Am J Physiol Lung Cell Mol Physiol 293: L383–L392, 2007.
First published May 25, 2007; doi:10.1152/ajplung.00024.2007.

Clara cells impact the pulmonary innate immune response to LPS

Arnon Elizur,[1]* Tracy L. Adair-Kirk,[2]* Diane G. Kelley,[2] Gail L. Griffin,[2] Daphne E. deMello,[3] and Robert M. Senior[2,4]

[1]*Division of Allergy and Pulmonary Medicine, Department of Pediatrics, and* [2]*Division of Pulmonary and Critical Care Medicine, Department of Medicine, and* [4]*Department of Cell Biology and Physiology, Washington University School of Medicine, and* [3]*Department of Pathology, St. Louis University Health Science Center, St. Louis, Missouri*

Submitted 18 January 2007; accepted in final form 21 May 2007

Elizur A, Adair-Kirk TL, Kelley DG, Griffin GL, deMello DE, Senior RM. Clara cells impact the pulmonary innate immune response to LPS. *Am J Physiol Lung Cell Mol Physiol* 293: L383–L392, 2007. First published May 25, 2007; doi:10.1152/ajplung.00024.2007.—Airway epithelial cells secrete proinflammatory mediators in response to LPS, but cytokine production by a prominent nonciliated bronchiolar epithelial cell, the Clara cell, specifically, is unknown. To investigate Clara cell cytokine production in response to LPS, we used a transformed murine Clara cell line, C22, and isolated Clara cells from C57Bl/6 mice. Stimulation of both cell types with LPS resulted in significant upregulation of keratinocyte-derived chemokine (KC) and monocyte chemoattractant protein-1, but did not induce TNF-α production. To determine whether LPS induces cytokine production by Clara cells in vivo, LPS was instilled intratracheally into mice. KC was expressed by Clara cells, alveolar type 2 cells, and alveolar macrophages, 2 h after LPS administration, as determined by in situ hybridization. TNF-α, although not expressed in airway epithelial cells, was expressed primarily in alveolar macrophages in response to LPS. To assess the impact of Clara cells on KC and TNF-α production in the lung in the early response to LPS, mice were treated with naphthalene to selectively induce Clara cell injury before LPS stimulation. KC expression in the airways and the lung periphery, and KC and TNF-α levels in the bronchoalveolar lavage fluid, were significantly reduced in naphthalene-treated vs. vehicle-treated mice after LPS stimulation. Furthermore, transwell cocultures of C22 cells and RAW264.7 macrophages indicated that C22 cells released a soluble factor(s) in response to LPS that enhanced macrophage production of TNF-α. These results indicate that Clara cells elaborate cytokines and modulate the lung innate immune response to LPS.

airway; cytokines; epithelium; inflammation; lung

THE AIRWAY EPITHELIUM is composed of several cell types that differ in their structure and function. The major cell types are ciliated cells, mucus cells, and Clara cells (17). Over the past decade, the traditional view of the airway epithelium as only a structural barrier has changed. It is now recognized that cells lining the airways are capable of producing a variety of proinflammatory cytokines that participate in innate immune responses (22, 41, 51, 53, 65). Specifically, it has been demonstrated that human and animal airway epithelial cell lines produce proinflammatory factors [leukotriene B_4, 12-hydroxyeicosatetraenoic acid, IL-6, IL-8, TNF-α, ICAM-1, monocyte chemoattractant protein (MCP)-1, granulocyte colony-stimulating factor (G-CSF), and granulocyte/macrophage colony-stimulating factor (GM-CSF)] in response to endotoxin or bacteria (23, 25, 46). Airway epithelium also produces cytokines [transforming growth factor (TGF)-β1, eotaxin-2 and -3, and regulated upon activation T cell expressed and secreted (RANTES)] in allergic airway inflammation and remodeling (15, 38, 57), and bronchial epithelial cells obtained by bronchial biopsy exhibit differential expression of cytokines (specifically MCP-1) between asthmatics and healthy controls (52). In response to cigarette smoke in patients with chronic obstructive pulmonary disease, airway epithelium expresses proinflammatory cytokines [IL-8, MCP-1, macrophage inflammatory protein (MIP)-1α, ICAM-1, and TGF-β1], and increased cytokine expression by human bronchial epithelial cells has been correlated with airflow limitation (12, 16, 31, 53, 54).

Further support for airway epithelium as a source of inflammatory mediators comes from studies of mice with alterations of airway epithelial NF-κB activity. Constitutive overexpression of IκB kinase selectively in murine airway epithelium results in phosphorylation of IκBα, NF-κB activation, inflammatory mediator production, and neutrophilic lung inflammation (47), suggesting that overproduction of inflammatory cytokines by airway epithelial cells is sufficient to drive innate immune responses. Conversely, targeted inhibition of NF-κB in murine distal airways but not in alveolar macrophages, by expressing the inhibitory protein IκBα under the Clara cell 10-kDa protein/uteroglobin (CC10) promoter, has led to marked reductions in secretion of inflammatory cytokines and influx of neutrophils into the lungs in response to LPS. These data show that airway epithelial cells, and specifically Clara cells, produce cytokines that are necessary for the neutrophilic inflammatory response to LPS (42, 50). Additional data that both alveolar macrophages and airway epithelial cells are required for the full lung innate immune response to LPS or bacteria was generated using radiation bone marrow chimera between mice deficient in MyD88, an adapter protein involved in LPS-induced NF-κB activation, and wild-type mice. Whereas TNF-α production was exclusively dependent on cells of hematopoietic origin, the levels of CXC chemokines, keratinocyte-derived chemokine (KC) and MIP-2, and lung inflammation were dependent on airway epithelial cells as well (31).

Clara cells are nonciliated, non-mucus producing secretory cells present in distal airways. In human lungs, Clara cells comprise 15–20% of distal airway epithelial cells, whereas in mice, Clara cells account for 70–90% (39). Clara cells secrete a variety of proteins, including CC10, Clara cell 55-kDa protein, surfactant proteins A, B, and D (SP-A, SP-B, and SP-D, respectively), Clara cell tryptase, and β-galactoside-binding lectin (48). Clara cells are also capable of secreting

*A. Elizur and T. L. Adair-Kirk contributed equally to this work.

Address for reprint requests and other correspondence: R. M. Senior, Dept. of Medicine, Washington Univ. School of Medicine, Box 8052, 660 South Euclid Ave., St. Louis, MO 63110 (e-mail: rsenior@im.wustl.edu).

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

proinflammatory cytokines in response to TNF-α (36), but the significance of cytokine expression by Clara cells in response to LPS is not known.

Considering the prominence of Clara cells in the distal airways, we speculate that Clara cells play an important role in the innate immune responses of the airways. To evaluate cytokine production by Clara cells, we have examined freshly isolated Clara cells and the recently developed C22 Clara cell line (13) for cytokine responses to LPS. The C22 cell line was developed by isolating Clara cells from the Immortomouse (21), which harbors a transgenic, temperature-sensitive, major histocompatibility complex (MHC)-driven, large T-antigen. When grown in non-permissive conditions, these cells display many characteristics of mature Clara cells, including growth in monolayers, dense secretory granules, and production of CC10 and SP-A, SP-B, and SP-D, but not SP-C. Stimulation of either isolated or C22 Clara cells with LPS resulted in significant upregulation of KC, but not TNF-α. These observations were complemented by observations of Clara cells in vivo following instillation of LPS.

MATERIALS AND METHODS

Cell Culture

C22 cells. C22 cells were obtained and maintained as previously described (13). Briefly, the cells were maintained in permissive conditions [DMEM supplemented with 2% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (250 μg/ml), endothelin-1 (0.25 μg/ml), interferon-γ (100 U/ml), insulin (10 μg/ml), transferrin (5 μg/ml), endothelial cell growth supplement (7.5 μg/ml), epidermal growth factor (0.025 μg/ml), hydrocortisone (0.36 μg/ml), and T_3 (0.02 μg/ml)] at 33°C.

Clara cells. Clara cells were isolated from C57Bl/6 mice using a protocol modified from Corti et al. (11, Atkinson JJ and Senior RM, unpublished data). Following perfusion of the lungs with saline via the right ventricle, 0.5 ml of 1% low-melting agarose in PBS was instilled through the trachea followed by 0.5 ml of 0.25% bovine pancreatic trypsin (T-8003; Sigma, St. Louis, MO) in HBSS without Ca^{2+} or Mg^{2+}. After a 2-min incubation on ice, the lungs were excised and incubated at 37°C for 10 min. The tissue was diced in DMEM + 250 μg/ml DNase + 2× antibiotic/antimycotic (AB/AM), filtered sequentially through 100- and 40-μm filters, transferred to 50-ml conical tubes with 2 ml FBS to inhibit the trypsin, and spun three times at 32 *g* for 6 min at 10°C. After each spin, the supernatant was discarded, and pellets were resuspended in 20 ml of DMEM + 2× AB/AM. The final pellet was resuspended in 8 ml of DMEM + 2× AB/AM, plated on a 100-mm mouse IgG-coated Petri plate, and incubated for 1 h at 37°C to remove contaminating macrophages. Nonadherent cells were subsequently collected, and aliquots were stained with nitroblue tetrazolium (NBT) and counted to determine Clara cell purity, as previously described (35).

RAW264.7 macrophages. Murine RAW264.7 macrophages, obtained from American Type Culture Collection (Rockville, MD), were maintained in low bicarbonate (1.5 g/l) DMEM, supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 4 mM L-glutamine.

Mice

Six- to eight-week-old C57Bl/6 mice (Taconic, Hudson, NY) were housed in a barrier facility under veterinary care of the Department of Comparative Medicine at Washington University School of Medicine. All procedures involving mice were approved by the Washington University School of Medicine Animal Studies Committee and were performed in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.

Stimulation of C22 and Clara Cells with LPS

C22 cells were plated at a density of 4.5×10^5 cells/60-mm plate and incubated overnight in non-permissive conditions (media not containing interferon-γ at 39°C to inactivate the MHC-driven large T-antigen) in a 10% CO_2 incubator (13). On the basis of NBT staining, the number of isolated Clara cells was plated at a density of 3.75×10^5 cells/well in DMEM + 2× AB/AM + 10% FBS in a 24-well plate. Following overnight incubation, C22 or Clara cells were washed three times in serum-free media and incubated for various times with serum-free media alone or media containing 0.1 μg/ml *Escherichia coli* LPS (L-4005, Sigma), a concentration that achieves maximal cytokine production (data not shown). In some experiments, cells were treated with 10 μg/ml cyclohexamide 30 min before and during LPS treatment.

Detection of Cytokine Production

Conditioned media of C22 and Clara cell cultures treated with LPS for 24 h were collected, and cytokine levels were assayed using the Raybio Mouse Cytokine Antibody Array (MA6060, TranSignal) according to the manufacturer's recommendations. Blots were scanned, and analysis of optical densities (OD) was performed on a PC using the public domain NIH Image program (developed at National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). The average OD of each cytokine was normalized to the average OD of the biotin-conjugated controls (top left and bottom right corners of each membrane) on the same membrane, to eliminate loading differences between membranes. Fold change was then determined as the difference between normalized untreated and LPS-treated conditions. Quantities of KC, MCP-1, and TNF-α in the conditioned media were determined by ELISA according to the manufacturers' recommendations (Mouse KC Duoset DY453, R&D Systems, Minneapolis, MN; OptEIA Sets for Mouse MCP-1 and TNF-α, BD Biosciences, San Jose, CA). ELISA data represent at least three independent experiments performed in triplicate.

RNA Isolation and Quantitative Real-Time PCR

RNA was isolated from C22 cells using the ToTALLY RNA isolation kit (Ambion, Austin, TX). One microgram of total RNA was reverse transcribed using random hexamers and the *Taq*man Reverse Transcription Reagents kit (Roche, Branchburg, NJ). After reverse transcription, real-time PCR reactions were performed using a Mx3000p thermocycler (Stratagene, La Jolla, CA), CYBR green, and primers specific for KC, MCP-1, or TNF-α. The primers for mouse KC were 5′-GGGCGCCTATCGCCAAT-3′ and 5′-ACCTTCAAGCTCTGGATGTTCTTG-3′ (64). The primers for mouse MCP-1 were 5′-ACTGAAGCCAGCTCTCTCTTCCTC-3′ and 5′-TTCCTTCTTGGGGGTCAGCACAGAC-3′ (20). The primers for mouse TNF-α were 5′-CCAGGCGGTGCCTA-3′ and 5′-GGCCATTTGGGAAC-3′ (45). The primers for the mouse housekeeping gene L32 were 5′-CAGGGTGCGGAGAAGGTTCAAGGG-3′ and 5′-CTTAGAGGACACGTTGTGAGCAATC-3′ and were used as a control. All samples were run in duplicate. Samples were run for 40 cycles at 95°C for 15 s and 60°C for 1 min. The cycle threshold (C_T) for each condition was determined and normalized to that of the L32 housekeeping gene for loading. Differences between C_T values (ΔC_T) of LPS-treated and untreated samples were used to calculate the fold change (fold change = $2^{-\Delta\Delta CT}$) (28).

Intratracheal Administration of LPS

Mice were anesthetized by intraperitoneal injection of 87 mg/kg ketamine + 13 mg/kg xylazine. Under sedation, the trachea was exposed, and 50 μl of PBS alone or containing 200 μg of LPS was

instilled directly into the trachea via an insulin syringe. After 2 h, mice were killed by carbon dioxide narcosis, and bronchoalveolar lavage (BAL) fluids were obtained, as previously described (1). The BAL fluids were assayed for cytokines by ELISA. The lungs were inflation-fixed with formalin and paraffin-embedded as previously described (3). In some experiments, 2.5 h before intratracheal LPS, mice were treated with naphthalene (300 mg/kg body wt) in corn oil or with equivalent volume of vehicle alone, by intraperitoneal injection, as previously described (27).

In situ Hybridization

The cell-type patterns of expression of KC and TNF-α in response to LPS were determined by in situ hybridization (ISH). A 315-bp fragment corresponding to nucleotides 533–847 of the mouse KC gene (34) was amplified by PCR using the full-length mouse KC cDNA (provided by Dr. Tom Hamilton, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH) (2) as the template. A 232-bp fragment corresponding to nucleotides 6180–6411 (14) of the mouse TNF-α gene was amplified by PCR using the full-length mouse TNF-α cDNA (provided by Dr. Christoph Muller, Institute of Pathology, Bern, Switzerland) (10) as the template. The resulting PCR products were subcloned into the pCRII-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) as per the manufacturer's recommendations. Following vector linearization, sense and antisense digoxigenin (DIG)-labeled probes were generated using the DIG RNA Labeling Mix (Roche) and T7 or SP6 RNA polymerase. Hybridization of the DIG-labeled probe to 5-μm lung sections was performed as previously described (56), and DIG was detected using the alkaline phosphate-conjugated anti-DIG antibody (Roche) and the BM purple alkaline phosphate substrate solution (Roche) as per the manufacturer's recommendations. Slides were counterstained with tartrazine yellow for contrast. To determine the number of peripheral lung cells expressing KC, DIG-positive cells were counted in five random high-power fields (HPF, ×40) in one lung section, obtained from at least 10 mice per condition. Results are expressed as the average number of KC-expressing cells per HPF.

Immunohistochemistry

Five-micrometer-thick sections were immunostained for Clara cells (anti-CC10, 1:25,000; provided by Gurmukh Singh, Univ. of Pittsburgh, Veterans Affairs Hospital) (49), for type 2 cells (anti-SP-C, 1:5,000; Millipore, Temecula, CA), or for macrophages (anti-Mac3, 1:500; BD Biosciences). Immunostaining was developed using a goat, rabbit, or rat ABC Elite Kit (Vector Laboratories, Burlingame, CA), respectively, followed by DAB (for CC10 or SP-C) or Vector NovaRed (for Mac3) Substrate Kit (Vector Laboratories). All slides were then counterstained with hematoxylin.

Alveolar Macrophages Isolation and Treatment

C57Bl/6 mice in groups of five were treated with naphthalene (300 mg/kg body wt) in corn oil or with equivalent volume of vehicle alone by intraperitoneal injection. After 2.5 h, BAL fluids obtained from the five mice per condition were pooled. Cells were cytospun and differentially stained, and equivalent numbers of alveolar macrophages were plated in a 24-well plate. The cells were allowed to adhere for 2 h and then were treated with LPS (0.1 μg/ml) in serum-free media for 4 h. The cell-conditioned media were assessed for TNF-α content by ELISA, as described above. Data represents at least three independent experiments in triplicate.

Transwell Coculture System and LPS Stimulation

RAW264.7 macrophages were plated at a density of 1×10^6 in the top chamber of 22-mm-diameter, 0.4-μm pore size Costar Transwell Permeable Supports (Corning, Corning, NY), and C22 cells were plated in non-permissive media not containing interferon-γ at a density of 2.5×10^5 cells in the bottom chamber. Following an overnight incubation at 37°C, the media in the top chamber was replaced with serum-free media alone, whereas the media in the lower chamber was replaced with either serum-free media alone or containing 0.01 μg/ml LPS. After 24 h, the conditioned media from both the bottom and the top chambers were pooled and assayed for TNF-α by ELISA as described above. RAW264.7 and C22 cells treated with LPS in monocultures served as controls. Total RNA was isolated from C22 cells, reverse transcribed, and analyzed for TNF-α mRNA using quantitative real-time PCR (qPCR) as described above.

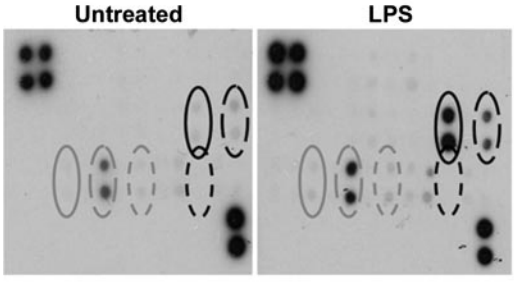
Statistical Analysis

All analysis was performed using SPSS 12.0 for Windows. An independent two-sided *t*-test was used to analyze the relationship between continuous variables. A *P* value of less than 0.05 was considered significant. All data represent at least three independent experiments performed in triplicate.

RESULTS

Cytokine Profile of C22 Cells in Response to LPS

To determine whether C22 cells express cytokines in response to LPS, we used a cytokine antibody array containing antibodies to 32 cytokines, including CXC chemokines (KC and MIP-2), CC chemokines [RANTES, MIP-1α, MIP-3β, MCP-1, MCP-5, 6-cysteine chemokine (6Ckine), cutaneous T cell-attracting chemokine (CTACK), eotaxin, thymus and activation-regulated chemokine (TARC)], colony-stimulating factors [G-CSF, GM-CSF, stem cell factor (SCF), VEGF, thrombopoietin (TPO)], pleiotropic cytokines (TNF-α), and receptors (sTNFri), interferons (IFN-γ), interleukins (IL-2, -3, -4, -5, -6, -9, -10, -12p40, -12p70, -13, -17), metalloproteinase inhibitors (TIMP-1), and hormones (leptin). Several NF-κB-dependent cytokines were upregulated by C22 cells in response to LPS. KC production was markedly upregulated in response to 24-h treatment with LPS (14.6-fold; Fig. 1). MCP-1 and MIP-2 were also upregulated by C22 cells in response to LPS, but much less than KC (2.8- and 2.3-fold, respectively). RANTES was constitutively produced by C22 cells, but the



Cytokine	Fold change
KC	14.55
MCP-1	2.80
MIP-2	2.32
RANTES	1.38
TNF-α	0.76
sTNFri	1.71

Fig. 1. Cytokine profile of C22 cells in response to LPS. C22 cells were treated with LPS for 24 h, conditioned media from 3 independent experiments were collected, and cytokine production was assayed using a mouse antibody array. Blots were scanned, optical density was adjusted based on positive controls (*top left, bottom right*), and the fold-change, from untreated to LPS-treated, was calculated as described in MATERIALS AND METHODS. KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2.

response to LPS was minimal. Many other NF-κB-dependent cytokines, and specifically TNF-α, were not detected in the conditioned media at baseline or in response to LPS. This absence of TNF-α was surprising given the ubiquitous nature of TNF-α and its documented secretion by other cells in response to LPS (23). Moreover, soluble TNF-α receptor (sTNFR) was detected in the culture media at baseline and was upregulated in response to LPS (1.7-fold), suggesting that the lack of TNF-α secretion was not due to an inability of C22 cells to cleave membrane bound TNF-α, a step catalyzed by TNF-α converting enzyme for both substrates (6, 32, 43).

Timing of Cytokine Secretion by C22 Cells in Response to LPS

As the cytokine antibody array provides only semiquantitative data, we quantified the secretion of several of the cytokines that were found to be induced by LPS using ELISA. KC and MCP-1 secretion in response to 24 h of LPS treatment reached 40–60 ng/ml, but MIP-2 levels were much lower, reaching 0.3 ng/ml (Fig. 2*A*). We also assayed for TNF-α in the culture media of LPS-treated C22 cells and found that it was absent, confirming the results obtained using the antibody array (Fig. 1). Next, we determined the kinetics of KC and MCP-1 secretion during 24 h of exposure to LPS. A gradual increase in MCP-1 and KC, to a lesser extent, was detected in the media of untreated C22 cells over the 24-h period (Fig. 2*B*). However, the secretion of KC and MCP-1 was significantly elevated by 2 h of LPS stimulation and continued to increase over the entire 24-h period of study.

KC Secretion by C22 Cells in Response to LPS is Due to New Synthesis

To determine whether the increased secretion of KC and MCP-1 in response to LPS reflected increased gene expression, qPCR was performed on RNA isolated from C22 cells stimulated with LPS for 24 h. It demonstrated increases in mRNA for both KC and MCP-1 in response to LPS (Fig. 3). In contrast, qPCR for TNF-α revealed virtually no TNF-α mRNA in C22 cells at baseline ($C_T = 36.3+/-1.1$) or in response to LPS ($C_T = 36.9+/-0.7$), excluding the possibility of rapid uptake and turnover of TNF-α as the basis for its absence in the media.

As Clara cells contain cytoplasmic granules that can be secreted upon stimulation (63), we examined whether KC is primarily synthesized or secreted in response to LPS stimulation. Accordingly, C22 cells were treated with cyclohexamide, a protein synthesis inhibitor, before and during 6 h of LPS stimulation. In the presence of 10 μg/ml cyclohexamide, LPS failed to induce production of KC by C22 cells (data not shown). Thus, LPS induces new synthesis of KC, and Clara cells do not store KC in granules.

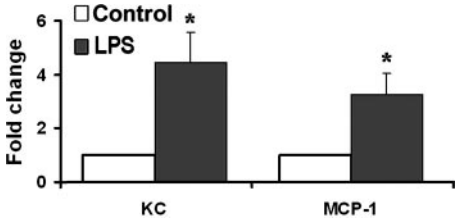


Fig. 3. Quantitative RT-PCR for KC and MCP-1 expression by C22 cells in response to LPS (24 h). C22 cells were treated with LPS for 24 h. RNA was isolated from untreated and LPS-treated C22 cells and reverse transcribed. Real-time PCR reactions were performed for KC and MCP-1. Data represents at least 3 independent experiments done in triplicate ± SE. $*P < 0.05$.

Isolated Clara Cells Secrete KC and MCP-1 But Not TNF-α in Response to LPS

C22 cells have many characteristics of mature Clara cells (13). To determine whether C22 cytokine production in response to LPS is similar to that of normal mouse Clara cells, we isolated Clara cells from C57Bl/6 mice. The cells were incubated in serum-free media alone or containing LPS for 24 h. Like C22 cells, Clara cells secreted both KC and MCP-1 under basal conditions, and upregulated their secretion in response to LPS (Fig. 4). Also, similar to C22 cells, Clara cells did not secrete TNF-α in response to LPS stimulation. The lack of TNF-α in the culture media of LPS-treated Clara cells confirms that our isolated cells are free of inflammatory cells. Thus cytokine production by Clara cells and C22 cells is similar with regard to production of these cytokines.

KC Expression in Response to LPS In Vivo

To evaluate whether the Clara cell response to LPS in vitro reflects in vivo responses, we administered LPS intratracheally to C57Bl/6 mice. By ISH, KC expression was prominent within 2 h of LPS stimulation in both airway epithelial and parenchymal lung cells (Fig. 5*B*), whereas it was only minimally expressed in the lungs of mice treated with PBS alone (Fig. 5*A*). An increase in TNF-α expression was also detected 2 h after LPS stimulation, but only by cells in the periphery of the lung, and not by airway epithelial cells (Fig. 5*E*). Sense controls did not show KC (Fig. 5*C*) or TNF-α (Fig. 5*F*)

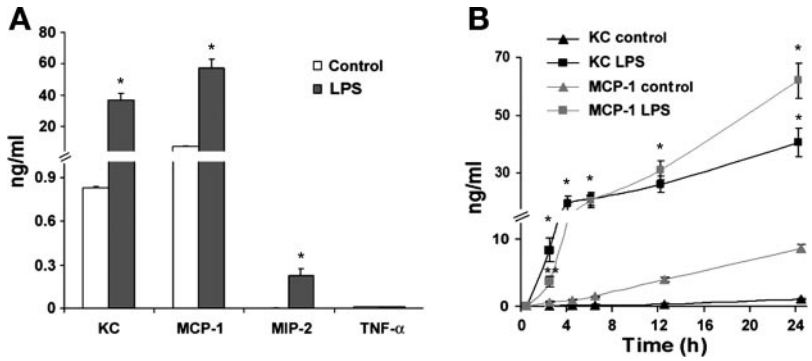


Fig. 2. Quantitative and time-dependent secretion of cytokines by C22 cells in response to LPS. C22 cells were treated with LPS for 24 h, and the levels of KC, MCP-1, MIP-2, and TNF-α in the conditioned media were measured by ELISA (*A*). C22 cells were treated with LPS, and KC and MCP-1 production in the conditioned media was measured by ELISA at selected time points (*B*). Data represents at least 3 independent experiments done in triplicate ± SE. $*P < 0.001$, $**P = 0.02$.

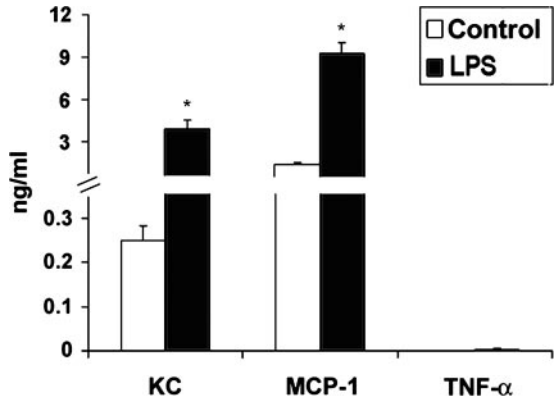


Fig. 4. Isolated Clara cells secrete KC and MCP-1, but not TNF-α, in response to LPS. Clara cells were isolated from C57Bl/6 mice, as described in MATERIALS AND METHODS, and treated with LPS for 24 h. The levels of KC, MCP-1, and TNF-α in the conditioned media were measured by ELISA. Data represents at least 3 independent experiments done in triplicate ± SE. *$P < 0.001$.

expression. These results indicate that the in vitro responses of Clara cells reflect the responses in vivo.

To identify the cells that express KC after LPS stimulation, immunohistochemistry was performed on serial sections using antibodies to cell-specific markers for Clara cells (CC10), alveolar type 2 cells (SP-C), or macrophages (Mac3). The majority of the KC-expressing cells in the airways also express CC10, indicating that Clara cells express KC in vivo in response to LPS (Fig. 6, *A* and *B*). In the lung periphery, the KC-expressing cells were identified as either alveolar macrophages (Fig. 6, *D* and *E*) or alveolar type 2 epithelial cells (Fig. 6, *G* and *H*). Secondary antibody-alone controls showed no staining (Fig. 6, *C*, *F*, and *I*). With the same approach, the peripheral lung cells that expressed TNF-α in response to LPS were identified to be almost exclusively alveolar macrophages (data not shown).

Naphthalene-Treated Mice Express Less KC in Both Airway Epithelial Cells and Parenchymal Lung Cells in Response to LPS

Naphthalene is a polycyclic aromatic hydrocarbon that becomes converted into a toxic intermediate by cytochrome *P*-450 monooxygenase (40). Cells that lack the specific cytochrome *P*-450 that converts naphthalene to its toxic metabolite, such as alveolar macrophages (19), would not be directly affected by naphthalene treatment. However, Clara cells, the principal site in the lung of xenobiotic metabolism by the cytochrome *P*-450 monooxygenase system (58), become selectively targeted in this model of lung injury. After only 2–3 h of naphthalene exposure, many Clara cells exhibit apical membrane blebbing, heavy consolidation of cytoplasmic spaces, and swollen smooth endoplasmic reticulum (SER), but there are only few permeable cells (59). Within 6 h of naphthalene exposure, the majority of Clara cells demonstrates increased permeability (59), and within 48 h, naphthalene induces exfoliation of Clara cells into the airway lumen (58). To determine the significance of Clara cell cytokine production in response to LPS, we intratracheally administered LPS to mice 2.5 h after naphthalene treatment, a point in which the epithelial barrier function should still be intact.

Naphthalene alone did not induce KC expression of PBS-treated mice (Fig. 7*B*) nor did treatment with the corn oil vehicle used to solubilize naphthalene (Fig. 7*A*). Furthermore, pretreatment with corn oil had no affect on LPS-induced KC expression (Fig. 7*C*). However, naphthalene-treated mice given LPS did not express KC in the airways, further demonstrating that Clara cells are the KC-expressing cells in the airways (Fig. 7*D*). Interestingly, the number of peripheral lung cells expressing KC in response to LPS was significantly lower in naphthalene-treated mice (29.3+/−1.9 KC-expressing cells) compared with corn oil-treated mice (41+/−2.4 KC-expressing cells; $P < 0.001$; Fig. 7*E*). To ensure that naphthalene does not exert a cytotoxic effect on macrophages, alveolar macrophages were isolated from naphthalene and corn oil-treated mice and stimulated with LPS in vitro. The cells from both groups of mice were viable as determined by trypan blue exclusion (data not shown), and their TNF-α production was similar (Fig. 7*F*). Since naphthalene selectively injures Clara cells, these data suggest that Clara cell stimulation by LPS affects KC production by cells in the lung periphery.

KC and TNF-α in the BAL Fluids of LPS-Treated Mice Are Reduced Following Naphthalene-Induced Clara Cell Injury

To determine the contribution of Clara cells to the quantity of cytokines in the BAL fluid, we examined the BAL fluids of

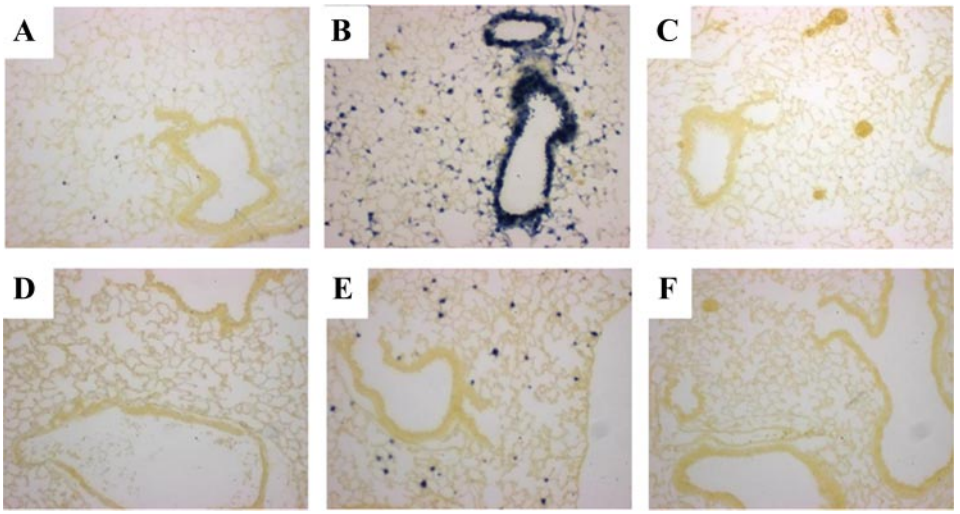


Fig. 5. In situ hybridization for KC and TNF-α mRNA following intratracheal LPS administration. C57Bl/6 mice were treated with intratracheal PBS (*A*, *D*) or PBS containing 200 μg of LPS (*B, C, E, F*). After 2 h, lungs were inflation-fixed with formalin and paraffin embedded. Lung sections were probed for KC (*A* and *B*) or TNF-α (*D* and *E*) expression by in situ hybridization (ISH) using digoxigenin (DIG)-labeled RNA probes. Sense probes for KC (*C*) and TNF-α (*F*) were used as negative controls. Blue staining represents positive hybridization. Sections were counterstained with tartrazine yellow for contrast. Images represent at least 3 independent experiments using at least 2 mice/condition.

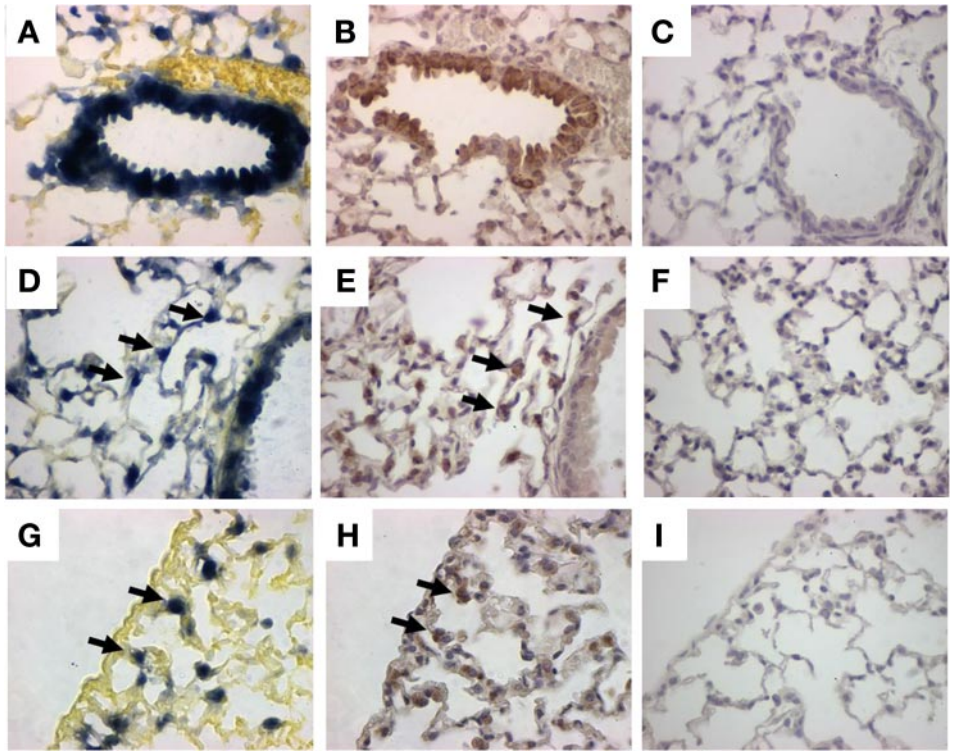


Fig. 6. Clara cells, alveolar type 2 cells, and alveolar macrophages express KC in response to LPS. C57Bl/6 mice were treated with intratracheal LPS. Serial lung sections were examined for KC expression by ISH (*A*, *D*, *G*) and for CC10 (*B*), surfactant protein C (*E*), or Mac3 (*H*) production using immunohistochemistry. Secondary antibody alone served as negative controls (*C*, *F*, and *I*). Images represent at least 3 independent experiments using at least 2 mice/condition. Arrows point to positive hybridization or immunostaining.

mice treated with corn oil alone or containing naphthalene before LPS stimulation, for KC and TNF-α. Whereas the amounts of KC in the BAL fluid of untreated and corn oil-treated mice were similar 2 h after LPS stimulation, KC was significantly reduced in the BAL fluid of naphthalene-treated mice compared with untreated mice (3.4-fold) or mice given corn oil (3.5-fold; Fig. 8). TNF-α was also significantly reduced in the BAL fluid in naphthalene-treated mice compared with untreated (2.7-fold) and corn oil-treated mice (3.2-fold). As TNF-α is expressed almost exclusively by macrophages after LPS administration, these data suggest that Clara cells can modulate the pulmonary innate immune response to LPS by regulating the production of cytokines by resident macrophages.

LPS-Stimulated C22 Cells Release A Diffusible Factor That Augments Macrophage Cytokine Production

Our data indicate that Clara cells in the airways of mice can modulate the cytokine production by macrophages in the lung periphery. To determine whether Clara cells release a diffusible factor in response to LPS to exert their effects on macrophages, we used a transwell coculture system in which RAW264.7 macrophages were grown in the top chamber and C22 cells were grown in the bottom chamber. LPS was added to the media in the bottom chamber, and following 24-h incubation, the conditioned media from both chambers were pooled and assayed for the presence of TNF-α. No TNF-α was detected in the conditioned media when RAW264.7 macrophages and C22 cells were grown in this transwell coculture system in the absence of LPS (Fig. 9). In addition, no TNF-α was detected in the conditioned media from C22 cells grown alone in the presence of LPS, as also seen in Fig. 2. However, treatment of RAW264.7 macrophages alone with LPS induced production of TNF-α (Fig. 9). Furthermore, following LPS treatment, a marked increase in the amount of TNF-α was detected in the conditioned media from transwell cocultures of C22 and RAW264.7 cells compared with that detected in the conditioned media from RAW264.7 cells alone. qPCR for TNF-α in untreated C22 cells alone and in LPS-treated C22 cells that were cocultured with macrophages revealed essentially undetectable amounts of transcripts under both conditions. Thus these data suggest that C22 cells secrete a diffusible factor(s) that significantly augments TNF-α secretion by RAW264.7 macrophages in response to LPS.

DISCUSSION

Our study sheds light on the cellular sources of the major proinflammatory cytokines (KC and TNF-α) in LPS-induced lung inflammation. We report that Clara cells produce cytokines in response to LPS, including KC, a potent neutrophil chemoattractant, and the human IL-8 homolog, both in vitro and in vivo. In addition, we observed that whereas LPS induces the expression of several NF-κB-regulated cytokines by Clara cells, others, among which is TNF-α, are not induced. Our data further suggest that the alveolar macrophage expression of TNF-α in response to LPS is modulated by a secreted factor(s) produced by the airway epithelial Clara cell.

Until recently, Clara cell research was hampered by relatively low yields from cell isolations and a rapid loss of phenotypic characteristics in culture (4, 7, 8, 29, 35, 60). Development of a murine Clara cell line (C22) from the Immortomouse helps to overcome these problems (13). Using isolated Clara cells, we confirmed that the cytokine profile produced by C22 cells replicated the profile produced by normal Clara cells in response to LPS. Thus it appears that C22

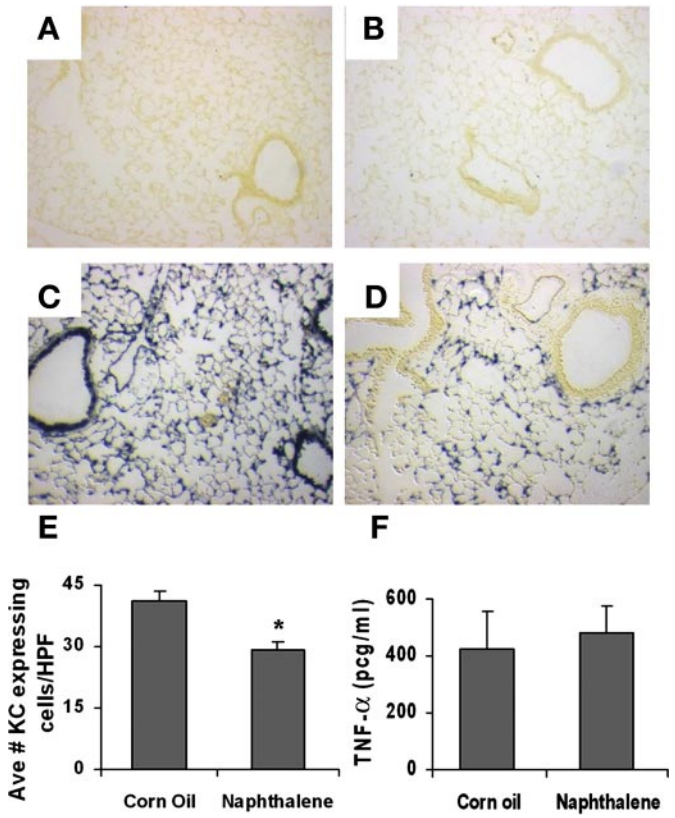


Fig. 7. Naphthalene-treated mice express less KC in both airway epithelial and parenchymal lung cells in response to LPS. Mice were pretreated with intraperitoneal corn oil alone (*A* and *C*) or containing naphthalene (*B* and *D*) 2.5 h before intratracheal administration of PBS (*A* and *B*) or LPS (*C* and *D*). Lung sections were examined for KC expression by ISH. Images represent at least 3 independent experiments using at least 2 mice/condition. The number of peripheral lung cells expressing KC was determined by manual counting of the number of DIG-positive cells in 5 high-power fields (HPF, ×40) in each lung section of at least 10 corn oil- or naphthalene-treated mice ± SE (*E*). $*P < 0.001$. Alveolar macrophages were isolated from groups of 5 naphthalene- and 5 corn oil-treated mice and stimulated with LPS for 2 h (*F*). The levels of TNF-α in the conditioned media were measured by ELISA. Data represents at least 3 independent experiments done in triplicate ± SE.

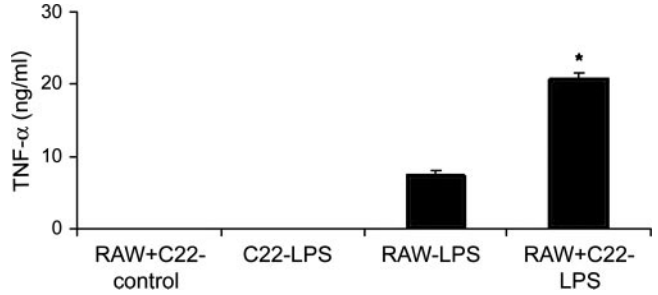


Fig. 9. C22 cells enhance the macrophage response to LPS. RAW264.7 cells were grown in the top chamber of a transwell system alone or with C22 cells in the bottom chamber, as described in MATERIALS AND METHODS, and were treated with LPS for 24 h. The levels of TNF-α in the conditioned media were measured by ELISA. Data represents at least 3 independent experiments done in triplicate ± SE. $*P < 0.0001$.

cells may serve as a tool to study cytokine production by Clara cells.

TNF-α, a pleiotropic cytokine that plays a critical role in inflammation (5), is regulated by NF-κB (61). Interestingly, we found that although Clara cells produce other NF-κB-regulated cytokines (KC and MCP-1), they do not express TNF-α. Previous studies have shown variable results as to the sources of TNF-α in LPS-induced lung inflammation. In some studies, TNF-α appears to be secreted by airway epithelial cells (25, 42), whereas in others, it appears to be derived from macrophages (33). Our results support the latter. By ISH, we found that airway epithelial cells, including Clara cells, do not express TNF-α in response to LPS stimulation in vivo, whereas macrophages in the same tissue section express TNF-α (Fig. 5). It is not clear why airway epithelial cells, although clearly being major participants in the lung inflammatory response to LPS, do not express TNF-α.

Intratracheal administration of LPS does not preclude LPS from entering the alveolar region, and therefore, at least some of the increased cytokine production could be due to a direct effect of LPS on the cells in the lung periphery. To determine the relative contribution of Clara cells to the overall KC production in the lung, we selectively injured the Clara cells with naphthalene shortly before LPS stimulation. In mice treated with naphthalene, we found that the LPS-induced expression of KC by airway epithelial cells was significantly reduced compared with vehicle-treated mice (Fig. 7). Moreover, the LPS-induced KC expression by cells in the peripheral zones of the lung was also reduced. Since intraperitoneally injected naphthalene accumulates preferentially in the bronchi and bronchioles and not in peripheral lung cells (44), and as we were able to demonstrate that a 2.5-h exposure to naphthalene does not affect cytokine production by alveolar macrophages in response to LPS in vitro, these data indicate that the effect of naphthalene on KC expression by peripheral lung cells is via its effect on Clara cells. Nevertheless, the reduction in the number of peripheral lung cells expressing KC in response to LPS following naphthalene injury, although statistically significant, is too small to account for the significant reduction in the

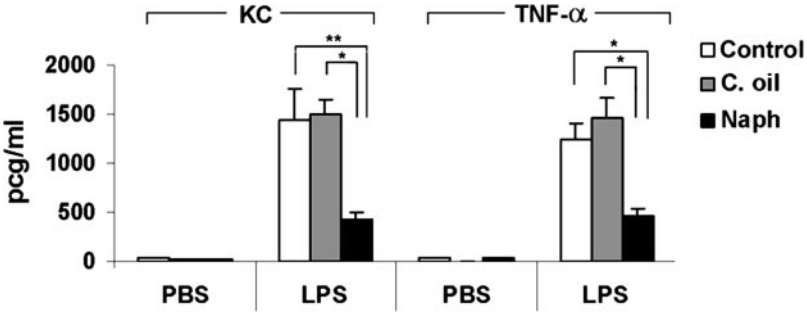


Fig. 8. The quantity of KC and TNF-α in the bronchoalveolar lavage (BAL) fluid of LPS-treated mice is significantly reduced following naphthalene-induced injury. BAL fluid was obtained from untreated and naphthalene- or corn oil-treated mice 2 h after LPS stimulation and evaluated for KC and TNF-α by ELISA. Data represents at least 3 independent experiments using at least 3 mice/condition done in triplicate ± SE. $*P < 0.001$, $**P = 0.005$.

quantity of KC in the BAL fluid (Fig. 8). Together, these data suggest that Clara cells themselves are significant producers of KC in response to LPS and that they modulate the expression of KC by peripheral lung cells.

The idea that activation of Clara cells in the airways affects the cytokine production of cells in the lung periphery was further supported by the significant reduction of LPS-induced TNF-α in the BAL fluid in naphthalene-treated mice compared with vehicle-treated mice (Fig. 8). Since TNF-α is produced almost exclusively by alveolar macrophages in response to LPS, and alveolar macrophages are not directly affected by naphthalene (19), these data suggest an indirect effect of LPS on macrophage expression of TNF-α. Thus, not only is the Clara cell a major producer of cytokines in response to LPS, but our findings indicate that the Clara cell modulates the response of resident lung inflammatory cells to LPS.

Several in vitro studies have demonstrated that bronchial epithelial cells produce cytokines that may influence inflammatory cells (9, 22, 23, 25, 30, 46, 65). Furthermore, studies that prevented NF-κB activation specifically in CC10-producing airway epithelial cells in vivo showed a significant reduction in cytokine levels in the BAL fluid and markedly reduced lung inflammation in response to LPS (42). Others have demonstrated that neutrophil recruitment to the lung in response to LPS is nearly blocked in the presence of MyD88-deficient alveolar macrophages or following depletion of alveolar macrophages (24, 31). Our data is novel because we show that Clara cells and alveolar macrophages are required for a full inflammatory response to LPS, not only because of the combined cytokine production, but more importantly because Clara cells augment the cytokine production of the inflammatory cells. In the absence of airway epithelial cells triggering, the macrophage response to LPS is reduced.

Cross talk between pulmonary epithelial cells and inflammatory cells has been investigated using coculture systems by other investigators. However, most utilize a contact coculture system with monocytes/macrophages and alveolar epithelial cells (18, 26, 37, 55, 62). Our data indicate that Clara cells in the airways of mice can modulate the cytokine production by macrophages in the lung periphery. Therefore, we hypothesized that the Clara cells produce a soluble factor(s) that affects distal inflammatory cells. To examine how the Clara cell augments the macrophage response to LPS, we cocultured the two cell types using a transwell system. Our data that C22 cells do not produce TNF-α in response to LPS (Fig. 2) allowed us to monitor the macrophage response specifically. We found that the macrophage response to LPS, as reflected by TNF-α production, was significantly elevated in the presence of C22 cells, indicating that LPS-stimulated Clara cells released a diffusible factor(s), which was capable of passing through the transwell membrane and activating macrophages. Our findings emphasize the magnitude to which airway epithelial cells enhance the inflammatory cell response to LPS.

In conclusion, we have found that Clara cells produce KC and that they affect KC expression by peripheral lung cells shortly after LPS stimulation. Also, we report that Clara cells, although not producers of TNF-α, impact TNF-α production by alveolar macrophages in response to LPS in vitro and in vivo. Together, our data suggest that LPS-exposed airway epithelial cells can secrete proinflammatory factor(s) that in turn activate resident macrophages and orchestrate the innate immune response throughout the lung. These findings should be considered in designing measures to treat acute lung injury.

ACKNOWLEDGMENTS

We thank Tom Hamilton, Dept. of Immunology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio, for the KC cDNA; Christoph Muller, Div. of Immunopathology, Institute of Pathology, Bern, Switzerland, for the TNF-α cDNA; Gurmukh Singh, Univ. of Pittsburgh, Veterans Affairs Hospital for the CC10 antibody; and Jeffery J. Atkinson for advice on Clara cell isolation and critical review of the paper.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-29594 and HL-47328 (R. M. Senior), by the Francis Family Foundation (T. L. Adair-Kirk), and by the Alan A. and Edith L. Wolff Charitable Trust/Barnes-Jewish Hospital Foundation (R. M. Senior).

REFERENCES

1. **Adair-Kirk TL, Atkinson JJ, Broekelmann TJ, Doi M, Tryggvason K, Miner JH, Mecham RP, Senior RM.** A site on laminin alpha 5, AQARSAASKVKVSMKF, induces inflammatory cell production of matrix metalloproteinase-9 and chemotaxis. *J Immunol* 171: 398–406, 2003.
2. **Armstrong DA, Major JA, Chudyk A, Hamilton TA.** Neutrophil chemoattractant genes KC and MIP-2 are expressed in different cell populations at sites of surgical injury. *J Leukoc Biol* 75: 641–648, 2004.
3. **Atkinson JJ, Holmbeck K, Yamada S, Birkedal-Hansen H, Parks WC, Senior RM.** Membrane-type 1 matrix metalloproteinase is required for normal alveolar development. *Dev Dyn* 232: 1079–1090, 2005.
4. **Belinsky SA, Lechner JF, Johnson NF.** An improved method for the isolation of type II and Clara cells from mice. In Vitro *Cell Dev Biol Anim* 31: 361–366, 1995.
5. **Beutler BA.** The role of tumor necrosis factor in health and disease. *J Rheumatol Suppl* 57: 16–21, 1999.
6. **Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP.** A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 385: 729–733, 1997.
7. **Blundell RA, Harrison DJ.** Integrin characterization in pulmonary bronchioles. *Exp Mol Pathol* 79: 74–78, 2005.
8. **Boogaard PJ, de Kloe KP, Wong BA, Sumner SC, Watson WP, van Sittert NJ.** Quantification of DNA adducts formed in liver, lungs, and isolated lung cells of rats and mice exposed to ^{14}C-styrene by nose-only inhalation. *Toxicol Sci* 57: 203–216, 2000.
9. **Cerri C, Chimenti D, Conti I, Neri T, Paggiaro P, Celi A.** Monocyte/macrophage-derived microparticles up-regulate inflammatory mediator synthesis by human airway epithelial cells. *J Immunol* 177: 1975–1980, 2006.
10. **Corazza N, Eichenberger S, Eugster HP, Mueller C.** Nonlymphocyte-derived tumor necrosis factor is required for induction of colitis in recombination activating gene (RAG)2(−/−) mice upon transfer of CD4(+)CD45RB(hi) T cells. *J Exp Med* 190: 1479–1492, 1999.
11. **Corti M, Brody AR, Harrison JH.** Isolation and primary culture of murine alveolar type II cells. *Am J Respir Cell Mol Biol* 14: 309–315, 1996.
12. **de Boer WI, Sont JK, van Schadewijk A, Stolk J, van Krieken JH, Hiemstra PS.** Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *J Pathol* 190: 619–626, 2000.
13. **Demello DE, Mahmoud S, Ryerse J, Hoffmann JW.** Generation and characterization of a conditionally immortalized lung clara cell line from the H-2Kb-tsA58 transgenic mouse. In Vitro *Cell Dev Biol Anim* 38: 154–164, 2002.
14. **Fransen L, Muller R, Marmenout A, Tavernier J, Van der Heyden J, Kawashima E, Chollet A, Tizard R, Van Heuverswyn H, and Van Vliet A.** Molecular cloning of mouse tumour necrosis factor cDNA and its eukaryotic expression. *Nucleic Acids Res* 13: 4417–4429, 1985.
15. **Fujisawa T, Kato Y, Atsuta J, Terada A, Iguchi K, Kamiya H, Yamada H, Nakajima T, Miyamasu M, Hirai K.** Chemokine production by the BEAS-2B human bronchial epithelial cells: differential regulation of eotaxin, IL-8, and RANTES by TH2- and TH1-derived cytokines. *J Allergy Clin Immunol* 105: 126–133, 2000.

16. **Fuke S, Betsuyaku T, Nasuhara Y, Morikawa T, Katoh H, Nishimura M.** Chemokines in bronchiolar epithelium in the development of chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 31: 405–412, 2004.
17. **Gail DB, Lenfant CJ.** Cells of the lung: biology and clinical implications. *Am Rev Respir Dis* 127: 366–387, 1983.
18. **Hjort MR, Brenyo AJ, Finkelstein JN, Frampton MW, LoMonaco MB, Stewart JC, Johnston CJ, D'Angio CT.** Alveolar epithelial cell-macrophage interactions affect oxygen-stimulated interleukin-8 release. *Inflammation* 27: 137–145, 2003.
19. **Hook GE, Bend JR, Fouts JR.** Mixed-function oxidases and the alveolar macrophage. *Biochem Pharmacol* 21: 3267–3277, 1972.
20. **Ikejima S, Sasaki S, Sashinami H, Mori F, Ogawa Y, Nakamura T, Abe Y, Wakabayashi K, Suda T, Nakane A.** Impairment of host resistance to *Listeria* monocytogenes infection in liver of db/db and ob/ob mice. *Diabetes* 54: 182–189, 2005.
21. **Jat PS, Noble MD, Ataliotis P, Tanaka Y, Yannoutsos N, Larsen L, Kioussis D.** Direct derivation of conditionally immortal cell lines from an H-2Kb-tsA58 transgenic mouse. *Proc Natl Acad Sci USA* 88: 5096–5100, 1991.
22. **Kao CY, Huang F, Chen Y, Thai P, Wachi S, Kim C, Tam L, Wu R.** Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF-kappaB-dependent signaling pathway. *J Immunol* 175: 6676–6685, 2005.
23. **Khair OA, Davies RJ, Devalia JL.** Bacterial-induced release of inflammatory mediators by bronchial epithelial cells. *Eur Respir J* 9: 1913–1922, 1996.
24. **Koay MA, Gao X, Washington MK, Parman KS, Sadikot RT, Blackwell TS, Christman JW.** Macrophages are necessary for maximal nuclear factor-kappa B activation in response to endotoxin. *Am J Respir Cell Mol Biol* 26: 572–578, 2002.
25. **Koyama S, Rennard SI, Leikauf GD, Shoji S, Von Essen S, Claassen L, Robbins RA.** Endotoxin stimulates bronchial epithelial cells to release chemotactic factors for neutrophils. A potential mechanism for neutrophil recruitment, cytotoxicity, and inhibition of proliferation in bronchial inflammation. *J Immunol* 147: 4293–4301, 1991.
26. **Krakauer T.** Stimulant-dependent modulation of cytokines and chemokines by airway epithelial cells: cross talk between pulmonary epithelial and peripheral blood mononuclear cells. *Clin Diagn Lab Immunol* 9: 126–131, 2002.
27. **Lawson GW, Van Winkle LS, Toskala E, Senior RM, Parks WC, Plopper CG.** Mouse strain modulates the role of the ciliated cell in acute tracheobronchial airway injury-distal airways. *Am J Pathol* 160: 315–327, 2002.
28. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2[-Delta Delta C(T)] method. *Methods* 25: 402–408, 2001.
29. **Massey TE, Geddes BA, Forkert PG.** Isolation of nonciliated bronchiolar epithelial (Clara) cells and alveolar type II cells from mouse lungs. *Can J Physiol Pharmacol* 65: 2368–2372, 1987.
30. **Mathew B, Park GY, Cao H, Azim AC, Wang X, Van Breemen RB, Sadikot RT, Christman JW.** Inhibitory κB kinase 2 activates airway epithelial cells to stimulate bone marrow macrophages. *Am J Respir Cell Mol Biol* 36: 562–572, 2007.
31. **Mio T, Romberger DJ, Thompson AB, Robbins RA, Heires A, Rennard SI.** Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. *Am J Respir Crit Care Med* 155: 1770–1776, 1997.
32. **Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, Hoffman CR, Kost TA, Lambert MH, Leesnitzer MA, McCauley P, McGeehan G, Mitchell J, Moyer M, Pahel G, Rocque W, Overton LK, Schoenen F, Seaton T, Su JL, Warner J, Willard D, and Becherer JD.** Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* 385: 733–736, 1997.
33. **Noulin N, Quesniaux VF, Schnyder-Candrian S, Schnyder B, Maillet I, Robert T, Vargaftig BB, Ryffel B, Couillin I.** Both hemopoietic and resident cells are required for MyD88-dependent pulmonary inflammatory response to inhaled endotoxin. *J Immunol* 175: 6861–6869, 2005.
34. **Oquendo P, Alberta J, Wen DZ, Graycar JL, Derynck R, Stiles CD.** The platelet-derived growth factor-inducible KC gene encodes a secretory protein related to platelet alpha-granule proteins. *J Biol Chem* 264: 4133–4137, 1989.
35. **Oreffo VI, Morgan A, Richards RJ.** Isolation of Clara cells from the mouse lung. *Environ Health Perspect* 85: 51–64, 1990.
36. **Park MS, Zhao B, Ramsay PL, Chang AS, Reardon MJ, DeMayo FJ.** Expression of inflammatory cytokines in a mouse transformed Clara cell line by tumor necrosis factor-alpha. *Ann NY Acad Sci* 923: 336–337, 2000.
37. **Pechkovsky DV, Zissel G, Stamme C, Goldmann T, Ari Jaffe H, Einhaus M, Taube C, Magnussen H, Schlaak M, Muller-Quernheim J.** Human alveolar epithelial cells induce nitric oxide synthase-2 expression in alveolar macrophages. *Eur Respir J* 19: 672–683, 2002.
38. **Perng DW, Wu YC, Chang KT, Wu MT, Chiou YC, Su KC, Perng RP, Lee YC.** Leukotriene C4 induces TGF-beta1 production in airway epithelium via p38 kinase pathway. *Am J Respir Cell Mol Biol* 34: 101–107, 2006.
39. **Plopper CG, Hill LH, Mariassy AT.** Ultrastructure of the nonciliated bronchiolar epithelial (Clara) cell of mammalian lung. III. A study of man with comparison of 15 mammalian species. *Exp Lung Res* 1: 171–180, 1980.
40. **Plopper CG, Macklin J, Nishio SJ, Hyde DM, Buckpitt AR.** Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. III. Morphometric comparison of changes in the epithelial populations of terminal bronchioles and lobar bronchi in mice, hamsters, and rats after parenteral administration of naphthalene. *Lab Invest* 67: 553–565, 1992.
41. **Polito AJ, Proud D.** Epithelia cells as regulators of airway inflammation. *J Allergy Clin Immunol* 102: 714–718, 1998.
42. **Poynter ME, Irvin CG, Janssen-Heininger YM.** A prominent role for airway epithelial NF-kappa B activation in lipopolysaccharide-induced airway inflammation. *J Immunol* 170: 6257–6265, 2003.
43. **Reddy P, Slack JL, Davis R, Cerretti DP, Kozlosky CJ, Blanton RA, Shows D, Peschon JJ, Black RA.** Functional analysis of the domain structure of tumor necrosis factor-alpha converting enzyme. *J Biol Chem* 275: 14608–14614, 2000.
44. **Reid WD, Ilett KF, Glick JM, Krishna G.** Metabolism and binding of aromatic hydrocarbons in the lung. Relationship to experimental bronchiolar necrosis. *Am Rev Respir Dis* 107: 539–551, 1973.
45. **Rube CE, Wilfert F, Palm J, Konig J, Burdak-Rothkamm S, Liu L, Schuck A, Willich N, Rube C.** Irradiation induces a biphasic expression of pro-inflammatory cytokines in the lung. *Strahlenther Onkol* 180: 442–448, 2004.
46. **Saba S, Soong G, Greenberg S, Prince A.** Bacterial stimulation of epithelial G-CSF and GM-CSF expression promotes PMN survival in CF airways. *Am J Respir Cell Mol Biol* 27: 561–567, 2002.
47. **Sadikot RT, Han W, Everhart MB, Zoia O, Peebles RS, Jansen ED, Yull FE, Christman JW, Blackwell TS.** Selective I kappa B kinase expression in airway epithelium generates neutrophilic lung inflammation. *J Immunol* 170: 1091–1098, 2003.
48. **Singh G, Katyal SL.** Clara cell proteins. *Ann NY Acad Sci* 923: 43–58, 2000.
49. **Singh G, Katyal SL.** Clara cells and Clara cell 10 kD protein (CC10). *Am J Respir Cell Mol Biol* 17: 141–143, 1997.
50. **Skerrett SJ, Liggitt HD, Hajjar AM, Ernst RK, Miller SI, Wilson CB.** Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin. *Am J Physiol Lung Cell Mol Physiol* 287: L143–L152, 2004.
51. **Smith SM, Lee DK, Lacy J, Coleman DL.** Rat tracheal epithelial cells produce granulocyte/macrophage colony-stimulating factor. *Am J Respir Cell Mol Biol* 2: 59–68, 1990.
52. **Sousa AR, Lane SJ, Nakhosteen JA, Yoshimura T, Lee TH, Poston RN.** Increased expression of the monocyte chemoattractant protein-1 in bronchial tissue from asthmatic subjects. *Am J Respir Cell Mol Biol* 10: 142–147, 1994.
53. **Takizawa H.** Airway epithelial cells as regulators of airway inflammation (Review). *Int J Mol Med* 1: 367–378, 1998.
54. **Takizawa H, Tanaka M, Takami K, Ohtoshi T, Ito K, Satoh M, Okada Y, Yamasawa F, Nakahara K, Umeda A.** Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). *Am J Respir Crit Care Med* 163: 1476–1483, 2001.
55. **Tao F, Kobzik L.** Lung macrophage-epithelial cell interactions amplify particle-mediated cytokine release. *Am J Respir Cell Mol Biol* 26: 499–505, 2002.
56. **Tian H, Jeong J, Harfe BD, Tabin CJ, McMahon AP.** Mouse Disp1 is required in sonic hedgehog-expressing cells for paracrine activity of the cholesterol-modified ligand. *Development* 132: 133–142, 2005.

57. **van Wetering S, Zuyderduyn S, Ninaber DK, van Sterkenburg MA, Rabe KF, Hiemstra PS.** Epithelial differentiation is a determinant in the production of eotaxin-2 and -3 by bronchial epithelial cells in response to IL-4 and IL-13. *Mol Immunol* 44: 803–811, 2007.
58. **Van Winkle LS, Buckpitt AR, Nishio SJ, Isaac JM, Plopper CG.** Cellular response in naphthalene-induced Clara cell injury and bronchiolar epithelial repair in mice. *Am J Physiol Lung Cell Mol Physiol* 269: L800–L818, 1995.
59. **Van Winkle LS, Johnson ZA, Nishio SJ, Brown CD, Plopper CG.** Early events in naphthalene-induced acute Clara cell toxicity: comparison of membrane permeability and ultrastructure. *Am J Respir Cell Mol Biol* 21: 44–53, 1999.
60. **Walker SR, Hale S, Malkinson AM, Mason RJ.** Properties of isolated nonciliated bronchiolar cells from mouse lung. *Exp Lung Res* 15: 553–573, 1989.
61. **Werner SL, Barken D, Hoffmann A.** Stimulus specificity of gene expression programs determined by temporal control of IKK activity. *Science* 309: 1857–1861, 2005.
62. **Wottrich R, Diabate S, Krug HF.** Biological effects of ultrafine model particles in human macrophages and epithelial cells in mono- and co-culture. *Int J Hyg Environ Health* 207: 353–361, 2004.
63. **Yoneda K.** Pilocarpine stimulation of the bronchiolar Clara cell secretion. *Lab Invest* 37: 447–452, 1977.
64. **Zhang P, Quinton LJ, Gamble L, Bagby GJ, Summer WR, Nelson S.** The granulopoietic cytokine response and enhancement of granulopoiesis in mice during endotoxemia. *Shock* 23: 344–352, 2005.
65. **Zhong H, Wu Y, Belardinelli L, Zeng D.** A2B adenosine receptors induce IL-19 from bronchial epithelial cells, resulting in TNF-alpha increase. *Am J Respir Cell Mol Biol* 35: 587–592, 2006.