Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin

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Skerrett, Shawn J., H. Denny Liggitt, Adeline M. Hajjar, Robert K. Ernst, Samuel I. Miller, and Christopher B. Wilson. Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin. Am J Physiol Lung Cell Mol Physiol 287: L143–L152, 2004. First published March 26, 2004; 10.1152/ajplung.00030.2004.—To determine the role of respiratory epithelial cells in the inflammatory response to inhaled endotoxin, we selectively inhibited NF-κB activation in the respiratory epithelium using a mutant IκB-α construct that functioned as a dominant negative inhibitor of NF-κB translocation (dIκB-α). We developed two lines of transgenic mice in which expression of dIκB-α was targeted to the distal airway epithelium using the human surfactant apoprotein C promoter. Transgene expression was localized to the epithelium of the terminal bronchioles and alveoli. After inhalation of LPS, nuclear translocation of NF-κB was evident in bronchiolar epithelium of nontransgenic but not of transgenic mice. This defect was associated with impaired neutrophilic lung inflammation 4 h after LPS challenge and diminished levels of TNF-α, IL-1β, macrophage inflammatory protein-2, and KC in lung homogenates. Expression of TNF-α within bronchial epithelial cells and of VCAM-1 within peribronchial endothelial cells was reduced in transgenic animals. Thus targeted inhibition of NF-κB activation in distal airway epithelial cells impaired the inflammatory response to inhaled LPS. These data provide causal evidence that distal airway epithelial cells and the signals they transduce play a physiological role in lung inflammation in vivo.

lipopolysaccharide; cytokines; nuclear factor-κB; transgenic mice

THE INHALATION OF LPS RESULTS in acute, neutrophilic inflammation of the distal air spaces of the lungs. The molecular mechanisms underlying the inflammatory response to LPS involve the detection of LPS by pattern recognition receptors followed by the coordinated expression of cytokines, chemokines, and adhesion molecules that direct the emigration of neutrophils across the endothelial and epithelial barriers that separate the bloodstream from the pulmonary air spaces (35, 39). The recognition of LPS is modulated by soluble factors, such as LPS binding protein and surfactant proteins, that are present in airway lining fluid and influence the presentation of LPS to membrane-bound CD14 (35, 38). Binding of LPS to CD14 triggers intracellular signaling that is mediated by Toll-like receptor 4 (TLR4) in association with a secreted cofactor, MD-2 (1, 2). LPS-induced signal transduction leads to activation of the NF-κB family of transcription factors, which in turn directs the expression of proinflammatory cytokines, chemokines, and adhesion molecules (30, 57). Early response cytokines, such as TNF-α and IL-1β, can amplify this response by stimulating the NF-κB-dependent induction of proinflammatory mediators in cells that lack components of the CD14/TLR4/MD-2 receptor complex and thus cannot respond directly to LPS (30, 57). Local production of CXC chemokines bearing the glutamic acid-leucine-arginine (ELR) triplet establishes the chemotactic gradient for the emigration of neutrophils from the bloodstream (57). The upregulation of leukocyte integrins and the increased expression of adhesive counterligands on the surfaces of endothelial, interstitial, and epithelial cells permits neutrophil extravasation along the chemotactic gradient (39).

Murine models of LPS-induced lung inflammation support this paradigm. Bronchoalveolar accumulation of neutrophils after inhalation of LPS is markedly blunted in mice with mutations of TLR4 (34). Intrapulmonary activation of NF-κB follows airway challenge with LPS (8, 22, 40, 49), and LPS-induced lung inflammation is impaired in mice lacking the RelA component of NF-κB (3). Pulmonary neutrophil recruitment in response to LPS is also blunted in mice lacking the type 1 TNF receptor (TNFR1) (52) and in animals depleted of the ELR+ CXC chemokines KC or macrophage inflammatory protein-2 (MIP-2) (20, 50). Similarly, depletion studies have documented the roles of β1- and β2-integrins and their ligands in the airway inflammatory response to LPS (4, 10, 18, 28, 31, 46).

Although the importance of individual molecules in the bronchoalveolar inflammatory response to LPS has been recognized, the cellular participants in this cascade are incompletely understood. Multiple cells may be involved in the initiation and regulation of air space inflammation, including alveolar macrophages, epithelial cells, fibroblasts, and endothelial cells (57). There is strong evidence supporting the role of alveolar macrophages in the inflammatory response to inhaled LPS. Alveolar macrophages are sensitive to the presence of LPS in vitro (36) and are activated in response to airway challenge with LPS in vivo (22). Furthermore, depletion of alveolar macrophages results in diminished NF-κB activation, cytokine responses, and neutrophilic inflammation after inhalation of LPS (7, 24).

The role of alveolar epithelial cells in the regulation of lung inflammation is less clear. In vitro, respiratory epithelial cells can secrete proinflammatory mediators and upregulate adhesion molecules in response to bacterial stimuli or endogenous factors such as IL-1β and TNF-α (15). In vivo studies have provided evidence of NF-κB activation and increased ICAM-1

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expression by bronchiolar and alveolar epithelial cells after airway challenge with LPS (4, 5, 9, 22). Moreover, targeted expression of KC or constitutive activation of NF-κB in airway epithelial cells is sufficient to elicit pulmonary inflammation in the absence of exogenous stimuli (48, 59). Furthermore, Poynter and colleagues (44) recently reported that targeted expression of a dominant negative IκB-α in proximal airway epithelial cells under the control of the rat CC10 promoter impaired lung inflammation after intranasal administration of LPS.

The goal of the present study was to evaluate the contribution of the distal airway epithelium to the regulation of lung inflammation induced by aerosolized LPS. Our strategy was to selectively block NF-κB activation in terminal bronchiolar and alveolar epithelial cells while leaving the signaling responses of proximal airway epithelial cells and alveolar macrophages intact. To achieve this end, we constructed transgenic mice in which a mutant IκB-α resistant to proteosomal degradation, because of alanine substitutions at Ser32 and Ser36 that block inducible phosphorylation (16), was targeted to the distal airway epithelium under the control of the human surfactant apoprotein C promoter (25). The mutant IκB-α functions as a dominant negative inhibitor of NF-κB translocation (dnIκB-α) in cells in which the transgene is expressed. We found that local cytokine responses and neutrophil recruitment in response to LPS were significantly reduced in the transgenic animals. These data provide causal evidence that distal airway epithelial cells and the signals they transduce play a physiological role in lung inflammation in vivo.

MATERIALS AND METHODS

Adenoviral vector-mediated transduction of A549 cells with dnIκB-α. The adenoviral vector directing the expression of human dnIκB-α, in which Ser32 and Ser36 have been mutated to alanine and a triple influenza hemagglutinin (HA) tag added to its 5′ end, and the adenoviral vector directing expression of β-galactosidase, have been described previously (32). A549 cells (American Type Culture Collection CCL-185) were grown in RPMI 1640 medium plus 10% fetal calf serum (GIBCO BRL, Grand Island, NY) in 24-well tissue culture trays and transduced with one or the other adenoviral vector. Forty-eight hours later, cells were exposed to recombinant TNF-α (200 U/ml throughout the period of culture), Pseudomonas aeruginosa (strain PAK, 107 CFU/ml for 1 h, after which cells were washed and refed with fresh medium containing gentamicin at 20 μg/ml to prevent bacterial overgrowth), or medium alone. After 6 or 24 h, supernatants were harvested and stored at −80°C. Levels of immunoreactive IL-8 were measured by ELISA using reagents from R&D Systems (Minneapolis, MN).

Generation and characterization of surfactant protein C dnIκB-α transgenic mice. The cDNA encoding the HA-tagged human dnIκB-α was identical to that used to make the recombinant adenovirus noted above (32). Transgenic mice were generated by inserting this cDNA into a vector under the control of the human surfactant protein C (SP-C) promoter, originally provided by Dr. Jeffrey Whitsett (Cincinnati Children’s Hospital), as previously described (25, 55). Five lines of SPCdnIκB-α mice were generated, and all founders and their offspring were healthy. Tissue expression of the transgene was analyzed by Northern blot, as previously described (55), using probes for both the coding region and the simian virus 40 (SV40) 3 untranslated region. Results with both probes were similar, and those obtained with the SV40 probe are shown in RESULTS. Expression of transgene-encoded protein was determined by Western blot. Briefly, lungs were homogenized on ice in PBS containing Pefabloc (Roche Diagnostics, Indianapolis, IN), 50 μg of lung protein were electrophoresed on reducing 10% SDS-polyacrylamide gels and transferred to nitrocellulose, and HA-tagged dnIκB-α was detected using a rabbit polyclonal antibody to HA (Santa Cruz Biotechnology, Santa Cruz, CA) and the Amersham ECL system (Amersham Biosciences, Piscataway, NJ). The same antibody was used for transgene detection by immunohistochemistry, using an alkaline phosphatase-conjugated secondary antibody and the ABC development system (Vector Laboratories, Burlingame, CA) (55). Two lines (5990 and 6006) that exhibited strong, lung-specific transgene expression were used for further studies. These transgenic mice were backcrossed to inbred strains of mice (C57BL/6J or C57BL/6N) between 8 and 14 times to C57BL/6 mice before they were used in the LPS challenge experiments. Transgene-negative littersmates were used as controls in all experiments; locally bred C57BL/6 mice were used as supplemental controls where specified. All mice were bred at the University of Washington Animal Facility. Mice were housed under specific pathogen-free conditions in filtered cages and were permitted unlimited access to sterile food and water. All animal experiments were approved by the Animal Care Committee of the University of Washington.

Endotoxins. Escherichia coli 0111:B4 LPS was purchased from Sigma (St. Louis, MO), reconstituted to 5 mg/ml in 20 mM EDTA, clarified in an ultrasonic water bath (Cole-Parmer, Vernon Hills, IL), aliquoted, and stored at −80°C. For each experiment, an aliquot was thawed, sonically dispersed, and diluted in sterile, endotoxin-free PBS (Mediatech, Herndon, VA) to 100 μg/ml LPS was prepared from P. aeruginosa strain PAK by magnesium-ethanol precipitation, as described (14, 19). For aerosolization, the lyophilized material was reconstituted in sterile water, clarified ultrasonically, and diluted to 100 μg/ml.

Exposure to aerosolized LPS. Mice were exposed to aerosolized LPS in a whole animal exposure system, as described (52). The animals were placed in wire mesh cages within a 55-l Plexiglas cylinder connected via 10-cm ducting to a 16-l aerosol chamber. Airflow through the system was maintained at 20 l/min by negative pressure. Aerosols were generated from twin jet nebulizers (Salter Labs, Arvin, CA), each containing 8 ml of LPS suspension and driven by forced air at 15–18 psi. After 30 min, the chamber was purged with ambient air, and the mice were returned to their microisolation cages.

Bronchoalveolar lavage. At each time point, 4 and 24 h after aerosol exposure, four to six transgenic mice and four to six nontransgenic control mice were killed with an overdose of pentobarbital (10 mg ip) and then exsanguinated by cardiac puncture. The trachea was exposed and cannulated with a 20-g polyethylene catheter before the chest was opened. The left hilum was clamped, and then the right lung was lavaged with four separate 0.5-ml volumes of 0.85% saline containing 0.6 mM EDTA, each retrieved after a 30-s dwell time. The aliquots from individual mice were combined and centrifuged at 300 g, and the supernatants were stored at −80°C. The cell pellets were resuspended in RPMI containing 5% heat-inactivated fetal calf serum (Hyclone, Logan, UT), and the cells were counted in a hemacytometer. Differentials were performed on slides prepared in a Shandon Cytospin (ThermoShandon, Pittsburgh, PA) and stained with a modified Wright-Giemsa technique (Diff-Quik; Dade Behring, Dudingen, Switzerland).

Myeloperoxidase activity. Myeloperoxidase (MPO) activity was measured in lung tissue after a method used in the laboratory of Theodore Standiford (Univ. of Michigan) (11). The entire left lung or individual lobes of the right lung were flash-frozen in liquid nitrogen and stored at −80°C. The thawed tissue was homogenized in 2% protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and then incubated in 1:1 with 100 mM 3,3′-diaminobenzidine buffer composed of 3.7 mM hexadecyltrimethylammonium bromide, 0.5 mM EDTA, 0.44 M monobasic potassium phosphate, and 0.06 M dibasic potassium phosphate (all Sigma). The mixture was sonically dispersed and
then centrifuged at 12,000 g for 15 min at 4°C. Supernatants were transferred in duplicate 10-μl volumes to 96-well plates and then mixed with 140 μl of MPO assay buffer composed of 88 mM monobasic potassium phosphate, 1.24 mM dibasic potassium phosphate, 0.005% hydrogen peroxide, and 0.53 mM O-dianisidine hydrochloride. MPO activity was read as the rate of change in absorbance at 490 nm over 2 min and corrected to lung weight.

**Histopathology.** Lungs were inflated in situ to ~20 cm of pressure with 4% paraformaldehyde and then removed en bloc and stored at 4°C in the same fixative. The tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A veterinary pathologist reviewed two to four sections from individual mice in a manner blinded to genotype and time from LPS exposure (54). The intensity of peribronchial and perivascular inflammation was scored on a scale of 0–4, with a score of 0 representing normal tissue and a score of 4 representing maximal accumulation relative to other sections. The ratio of mononuclear and polymorphonuclear cells in these inflammatory foci was noted. The degree of parenchymal inflammation was also scored on an all-inclusive scale of 0–4, with a score of 0 representing normal tissue and a score of 4 representing widespread interstitial and/or alveolar infiltration. Lung tissue harvested from unexposed mice representing each genotype served as controls.

**Immunohistochemistry.** Immunohistochemical procedures were performed on frozen sections or on treated paraffin sections. Before lung tissue was frozen, individual lobes were infiltrated under gentle pressure with a mixture of PBS and Optimum Cutting Temperature (OCT) compound (Miles, Elkhart, IN). Lobes were then embedded in OCT and flash-frozen in isopentane chilled in liquid nitrogen. Sections were cut at ~5 μm and placed on charged slides. After brief fixation in cold acetone, sections were incubated with appropriate antibodies overnight at 4°C. Paraffin sections of tissue fixed in 4% paraformaldehyde were used for detection of RelA. Antigen retrieval was performed by boiling slides for 10 min in 10 mM citrate buffer (pH 6.0), after which sections were washed and incubated with antibody overnight at 4°C. Bound antibodies were detected after being washed with appropriate Vectastain ABC kits labeled with either alkaline phosphatase (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate) or peroxidase (diaminobenzidine substrate; Vector). Goat anti-mouse TNF-α and goat anti-mouse VACM-1 were purchased from R&D Systems. Rabbit anti-mouse RelA was purchased from Research Diagnostics (Flanders, NJ). Antisera to irrelevant antigens were used as controls. To heighten contrast, no counterstains were used.

**Cytokine assays.** Cytokine levels were measured in lung homogenates of mice. ELISA, using antibody pairs and recombinant standards purchased from R&D Systems. Freshly isolated left lungs were homogenized in PBS and then diluted 1:1 in 4°C lysis buffer containing 2X protease inhibitor cocktail. After 30 min of incubation on ice, the mixture was centrifuged at 1,200 g, and supernatants were stored at −80°C until assayed.

**Data analysis.** Data are expressed as means ± SE. Statistical comparisons among groups for continuous variables measured at multiple time points were made by one-way ANOVA with Tukey’s post hoc test. Comparisons of ordinal lung inflammation scores were made by Mann-Whitney’s U-test. A P value of <0.05 was considered significant.

## RESULTS

**DnIkB-α inhibits IL-8 production in respiratory epithelial cells.** The dnIkB-α construct used to generate SPCdnIkB-α transgenic mice inhibits NF-κB activation in vitro (16), and we have previously shown that a recombinant adenovirus encoding dnIkB-α inhibits NF-κB activation, cytokine production, and inflammation in the liver in vivo (32). To determine whether this dnIkB-α construct could also block proinflammatory cytokine production by respiratory epithelial cells, we transduced type II-like A549 cells with a recombinant adenovirus encoding dnIkB-α or with a control adenovirus encoding β-galactosidase and then stimulated these cells with TNF-α or live *P. aeruginosa*. IL-8 production in response to these stimuli was abolished in cells transduced with the dnIkB-α adenovirus but not in cells transduced with the control β-galactosidase adenovirus (Table 1).

**Characterization of SPCdnIkB-α transgenic mice.** DnIkB-α expressed under the control of the human SP-C promoter (Fig. 1A) was used to generate five lines of SPCdnIkB-α transgenic mice, two of which (lines 5990 and 6005) were selected for further study after initial screening. Transgene-positive mice from these lines expressed the transgene in their lungs but not in other tissues, as shown by Northern blot (Fig. 1B). The protein encoded by the transgene was detected in lung homogenates by Western blot (Fig. 1C) and by immunohistochemical staining of lung sections using a rabbit polyclonal IgG antibody to the HA tag (Fig. 2). In line 6006, there was substantial cytoplasmic and some nuclear staining in the majority of cells in the terminal bronchioles (Fig. 2, A and B). Staining of alveolar epithelial cells was detected but was not as distinct as the staining of the terminal airway epithelial cells (not shown). Expression of the transgene was not detected in other lung cells. Transgene-negative littermate controls showed minimal background staining (Fig. 2C). Similar results were obtained in the 5990 line, although the expression in the terminal bronchioles was not as intense (not shown). Thus dnIkB-α was selectively expressed in the distal lung epithelium of SPCdnIkB-α transgenic mice.

**LPS-induced bronchiolar NF-κB activation is impaired in SPCdnIkB-α mice.** Nuclear translocation of NF-κB was detected by immunohistochemistry using an antibody to RelA. In unchallenged mice, RelA was not found in the nuclei of airway epithelial cells but was distributed diffusely and faintly in the cytosol (Fig. 3A). After inhalation of LPS, nuclear localization of RelA was evident in bronchiolar epithelial cells of transgene-negative littermate control mice (Fig. 3B), but RelA

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**Table 1. IL-8 (ng/ml) secretion by transduced A549 cells stimulated with *Pseudomonas aeruginosa* or TNF-α**

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<th>Medium</th>
<th><em>P. aeruginosa</em></th>
<th>TNF-α</th>
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<tr>
<td>Ad−β-gal</td>
<td>11 ± 1</td>
<td>180 ± 36</td>
<td>195 ± 48</td>
</tr>
<tr>
<td>AddnIkB-α</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>8 ± 1</td>
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Data are means ± SE of triplicate wells. A549 cells were transduced with adenoviral vectors expressing β-galactosidase (Ad−β-gal) or dominant negative IkB-α (AddnIkB-α), and then stimulated with TNF-α (200 U/ml) or *Pseudomonas aeruginosa* (10⁷ CFU/ml) as described in MATERIALS AND METHODS. IL-8 was measured in supernatants by ELISA.
translocation was not observed in the airway epithelium of SPCdnI/H9260B- and SPCdnI/H9251B- mice (Fig. 3C). Nuclear RelA was detected following LPS administration in some alveolar macrophages in both transgene-negative littermate controls and in SPCdnI/H9260B- and SPCdnI/H9251B- mice (Fig. 3, B and C). It was technically difficult to localize RelA in alveolar epithelial cells by this method, which precluded determination of whether or not NF-κB activation occurred in type I or type II cells following inhalation of LPS. However, aerosolized LPS did not induce the nuclear translocation of RelA in proximal conducting airway epithelial cells of either control or transgenic mice (not shown). Minimal background staining was evident in sections incubated with the isotype control antibody (not shown). These findings indicate that inhalation of LPS was followed by NF-κB activation in bronchiolar epithelial cells and that dnI/H9260B inhibited LPS-induced NF-κB translocation in these cells.

Reduced bronchoalveolar inflammatory response to aerosolized LPS in SPCdnI/H9260B- mice. We used three complementary approaches to determine whether inhibition of NF-κB activation in the distal lung epithelium impaired pulmonary inflammation in response to inhaled LPS: enumeration of neutrophils in bronchoalveolar lavage (BAL) fluid, measurement of MPO activity in lung homogenates, and histopathological analysis of fixed lung tissue. As shown in Fig. 4, the inhalation of LPS resulted in prompt and sustained neutrophilic airway inflammation. However, the increase in the numbers of neutrophils and total cells in BAL samples harvested 4 h after inhalation of E. coli LPS was significantly blunted in SPCdnI/H9260B- mice from both the 5990 (Fig. 4A) and 6006 (Fig. 4B) transgenic lines compared with nontransgenic littermate controls. The increase in total cells after LPS exposure was attributable to the increase in neutrophils; there were no significant differences between transgenic and nontransgenic mice in the number of mononuclear cells in BAL specimens at either time point after inhalation of LPS.

We also measured the inflammatory responses to aerosolized P. aeruginosa LPS because P. aeruginosa is an important

Fig. 1. Generation and assessment of transgene expression in surfactant protein C dominant negative inhibitor of NF-κB translocation (SPCdnI/H9260B-) transgenic mice. A: transgene construct. B: representative Northern blots of tissues from a transgene-negative mouse (left) and from a transgenic mouse from lines 6006 (middle) and 5990 (right) hybridized with a probe from the 3′ untranslated region. The lanes contain 20 μg of RNA from kidney (lane 1), liver (lane 2), spleen (lane 3), testis (lane 4), or thymus (lane 5) or 10 μg of RNA from lung (lane 6). Similar results were obtained with a probe for the coding region of human dnI/H9260B-. C: Western blots of lung tissue from transgene negative (Tg−) and transgene positive (Tg+) littermate from lines 6006 (left) and 5990 (right) and from two additional transgene negative controls (middle) were probed with a polyclonal antibody to the hemagglutinin (HA) tag.

Fig. 2. Detection of the transgene-encoded product in lung tissue by immunohistochemistry. A and B show results from a transgene-positive mouse from line 6006 and from a transgene-negative littermate control (C). Tissue sections were exposed to an antibody to the HA tag and processed as described in MATERIALS AND METHODS. Minimal background staining was evident in transgene-negative littermate controls (C) and in sections incubated with an irrelevant primary antibody (not shown). Original magnification, A = ×20; B and C = ×40.
airway pathogen, particularly in the setting of cystic fibrosis, and because *P. aeruginosa* LPS has been shown to be less stimulatory than *E. coli* LPS in some settings (21). As with *E. coli* LPS, the accumulation of bronchoalveolar neutrophils in response to aerosolized *P. aeruginosa* LPS was significantly reduced in the SPCdnIkB-α mice (not shown). Thus the defective inflammatory response to inhaled endotoxin in SPCdnIkB-α mice was not limited to a particular type of LPS.

**Lung MPO activity after inhalation of LPS is blunted in SPCdnIkB-α mice.** MPO activity, representing the total number of neutrophils present in lung tissue, was significantly diminished in SPCdnIkB-α mice compared with nontransgenic controls after inhalation of either *E. coli* LPS (Fig. 4C) or *P. aeruginosa* LPS (not shown). Thus the impaired accumulation of bronchoalveolar neutrophils in the SPCdnIkB-α mice reflects a reduction in the emigration of neutrophils from the blood, not merely defective migration across the epithelium.

**Reduced lung tissue inflammation in response to LPS in SPCdnIkB-α mice.** No structural abnormalities or inflammation were evident in the lungs of unchallenged SPCdnIkB-α mice or transgene-negative controls as determined by histopathological examination of tissue sections (not shown). Inhalation of LPS induced robust neutrophilic inflammation in control animals, but this response was substantially reduced in the SPCdnIkB-α mice. Perivascular and peribronchial cuffs of leukocytes were less prominent and contained a lower proportion of neutrophils in SPCdnIkB-α mice (Fig. 5, B and D) than in the nontransgenic controls (Fig. 5, A and C). In more severe cases, the nontransgenic controls displayed peribronchiolar hemorrhage and, occasionally, small vessel thrombosis, features not observed in SPCdnIkB-α mice. Alveolar inflammation was mild in all animals but was less apparent in the transgenic mice. Engorgement of pulmonary lymphatics, a marker of edema formation, was more evident in sections from the nontransgenic mice than in sections from the SPCdnIkB-α mice.

The differences between SPCdnIkB-α and control mice in histological measures of inflammation are represented quantitatively in Fig. 6. The degree of perivascular and peribronchial cellular infiltration (Fig. 6A) and the ratios of neutrophils to mononuclear cells in these infiltrates (Fig. 6B) were signifi-
tantly reduced both 4 and 24 h after inhalation of LPS in the SPCdnIkB-α mice. The alveolar inflammation scores were lower in the SPCdnIkB-α mice than in nontransgenic controls, but the trends did not reach statistical significance (Fig. 6C, \( P = 0.057 \) at 24 h).

**Blunted intrapulmonary cytokine responses after inhalation of LPS in SPCdnIkB-α mice.** To explore mechanisms underlying the observed impairment in lung inflammation after inhalation of LPS in SPCdnIkB-α mice, we measured whole lung levels of early response cytokines known to be important in neutrophil recruitment, including the pluripotential mediators TNF-α and IL-1β and the ELR+ CXC chemokines MIP-2 and KC (Fig. 7). Low levels of these proteins were measured in lung tissue from unexposed animals. After exposure to aerosolized LPS, increased concentrations of TNF-α, IL-1β, MIP-2, and KC were detected in lung homogenates from both groups of mice 4 h later, but levels of all cytokines were significantly lower in lungs harvested from SPCdnIkB-α mice compared with nontransgenic animals. By 24 h after inhalation of LPS, pulmonary levels of these cytokines had declined to near the levels measured in unexposed mice. Thus the intrapulmonary cytokine response to LPS was significantly impaired in SPCdnIkB-α mice.

**Impaired tissue expression of TNF-α and VCAM-1 after LPS in SPCdnIkB-α mice.** The defect in LPS-induced lung inflammation was paralleled by impaired induction of TNF-α in the bronchiolar epithelium and reduced adhesion molecule expression on lung vascular endothelium of SPCdnIkB-α mice. TNF-α was not detected by immunohistochemistry in the lungs of SPCdnIkB-α or littermate control mice before administration of LPS (not shown). However, after aerosol exposure to LPS, TNF-α was strongly expressed in bronchiolar epithelial cells of controls (Fig. 8A); this was not observed in SPCdnIkB-α mice (Fig. 8B). Similarly, lung vascular endothelial activation, as measured by the induction of VCAM-1, was clearly evident in the peribronchiolar vessels of controls following administration of LPS (Fig. 8C) but not in the peribronchiolar vasculature of SPCdnIkB-α mice (Fig. 8D). TNF-α expression by alveolar macrophages was evident in both transgenic and nontransgenic mice after LPS challenge (not shown).

**DISCUSSION**

The major finding of these studies is that targeted inhibition of NF-κB activation in distal airway epithelial cells leads to blunted local cytokine responses and impaired pulmonary inflammation after inhalation of LPS. To investigate the role of epithelial cells in the regulation of lung inflammation in vivo, we developed novel transgenic mice in which dnIkB-α was expressed under the control of the human SP-C promoter. Expression of the transgene in these mice was lung specific and localized to the epithelial cells lining the terminal bronchioles and the alveoli. SPCdnIkB-α mice exhibited impaired activation of NF-κB in distal airway epithelial cells after exposure to aerosolized LPS compared with nontransgenic littermates. This defect was accompanied by diminished LPS-induced neutrophilic inflammation of the lungs. The blunted inflammatory response in the transgenic mice was associated with lower whole lung levels of TNF-α, IL-1β, MIP-2, and KC as well as diminished epithelial cell production of TNF-α and reduced VCAM-1 expression by lung vascular endothelial cells. These data constitute conclusive evidence that distal airway epithelial
cell activation contributes to the regulation of lung inflammation in vivo.

Our findings support earlier investigations that predicted a role for respiratory epithelial cells in regulating the pulmonary inflammatory response to LPS. First, respiratory epithelial cells can produce proinflammatory cytokines, release neutrophil chemotactic factors, and upregulate adhesion molecules upon stimulation in vitro. Several investigators have shown that A549 cells, a type II-like cell line derived from a human alveolar cell carcinoma (33), release CXC chemokines and neutrophil chemotactic activity upon stimulation with TNF-α or IL-1β in vitro (29, 53, 56). Tracheobronchial epithelial cells also can express IL-8 and other cytokines and release neutrophil chemotactic factors when stimulated with TNF-α and IL-1β (12, 13, 29, 37, 41). It is less clear that respiratory epithelial cells respond directly to LPS. Some investigators have reported that A549 cells (43, 45, 56) and tracheobronchial epithelial cells (17, 37, 41) do not respond to LPS in vitro, whereas others have described cytokine and neutrophil chemotaxin release by A549 cells and tracheobronchial epithelial cells stimulated with high concentrations of *P. aeruginosa* or *E. coli* LPS, particularly in the presence of serum (26, 27, 43, 51). Rat type II cells in primary culture have been shown to express monocyte chemotactic peptide-1 (42) and to upregulate ICAM-1 (4) on exposure to *E. coli* LPS, IL-1β, or TNF-α in vitro, although their responsiveness to LPS is limited to stimulation of the apical surface (47). One factor in these discrepant reports concerns the expression of LPS recognition molecules by the target cells that were studied. Some investigators have found that A549 cells and tracheobronchial epithelial cells express mRNA for TLR4 (6, 51), the signaling component of the LPS receptor complex (2). Other investigators have been unable to detect TLR4 expression by A549 cells (60). Nonetheless, these studies indicate that respiratory epithelial cells have the capacity to participate in the inflammatory response to LPS either by direct recognition of LPS or by amplifying signals initiated by other cells, such as alveolar macrophages, that are more sensitive to the presence of LPS. Our studies provide compelling evidence that distal airway epithelial cells contribute to LPS-induced lung inflammation but do not establish whether the epithelial cells respond directly to LPS or to secondary signals in vivo.

Fig. 6. Lower lung inflammation scores in SPCdnlB-α mice exposed to aerosolized LPS. Lung sections obtained from line 5990 SPCdnlB-α mice (TG+) and nontransgenic littermates (TG−) 4 and 24 h after inhalation of *Escherichia coli* LPS were stained with hematoxylin and eosin and scored as described in MATERIALS AND METHODS. *A*: perivascular (perivasc) and peribronchial (peribronch) inflammation scores. *B*: neutrophil to mononuclear (mononuc) cell ratios in perivascular and peribronchial tissue. *C*: alveolar air space and interstitial inflammation scores. Similar differences were obtained with line 6006. Data are means ± SE, n = 4. *P < 0.05 compared with nontransgenic mice by Mann-Whitney’s *U*-test.

Fig. 7. Blunted intrapulmonary cytokine responses to LPS in SPCdnlB-α mice. Immunoactive TNF-α, IL-1β, macrophage inflammatory protein-2 (MIP-2), and KC were measured by ELISA in lung homogenates harvested from unexposed mice (No LPS) and mice exposed to aerosolized LPS. TG+, SPCdnlB-α mice from line 5990; TG−, nontransgenic mice (littermates 4 h after LPS, otherwise C57BL/6). Data are means ± SE, n = 6. *P < 0.01 compared with nontransgenic mice; †P < 0.001 compared with nontransgenic mice.
Additional evidence that respiratory epithelial cells play an active role in the pulmonary inflammatory response to LPS has come from in vivo observations that respiratory epithelial cells are stimulated after airway deposition of LPS and that artificial activation of NF-κB in the respiratory epithelium is sufficient to elicit neutrophilic pulmonary inflammation. Intratracheal instillation of LPS induces upregulation of ICAM-1 expression by alveolar and bronchiolar epithelium (4, 5, 9), suggesting proinflammatory activation of these cells. Airway challenge with LPS also induces IκB-α degradation and nuclear accumulation of NF-κB in lung tissue (8, 40, 49), and there is evidence for epithelial cell involvement in whole lung NF-κB activation. Using an NF-κB-luciferase reporter mouse, Hubbard and colleagues (22) found increased luciferase expression in alveolar macrophages and bronchiolar epithelium after intrapulmonary challenge with LPS. The important role of alveolar macrophages in the inflammatory response to LPS has been demonstrated: depletion of alveolar macrophages resulted in diminished whole lung NF-κB activation and neutrophilic inflammation after exposure of mice to aerosolized LPS (7, 24). However, a role for parenchymal cells in pulmonary NF-κB activation after inhalation of LPS was suggested by Alcamo et al. (3), using neonatal mice lacking both RelA and TNFR1. These animals were protected from the embryonic lethality of RelA deficiency but exhibited a severe defect in bronchoalveolar neutrophil accumulation after exposure to aerosolized LPS that was not evident in neonatal mice lacking only TNFR1. Interestingly, replacement of the alveolar macrophage population of lethally irradiated wild-type mice with hematopoietic progenitor cells harvested from RelA- or RelA/TNFFR1-deficient mice yielded animals in which the inflammatory response to LPS was not significantly impaired. These studies suggested that pulmonary inflammation after inhalation of LPS is not dependent on RelA expression by alveolar macrophages but did not identify the lung parenchymal cells in which NF-κB activation was required. Finally, Sadikot et al. (48) reported that transduction of airway epithelial cells in vivo by intratracheal administration of recombinant adenoviral vectors encoding inhibitor of NF-κB kinase 1 (IκK1) or IκK2 resulted in constitutive expression of NF-κB-dependent luciferase activity, increased cytokine expression, and neutrophilic alveolar inflammation. Observations in this model system indicated that direct activation of NF-κB in airway epithelium can drive pulmonary inflammation. Our data show that this is physiologically relevant and that activation of NF-κB in distal airway epithelial cells is an important component of the coordinated inflammatory response to inhaled LPS.

Poynter and colleagues (44), using an approach similar to our own, recently reported that transgenic mice expressing a dominant negative IκB-α under control of the rat CC10 promoter exhibited impaired airway inflammation in association with reduced levels of MIP-2 and TNF-α in BAL fluid after nasal challenge with LPS. The rat CC10 promoter is expressed in transgenic mice in epithelial cells lining the trachea, bronchi, and larger bronchioles (58), whereas transgenes controlled by the human SP-C promoter are expressed in the epithelium of the terminal bronchioles and alveoli (62). Although overlap in the sites of transgene expression in these two studies cannot be excluded, our observations and those reported by Poynter et al. (44) suggest that both proximal and distal airway epithelial cells actively contribute to the inflammatory response to LPS. Our results and those of Poynter et al. (44) suggest that NF-κB activation in respiratory epithelial cells contributes to the lung inflammatory response to inhaled LPS through the...
induction of proinflammatory cytokines, which in turn act to upregulate the expression of adhesion molecules on the vascular endothelium. We found that intrapulmonary production of early response cytokines (TNF-α and IL-1β) and of ELR+ CXC chemokines (MIP-2 and KC) were significantly blunted in transgenic mice after inhalation of LPS. Furthermore, by immunohistochemical analysis, we detected TNF-α in the bronchiolar epithelial cells of control mice after LPS exposure but not in the epithelium of SPCdnI-Hα mice. This finding is consistent with reports that bronchial epithelial cells can secrete TNF-α in response to Haemophilus influenzae LPS in vitro (23) and that lung infection with H. influenzae induces the expression of TNF-α in airway epithelial cells in vivo in a TLR4-dependent manner (61). We have previously shown that pulmonary inflammation in response to inhaled LPS is partially dependent on TNFR1-mediated signaling (52, 55). TNF-α promotes inflammation, in part, by upregulating the expression of adhesion molecules, including ICAM-1 and VCAM-1, which are involved in leukocyte emigration from the vascular compartment into the air spaces of the lungs (4, 5, 9, 10, 18, 28, 31, 46). We observed that expression of VCAM-1 on the peribronchiolar endothelial was markedly induced in response to LPS in control but not in SPCdnI-Hα mice. VCAM-1 is a vascular endothelial ligand for β1-integrins, which act in concert with β2-integrins and their ligands to mediate neutrophil migration into the air spaces in response to LPS (10, 18, 28, 31, 46). By contrast, only β1-integrins appear to be involved in neutrophil recruitment to the lungs in response to the CXC chemokine KC (46). Both KC and MIP-2 participate in pulmonary neutrophil recruitment in response to LPS (20, 50), and the low levels of these chemokines in SPCdnI-Hα mice after inhalation of LPS may have contributed to the blunted inflammatory response in these animals.

For several reasons, our findings are likely to underestimate the relative contribution of the respiratory epithelium in the pulmonary inflammatory response to inhaled LPS. First, NF-κB activation was impeded only in the bronchiolar and alveolar epithelial cells in which the transgene was expressed. The contribution of tracheobronchial epithelial cells to the inflammatory response to LPS would not have been detected by this approach. Second, the inhibition of NF-κB activation in the targeted epithelial cells is likely to have been incomplete. Finally, only events that are dependent on NF-κB activation would have been impaired. Despite these limitations, our studies provide strong support for the conclusion that the respiratory epithelium plays an active role in the pulmonary inflammation induced by inhalation of LPS.

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