS</td>
<td>2,143.95±397.02</td>
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sCD14, soluble CD14.
receiving wild-type macrophages. However, LPS-exposed C57BL/6CD14−/− mice receiving wild-type macrophages had pulmonary inflammation that was significantly elevated compared with C57BL/6CD14−/− mice receiving either wild-type macrophages and exposed to air alone or vehicle alone and exposed to LPS (Fig. 4A).

We next asked whether CD14 expression on macrophages alone is sufficient to reconstitute the physiological response to inhaled LPS. C57BL/6CD14−/− mice receiving wild-type macrophages and exposed to inhaled LPS demonstrate a trend toward increased response to MCh but were not statistically different from C57BL/6CD14−/− mice receiving wild-type macrophages exposed to air or from LPS-exposed C57BL/6CD14−/− mice receiving vehicle alone in their responsiveness to inhaled MCh (Fig. 4B). C57BL/6CD14−/− mice receiving wild-type macrophages and exposed to LPS had no detectable TNF-α in whole lung lavage fluid (Table 4), and neither did C57BL/6CD14−/− mice receiving wild-type macrophages and exposed to air nor C57BL/6CD14−/− mice receiving vehicle alone and exposed to LPS (Table 4).

**DISCUSSION**

The findings presented here demonstrate that CD14 is required for the physiological and biological response to inhaled LPS and that exogenously added sCD14 is sufficient to reconstitute the physiological (AHR) and biological (inflammation and cytokines) response to inhaled LPS in C57BL/6CD14−/− mice. Furthermore, although CD14 expression on macrophages alone can partially reconstitute the ability of C57BL/6CD14−/− mice to mount a neutrophilic inflammatory response to inhaled LPS, in our hands, macrophage expression of CD14 is not sufficient for the development of AHR or TNF-α production. We also show that augmenting the endogenous concentration of sCD14 enhances neutrophilic inflammation in response to inhaled LPS.

The role of endogenous mCD14 and sCD14 in the lung in the response to inhaled LPS has not been fully elucidated. Systemically, it has been shown that transgenic mice that overexpress human mCD14 have significantly increased sensitivity to LPS (8). Our dose response experiment in wild-type mice is consistent with this result (Fig. 3). Additionally, CD14-deficient mice are hyporesponse to intraperitoneal LPS and resistant to experimental gram-negative sepsis (16). In the lung, it has recently been shown that the response to low-dose LPS instilled intratracheally is completely dependent on CD14, whereas at higher doses, the response is also dependent on CD11b (24). Our low-dose study is completely consistent with this finding. Additionally, it has also been demonstrated that intravenous injection of an anti-CD14 antibody attenuates LPS-induced lung injury (41). Two opposing functions have been described for sCD14. It has been shown that sCD14 can either compete with mCD14 for LPS binding (17, 18, 34, 35), thus limiting the signaling capability of LPS, or it can enable LPS-induced activation of cells that do not express mCD14 such as endothelial, epithelial, and smooth muscle cells (11, 19, 28, 32, 33). In vitro sCD14 can either enhance or inhibit the responses of cells in a concentration-dependent manner (13, 17, 35), and in vivo it has been shown that recombinant bovine or recombinant human sCD14 injected intratracheally protects mice from experimental LPS-induced shock (26, 39) suggesting that this sCD14 competes with endogenous mCD14 for LPS binding. We show that sCD14 renders C57BL/6CD14−/− mice sensitive to inhaled LPS and also enhances the sensitivity of C57BL/6 mice, and thus these observations and

![Fig. 4. Oropharyngeal instillation of alveolar macrophages from C57BL/6 mice into C57BL/6CD14−/− mice only partially restores responsiveness to inhaled LPS. A: total cells (left) and %PMN (right) in whole lung lavage fluid from LPS-exposed C57BL/6 mice and LPS-exposed and air-exposed C57BL/6CD14−/− mice receiving either wild-type macrophages (w/t Mφ) via oropharyngeal aspiration or vehicle alone. B: whole body plethysmography (Penh) from LPS-exposed C57BL/6 mice and LPS-exposed and air-exposed CD14−/− mice receiving either wild-type macrophages via oropharyngeal aspiration or vehicle alone. n = 5 mice per group. Data are means ± SE. *P < 0.05 significantly elevated vs. air-exposed CD14−/− mice receiving vehicle alone; **P < 0.05 significantly elevated vs. air-exposed CD14−/− mice receiving wild-type macrophages or LPS-exposed CD14−/− mice receiving vehicle alone; #P < 0.05 significantly elevated vs. all other groups.

**Table 4. TNF-α in whole lung lavage fluid measured by ELISA**

<table>
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<th>Treatment</th>
<th>TNF-α, pg/ml</th>
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<tbody>
<tr>
<td>CD14−/− + w/t Mφ</td>
<td>Air</td>
<td>ND</td>
</tr>
<tr>
<td>CD14−/− + w/t Mφ</td>
<td>LPS</td>
<td>ND</td>
</tr>
<tr>
<td>CD14−/− + Vehicle</td>
<td>LPS</td>
<td>ND</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>LPS</td>
<td>1,448.87 ± 140.18</td>
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w/t Mφt, wild-type macrophages.
our data taken together suggest that CD14 is an enhancer of the response to low-dose inhaled LPS.

Macrophages are the first line of defense against pathogenic stimuli in the lung. The elaboration of cytokines such as TNF-α, IL-1, and IL-6 serve to amplify and regulate this response (27, 42). However, little is known about the exact mechanism by which macrophages recruit PMNs and other inflammatory cells to the lung, and even less is known about the relationship between these events and AHR. In a previous study (22) and in the present work, we have used adoptive transfer to attempt to address this question. In our hands, we typically recover up to $3 \times 10^5$ alveolar macrophages from whole lung lavage of naïve C57BL/6 mice; thus in our studies, we instilled $2 \times 10^5$ wild-type alveolar macrophages. In this study in C57BL/6CD14−/− mice and in our previous study, instilling $2 \times 10^5$ wild-type macrophages into TLR4-deficient mice (22) showed the same response, that is, significantly elevated total inflammatory cells (Fig. 4A, left), of which the majority are PMN (Fig. 4A, right), but no TNF-α in whole lung lavage fluid. It is well known that alveolar macrophages are capable of producing TNF-α when stimulated in culture (31). That we were not able to detect TNF-α in whole lung lavage from these animals suggests that either 1) these macrophages are not fully functional, or 2) the amount of TNF-α they are able to produce is below the level of detection of our assays. Our data neither confirm nor refute either possibility. However, the recruitment of PMNs to the lung in both this and our previous study suggests that these macrophages are able to initiate the inflammatory response and hence are normally active. Whether more wild-type macrophages would be required to sustain the response or whether CD14 in this study and TLR4 in our previous study must interact with cells other than macrophages for the progression of the inflammatory response remains to be determined. Likewise, whether more wild-type macrophages would lead to increased AHR in C57BL/6CD14−/− mice also remains to be determined.

In support of the hypothesis that more cell types must interact with CD14 for a whole response to inhaled LPS (inflammatory cells, cytokines, and AHR), epithelial cells, which have been shown to express cell surface CD14 in vitro (14) and ex vivo (4), produce proinflammatory cytokines, release neutrophil chemotactic factors, and increase expression of adhesion molecules on LPS stimulation in vitro. As a model of epithelial cell behavior, it has been shown that A549 cells can release CXC chemokines with neutrophil chemotactic activity on stimulation with TNF-α or IL-1β in vitro (25, 37, 38), and primary cultures of human pulmonary epithelial cells also can express IL-8 and other neutrophil chemotactic factors when stimulated with TNF-α and IL-1β (7, 25). Supporting the importance of these cells in the response to inhaled LPS, Skerrett et al. (36) have recently demonstrated that selective inhibition of NF-κB in distal epithelium during an LPS inhalation challenge can attenuate the biological response (neutrophilic inflammation and cytokine production). Cumulatively, these observations suggest complex interactions between cell types required to activate the participating cells to reconstitute the whole physiological and biological response to inhaled endotoxin.

In summary, CD14 is required for the biological response to inhaled endotoxin, and sCD14 can reconstitute the whole (physiological and biological) response to inhaled LPS in the lung. Macrophage expression of CD14 is sufficient to initiate the inflammatory response to inhaled LPS, whereas it appears that CD14 interaction with multiple cell types may be required for progression of this response.

GRANTS

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REFERENCES


