Inhibitory κ B Kinase 2 Activates Airway Epithelial Cells to Stimulate Bone Marrow Macrophages

Biji Mathew, Gye Young Park, Hongmei Cao, Anser C. Azim, Xuerong Wang, Richard B. Van Breemen, Ruxana T. Sadikot, and John W. Christman

Section of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine; Department of Medicinal Chemistry and Pharmacognosy, University of Illinois; and Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois

It has not been resolved whether macrophages or airway epithelial cells primarily respond to infectious and inflammatory stimuli and initiate a cell-to-cell inflammatory interaction within the airways. We hypothesized that the airway epithelial cells are primary responders that activate macrophages in response to environmental stimuli. To investigate the unilateral contribution of airway epithelial cells in the activation of macrophages, we developed an *in vitro* system in which the primary mouse tracheal epithelial cells (MTEC) and primary bone marrow-derived macrophages (BMDM) were incubated together for a brief period of time in a Transwell culture plate. MTEC were transfected with adenoviral vectors that express a constitutively active form of IKK2 (Ad-cIKK2), Ad- β -Gal, or PBS for 48 h before incubating with the macrophages. Macrophage activation was determined by measuring surface expression of CD11b, activation of NF- κ B, phagocytic activity and production of reactive oxygen species, and cyclooxygenase (COX)-2 gene expression and production of prostaglandins. Macrophage adherence to epithelial layer was confirmed by CD68 immunostaining and scanning electron microscopy. MTEC cells transfected with Ad-cIKK2 produced increased amounts of IL-6, mouse GRO- α , TNF- α , and prostaglandin (PG)E₂. Exposure of BMDM to MTEC, transfected with Ad-cIKK2, led to an increase in the CD11b expression and increased adherence of macrophages to the epithelial cell layer. NF- κ B activation, COX-2 gene expression, and PGD₂ synthesis were also increased in BMDM that were incubated with MTEC transfected with Ad-cIKK2. These data suggest that airway epithelial cells potentially play a primary role in generating inflammatory signals that result in activation of macrophages.

Keywords: airway epithelium; macrophages; lung; inflammation

Airway epithelial cells were once considered to act simply as structural barriers within the airways (1). In addition to a role of protecting the internal milieu of the lungs from inhaled environmental pathogens and toxins, airway epithelium forms an integral part of the innate immune system and plays an active role in inflammatory response (2, 3). Airway epithelial cells recognize environmental stimuli through pattern recognition receptors like Toll-like receptors (TLRs) and result in the activation of cells of the immune systems (4, 5). Activation of several signaling pathways such as NF- κ B, signal transducer and activators of transcription (STAT), and mitogen-activated protein kinase (MAPK) has been demonstrated in airway epithelial cells as well. Soluble mediators produced by activation of these path-

CLINICAL RELEVANCE

Our study helps elucidate the mechanisms related to the initiation and development of inflammation and its progression in the airways and suggests that therapies directed at epithelial cells could modulate macrophage function.

ways in airway epithelial cells could contribute to the initiation, intensity, or duration of inflammation.

Recent studies of lung inflammation and asthma have suggested that airway epithelial cells are the first cell type to respond to endotoxin in the lung. For example, after 30 min of LPS treatment, STAT3 activation is markedly increased in the airway epithelial cells, and this occurs before activation and recruitment of other inflammatory cells (6). In a *Pseudomonas aeruginosa*-induced lung inflammation model, NF- κ B activation occurs in epithelial cells at 4 h after intratracheal administration of the bacteria—a much earlier time point than in other cell types in the lung, including alveolar macrophages (7). Together, these observations suggest that airway epithelial cells are activated before other cell types in the lung and could contribute to the generation and orchestration of inflammatory processes.

We have already reported that airway epithelial cells are fully capable of inducing an acute respiratory distress syndrome (ARDS)-like inflammatory response mediated through NF- κ B activation (7). Without any other stimuli, selective activation of NF- κ B in airway epithelial cells in mice was induced by administration of adenoviral vectors that constitutively express an active form of IKK1 or IKK2 (cIKK1/cIKK2). This was sufficient to induce severe lung injury, including production of inflammatory mediators and accumulation of neutrophils in the airspace. This response was abrogated by co-administration of adenoviral vectors expressing a dominant inhibitor of NF- κ B, suggesting that activation of airway epithelial cells is also necessary for full-blown lung inflammation (7). Similarly, selective ablation of epithelial NF- κ B activation also diminishes an allergen-induced inflammatory response (8, 9). Although it has been clearly demonstrated that activation of NF- κ B in epithelial cells can result in lung inflammation, there are no published reports that address the issue of how NF- κ B-activated epithelial cells initiate and maintain lung inflammation by interacting with inflammatory cells.

NF- κ B pathway regulates the expression of adhesion molecules and chemokines, which play a critical role in the cell-to-cell interaction and generation of inflammation in the lung. Airway epithelial cells and alveolar macrophages are in close contact within the airspace. The interaction between these two cell types may be one of the pathways involved in regulating local inflammatory response in the lung (10). Also, the mechanism of cell-to-cell interaction between epithelial cells and alveolar macrophages in the context of initiation of inflammatory signaling

(Received in original form July 7, 2006 and in final form November 21, 2006)

This work was supported by the Department of Veterans Affairs and National Institutes of Health Grants HL66196 and HL-075557 (J.W.C.).

Correspondence and requests for reprints should be addressed to John W. Christman, M.D., Room 920-N, Clinical Science Building, 840 South Wood Street, Chicago, IL 60612. E-mail: jwc@uic.edu

Am J Respir Cell Mol Biol Vol 36, pp 562–572, 2007

Originally Published in Press as DOI: 10.1165/rcmb.2006-0245OC on January 4, 2007

Internet address: www.atsjournals.org

and its transmission to neighboring cells remains unclear. There are no studies focused on the unidirectional signal transmission from epithelial cells to macrophages by using a model in which epithelial cells function as primary responders in the alveolar milieu. No other studies specifically targeted particular pathways, like NF- κ B, in the epithelial cells and looked at the cell-to-cell interaction signaled from epithelial cells to macrophages using a reliable model to generate the inflammatory processes that is not mediated through surface receptors shared by the two cell types. Other studies have used stimuli, like endotoxin and TNF- α , that stimulate both epithelial cells and macrophages, making it difficult to discern the directionality of cell-to-cell interactions because both cell types have surface receptors for these stimuli. To avoid the nuances of immortalized cell lines, we used primary mouse tracheal epithelial cells (MTEC) and bone marrow-derived macrophages (BMDM) for these studies. In the present study, we hypothesized that airway epithelial cells are primary responders to environmental stimuli and initiate signals that are necessary to activate the macrophages, thereby mounting an effective inflammatory response in the lung. Using *in vitro* cultured primary mouse tracheal epithelial cells, we have demonstrated that NF- κ B activation in airway epithelial cells generate signals that contribute to an inflammatory macrophage phenotype.

MATERIALS AND METHODS

Animals

Wild-type C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). Intercellular adhesion molecule (ICAM)-1 knockout mice were a gift from Dr. Asrar Malik (Univ. of Illinois, Chicago, IL). HLL transgenic mice (NF- κ B reporter mice expressing *Photinus* luciferase cDNA under the control of proximal 5' HIV-LTR promoter) have been described previously (11, 12). All procedures and protocols using mice were approved by the animal care committee of the University of Illinois at Chicago and the Jesse Brown VAMC.

MTEC Isolation

For isolating primary MTEC, we have followed the previous published protocol of Steven Brody (Washington University, St. Louis, MO) with minimal modifications (13). Briefly, the tracheas were opened longitudinally and incubated in 1.5 mg/ml pronase (Roche Molecular Biochemicals, Indianapolis, IN) for 18 h at 4°C. Epithelial cells were dislodged from tracheas, collected by centrifugation, and resuspended in DNase solution to avoid clumping together. These cells were centrifuged and resuspended in MTEC basic media (Dulbecco's modified Eagle's medium [DMEM]-Ham's F-12 [1:1 vol/vol], 15 mM HEPES, 3.6 mM sodium bicarbonate, 4 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml fungizone). After incubating in tissue culture plates (Primera; Becton-Dickinson Labware, Franklin Lakes, NJ) for 3–4 h at 37°C, floating epithelial cells were collected by centrifugation. MTEC cells were seeded on a 12-mm-diameter, 0.4- μ m pore size polycarbonate semipermeable membrane (Corning, Cambridge, MA). The cells were cultured for 5 d with the culture medium MTEC plus, which is MTEC basic media supplemented with 10 μ g/ml insulin, 5 μ g/ml transferrin, 0.1 μ g/ml cholera toxin, 25 ng/ml epidermal growth factor (Becton-Dickinson, Bedford, MA), 30 μ g/ml bovine pituitary extract, 5% fetal bovine serum [FBS], and 0.01 μ M retinoic acid. Medium was changed every 2 d until the transmembrane resistance (R_t) was $> 1,000 \Omega \cdot \text{cm}^2$, as measured by an epithelial Ohm-voltmeter (World Precision Instruments, Sarasota, FL). Media was removed from the upper chamber to establish an air-liquid interface (ALI), and lower chambers were provided with fresh MTEC/NS medium, which is MTEC basic medium supplemented with 2% Nu Serum (Becton-Dickinson) and 0.01 μ M retinoic acid every 2 d.

Preparation of BMDM

BMDM were obtained from mice following the method of Celada and coworkers (14) with certain modifications. After mice were killed, bone

marrow was flushed from the femurs. The cells were washed and resuspended in DMEM, with 10% endotoxin-free FBS and 10% (vol/vol) L929 cell-conditioned medium as a biological source of macrophage colony-stimulating factor. The medium was replenished at Day 4, and nonadherent cells are removed. The cells were used for the experiments after Day 7, which is a time point at which purine rich box-1 (PU.1) is maximally expressed, corresponding to a mature macrophage phenotype (J.W.C., unpublished observation).

Adenovirus Transfection into MTEC

We used adenoviral vectors carrying constitutively active form of IKK2 (Ad-cIKK2) and β -gal (Ad- β -gal), which were described in our previous publication (7). IKK2 were made constitutively active by Ser-Glu mutations in critical serine residues that are phosphorylated in the active kinase (15). To be transfected by adenoviral vectors, MTEC cells were washed with PBS and kept on serum-free media overnight before adding adenoviral vectors. The cells were then transfected with Ad-cIKK2, Ad- β -gal at a multiplicity of infection (MOI) of 1:10, or medium alone, washed after 3 h with $1 \times$ PBS, and replenished with serum containing medium and incubated at 37°C for 48 h. After incubation, cIKK2 expression was confirmed by Western blot analysis and immunostaining with anti-flag antibody.

NF- κ B Reporter Assay for MTEC

MTEC were isolated from HLL mice were used to measure NF- κ B activation on epithelial cells followed by cIKK2 activation. After 48 h of transduction with Ad-cIKK2, Ad- β -gal, or media alone, the MTEC were collected, and lysed with freshly reconstituted luciferase assay buffer. The luciferase activity is measured by Luciferase Reporter Assay System (Promega Corporation, Madison, WI) following manufacturer's instructions. Luciferase activity was normalized for total protein content.

Cytokine/Chemokine Assay

To measure cytokine and chemokine levels, the culture media was assayed with Bio-plex mouse cytokine kit (Bio-Rad, Hercules, CA) by carefully following the manufacturer's instructions.

PGE₂, PGD₂ Assay

After 48 h of transfection of epithelial cells (with Ad-cIKK2, Ad- β -gal, or media alone) and incubation, the MTEC culture supernatant was collected and stored at -80°C . Similarly, the co-culture supernatants collected after incubating the activated MTEC with BMDM for 6 h were also collected and saved at -80°C . PGE₂ and PGD₂ were measured by liquid chromatography in conjunction with mass spectrometry (LC-MS/MS).

Western Blot Analysis for ICAM-1

After 48 h of cIKK2 transfection, MTEC were washed once in ice-cold PBS and trypsinized. Cells were lysed using M-PER mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL). Protein content in cell lysate was determined by Bio-Rad Protein assay reagent (Bio-Rad). Cell lysates containing equal amounts of protein following dissociation in Laemmli buffer by boiling for 5 min were subjected to SDS-PAGE. After SDS PAGE, the proteins were transferred to PVDF membrane by electro-blotting, blocked for 1 h with TBST (Tris-buffered saline with 0.1% Tween 20) containing 0.5% bovine serum albumin (BSA), and probed with primary and secondary antibodies and immunodetected by enhanced chemiluminescence kit (Amersham, Pittsburgh, PA).

CD11b Expression on Macrophages

For quantitative evaluation of CD11b antigen density on macrophages, flow cytometry analysis was performed. BMDM were collected, resuspended in culture medium, and directly placed over on the apical side of the epithelial cell monolayer growing on the Transwell inserts (transfected with Ad-cIKK2, Ad- β -gal, or medium). The macrophages were added at a ratio of 1:10 (epithelial cell:macrophage) and incubated at 37°C for 1 h. After a 1-h incubation period, macrophages were collected, centrifuged, and resuspended in PBS containing 0.2% BSA and 2 mM EDTA. The cells were distributed to 50 μ l each into Eppendorf tubes

and incubated with PE anti-mouse –CD11b (BD Bioscience, San Jose, CA) (0.05 μ g/million cells as determined by titration) on ice in dark and normal rabbit IgG as isotype control. The cells were analyzed by FACS analyzer (Elite Epics ESP; Beckman Coulter, Miami, FL) soon after incubation.

Blocking ICAM-1 on Epithelial Cells

To evaluate whether ICAM-1 expression on epithelial cells up-regulate CD11b on macrophages, we have used epithelial cells from ICAM-1-deficient mice transfected with cIKK α co-incubated with wild type macrophages as described above and analyzed for CD11b. This experiment was repeated with neutralizing anti-mouse CD54 (ICAM-1; BD Bioscience) functional blocking antibody (10 μ g/ml) by adding to the cIKK2 overexpressed MTEC for 1 h before incubating with the macrophages.

Phagocytosis Assay

Macrophages were collected after incubating on epithelial cell monolayer for 1 h with medium containing 1% serum for 1 h. Then they were incubated with fluorescein isothiocyanate (FITC)-labeled latex beads (0.9 μ m, 10 beads/macrophage; Spherotech, Libertyville, IL) for 2 h at 37°C. Fluorescent beads can be used to measure phagocytosis because they are easy to manipulate and show an exceptionally intense fluorescence after laser excitation (16). Internalized FITC-labeled latex beads were measured as mean fluorescent intensity after external quenching with trypan blue (1.2 mg/ml). Cells kept at 4°C with beads added and washed immediately were used as control. Approximately 10,000 cells from each sample were analyzed by flow cytometry using an Epics Elite ESP cytometer (Beckman Coulter).

Superoxide Measurement

Superoxide production was measured from BMDM after incubating with epithelial cells using a commercially available Lumimax superoxide kit from Stratagene (La Jolla, CA).

Electron Microscopy

Epithelial cells were transfected with Ad-cIKK2 or Ad- β -gal as described above. After 48 h of incubation, the membranes were washed. Macrophages were added to the epithelial monolayer on the apical side of the cells by placing them in the inner Transwell chamber and incubated at 37°C for 1 h. The unattached macrophages were washed off with 1 $\times$ PBS and the Transwell membrane was fixed with 2.5% glutaraldehyde and processed and visualized on a Hitachi S-3000N microscope (Tokyo, Japan).

CD68 Staining of Macrophages

To quantify the macrophage adherence on the epithelial monolayer, the macrophage-specific CD68 staining was used. The epithelial membrane after incubation with macrophages for 1 h was washed with PBS and fixed with 4% paraformaldehyde for 10 min. After fixation the cells were permeabilized with 0.2% Triton X. After blocking with 3% BSA for 60 min, the membrane was incubated with primary antibody CD68 (Santa Cruz Biotechnology, Santa Cruz, CA) with 1:200 dilution at 4°C overnight. Membranes were incubated with TRITC anti-rabbit secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA; A) for 30 min. The membrane was mounted on slides in mounting media with DAPI (Vector Laboratories, Burlingame, CA). CD68-immunostained macrophages were imaged using the LSM 510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Ten random fields were selected at low-power magnification, and the number of blue DAPI-stained cells as well as red CD68-positive macrophages was counted from both experimental and control membranes.

RT-PCR

After incubating with the epithelial monolayer for 2 h, total RNA was purified from macrophages by using TRIZOL reagent (Invitrogen, Carlsbad, CA). Three micrograms of total RNA were reverse transcribed by using first-strand cDNA synthesis kit (MBI Fermentas, Hanover, MA). Single-stranded cDNA was then amplified by PCR with specific primers of COX-2 and hypoxanthine phospho ribosyl trans-

ferase (HPRT). The primers used were as follows: COX-2, 5'-TGAAG ACCAGGAGTACAGC-3' and 5'-GGTACAGTTCCATGACATCG-3'; HPRT, 5'-GTTGGATACAGGCCAGACTTTGTTG-3' and 5'-AG CCTATAGCCAGCCT ACCCTC-3'. HPRT was used as an internal control to evaluate relative expression of COX-2.

NF- κ B Reporter Assay on Macrophages

Macrophages were isolated from HLL mice. After 4 h of incubation with MTEC transfected with Ad-cIKK2 and Ad- β -gal, cells were collected, and lysed with freshly reconstituted luciferase assay buffer. The luciferase activity is measured by Luciferase Reporter Assay System (Promega Corporation) following manufacturer's instructions. Luciferase activity was normalized for total protein content. Since the MTEC cells in our experiments do not carry the NF- κ B-driven luciferase transgene, all of the luciferase activity is attributed to NF- κ B activation in the BMDM from the HLL mice in these experiments.

PGD $_2$ Assay

After 6 h of macrophage incubation with MTEC transfected with Ad-cIKK2 and Ad- β -gal, the culture supernatant was collected and stored at –80°C. PGD $_2$ was determined by LC/MS-MS.

Statistical Analysis

Our statistical analyses were performed with Graph Pad InStat (Graph Pad Software, San Diego, CA), using an unpaired *t* test and ANOVA.

RESULTS

Experimental Design

Using primary MTEC and BMDM, we aimed to better understand the role of airway epithelial cells in initiating inflammatory responses and activating macrophages upon transmission of signals generated by epithelial cells. As summarized in Figure 1, we have activated MTEC cells through specific activation of NF- κ B pathway with Ad-cIKK2 transfection and generated inflammatory signals. To evaluate the activation status of macrophages upon exposure to activated epithelial cells, we have examined CD11b expression on macrophages, macrophage adherence to epithelial cell monolayer, phagocytic capacity and production of reactive oxygen species, NF- κ B activation, and NF- κ B-dependent gene expression in macrophages. To evaluate the involvement of adhesion molecule in the macrophage–epithelial cell interaction, we used the ICAM-1 knockout epithelial cells and anti-ICAM antibody treatment on epithelial cells to block ICAM-1.

cIKK2 Induces NF- κ B-Dependent Gene Expressions in Airway Epithelial Cells

NF- κ B pathway can be activated by delivering cIKK2 to the epithelial cells by adenoviral vector. Forty-eight hours after transfection with adenoviral vectors of MTEC (MOI of 100), expression of flag-tagged cIKK2 was observed by Western blot as well as by fluorescent immunohistochemistry (Figure 2A). Most of flag-tagged cIKK2 was expressed in the cytoplasm and cell membrane of the MTEC. The transfection efficiency of adenoviral vector to epithelial cells was more than 90%, which is comparable to other reports (7). To determine the expression of NF- κ B-dependent genes in airway epithelial cells after transgene expression, we measured the levels of mouse GRO- α (KC), TNF- α and IL-6, which are known to be NF- κ B-dependent genes, in supernatants of MTEC at 48 h after viral transfection with Ad-cIKK2 or control viruses (Ad- β -gal) (Figures 2B–2D). As shown, the expression of KC, IL-6, and TNF- α was increased significantly (*P* < 0.05) in cIKK2-activated epithelial cells compared with the controls.

Airway epithelial cells are one of the major sources of inflammatory mediators, such as PGE $_2$ in airway inflammatory

Figure 1. Illustration of experimental design.

diseases. COX-2 is the rate-limiting enzyme of inducible PGE₂ production (17, 18). NF- κ B is one of the key transcriptional factors involved in the expression of COX-2. We measured the PGE₂ levels in MTEC culture supernatant as an indicator of COX-2 induction and NF- κ B activation using LC/MS-MS. There was a substantial amount of PGE₂ production at basal level both in control epithelial cells and Ad- β -gal-transfected cells, which was expected, since it is already known that airway epithelial cells have a constitutive expression of COX-2 expression. However, cIKK2-transfected airway epithelial cells produced nearly two times the amount of PGE₂ compared with Ad- β -gal-transfected or untreated cells (Figure 2E). We were unable to detect any PGD₂ in the MTEC culture supernatant (data not shown). To evaluate activation of NF- κ B in epithelial cells, we used MTEC from HLL mice, in which luciferase activity serves as an *in vitro* surrogate marker of NF- κ B activation. cIKK2-transfected MTEC showed a significant increase in luciferase activity ($P < 0.05$, $n = 3$) compared with the control MTEC that were transduced with Ad- β -gal or with media (Figure 2F). These data assure the efficacy and fidelity of cIKK2 transfection and activation of airway epithelial cells and its subsequent signaling and interaction with macrophages in our system.

Airway Epithelial Cells Induced CD11b Expression on Macrophages

CD11b expression by macrophages is functionally crucial for recruitment of macrophages during inflammation (19) and can be used as a marker of macrophage activation (20, 21). Increased expression of CD11b is an effector mechanism that enhances macrophage participation in specific immune response by co-operation with other immune cells. Induction of CD11b is also associated with cell-to-cell interactions between macrophages and epithelium (20). First, as a positive control, we directly stimulated macrophages with LPS and measured CD11b expression at 30 and 60 min. CD11b expression by LPS-stimulated BMDM was elevated significantly at both time points, compared with baseline (Figure 3A). Next, we incubated macrophages with cIKK2-activated MTEC that also showed significantly increased expression of CD11b, compared with the control macrophages that were incubated with MTEC transfected with Ad- β -gal (Figures 3B and 3C).

Macrophage–Epithelial Cell Crosstalk in the Airways Is through Interaction of ICAM-1/CD11b

From the above data, it is evident that some epithelial ligands produced by NF- κ B activation on epithelial cells triggered the CD11b receptor expression on macrophages. Next, we investigated whether the epithelial counter part of macrophage CD11b, ICAM-1, was expressed by MTEC cell in response to cIKK-2 activation of NF- κ B. ICAM-1 is already known to be expressed in airway epithelial cells in an NF- κ B-dependent manner (22, 23) and is a key determinant of epithelial and immune cell adhesion (24, 25). Initial studies were done to show that there was induction of ICAM-1 gene expression in MTEC cells after NF- κ B activation through transfection with Ad-cIKK2 by showing increased production of immunoreactive ICAM-1 by Western blot (Figure 4D). Hence, we examined the involvement of epithelial ICAM-1 on the expression of CD11b on macrophages during cellular contact. MTEC cells from ICAM-1 null or wild-type mice were co-cultured with wild-type BMDM. The epithelial cells were transfected and activated with Ad-cIKK2, and after transgene expression the BMDM and MTEC were incubated together for 1 h. CD11b expression on macrophages was measured as mean fluorescent intensity. The macrophages incubated with ICAM-1 knockout epithelial cells showed a significant decreased CD11b expression than those with wild-type epithelial cells (Figures 4A and 4C), suggesting that the communication between these two cell types depends on interaction of CD11b and ICAM-1 on their cell surface. Similar data were observed when this experiment was repeated with ICAM-1-blocking antibody (Figure 4B).

Activated Epithelial Cells Lead to Macrophage Adherence to Epithelial Monolayer

The interaction of CD11b on macrophages and ICAM-1 on the epithelial cells is one of the reasons responsible for the firm sticking of macrophages to the epithelium. Firm adhesion of macrophage is the physiologic consequence of the CD11b/ICAM-1 interaction. In our experiment, macrophages showed an increase in adhesion to activated epithelial monolayer, by scanning electron microscopy, even after a very short time period of 1 h exposure (Figures 5A and 5B). We quantified the adherent macrophages to the MTEC monolayer by CD68 staining of the Transwell membrane. Macrophages were counted in 10 randomly

Figure 2. Ad-clKK2 transfection activates NF- κ B pathway in airway epithelial cells. MTEC cells were cultured for 14 d in Transwell inserts and transfected with ad-clKK2, ad- β gal (MOI 1:10) or medium alone. (A) After 48 h of transfection, flag-tagged cIKK2 was detected by Western blot and immunohistochemistry. Flag-tagged cIKK2 expression (red) is observed in the cytoplasm and on the cell membrane. The blue color here is the DAPI-stained nucleus. (B–D) At 48 h after transfection with Ad-clKK2, Ad- β gal, or medium alone, production of KC (B), IL-6 (C), and TNF- α (D) was assayed from culture media by using Bio-plex mouse cytokine kit (Bio-Rad). PGE₂ production (E) was measured from MTEC culture supernatants by LC/MS-MS method. Luciferase activity (F) was measured from MTEC after viral transduction as a measure of NF- κ B activation status in epithelial cells. The data are the mean $\pm$ SD of samples from three separate experiments. (* P < 0.05, n = 3).

selected low-power fields. The percentage of macrophages adhering to epithelial cells was calculated by counting the total number of cells to those that were positive for CD68. The percentage of labeled macrophages adhering to cIKK2-activated epithelial cells was about three times higher than that of macrophages adhering to the control epithelial cells (Figures 5C and 5D). These data demonstrate that macrophages preferentially adhere to activated airway epithelial cells, which could have a role in mediating the inflammatory processes.

Airway Epithelial Cells Activate the NF- κ B Pathway in Macrophages, Resulting in COX-2 Gene Expression and Production of PGD₂

COX-2 protein is not constitutively expressed but can be induced by treatment with LPS in macrophages. Unlike airway epithelial cells, which produced mostly PGE₂ (Figure 2C), macrophages produced mainly PGD₂ in response to treatment with LPS, via the COX-2 enzymatic pathway (Figures 6A and 6B). Thus, we used COX-2 gene expression and PGD₂ production as an

Figure 3. Airway epithelial cells induced CD11b expression on macrophages by direct cellular contact. (A) The level of CD11b on RAW cells was measured at 30 min and 1 h after LPS stimulation as a positive control. The *left panel* is isotype control as a negative population. The *right panels* represent cells without LPS, and with LPS 30 min and 1 h, respectively. (B) The expression of CD11b on BMDM assayed after co-incubation with epithelial cells for 1 h. The *left panel* represents the control experiment, and *right panels* are vector control (Ad-β-gal) and Ad-cIKK2-activated groups, respectively. This figure is a representative diagram of three experiments with similar findings. (C) The graph shows the mean and SEM of the fluorescent intensity of CD11b expression of BMDM exposed to MTEC that were treated with control (Ad-β-gal) and Ad-cIKK2 vectors from three independent experiments * $P < 0.05$; each experiment used pooled cells from 2–3 mice.

indicator of NF-κB activation in macrophages. To evaluate activation of NF-κB in macrophages that were exposed to activated epithelial cells, we used BMDM from HLL mice, in which luciferase activity serves as an *in vitro* surrogate marker of NF-κB activation of macrophages (12, 26). HLL macrophages briefly exposed to cIKK2-transfected MTEC (wild-type MTEC without any luciferase activity) showed a significant increase in luciferase activity ($P < 0.05$, $n = 3$) compared with the control MTEC that were exposed to Ad-β-gal or were treated with PBS (Figure 6C).

Next, we measured expression of COX-2, an NF-κB-dependent gene, in macrophages. COX-2 is one of the key inflammatory genes in macrophages that are transcriptionally regulated by NF-κB. COX-2 mRNA was undetectable in macrophages incubated with untreated epithelial cells as well as in Ad-β-gal-transfected ones. However, macrophages that were incubated with Ad-cIKK2-transfected epithelial cells expressed increased steady-state COX-2 mRNA (Figure 6D), demonstrating epithelial cell and macrophage interaction. As a consequence of COX-2 activation of macrophages that were exposed to activated MTEC, we observed increased production of PGD₂ in co-culture supernatant (Figure 6E).

Phagocytic Activity and ROS Production by Macrophages Is Not Altered by Contact with Activated Epithelial Cells

Phagocytosis and ROS production are key innate immune functional responses of macrophages. We measured changes in

phagocytic activity of macrophages in the context of cellular interaction with epithelial cells by examining uptake of fluorescent beads. As a positive control, we first determined that the phagocytic activity of macrophages was effectively increased by stimulating the macrophages with LPS for a very short period (Figure 7A). Next we exposed macrophages to the MTEC that were treated with Ad-cIKK-2. Compared with macrophages treated with MTEC transfected with Ad-β-gal or treated with PBS, we were unable to detect an increase in phagocytosis in macrophages co-incubated with NF-κB-activated epithelial cells (Figures 7B and 7C). Similarly there was no significant increase in ROS production from macrophages co-cultured with MTEC transfected with ad-cIKK2 (Figure 7D). Thus, it appears that activation of macrophages by MTEC has some functional selectivity.

DISCUSSION

In the present study, we examined how airway epithelial cells communicate with macrophages in a biologically relevant *in vitro* system. Our data demonstrate here that airway epithelial cells direct an ICAM-1-dependent process of intercellular communication by initiating inflammatory signals that contribute to the activation of macrophages. In addition, the present study shows that epithelial cells are able to induce integrin expression on

Figure 4. The crosstalk between macrophages and airway epithelial cells is through ICAM-1. (A) Wild-type macrophages were incubated with ICAM-1-deficient epithelial cells, which were transfected with Ad-cIKK2 before exposure to macrophage. Macrophages were collected and analyzed for CD11b expression. (B) Similar experiment was repeated by using ICAM-1-blocking antibody on wild-type epithelial cells. After cIKK2 transfection, wild-type MTEC were treated with anti-ICAM-1 antibody for 1 h and then co-cultured with macrophages as explained above. (C) Graph here is the comparative representation of the mean fluorescent intensities of CD11b expression by WT BMDM co-cultured with WT MTEC and ICAM-1-deficient MTEC. Both the cell types were transfected and activated with Ad-cIKK2 before co-culture ($n = 3$, $*P = 0.05$). (D) WB showing ICAM-1 protein expression on MTEC after NF- κ B activation with Ad-cIKK2 (lane 3). LPS-treated total lung homogenate is loaded as positive control (lane 2). Lane 1 is cell lysate from untreated MTEC, and lane 4 from MTEC that were treated with the Ad- β -gal vector.

macrophages, resulting in the attachment of macrophages to the epithelial cell surface. Activated epithelial cells can trigger NF- κ B activation in macrophages, with subsequent proinflammatory gene expression. Interestingly, the interaction of epithelial cells with macrophages leads to an altered phenotype of the macrophages in a function-specific manner, such as expression of CD11b, adherence to the epithelial layer, expression of COX-2, and synthesis of PGD₂, but does not alter phagocytic activity or production of ROS. This suggests that the interaction between these two cell types is function specific. Our study could explain the underlying mechanism of our previous observations in which selective single-cell activation of airway epithelial cells is enough to generate full-blown lung inflammation like ARDS (7).

The experimental design is more relevant than previous models, in terms of using primary rather than immortalized cells, the method of activation of epithelial cells that avoids direct activation of both epithelial cells and macrophages, and use of biologically relevant endpoints. We have used mouse primary BMDM and airway epithelial cells and developed a model mimicking normal airspace. Since BMDM and alveolar macrophages have a common ancestry, we think that our studies using BMDM are biologically relevant. In addition, mature alveolar macrophages may not reflect the interactions of newly recruited monocytes in an inflammatory milieu. By exposing only the apical epithelial surface for macrophages and allowing them to commu-

nicate, interact, and adhere to epithelial cells, we could simulate the interaction between airway epithelial cells and macrophages in normal airspace, which allowed us to evaluate cellular functions in a manner that is similar to airways *in situ* by preserving their structural and functional characteristics. The methodology adopted here for epithelial cell culture has the advantage of preserving important biological properties of airway epithelial cells such as cilia formation, distinct polarization, tight junctions, and extensive cytoplasmic extensions (13).

There are only few studies that have investigated the interaction between airway epithelial cells and macrophages upon stimulation with various agents (20, 27–29). Most of these studies have focused on determining the synergistic effect of mixed cultures of both cell types in same liquid media. Fujii and colleagues co-cultured human bronchial epithelial cells and alveolar macrophages *ex vivo* and showed that exposure of these cells to particulate matter led to the production of several cytokines and chemokines (30). However, they did not address the issue of which cells were the primary responders to the stimuli. It is likely true that macrophages and epithelial cells make a joint contribution to the inflammatory milieu of the airway; but the direction of the interaction has important therapeutic implications.

In this study, we have focused mainly on the contact-dependent activation of macrophages with airway epithelial cells to evaluate

Figure 5. Macrophages showed increased adherence to NF- κ B-activated airway epithelial cells. (A) Scanning electron microscopy showing the cilia and microvilli of MTEC growing on Transwell membranes. (B) Adherent macrophages (indicated by arrows on top of MTEC after co-culture with NF- κ B-activated epithelial cells). (C and D) Immunolocalization of macrophages attached to epithelial membrane by using CD68 staining (red) after co-incubation with epithelial cells. (C) Unattached macrophages were washed off and the membrane was fixed and stained with CD68 antibody. (D) The data was quantified as the number of adherent CD68-staining cells attached to the epithelial monolayer in 10 low-power fields (LPF) (* $P < 0.05$).

the efficacy of epithelial cells to transmit signals and activate macrophages. The cell-to-cell contact is particularly important for modulating the phenotype and functions of human mononuclear phagocytes (31). We have studied unidirectional signaling pathway from airway epithelial cells to macrophages by activating cIKK2 expression in MTEC specifically before macrophage exposure, thereby turning on the NF- κ B-dependent gene expression in the epithelial cells, which could then interact with macrophages.

We have previously shown that adenovirus-mediated gene transfer is highly efficient and specific to airway epithelial cells (7). Adenoviral vectors are well known to have preferential tropism for epithelial cells, even though they may activate macrophages directly. In our previous study we have shown that transgene expression using adenoviral vectors is confined to airway epithelial cells and is not expressed in lung lavage cells in mice (7). Here, we have washed epithelial cells vigorously after transfection to make viral vector-free media during incubation, and macrophage exposure to epithelial cells was restricted to a very short time period (1–2 h), which is not a sufficient time period for expression of viral transgene in macrophages (this requires 48–72 h). Thus, the possibility for adenoviral vectors to express the transgene in macrophages and to modify the study endpoints was effectively ruled out.

NF- κ B-activated airway epithelial cells can be a source of proinflammatory mediators that are important for directing innate immune responses in the lungs (32–34). Interestingly, our data of mediator profile produced by the epithelial cells after cIKK2 activation followed the same pattern of that of the cytokines found to be up-regulated in the whole lung from Ad-cIKK2-transfected mice in our previous publication (5). This finding suggests that increased production of these mediators in lung is a direct result of epithelial cell NF- κ B activation. NF- κ B regulates the epithelial expression of multiple genes important to airway inflammation, including genes encoding cytokines, chemokines, and adhesion molecules (35). In addition to cytokines and chemokines, airway epithelial cells also produce lipid mediators such as PGE₂, as a result of activation of COX-2 in epithelial cells, creating a proinflammatory environment in the lung.

Induction of CD11b makes macrophages more receptive to provide effector mechanisms and helps them to participate with other cells in immune responses (20). In previous studies, the rapid influx of monocytes and neutrophils into the lungs and alveoli after exogenous stimulation has been shown to be dependent on CD11b expression (36, 37). CD11b blockade resulted in reduced number of alveolar macrophages in a pneumonia model (19), suggesting the role of CD11b in recruiting macrophages

Figure 6. COX-2 and PGD₂ synthesis from macrophages. (A and B) RAW cells were treated with LPS alone and LPS along with the selective COX-2 inhibitor, SC58236. COX-2 protein expression and prostaglandin synthesis were analyzed. As shown, more PGD₂ than PGE₂ is produced by COX-2 activation in RAW macrophages. (C) BMDM isolated from HLL mice (NF- κ B reporter mice) was co-incubated with epithelial cells transfected with either Ad-cIKK2 or Ad- β -gal. Bioluminescent detection of luciferase activity was measured to evaluate NF- κ B activity (* P < 0.05, n = 5). BMDM exposed to cIKK-transfected MTEC showed increased NF- κ B luciferase activity. (D) BMDM were co-incubated with tracheal epithelial cells, which were transfected either Ad-cIKK2 or Ad- β -gal as described in EXPERIMENTAL DESIGN. After 2 h co-incubation, the macrophages were harvested and analyzed for COX-2 mRNA by RT-PCR. HPRT mRNA was also measured as loading control. (E) PGD₂ was measured from supernatants of co-culture of macrophages and airway epithelial cells transfected either cIKK2 or β -gal by LC-MS/MS method. PGE₂ was not detected in the culture supernatant. The data are the mean $\pm$ SD of seven separate samples measured at two different times. * P < 0.05.

into lung. However, the mechanism of regulation of CD11b expression on macrophages in the context of cellular inflammation is not yet clear. Our results show that the activation status of epithelial cells can affect CD11b expression on macrophages. We believe this is through the crosstalk with adhesion molecules on epithelial cells. ICAM-1 is expressed on bronchiolar and alveolar epithelial cells after LPS challenge through NF- κ B activation, and expressed ICAM-1 is biologically active (32) and is associated with enhanced recruitment and transepithelial migration of neutrophils. Our results showed that depletion of ICAM-1 on epithelial cells diminished the induction of CD11b on macrophages, suggesting that ICAM-1 mediated CD-11b up-regulation

on macrophages. The number of macrophages attached to epithelial monolayer is increased in 60% when macrophages were exposed to NF- κ B-activated epithelial cells. It appears that airway epithelial cell-macrophage adhesion depends on the expression of ICAM-1 on the epithelial cell surface and the concomitant levels of expression and activation of CD11b on the macrophage surface. Even though we focused on contact-dependent interaction between the cells, there is the possibility of soluble mediators produced by MTEC after NF- κ B activation could have an influence on macrophages. To minimize this influence, we have washed the MTEC before co-culture and have limited the interactions to only 1 h.

Figure 7. Phagocytic activity and ROS production are not significantly altered by contact with activated epithelial cells. Phagocytosis was assayed by flow cytometry using FITC-labeled latex beads and expressed as mean fluorescent intensity. (A) BMDM treated with LPS (1 μ g/ml) for 30 min, is used here as a positive control for phagocytosis. (B) BMDM cells were incubated with epithelial cells transfected with Ad-clKK2, Ad- β -gal, or media alone for 1 h. Then macrophages were collected and assayed for phagocytosis. (C) The graph is a representation of phagocytic assays done with macrophages after co-culture ($n = 4$). The difference of phagocytic function between Ad- β -gal group and Ad-clKK2 group shown in B is statistically insignificant. (D) ROS production was measured after co-culturing BMDM with activated MTEC and expressed as relative light units (RLU). Statistically, there was no significant difference between groups ($n = 5$) with regard to ROS production.

The COX-2 promoter contains two putative NF- κ B-binding sites, and it has been shown that NF- κ B is a positive regulator of COX-2 expression in LPS-induced macrophages (38). In our system, contact-dependent activation of macrophages through activated epithelial cells showed enhanced NF- κ B expression evidenced through luciferase activity. This could explain the COX-2 mRNA expression in macrophages followed by PGD2 synthesis in the co-culture supernatants of clKK2-activated MTEC and macrophages.

Interestingly, phagocytic property of the macrophages and ROS production were not significantly changed by incubation with epithelial cells. This finding was intriguing, since macrophages are professional phagocytes and expected to be activated more with external stimuli as is seen with LPS stimulation in our experiments. We have exposed macrophages for 1 h, and this might not be sufficient to make a notable change in their activity with regard to phagocytosis and ROS production. We have used latex beads in our assay that presumably are phagocytized via scavenger receptors. We cannot generalize this observation to other particles, since phagocytosis is a receptor-specific phenomenon. Different types of receptors participate in recognition and internalization of particles during phagocytosis. We think that the amplified COX-2 message level and enhanced luciferase activity in macrophages clearly demonstrate here that macrophages are stimulated upon contact with activated epithelial cells.

Together, these data suggest that airway epithelial cells play an important role in stimulating and activating immune and inflammatory cells, rather than performing the barrier function in the lung.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

- Poynter ME, Irvin CG, Janssen-Heininger YA. A prominent role for airway epithelial NF-kappa B activation in lipopolysaccharide-induced airway inflammation. *J Immunol* 1995;170:6257–6265.
- Dale BA. Periodontal epithelium: a newly recognized role in health and disease. *Periodontol* 2002;30:70–78.
- Hedges SR, Agace WW, Svanborg C. Epithelial cytokine responses and mucosal cytokine networks. *Trends Microbiol* 1995;3:266–270.
- Greene CM, Carroll TP, Smith SG, Taggart CC, Devaney J, Griffin S, O'Neill SJ, McElvaney NG. TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. *J Immunol* 1995;174:1638–1646.
- Saito T, Yamamoto T, Kazawa T, Gejyo H, Naito M. Expression of toll-like receptor 2 and 4 in lipopolysaccharide-induced lung injury in mouse. *Cell Tissue Res* 2005;321:75–88.
- Severgnini M, Takahashi S, Rozo LM, Homer RJ, Kuhn C, Chung JW, Perides G, Steer M, Hassoun PM, Fanburg BL, et al. Activation of the STAT pathway in acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L1282–L1292.
- 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* 2003;170:1091–1098.
- Poynter ME, Cloots R, van Woerkom T, Butnor KJ, Vacek P, Taatjes DJ, Irvin CG, Janssen-Heininger YM. NF-kappa B activation in airways modulates allergic inflammation but not hyperresponsiveness. *J Immunol* 2004;173:7003–7009.
- Broide DH, Lawrence T, Doherty T, Cho JY, Miller M, McElwain K, McElwain S, Karin M. Allergen-induced peribronchial fibrosis and mucus production mediated by Ikkappa B kinase beta-dependent genes in airway epithelium. *Proc Natl Acad Sci USA* 2005;102:17723–17728.
- Kadowaki T, Nakayama K, Okamoto K, Abe N, Baba A, Shi Y, Ratnayake DB, Yamamoto K. Porphyromonas gingivalis proteinases as virulence determinants in progression of periodontal diseases. *J Biochem (Tokyo)* 2000;128:153–159.
- Blackwell TS, Yull FE, Chen CL, Venkatakrishnan A, Blackwell TR, Hicks DJ, Lancaster LH, Christman JW, Kerr LD. Multiorgan nuclear factor kappa B activation in a transgenic mouse model of systemic inflammation. *Am J Respir Crit Care Med* 2000;162:1095–1101.
- Sadikot RT, Jansen ED, Blackwell TR, Zoia O, Yull F, Christman JW, Blackwell TS. High-dose dexamethasone accentuates nuclear factor-kappa b activation in endotoxin-treated mice. *Am J Respir Crit Care Med* 2001;164:873–878.
- You YE, Richer J, Huang T, Brody SL. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L1315–L1321.
- Celada AP, Gray W, Rinderknecht E, Schreiber RD. Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity. *J Exp Med* 1984;160:55–74.
- Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, et al. IKK-1 and IKK-2:

- cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* 1997;278:860–866.
16. Djaldetti M, Salaman H, Bergman M, Djaldetti R, Bessler H. Phagocytosis—the mighty weapon of the silent warriors. *Microsc Res Tech* 2002; 57:421–431.
 17. Tanabe T, Tohnai N. Cyclooxygenase isozymes and their gene structures and expression. *Prostaglandins Other Lipid Mediat* 2002;68–69:95–114.
 18. Elder DJ, Halton DE, Hague A, Paraskeva C. Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clin Cancer Res* 1997;3:1679–1683.
 19. Kirby AC, Raynes JG, Kaye PM. CD11b regulates recruitment of alveolar macrophages but not pulmonary dendritic cells after pneumococcal challenge. *J Infect Dis* 2006;193:205–213.
 20. Striz I, Slavcev A, Kalanin A, Jaresova M, Rennard SI. Cell-cell contacts with epithelial cells modulate the phenotype of human macrophages. *Inflammation* 2001;25:241–246.
 21. Gonzalez-Juarrero M, Shim TS, Kipnis A, Junqueira-Kipnis AP, Orme IM. Dynamics of macrophage cell populations during murine pulmonary tuberculosis. *J Immunol* 2003;171:3128–3135.
 22. Lin FS, Lin CC, Chien CS, Luo SF, Yang CM. Involvement of p42/p44 MAPK, JNK, and NF-kappaB in IL-1beta-induced ICAM-1 expression in human pulmonary epithelial cells. *J Cell Physiol* 2005;202: 464–473.
 23. Krunkosky TM, Martin LD, Fischer BM, Voynow JA, Adler KB. Effects of TNF alpha on expression of ICAM-1 in human airway epithelial cells in vitro: oxidant-mediated pathways and transcription factors. *Free Radic Biol Med* 2003;35:1158–1167.
 24. Floreani AA, Wyatt TA, Stoner J, Sanderson SD, Thompson EG, Allen-Gipson D, Heires AJ. Smoke and C5a induce airway epithelial intercellular adhesion molecule-1 and cell adhesion. *Am J Respir Cell Mol Biol* 2003;29:472–482.
 25. Holtzman MJ, Morton JD, Shornick LP, Tyner JW, O'Sullivan MP, Antao A, Lo M, Castro M, Walter MJ. Immunity, inflammation, and remodeling in the airway epithelial barrier: epithelial-viral-allergic paradigm. *Physiol Rev* 2002;82:19–46.
 26. Sadikot RT, Zeng H, Yull FE, Li B, Cheng DS, Kernodle DS, Jansen ED, Contag CH, Segal BH, Holland SM, *et al.* p47phox deficiency impairs NF-kappa B activation and host defense in *Pseudomonas* pneumonia. *J Immunol* 2004;172:1801–1808.
 27. Bodet C, Chandad F, Grenier D. Modulation of cytokine production by *Porphyrromonas gingivalis* in a macrophage and epithelial cell co-culture model. *Microbes Infect* 2005;7:448–456.
 28. Rothen-Rutishauser BM, Kiama SG, Gehr P. A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. *Am J Respir Cell Mol Biol* 2005;32:281–289.
 29. Chakraborty A, Li L, Chakraborty NG, Mukherji B. Stimulatory and inhibitory differentiation of human myeloid dendritic cells. *Clin Immunol* 2000;94:88–98.
 30. Fujii T, Hayashi S, Hogg JC, Mukae H, Suwa T, Goto Y, Vincent R, van Eeden SF. Interaction of alveolar macrophages and airway epithelial cells following exposure to particulate matter produces mediators that stimulate the bone marrow. *Am J Respir Cell Mol Biol* 2002;27:34–41.
 31. DiMango E, Zar HJ, Bryan R, Prince A. Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. *J Clin Invest* 1995;96:2204–2210.
 32. Melotti P, Nicolis E, Tamanini A, Rolfini R, Pavirani A, Cabrini G. Activation of NF-kB mediates ICAM-1 induction in respiratory cells exposed to an adenovirus-derived vector. *Gene Ther* 2001;8:1436–1442.
 33. Poynter ME, Irvin CG, Janssen-Heininger YM. Rapid activation of nuclear factor-kappaB in airway epithelium in a murine model of allergic airway inflammation. *Am J Pathol* 2002;160:1325–1334.
 34. Karin M, Yamamoto Y, Wang QM. The IKK NF-kappa B system: a treasure trove for drug development. *Nat Rev Drug Discov* 2004;3: 17–26.
 35. Maus UA, Koay MA, Delbeck T, Mack M, Ermert M, Ermert L, Blackwell TS, Christman JW, Schlondorff D, Seeger W, *et al.* Role of resident alveolar macrophages in leukocyte traffic into the alveolar air space of intact mice. *Am J Physiol Lung Cell Mol Physiol* 2002; 282:L1245–L1252.
 36. Mizgerd JP, Kubo H, Kutkoski GJ, Bhagwan SD, Scharffetter-Kochanek K, Beaudet AL, Doerschuk CM. Neutrophil emigration in the skin, lungs, and peritoneum: different requirements for CD11/CD18 revealed by CD18-deficient mice. *J Exp Med* 1997;186:1357–1364.
 37. Xing Z, Ohtoshi T, Ralph P, Gaudie J, Jordana M. Human upper airway structural cell-derived cytokines support human peripheral blood monocyte survival: a potential mechanism for monocyte/macrophage accumulation in the tissue. *Am J Respir Cell Mol Biol* 1992;6:212–218.
 38. D'Acquisto F, Iuvone T, Rombola L, Sautebin L, Di Rosa M, Carnuccio R. Involvement of NF-kappaB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. *FEBS Lett* 1997;418:175–178.