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Outer Membrane Protein A from *Klebsiella pneumoniae* Activates Bronchial Epithelial Cells: Implication in Neutrophil Recruitment

Muriel Pichavant,* Yves Delneste,† Pascale Jeannin,‡ Catherine Fourneau,* Anne Brichet,† André-Bernard Tonnel,*‡ and Philippe Gosset2*  

Aside from its mechanical barrier function, bronchial epithelium plays an important role both in the host defense and in the pathogenesis of inflammatory airway disorders. To investigate its role in lung defense, the effect of a bacterial cell wall protein, the outer membrane protein A from *Klebsiella pneumoniae* (kpOmpA) on bronchial epithelial cells (BEC) was evaluated on adhesion molecule expression and cytokine production. Moreover, the potential implication of this mechanism in kpOmpA-induced lung inflammation was also determined. Our in vitro studies demonstrated that kpOmpA strongly bound to BEAS-2B cells, a human BEC line, and to BEC primary cultures, resulting in NF-kB signaling pathway activation. Exposure to kpOmpA increased ICAM-1 mRNA and cell surface expression, as well as the secretion of IL-6, CXC chemokine ligand (CXCL)1, CXCL8, C-C chemokine ligand 2, CXCL10 by BEAS-2B cells, and BEC primary cultures \( (p < 0.005) \). We analyzed in vivo the consequences of intratracheal injection of kpOmpA to BALB/c mice. In kpOmpA-treated mice, a transient neutrophilia (with a maximum at 24 h) was observed in bronchoalveolar lavage and lung sections. In vivo kpOmpA priming induced bronchial epithelium activation as evaluated by ICAM-1 and CXCL1 expression, associated with the secretion of CXCL1 and CXCL5 in bronchoalveolar lavage fluids. In the lung, an increased level of the IL-6, IL-11, CXCL1, CXCL5, CXCL10 mRNA was observed with a maximum at 6 h. These data showed that kpOmpA is involved in host defense mechanism by its ability to activate not only APC but also BEC, resulting in a lung neutrophilia. *The Journal of Immunology,* 2003, 171: 6697–6705.
In contrast, OmpA involvement in lung inflammation, and particularly its action on BEC, is not documented. The purpose of this work was to identify potential interactions between kpOmpA and BEC in vitro and in vivo and secondarily the development of lung inflammation. Our results demonstrated that kpOmpA binds to BEC and induces adhesion molecule and cytokine expression by these cells. In vivo, kpOmpA triggers BEC activation and a neutrophil influx.

Materials and Methods

Cytokines, Abs, and other reagents

kpOmpA was expressed in *Escherichia coli* and purified as described as an endotoxin-free preparation (14). The following materials were purchased: DMEM/F12 medium (Invitrogen, Cergy Pontoise, France), airway epithelial cell growth and basal medium (Promocell, Heidelberg, Germany), antibiotics (penicillin G sodium, 10,000 U/ml; streptomycin sulfate, 10,000 mg/ml; and amphotericin B, 25 µg/ml); and 2 mM l-glutamine (Invitrogen), collagen G (3 mg/ml in 12 mM HCl, Biochrom, Berlin, Germany), FCS, Ultrastructure G, trypsin (containing 1 mM EDTA); agarose, PBS, TRIZol reagent (Life Technologies, Grand Island, NY), chloroform (Merck, Fenayen sous Bois, France) and isopropanol (Carlo Erba, Milan, Italy), gelstar (FMC bioproducts, Rockland, ME); and recombinant human TNF-α, IFN-γ (R&D Systems, Abingdon, U.K.); SB203580, RO318220, PD98059, genistein (Calbiochem, San Diego, CA), LY294002 (Sigma-Aldrich, St. Louis, MO). The following mouse mAb were used: anti-cytokeratin 5/6/18 Ab (NeoMarkers, Fremont, CA); anti-ICAM-1 mAb (IgG1), clone B159 and the isotype control (IgG1), clone MOPC 21 (BD Biosciences, Erembodegem, Belgium), as well as the secondary Abs FITC (IgG1), clone B159 and the isotype control (IgG1), clone MOPC 21 (BD Biosciences, Erembodegem, Belgium), as well as the secondary Abs FITC or PE-labeled streptavidin (Sigma-Aldrich). Neutralizing anti-human Toll-like receptor 2 (TLR)-2 and TLR-4 mouse mAb were purchased by eBioscience (San Diego, CA).

Cell culture

Human bronchial epithelial biopsies were obtained by fiberoptic bronchoscopy from patients who were being investigated for bronchopulmonary carcinoma. Biopsies were taken largely at a distance from the tumor. Histologic features of the bronchial mucosa were normal in all specimens. All procedures were reviewed and approved by Hospital Institutional Review Board and written informed consent was obtained from all subjects included in the study.

BEC were cultured as previously described (17). Briefly, one explant (~0.5 × 0.5 mm in size) was placed on sterile plastic dishes coated with collagen G matrix (type I and III collagen). After an adherence phase, explants were cultured in DMEM/F12 medium containing 2% Ultroser G, 1% antibiotics; and 2 mM l-glutamine. Culture medium was changed every 3–4 days. Explants were cultured until BEC were confluent. Then, explants were transferred three times to new dishes to initiate new BEC primary cultures. After transfer, confluent epithelial cells were incubated for 24 h with medium alone (negative control) and then incubated with kpOmpA in fresh medium. Epithelial phenotype (>96%) was confirmed by staining with anti-cytokeratin 5/6/18 Ab (data not shown).

BEAS-2B cells were obtained from the American Type Culture Collection (Manassas, VA). This cell line was derived from human bronchial epithelium transformed by an adenosivirus SV-40 hybrid virus. BEAS-2B cells were cultured in 75-cm² culture flasks, until passage 20, and maintained in BEC growth medium, containing 1% antibiotic solution. After seeding, 5 × 10⁴ cells were plated on six-well cluster plates (Costar, Cambridge, MA) with collagen G coating. Confluent cells were then cultured in BEC basal medium for 24 h.

Cell activation was achieved by addition of endotoxin-free recombinant kpOmpA (2–40 µg/ml) or a control glycoprotein such as BSA. In some experiments, TNF-α (200 U/ml) with or without IFN-γ (100 U/ml) were added in the presence or not of kpOmpA. Activation by LPS (serotype 055B5; 100 ng/ml) and kpOmpA in the presence or not of polymyxin B (50 U/ml) (Sigma-Aldrich) was also performed as a control. Supernatants were collected after 6 or 24 h incubation and cells were lysed by TRIZol reagent to prepare RNA.

Quantification of mRNA expression

Total RNA were purified and reverse transcription performed using oligo-dT primers and Superscript reverse transcriptase (Invitrogen). In BEAS-2B cells, mRNA expression for human IL-6, IL-18, CCL2, CCL5, CXCL8, CXCL10, and ICAM-1 was evaluated by real-time RT-PCR using GAPDH mRNA as a reference. In mouse lungs, mRNA expression for human IL-6, IL-18, CCL2, CXCL1, CXCL5, CXCL10, and ICAM-1 mRNA expression was determined by the same method using β-actin mRNA as a reference. The primer sequences and the size of the product are reported in Table I. After gel electrophoresis and staining with gelstar, the intensity of each band was determined by the same method using β-actin mRNA as a reference.

Table I. Primers for RT-PCR analysis of cytokine, chemokine, and adhesion molecule expression

<table>
<thead>
<tr>
<th>Target</th>
<th>Product (bp)</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGAPDH</td>
<td>206</td>
<td>5′-GTC TTC ACC ACC ATG GAG A-3′</td>
</tr>
<tr>
<td>mβ-actin</td>
<td>353</td>
<td>5′-GTC GGG GGG CCC CAG GCA CCA-3′</td>
</tr>
<tr>
<td>hIL-6</td>
<td>260</td>
<td>5′-GTC GGG GGG CCC CAG GCA CCA-3′</td>
</tr>
<tr>
<td>hIL-18</td>
<td>512</td>
<td>5′-GTC GGG GGG CCC CAG GCA CCA-3′</td>
</tr>
<tr>
<td>hCCL2</td>
<td>274</td>
<td>5′-GTC GGG GGG CCC CAG GCA CCA-3′</td>
</tr>
<tr>
<td>hCXCL8</td>
<td>247</td>
<td>5′-GTC GGG GGG CCC CAG GCA CCA-3′</td>
</tr>
<tr>
<td>hCXCL10</td>
<td>210</td>
<td>5′-GTC GGG GGG CCC CAG GCA CCA-3′</td>
</tr>
<tr>
<td>hICAM-1</td>
<td>375</td>
<td>5′-GTC GGG GGG CCC CAG GCA CCA-3′</td>
</tr>
<tr>
<td>mIL-6</td>
<td>352</td>
<td>5′-GTC GGG GGG CCC CAG GCA CCA-3′</td>
</tr>
<tr>
<td>mCCL2</td>
<td>273</td>
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</tr>
<tr>
<td>mCXCL1</td>
<td>530</td>
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<tr>
<td>mCXCL5</td>
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<td>mCXCL10</td>
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</tr>
<tr>
<td>mICAM-1</td>
<td>432</td>
<td>5′-GTC GGG GGG CCC CAG GCA CCA-3′</td>
</tr>
</tbody>
</table>

a. h, human; m, mouse.
were expressed as a ratio: gene of interest/GAPDH or β-actin mRNA for the human or the mouse genes, respectively.

**Flow cytometric analysis**

BEC were briefly treated with trypsin solution and, after neutralization of proteinase activity, removed from plates by repeated pipetting. The binding of kpOmpA on BEC was evaluated by their incubation for 30 min at 37°C with biotinylated-kpOmpA (20 μg/ml) or -tetanus toxin C (20 μg/ml) as a control. After washings, cells were incubated with PE-labeled streptavidin for another 30 min and then washed twice. To block the binding of kpOmpA, neutralizing anti-TLR-2 and anti-TLR-4 mAbs (20 μg/ml) were preincubated with BEAS-2B cells for 30 min before the addition of biotinylated kpOmpA.

To assess the modulation of ICAM-1 expression on BEAS-2B cells, cells were stimulated in presence or not of kpOmpA (8, 20, or 40 μg/ml) for 24 h. FITC-labeled anti-ICAM-1 mAb and the isotype control were added for 30 min to cells, and then washed twice. Cells were resuspended and fixed in PBS with 0.25% paraformaldehyde. Fluorescence was analyzed on 10,000 events using a flow cytometer (FACSCalibur; BD Biosciences). Previous experiments showed that BEC treatment with trypsin did not affect ICAM-1 expression compared with cells detached with PBS plus EDTA 2 mM (data not shown).

Results were expressed as difference mean fluorescent intensity (MFI) with specific Ab minus the isotype control MFI (ΔMFI).

**Cytokine measurement**

Concentrations of human IL-6, CCL2, CCL5, CXCL1, CXCL8, and CXCL10 in BEC culture supernatants were determined by sandwich enzyme immunoassay (R&D Systems). Levels of murine IL-10, IL-12, IFN-γ, TNF-α, CXCL1, and CXCL5 (R&D Systems) were determined by ELISA in bronchoalveolar lavage (BAL) fluids.

**EMSA**

BEAS-2B cells were cultured in medium alone, or in presence of TNF-α (200 U/ml) (positive control) or kpOmpA (5, 20, and 40 μg/ml) for 2 h. After washing, nuclear proteins were extracted by standard procedures (18). The probes used for the gel retardation assay contained the consensus κB (5′-CAG CGG CAG GGG AAT TCC CCT CTC CTT AGG TT-3′) binding site. The 5′ end 13P-labeling of the double-stranded oligonucleotide and EMSA were performed by standard procedures. Membranes were exposed to a PhosphoImager screen (Molecular Dynamics, Sunnyvale, CA) and the intensity of the bands were quantified by using a computer image-analysis system.

**Transient transfection assays**

BEAS-2B cells, grown to 70–80% confluence in BEC growth medium and starved during 24 h in BEC basal medium, were transiently transfected by lipofection using lipofectamin (Invitrogen) technique with reporter and expression GL3 plasmids containing or not a tandem repeat of an NF-κB site and luciferase promoter. Luciferase assays were performed 24 h after transfection as previously described (19).

**kpOmpA intratracheal injection in mice**

Six- to 10-wk female BALB/c mice were anesthetized by i.p. injection of avertin (Sigma-Aldrich) (2.5% v/v in PBS). Fifty microliters of BSA (100 μg) or kpOmpA solution (20 or 100 μg) were administrated intratracheally under direct vision through the opening vocal cords using a 23G metal catheter connected to the outlet of a micropipette. Mice were sacrificed after 6 h, 1, 2, 3, and 7 days, and BAL fluids were performed by PBS instillation (1 ml).

**Immunohistochemistry for ICAM-1 and CXCL1 expression in mice lung tissue**

Lung specimens were fixed with Immunohistofix and embedded in Immunoahistowax (Aphase, Mornmont, Belgium). After permeabilization, sections were overlaid overnight with rabbit anti-mouse ICAM-1 (BD Biosciences) or CXCL1 Abs (R&D Systems). Ab binding was detected after a 2-h incubation with biotin-conjugated goat anti-rabbit IgG Ab (dilution, 1/400) at room temperature, followed by extravidin-alkaline phosphatase incubation (dilution, 1/200; Sigma-Aldrich) for 30 min. Color development was obtained with a Fast-Red solution (Sigma-Aldrich). Slides were washed three times between each step. Gill’s hematoxylin was used to counterstain. Leukocyte infiltrate was shown by May-Grunwald Giemsa staining (Sigma-Aldrich) on lung sections.
higher than in medium alone, in the presence of 8, 20, and 40 μg/ml kpOmpA, respectively.

kpOmpA induces cytokine mRNA expression and secretion in BECs

Effect of kpOmpA on cytokine production by BEAS-2B cells and BEC primary cultures was analyzed. kpOmpA effects on mRNA expression for CCL2, CXCL8, CXCL10, IL-6, and IL-18 were quantified in BEAS-2B cells by RT-PCR. Cytokine mRNA transcripts were undetectable in resting BEAS-2B cells, except for IL-18, as shown in Fig. 3A. However, CCL2, CXCL8, CXCL10, and IL-6 mRNA levels increased in a dose-dependent manner after treatment with 8 and 20 μg/ml kpOmpA. Cytokine mRNA levels reached a maximum at 6 h postexposure, and persisted at 24 h. Such modulation was not observed for IL-18 (Fig. 3A).

kpOmpA-induced mRNA expression was associated with the dose-dependent production of CCL2, CXCL1, CXCL8, CXCL10, and IL-6 release, in BEAS-2B cell supernatants after 24 h of stimulation (p < 0.01 for these cytokines) (Fig. 3B). CXCL1 and CXCL8 production was strongly induced by 480- and 110-fold, respectively, in the presence of 20 μg/ml kpOmpA compared with medium alone. Moreover, treatment with polymyxin B (an inhibitor of endotoxin activation) did not affect CCL2 and CXCL8 production, whereas it neutralized the effect of 10 ng/ml LPS (data not shown). Similar results were obtained with BEC primary cultures: kpOmpA (20 μg/ml) increased significantly the production of CCL2, CXCL1, CXCL10, and IL-6 (p < 0.05), and CXCL8 (p < 0.01) compared with unstimulated cells after 24 h (Fig. 3C). Taken together, these data show that kpOmpA triggers CCL2, CXCL8, CXCL10, and IL-6 mRNA expression and secretion in BEC.

kpOmpA activates intracellular signaling pathway in BEAS-2B cells

To investigate transduction pathway involved in kpOmpA activation, different inhibitors of protein kinases were tested on chemo-kinase production (Table II). After kpOmpA stimulation, P38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580) reduced CCL2, CXCL8, and CXCL10 secretion by 39, 40, and 34%, respectively, whereas the extracellular signal-regulated kinase 1/2 MAPK inhibitor PD98059 had no effect. The inhibitor of phosphatidylinositol 3-kinase (PI3-K) LY294002 inhibited in a higher manner CCL2, CXCL8, and CXCL10 secretion (62, 49, and 60%, respectively). Protein kinase C (PKC) inhibitor, referred to as RO318220, also decreased CCL2 (45%) and CXCL10 (70%) production, but not CXCL8 (20%).

NF-κB nuclear translocation was also investigated in BEAS-2B cells by EMSA. In these experiments, NF-κB was activated in a dose-dependent manner with kpOmpA (Fig. 4A). TNF-α-induced translocation was used as a positive control.

To evaluate NF-κB promoter activity, the luciferase activity was measured in lysates of BEAS-2B cells transfected with the reporter plasmid pGL3, containing or not a tandem repeat of an NF-κB site (Fig. 4B). The data showed that kpOmpA (20 μg/ml) or TNF-α
induced NF-κB activation, as evidenced by the increase in luciferase activity (10- and 13-fold, respectively). No luciferase activity was observed when cells were transfected with plasmid without NF-κB site.

kpOmpA potentiates TNF-α and TNF-α + IFN-γ effects on CXCL8 and CCL2 production by BEAS-2B cells

To investigate the potential synergy between inflammatory cytokines and kpOmpA, BEAS-2B cells were activated by TNF-α and TNF-α + IFN-γ. CXCL8 and CCL2 production was up-regulated in the presence of these cytokines and kpOmpA alone (Fig. 5). BEC costimulation with kpOmpA and TNF-α, or TNF-α + IFN-γ had a synergistic effect on CXCL8 and CCL2 production. The dose-dependent effect of kpOmpA was also observed in the presence of TNF-α and TNF-α + IFN-γ. These data demonstrated that kpOmpA potentiates TNF-α and TNF-α + IFN-γ effects on CXCL8 and CCL2 production by BEAS-2B cells.

FIGURE 4. kpOmpA activates the NF-κB signaling pathway in BEAS-2B cells. A. BEAS-2B cells were incubated with medium alone (negative control), TNF-α (positive control), or kpOmpA (5, 20, and 40 μg/ml). After 2 h of incubation, nuclear protein extracts were prepared and EMSA for NF-κB were performed as described in Materials and Methods. Results are representative of one of three experiments. B, BEAS-2B cells were transiently transfected with GL3 reporter plasmids containing (-), or not ( ), a tandem repeat of an NF-κB site. Cells were incubated with medium alone (control), kpOmpA (20 μg/ml), or TNF-α (200 U/ml) for 24 h. Epithelial cells were harvested, and luciferase activity in protein equivalent cell lysates was assessed as described in Materials and Methods. Results are representative of one of three experiments.

Table II. Modulation of kpOmpA-induced chemokine production

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CXCL2</th>
<th>CXCL8</th>
<th>CXCL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>142.5 ± 52.3</td>
<td>103 ± 75.4</td>
<td>30.25 ± 13.3</td>
</tr>
<tr>
<td>kpOmpA + DMSO</td>
<td>1186.5 ± 274.5</td>
<td>755.5 ± 176.1</td>
<td>78.25 ± 27.9</td>
</tr>
<tr>
<td>+ SB203580</td>
<td>758 ± 129.5</td>
<td>487.25 ± 104.3</td>
<td>55.25 ± 12.4</td>
</tr>
<tr>
<td>+ LY294002</td>
<td>469 ± 44.4</td>
<td>414 ± 75.2</td>
<td>47.5 ± 16</td>
</tr>
<tr>
<td>+ RO318220</td>
<td>677 ± 64.8</td>
<td>614.5 ± 126</td>
<td>39 ± 10.8</td>
</tr>
<tr>
<td>+ PD98059</td>
<td>1337.5 ± 357.7</td>
<td>941.25 ± 313.8</td>
<td>70.25 ± 21.5</td>
</tr>
</tbody>
</table>

*Modulation of kpOmpA-induced chemokine production by inhibitors of the MAPK P38 (SB203580), extracellular signal-regulated kinase 1/2 (LY294002), PKC (RO318220), PI3-K (PD98059), or the vehicle (DMSO). Chemokine levels in BEAS-2B cell supernatants (24 h) were expressed in picograms per milliliter as the mean ± SEM of three separate experiments.

FIGURE 5. Synergistic effect of TNF-α or TNF-α + IFN-γ on kpOmpA-induced cytokine production by BEAS-2B cells. CXCL8 and CCL-2 levels were measured in 24-h supernatants, after addition of 2, 8, and 20 μg/ml kpOmpA on BEAS-2B cells cultured with medium alone (○), or activated with TNF-α (200 U/ml) (■) or TNF-α + IFN-γ (100 U/ml) (▲). Results are expressed as mean ± SEM of three experiments.

Intratracheal injection of kpOmpA induces lung inflammation and BEC activation

To investigate in vivo kpOmpA effects, mice were injected intratracheally. BAL and lung fragments were collected to analyze lung inflammation.

In mice primed with kpOmpA, there was a trend to an increase in total BAL cell number compared with the control BSA (104 ± 107 and 82 × 105 cells in comparison with 60 × 103 cells). The absolute number of macrophages is not significantly modified 6 and 24 h after intratracheal injection of 100 μg of kpOmpA (49 ± 100)

FIGURE 6. kpOmpA intratracheal injection in mice induces neutrophil influx in BAL fluids. Mice received an intratracheal injection of BSA (100 μg) or kpOmpA (20 or 100 μg). BAL cells were spun on slides and stained with May-Grinwald-Giemsa. These data showed percentages of macrophages and neutrophils in BAL fluids collected at 6 h, 1, 2, 3, and 7 days after instillation. Results are expressed as mean ± SEM for three mice and are representative of three experiments.
$10^3$ cells and $36 \times 10^3$ cells, respectively) compared with the number obtained after BSA instillation ($42 \times 10^3$ cells). In contrast, the absolute number of neutrophils was significantly increased 6 and 24 h after instillation of 100 μg of kpOmpA ($34 \times 10^3$ cells and $42 \times 10^3$ cells, respectively) in comparison with naive mice ($6 \times 10^3$ cells) ($p < 0.05$). This was confirmed by the analysis of BAL cell differential count in mice receiving kpOmpA (Fig. 6). Whereas BAL cells from control mice (receiving BSA) consisted predominantly of macrophages, a pronounced influx of neutrophils was found in BAL of mice primed with 100 μg of kpOmpA, with a maximum at day 1 (85% total cells). kpOmpA exhibited an in vivo dose-dependent effect as intratracheal injection of 20 μg induced a lower neutrophil influx on day 1 (55% total cells).

Neutrophil percentage progressively returned to baseline level at day 7. Lymphocyte, eosinophil, and epithelial cell counts were not markedly modified after kpOmpA injection (data not shown).

Neutrophil influx was also observed on lung sections collected 6 or 24 h after kpOmpA injection. Neutrophils are present in both alveolar spaces, bronchial walls, and airway lumen (Fig. 7, A and B). The cellular infiltrate reached its maximum at 24 h, and resolved after 7 days (data not shown). In contrast, few infiltrating leukocytes were observed in lung sections of mice primed with BSA (data not shown). To demonstrate in vivo BEC activation after intratracheal injection of kpOmpA, ICAM-1 expression and CXCL1 production was measured by immunohistochemistry on lung sections. kpOmpA up-regulated ICAM-1 expression within BEC but also within pneumocytes and endothelial cells (Fig. 7, E and F). ICAM-1 expression was similar at 6 and 24 h postexposure to kpOmpA. In contrast, a faint staining for ICAM-1 was detected within BEC after BSA exposure (Fig. 7D). Concerning CXCL1 production, BEC and alveolar macrophages were strongly positive for this chemokine after kpOmpA treatment (Fig. 7, I and J) in contrast with control mice receiving BSA (Fig. 7H). CXCL1 staining was mainly detected at 6 h (Fig. 7I) but decreased at 24 h (Fig. 7J).

In addition, lung inflammation was evaluated by the measurement of cytokine and chemokine production. In lung homogenates, mRNA expression for CXCL1, CXCL5, CXCL10, and IL-6 was transiently increased at 6 h after kpOmpA injection (Fig. 8), but CCL2 mRNA levels were not affected (Fig. 8). ICAM-1 mRNA expression was similarly up-regulated after kpOmpA exposure at 6 h. Expression of CXCL1 mRNA was associated with the concomitant release of this chemokine in BAL fluids at 6 h; CXCL5 production was also progressively induced (Fig. 9). In addition to chemokine production, kpOmpA instillation also triggered the secretion of inflammatory and immunomodulatory cytokines. kpOmpA intratracheal injection transiently up-regulated IL-10, IFN-γ, and TNF-α, (at 6 h) in BAL fluids whereas it did not increase IL-12 levels (Fig. 9).

**Discussion**

Effective lung defense against bacterial infection is primarily dependent on the rapid clearance of micro-organisms via the participation of two phagocytic cells: neutrophils and macrophages. Inflammatory cell recruitment and activation require complex interactions involving the secretion of activating and chemotactic
factors as well as adhesion molecule expression. Although macrophages may initially encounter bacteria, the micro-organisms are also able to colonize airway epithelium. Increasing evidence shows that BEC are also implicated in the control of the local inflammatory reaction (1, 2). In this way, among pathogen-associated molecular patterns, different bacterial membrane components, including LPS and glycoproteins, have been shown to activate BEC. In this study, we demonstrated that kpOmpA binds to and activates BEC particularly through the NF-κB pathway. This activation induces cytokine and chemokine production and enhances ICAM-1 expression. In vivo intratracheal kpOmpA injection induces an inflammatory reaction associated with BEC activation as shown by ICAM-1 and CXCL1 expression on epithelial cells.

First, we report that kpOmpA binds to BEC in a saturable fashion to similar levels as observed with monocyte-derived DC (data not shown), suggesting at least that one of its receptors is expressed on epithelial cells. The innate immune system recognizes diverse pathogen-associated molecular patterns by members of the TLR family, leading to an inflammatory reaction (21). TLR-2 and TLR-4 are expressed by numerous cell types such as monocytes, macrophages, DCs, endothelial cells, and mucosal epithelial cells. TLR-2 is involved in the recognition of diverse bacteria and their products, including Gram-positive bacteria and peptidoglycans, whereas TLR-4 has been identified as the receptor for LPS, an endotoxin of Gram-negative bacteria (22). Blocking anti-TLR-4 Abs did not inhibit kpOmpA binding to BEC. kpOmpA-mediated activation is not due to LPS because endotoxin is undetectable in kpOmpA preparation and polymyxin B does not neutralize kpOmpA effects on bronchial epithelium. Using TLR-2-transfected cells and DC from TLR-2-deficient mice, Jeannin et al. (14) demonstrated that TLR-2 is involved in kpOmpA-mediated signaling but is not required in kpOmpA binding to and endocytosis by DC (14, 20). We also show here that blocking anti-TLR-2 Abs do not affect kpOmpA binding to BEC. These observations suggest that endocytic receptor(s) might be responsible for the capture of kpOmpA and for the subsequent mobilization of TLR-2 to activate the signaling cascade (23). Nevertheless, activation of airway epithelial cells by TLR-2 ligands does not necessarily require TLR-2 signaling because lipoteichoic acid, another constituent of bacteria wall, activates platelet-activating factor receptor in these cells, resulting in the transactivation of the epidermal growth factor receptor (24). In our study, BEC exposure to kpOmpA activates the NF-κB pathway, a signaling pathway involved in TLR-2-mediated cell activation. This transcription factor has been identified as an inducer for the transcription of cytokine, chemokine, and adhesion molecule genes such as ICAM-1 (25). The present study also demonstrates that kpOmpA induces ICAM-1 expression and cytokine secretion by BEC through the activation of NF-κB and protein kinases such as P38 MAPK, PI_3-K, and PKC.

Mobilization of innate defense mechanisms involves the recruitment of effector cells, and particularly neutrophils and macrophages. This process requires the coordinate activity of proinflammatory cytokines, chemokines, and adhesion molecules. We showed that in vitro, kpOmpA induces proinflammatory cytokine production by BEC which implicate, at least in part, NF-κB signaling pathway activation. This included the production of the cytokine IL-6 and chemokines such as CCL2, CXCL1, CXCL8, or CXCL10. IL-6 was initially considered as a proinflammatory cytokine, chemokines, and adhesion molecule genes such as ICAM-1 (25). The present study also demonstrates that kpOmpA induces ICAM-1 expression and cytokine secretion by BEC through the activation of NF-κB and protein kinases such as P38 MAPK, PI_3-K, and PKC.

Mobilization of innate defense mechanisms involves the recruitment of effector cells, and particularly neutrophils and macrophages. This process requires the coordinate activity of proinflammatory cytokines, chemokines, and adhesion molecules. We showed that in vitro, kpOmpA induces proinflammatory cytokine production by BEC which implicate, at least in part, NF-κB signaling pathway activation. This included the production of the cytokine IL-6 and chemokines such as CCL2, CXCL1, CXCL8, or CXCL10. IL-6 was initially considered as a proinflammatory cytokine, but more recent data showed that IL-6 could participate to the resolution of inflammatory reactions (26). It induces the secretion of cytokine inhibitors such as IL-1 receptor antagonist and soluble TNFR by monocytes, and of protease inhibitors such as tissue inhibitor of metalloproteinase 1 or α2-macroglobulin. Among chemokines, CXCL1, CXCL5, and CXCL8, as well as other chemokines containing an AA motif ELR, are chemotactic.
kpOmpA ACTIVATES BECs

Adhesion molecule expression, in response to kpOmpA, specifically and secretion of chemokines including CXCL1 and CXCL5. Interestingly, CXCL5 production seems to be delayed (maximal at 24 h) compared with CXCL1 secretion (at 6 h), suggesting that these chemokines act sequentially in this model. Both CXCL1 and CXCL5 potently activate neutrophils via the murine CXCL8 receptor homologue, resulting in increased Mac-1 expression and in respiratory burst activity (28–30). The recruitment and the subsequent activation of neutrophils play a key role in lung bacterial clearance and improved survival (31, 32). CXCL5 is predominantly produced by fibroblasts and lung epithelial cells. Macrophages are reported to be the main source of CXCL1 (33, 34). However, immunohistochemistry analysis showed that both macrophages and BEC are strongly positive for CXCL1 expression after exposure to kpOmpA, suggesting that both cell types are involved in this process. The adhesion molecule ICAM-1 participates in leukocyte diapedesis by mediating firm adhesion to endothelial or epithelial cells, and this way is involved in host defense (35). We also reported that ICAM-1 expression is up-regulated after kpOmpA exposure in alveolar and bronchial epithelium and in endothelium, witness of the local inflammation. In human BEC, there is some specificity in the kpOmpA-derived adhesion molecule expression, because kpOmpA alone or in presence of TNF-α does not modulate VCAM-1 expression (data not shown) in contrast to ICAM-1. Therefore, these data demonstrated that chemokine secretion and adhesion molecule expression, in response to kpOmpA, specifically direct neutrophil migration across the epithelial barrier at infected mucosal sites.

The in vivo production of inflammatory and immunostimulatory cytokines, such as TNF-α and IL-10, was investigated in the BAL. In lung, TNF-α is predominantly produced by macrophages, but BEC may also secrete it (1). This cytokine improves lung bacterial clearance through an up-regulation of adhesion molecule expression and secretion of chemokines including CXCL1 (36). In our study, kpOmpA induces a weak production of TNF-α by BEC, whereas IFN-γ and IL-10 remain undetectable in BEC supernatants (data not shown). In addition, kpOmpA potentiates the effects of TNF-α, and TNF-α + IFN-γ on CXCL8 and CCL2 production by BEC. kpOmpA activates macrophages, which produce high amounts of TNF-α (16). In turn, these mediators may expand macrophage activation. So, in vivo, this may result in a proinflammatory amplification loop between macrophages and epithelial cells. In contrast, IL-10 is detrimental to innate and cell-mediated immunity in the lung, particularly by blocking the synthesis of proinflammatory cytokines, including IFN-γ, IL-1, TNF-α, IL-12, and chemokine production (37). In vivo, neutralization by anti-IL-10 Ab results in an enhanced bacterial clearance (38) whereas IL-10 secretion serves to limit the production of proinflammatory cytokines in this model (38, 39). During infection, IL-10 secretion seems to have a dual effect: detrimental on the bacterial clearance and beneficial on the inflammation as shown in septic peritonitis (40). In our study, IL-10 production reaches its maximum at 6 h and persists at 24 h, whereas the production of chemokines and proinflammatory cytokines returned to baseline. These demonstrated that kpOmpA-induced inflammatory burst was associated with the production of anti-inflammatory mediators (IL-6 and IL-10).

The different cell populations in the mucosal barrier are equipped to sense and respond to the molecular contents in the lumen and to translate this molecular information into signals that can reach local or distant sites within the body. In addition to its effect on BEC, kpOmpA interacts and activates DC, inducing their maturation and delivering Ag into the MHC class I presentation pathway (14). However, the IL-12 production induced by kpOmpA in macrophages and DC is modest compared with that obtained after LPS- or CD40-dependent activation (16). In this context, the lack of IL-12 secretion in BAL fluids, after kpOmpA treatment may be due to the capture of this cytokine by IL-12R-positive cells. In vivo IL-12 secretion by kpOmpA-activated APC may also require the action of a coactivator. Nevertheless, IFN-γ levels, whose production can be induced by IL-12, are increased after kpOmpA instillation. As IFN-γ production is observed after 6 h, kpOmpA presumably directly induces its production by immunocompetent cell activation. Further studies are required to elucidate this point. Because IFN-γ plays an essential role in the development of adaptive immunity (41), kpOmpA may orchestrate the transition from innate to adaptive immunity, and the polarization of this immune response to promote cell-mediated immunity.

In conclusion, the present data identify a new mechanism by which the bronchial epithelium is involved in anti-infectious defense. BEC exposed to kpOmpA shows an increased expression of adhesion molecules and an up-regulation of chemokine secretion, which are implicated in vivo in inflammatory cell recruitment toward the lung. In addition to endotoxin and glycoprotein, OmpA is also an important component of the bacterial wall implicated in the development of a defense mechanism.

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References


