Klebsiella pneumoniae Capsule Polysaccharide Impedes the Expression of β-Defensins by Airway Epithelial Cells

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Human β-defensins (hBDs) contribute to the protection of the respiratory tract against pathogens. It is reasonable to postulate that pathogens have developed countermeasures to resist them. Klebsiella pneumoniae capsule polysaccharide (CPS), but not the lipopolysaccharide O antigen, mediated resistance against hBD1 and hBD2. hBD3 was the most potent hBD against Klebsiella. We investigated the possibility that as a strategy for survival in the lung, K. pneumoniae may not activate the expression of hBDs. Infection of A549 and normal human bronchial cells with 52145-ΔwcaK2, a CPS mutant, increased the expression of hBD2 and hBD3. Neither the wild type nor the lipopolysaccharide O antigen mutant increased the expression of hBDs. In vivo, 52145-ΔwcaK2 induced higher levels of mBD4 and mBD14, possible mouse orthologues of hBD2 and hBD3, respectively, than the wild type. 52145-ΔwcaK2-dependent upregulation of hBD2 occurred via NF-κB and mitogen-activated protein kinases (MAPKs) p44/42, Jun N-terminal protein kinase (JNK)-dependent pathways. The increase in hBD3 expression was dependent on the MAPK JNK. 52145-ΔwcaK2 engaged Toll-like receptors 2 and 4 (TLR2 and TLR4) to activate hBD2, whereas hBD3 expression was dependent on NOD1. K. pneumoniae induced the expression of CYLD and MKP-1, which act as negative regulators for 52145-ΔwcaK2-induced expression of hBDs. Bacterial engagement of pattern recognition receptors induced CYLD and MKP-1, which may initiate the attenuation of proinflammatory pathways. The results of this study indicate that K. pneumoniae CPS not only protects the pathogen from the bactericidal action of defensins but also impedes their expression. These features of K. pneumoniae CPS may facilitate pathogen survival in the hostile environment of the lung.

The lung is a portal of entry for many pathogens, which can gain easy access to the bloodstream by crossing the alveolar-capillary membrane. Several mechanisms are devoted to protecting the lung, but the complement system and the antimicrobial peptides (APs) and proteins present on the airway surface make up the protective front (22, 39). The most abundant antibacterial agents in the airways are lysozyme and lactoferrin, which are secreted by submucosal glands, surface epithelia, and neutrophils (3, 22, 70). Other peptides found in the airway liquid are defensins (3), cathelicidins (3).

Several human BDs (hBDs) have been identified, of which hBD1 (DEFB1), hBD2 (DEFB4), and hBD3 (DEFB3) are the most studied (35, 63). BDs show antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi, and viruses. hBD3 appears to be the most potent hBD, since it kills a broad range of microbes at low peptide concentrations. Moreover, in contrast to hBD1 and hBD2, hBD3 displays potent antimicrobial activity at physiological salt concentrations (46, 57). Each hBD has a unique expression profile. hBD1 is constitutively expressed by epithelial cells lining the respiratory tract (47), whereas the expression of hBD2 and hBD3 by airway epithelial cells is induced by cytokines or by the presence of pathogens (27, 28, 47, 66). Thus, hBD2 and hBD3 play an important role in host defense as inducible components of the epithelial barrier. Indeed, hBD2 and hBD3 levels increase severalfold in the lung during pneumonia (29, 33). The importance of BDs in lung defense has been established by the use of knockout mice. Animals lacking mouse BD1 (mBD1) display a defect in the ability to clear Haemophilus influenzae from the lungs (49). However, BDs not only protect the lung against invading microbes but also modulate the host immune response by providing an interface between innate and adaptive immune responses (64, 76–78).

Klebsiella pneumoniae is one of the most common pathogens causing community-acquired respiratory infections, which are particularly devastating in immunocompromised patients (58, 62). Community-acquired pneumonia is a very severe illness with a rapid onset. Despite the availability of an adequate antibiotic regimen, the outcome is often fatal, with observed mortality rates around 50%. The high prevalence of multidrug-resistant isolates further complicates the treatment of these infections (69). Capsule polysaccharide (CPS) is recognized as one of the most important virulence factors of this pathogen. CPS mutants are unable to colonize pulmonary and systemic tissues (13, 41, 42). In vitro studies have shown that the presence of CPS inhibits the deposition of the complement component C3 onto the bacterium (5, 12, 16) and reduces adhesion and phagocytosis of the bacterium by macrophages and epithelial cells (12, 13, 18, 54). Taken together, these findings...
suggest that CPS plays an important role in the interplay between K. pneumoniae and the innate immune system.

Recently we have started to study whether K. pneumoniae expresses mechanisms of resistance against APs. We have shown that K. pneumoniae surface-bound CPS may act as a protective shield on the bacterial surface against APs (8), whereas released CPS traps APs, thereby blocking their bacterial activity (45). Moreover, sublethal concentrations of APs induce an increase in the transcription of the hpsA operon, which correlates with an increase in the amount of surface-bound CPS (8). Concentrations of APs in infected tissues (for example, those found in the surface liquid lining the airway epithelium) could be rather high due to the increased production of APs after recognition of the pathogen. Therefore, although K. pneumoniae is endowed with mechanisms against APs, these high levels of APs may render these bacterial countermeasures ineffective.

Here we explored the possibility that as a strategy for survival in the lung, K. pneumoniae may not activate the expression of hBDs by airway epithelial cells. We present evidence indicating that wild-type bacteria do not induce the expression of Bds in vitro or in vivo, in contrast to a CPS mutant. We also report that a K. pneumoniae CPS mutant engages Toll-like receptors 2 and 4 (TLR2 and TLR4) to induce the expression of hBD2, whereas the expression of hBD3 is dependent on the activation of NOD1. Finally, we investigate whether there are negative mechanisms regulating the expression of hBDs induced by a K. pneumoniae CPS mutant.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions, and reagents.** K. pneumoniae 52145 is a clinical isolate (serotype O1:K2) that has been described previously (50). The isogenic mutants 52145-ΔwaxK, which does not express CPS, and 52021, which does not express the lipopolysaccharide (LPS) O side chain, have also been described previously (13, 45). Bacteria were grown in Luria-Bertani (LB) medium at 37°C.

Recombinant hBD1, hBD2, and hBD3 were purchased from PeproTech. As reported by the manufacturer, the purities of defensins were greater than 98% by reverse-phase high-pressure liquid chromatography (HPLC) analysis. Caffeic acid phenethyl ester (CAPE), an NF-κB inhibitor, and SB203580, a p38 mitogen-activated protein kinase (MAPK) inhibitor, were purchased from Sigma, whereas U0126, a p44/42 MAPK inhibitor, and SP600125, a Jun N-terminal protein kinase (JNK) inhibitor, were purchased from Calbiochem.

**Antimicrobial peptide resistance assay.** The assay described by Campos et al. (8) was carried out with minor modifications. Briefly, bacteria were grown at 37°C in 5 ml LB medium, harvested (5,000 × g, 15 min, 5°C) at the exponential phase of growth, and washed three times with phosphate-buffered saline (PBS). A suspension containing approximately 10⁷ CFU/ml was prepared in 10 mM PBS (pH 6.5), 1% treptone soy broth (TSB; Oxoid), and 100 mM NaCl. Aliquots (5 μl) of this suspension were mixed in Eppendorf tubes with various concentrations of hBDs. In all cases, the final volume was 30 μl. After 3 h of incubation, the contents of the Eppendorf tubes were diluted 1:10 with PBS, and 100 μl was immediately plated on LB agar. Colony counts were determined, and results were expressed as percentages of the colony count of bacteria that were not exposed to antibacterial agents. The 50% inhibitory concentration (IC50) of hBDs was defined as the concentration producing a 50% reduction in the colony counts from that for bacteria not exposed to the antibacterial agent.

All experiments were conducted with duplicate samples on three independent occasions.

**Cell culture and infection.** Monolayers of A549 human lung carcinoma cells (ATCC CCL185) derived from type II pneumocytes were grown in RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS), and antibiotics (penicillin and streptomycin) in 25-cm² tissue culture flasks at 37°C under a humidified 5% CO2 atmosphere. Primary normal human bronchial epithelial (NHBE) cells (Lonza) were grown in a bronchial epithelial cell growth medium (bronchial epithelial cell basal medium [BBBM]; Lonza) supplemented with 0.5 ng/ml human epidermal growth factor, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodothyronine, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, 52 μg/ml bovine pituitary extract, and 0.1 ng/ml retinoic acid at 37°C under 5% CO2. Tissue culture flask (25 cm²) were coated with collagen from calf skin (Sigma).

For infections, A549 cells were seeded to 90% confluence (4 × 10⁷ to 5 × 10⁷ cells/well) in 24-well tissue culture plates. Cells were serum starved 16 h before infection by replacement of medium with supplemented RPMI medium lacking FCS. For NHBE cells, cells were seeded to 80% confluence (2 × 10⁷ cells/well) in collagen-coated 24-well tissue culture plates with 1 ml of BBM per well. Overnight-grown bacteria were subcultured and grown to exponential phase, harvested by centrifugation (5,000 × g, 15 min, 5°C), and resuspended in PBS. Before infection, cells were washed three times with PBS, and infection was performed using a multiplicity of infection of 100 bacteria per cell. To synchronize infection, plates were centrifuged at 200 × g for 5 min. After 2 h of infection, cells were washed three times with PBS and were then further incubated with RPMI 1640 containing 10% FCS, HEPES, and gentamicin (100 μg/ml), to kill extracellular bacteria. Cell viability, assessed by trypan blue dye exclusion, was >95%.

**RT-qPCR.** Cells seeded into 60-mm-diameter tissue culture dishes were infected. After infection, cells were washed with PBS, and total RNA was purified using a NucleoSpin RNA II kit (Macherey-Nagel) exactly as recommended by the manufacturer. cDNA was obtained by retrotranscription of 1.5 to 2 μg of total RNA using a commercial RT First Strand kit as recommended by the manufacturer (SA Biosciences). The reaction included one step to eliminate traces of genomic DNA. Real-time PCR (RT-PCR) analyses were performed with the 7500 Fast Real-Time PCR system (Applied Biosystems). cDNA (2 μl) was used as a template in a 25-μl reaction mixture containing 1× SYBR green RT2 qPCR Master Mix (SA Biosciences) and a primer mixture. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin were amplified as controls using 5 ng of cDNA as the template. The following intron-spanning primers were used: for GAPDH, 5'-GAAGATGAACTTGAGAAGCTG-3' (sense) and 5'-AAATGTTGAGTGTTGAGTTT-3' (antisense); for actin, 5'-AGAAATACTGGCACCAACC-3' (sense) and 5'-GGGGTGTTGAAGGCTTCAA-3' (antisense); for hBD1, 5'-GACAATCCACGTTCTTAAAT-3' (sense) and 5'-CAGAGTAAACACGAGAAG-3' (antisense); for CYLD, 5'-TATTGGGAAGGAGCATTGTGC-3' (sense) and 5'-CTGCTCTCCAGGTTCCTGCC-3' (antisense); and for MKP-1, 5'-GTCGTGGCAGAACACAGCCGA-3' (sense) and 5'-CGATTACCTCTCAATAGGTA-3' (antisense). To amplify hBD2 and hBD3, the RT-qPCR primer assay for DEFBI (catalog number PHH11010A; SA Biosciences) and the QuantiTect primer assay for DEFBI03A (catalog number QT02170104; Qiagen) were used, respectively. To amplify mBDs, similar amounts of cDNAs were used with the following primers: for Defb1, 5'-AACA CGGTACAAGGCTCC-3' (sense) and 5'-TCACAAGATGTCCTAGGACC-3' (antisense); for Defb3, 5'-CTCCACTTGACCCCTTACCC-3' (sense) and 5'-GGGATGATGCTCCCAGCCACACC-3' (antisense); for Hpdefb14, 5'-TTGTTCTTGAGTGGTT-3' (antisense) and 5'-GCTTCTTGCGACACCTT-3' (sense). For amplification of mouse actin, the primers used were 5'-TTTACCAACTGCGGACGACA-3' (sense) and 5'-CTGGTTGATCCTTCTACG-3' (antisense), whereas for the amplification of mouse GAPDH, the primers used were 5'-CCCCTAACCATACTGCGGG-3' (sense) and 5'-CCTCCAATGGCCAAACTT-3' (antisense). For gene detection (human or mouse), the thermocycling protocol was as follows: 95°C for 15 min for hot-start polymerase activation, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. SYBR green dye fluorescence was measured at 521 nm during the annealing phase. The threshold cycle (Ct) value reflects the cycle number at which the fluorescence generated in a reaction crosses a given threshold. The Ct value assigned to each well thus reflects the point during the reaction at which a sufficient number of amplicons have been accumulated. The relative amount of mRNA in each sample was calculated based on its Ct value in comparison with the Ct values of GAPDH and actin. The results were expressed as changes in gene expression, determined using the following formula: 2−[(Ct of target gene − Ct of housekeeping genes of treated cells or mice)/(Ct of target gene − Ct of housekeeping genes of untreated cells or mice)] (with Ct values in arbitrary units). The specificity of the PCR products was determined by melting curve analysis, and amplification products were resolved on 1% agarose gel containing 0.5% HEPES, 10% isopropanol, and 5% formaldehyde. After the injection of the antisera, the Jurkat cell line, positive for the respective target (human or mouse, antihuman [human actin, 149 bp; mouse actin, 138 bp; human GAPDH, 242 bp; mouse GAPDH, 275 bp; human DEFBI, 226 bp; mouse Defb1, 246 bp; human DEF4B, 92 bp; mouse Defb4, 271 bp; human DEFBI03A, 111 bp; mouse Defb14, 164 bp; human CYLD, 276 bp; human MKP-1, 431 bp), cDNAs were
obtained from three independent extractions of mRNA, and each cDNA was amplified by RT-quantitative PCR (qPCR) on two independent occasions.

**Intranasal infection model.**  Five- to 7-week-old female C57BL/6J OlaHsd mice (Hurlan) were anesthetized by intraperitoneal (i.p.) injection of a mixture containing ketamine (50 mg/kg) and xylazine (5 mg/kg). Overnight bacterial cultures were centrifuged (2,500 × g, 20 min, 22°C), resuspended in PBS, and adjusted to 5 × 10^7 CFU/ml. A 20-μl volume of the bacterial suspension (equivalent to 10^7 CFU) was inoculated intranasally in four 5-μl aliquots. Noninfected mice were inoculated intranasally with 20 μl of PBS in four 5-μl aliquots. To facilitate consistent inoculations, mice were held vertically during inoculation and were placed on a 45° incline during recovery from anesthesia. At the indicated times after infection, mice were euthanized by cervical dislocation; lungs were rapidly dissected, immediately frozen in liquid nitrogen, and stored at −80°C until the assays were performed. Mice were treated in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Directive 86/609/EEC) and in agreement with the Bioethical Committee of the University of the Balearic Islands.

For purification of RNA from lungs, lungs were quickly weighed and homogenized with 1 ml of TRI reagent (Ambion) using an Ultra-Turrax T25 basic homogenizer (IKA) on ice. First, total RNA was purified using a standard chloroform-isopropanol alcohol protocol; then the RNA obtained was further purified using a NucleoSpin RNA II kit (Machery-Nagel) exactly as recommended by the manufacturer. RNA integrity was verified using a formaldehyde-agarose gel, quantified spectrophotometrically with a NanoDrop spectrophotometer, and stored at −80°C. mRNA expression was measured by RT-qPCR analysis as described above.

**Plasmids, transfections, and luciferase assays.** The transfection vectors containing the 5′-flanking regions of the human DEFB4 (hBD2) and DEFB3 (hBD3) genes have been described previously (7, 74). The pRL-TK Renilla luciferase control reporter vector was purchased from Promega. A549 cells seeded in 24-well plates were transiently transfected when they reached 40 to 60% confluence. They were washed three times with PBS, and transfections were carried out in 500 μl of Opti-MEM reduced-serum medium (Invitrogen) by using the Lipofectamine 2000 transfection reagent according to the manufacturer’s recommendations (Invitrogen). One microgram of the hBD2 reporter plasmid or 500 ng of the hBD3 reporter plasmid was cotransfected with 20 ng of the pRL-TK Renilla luciferase plasmid. Forty-eight hours posttransfection, cells were infected as described above.

For luciferase assays, cells were lysed with Passive Lysis buffer (Promega). Luciferase activity was assayed using the Dual Luciferase reporter assay kit according to the manufacturer’s instructions (Promega). Firefly luciferase values were normalized to Renilla luciferase control values. Results were plotted as relative luciferase activity compared with the activity measured for nonstimulated control cells. The luciferase assay was carried out in triplicate on at least three independent occasions.

**siRNA.** RNA-mediated interference for downregulating MyD88, CYLD, and MKP-1 was carried out by the transfection of MyD88 small interfering RNA (siRNA) (5′-AACTGGAAACGACAAACTATC-3′), CYLD siRNA (Hs_CYLD_4 HP siRNA; catalog no. SI01100996), and MKP-1 siRNA (Hs_DUSPI_5 HP siRNA; catalog no. SI03100488), respectively, which were purchased from Qiagen. The AllStars Negative Control siRNA (Qiagen) was used as nonsilencing control interfering RNA. Interfering RNAs for TLR4 (catalog no. Hs11018114), TLR2 (catalog no. Hs11018153), and CARD4/NOD1 (catalog no. Hs1115906) were purchased from Invitrogen. Stealth RNAi Negative Control Med GC was used as control interfering RNA. Unless otherwise indicated, 20 nM siRNA per well was used for transfection with the Lipofectamine 2000 transfection reagent according to the manufacturer’s recommendations (Invitrogen). Forty-eight hours posttransfection, cells were infected as described above.

**Immunoblotting.** Cells were seeded on 6-well tissue culture plates at 1 × 10^6 per well. Cells were infected as described above, washed three times with cold PBS, scraped, and lysed with 100 μl lysis buffer (1× sodium dodecyl sulfate [SDS] sample buffer, 62.5 mM Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 10% glycerol, 50 mM dithiothreitol [DTT], 0.01% [wt/vol] bromophenol blue) on ice. Samples were sonicated, boiled at 100°C for 10 min, and cooled on ice. Proteins (15 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred by semidyed blotting to a nitrocellulose membrane, and blocked with 5% skim milk in PBS. Immunostaining for IκBα and MKP-1 was performed with a polyclonal rabbit anti-IκBα antibody (1:1,000) and an anti-phospho-p38, p44/p42, or JNK, purchased from Cell Signaling, respectively, all purchased from Cell Signaling. Blots were reprobed with a polyclonal antibody against p38, p44/p42, or JNK, purchased from Cell Signaling, in order to ensure that equal amounts of proteins were loaded in each lane. Immunostaining for CYLD and MKP-1 was performed with an anti-phospho-p44/42 (Thr202/Tyr204) antibody (1:1,000), and an anti-phospho-p38, p44/p42, or JNK, purchased from Cell Signaling, in order to ensure that equal amounts of proteins were loaded in each lane. Immunostaining for CYLD was performed with a polyclonal rabbit anti-CYLD antibody (1:1,000) (Imgenex Corp.). Immunoreactive bands were visualized by incubation with goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase (Pierce) (1:20,000) using the SuperSignal West Dura system (Pierce). To ensure that equal amounts of proteins were loaded in the lanes, blots were reprobed with mouse anti-human tubulin (Sigma) (1:3,000), and immunoreactive bands were visualized by incubation with goat anti-mouse immunoglobulins conjugated to horseradish peroxidase (Pierce) (1:1,000). Images were recorded with a GeneGnome HR imaging system (Syngene) as JPEG files, and they were exported to a personal computer for densitometry analysis using ImageJ software (http://rsb.info.nih.gov/ij/download.html). Bands in each lane were selected and analyzed using the Histogram analysis tool, and mean intensities were recorded. The results were expressed as relative levels of protein (mean intensity of protein/mean intensity of tubulin) × 100.

**Statistical methods.** Statistical analyses were performed using one-way analysis of variance (ANOVA) with Bonferroni contrasts of the one-tailed t test or, when the requirements were not met, by the Mann-Whitney U test. A P value of <0.05 was considered statistically significant. The analyses were performed using Prism4 for PC (GraphPad Software).

**RESULTS**

Human β-defensins kill Klebsiella pneumoniae. Previously we have shown that *K. pneumoniae* CPS mediates resistance to different antimicrobial peptides, including hBD1 (8, 45). However, in those studies, the sensitivities of *K. pneumoniae* to hBD2 and hBD3, whose levels increase in the lung during pneumonia, were not tested (29, 33). Survival assays (Fig. 1) demonstrated that wild-type *K. pneumoniae* strain 52145 (referred to below as Kp52145) was more resistant to hBD1 and hBD2 than strain 52145ΔωnC2, an isogenic CPS mutant strain. In our previous study, we found that the *K. pneumoniae* LPS O antigen also mediates resistance to certain APs (8). However, Kp52145ΔωnC2, a mutant lacking the LPS O antigen but expressing CPS, showed a level of resistance to hBD1 and hBD2 similar to that of Kp52145. Interestingly, all strains were more susceptible to hBD3 than to the other hBDs tested; furthermore, Kp52145 and Kp52145ΔωnC2 were as susceptible as 52145ΔωnC2 (Fig. 1). To further confirm these findings, we performed dose-response experiments that allowed us to de-
termine the 50% inhibitory concentrations (IC$_{50}$) of hBDs for these strains. The IC$_{50}$ of hBD1 for Kp52145 was 1.51 ± 0.03 μg/ml, similar to that for 52O12 (1.13 ± 0.05 μg/ml [P > 0.05]) but significantly higher than that for 52145-Δwca$_{K2}$ (0.42 ± 0.10 μg/ml [P < 0.05]). The IC$_{50}$ of hBD2 for 52145-Δwca$_{K2}$ (0.31 ± 0.01 μg/ml) was also lower than those for Kp52145 and 52O21 (1.04 ± 0.06 and 0.81 ± 0.03 μg/ml, respectively). In contrast, the IC$_{50}$ of hBD3 for Kp52145, 52O21, and 52145-Δwca$_{K2}$ were not significantly different (0.30 ± 0.04, 0.25 ± 0.02, and 0.26 ± 0.06 μg/ml, respectively).

In summary, these data indicate that CPS, but not the LPS O antigen, mediates resistance to hBD1 and hBD2 and that hBD3 is the most potent hBD against K. pneumoniae.

The K. pneumoniae CPS mutant induces the expression of human β-defensins in airway epithelial cells. Airway epithelial cells do express hBDs, and data indicate that hBD1 is constitutively expressed, whereas hBD2 and hBD3 are upregulated upon infection (27, 28, 47, 66). We sought to determine whether K. pneumoniae increases the expression of hBD mRNA in normal human bronchial epithelial (NHBE) cells. Analysis by RT-qPCR revealed that Kp52145 did not induce the expression of hBD1, hBD2, or hBD3 but that it caused a decrease in the expression of hBD1 at the first time point analyzed (Fig. 2A). Similar results were obtained when infections were performed with strain 52O21. Therefore, we asked whether the presence of CPS on the bacterial surface may prevent the activation of cellular signaling pathways responsible for the upregulation of hBD expression. Indeed, infection of NHBE cells with 52145-Δwca$_{K2}$ induced the expression of hBD2 in a time-dependent manner (Fig. 2A). 52145-Δwca$_{K2}$ also increased hBD3 mRNA levels 6 h postinfection (Fig. 2A).

Similar studies in which A549 airway epithelial cells were infected were carried out (Fig. 2B). 52145-Δwca$_{K2}$ increased mRNA levels of the three hBDs whereas neither Kp52145 nor 52O21 induced the expression of any hBDs, even at 10 h postinfection (Fig. 2B). Additionally, A549 cells were transiently transfected with a luciferase reporter construct controlled by the promoter regions of either hBD2 or hBD3. These plasmids have been used previously to monitor the expression of hBD2 and hBD3 in airway epithelial cells (7, 74).
good agreement with the data obtained by RT-qPCR, only 52145-ΔwcaK2 induced the activation of both reporter constructs (Fig. 2C).

Collectively, our data show that a CPS mutant, but neither the wild type nor an LPS O antigen mutant, increased the expression of hBD2 and hBD3 by NHBE and A549 cells.

The K. pneumoniae CPS mutant induces higher levels of β-defensins in vivo than the wild-type strain. We investigated whether 52145-ΔwcaK2 induces higher levels of β-defensins than Kp52145 in the lungs of infected mice. Although there is no clear consensus on the existence of clear mouse orthologues of hBD1 (6, 31, 36), hBD2 (35, 56), and hBD3 (mBDs) mBD1, mBD4, and mBD14 are the possible mouse orthologues of hDB2 and hBD3, reports suggest that mouse β-defensins (mBDs) mBD1, mBD4, and mBD14 are the possible mouse orthologues of hBD1 (6, 31, 36), hBD2 (35, 56), and hBD3 (61), respectively. Mice were infected intranasally and were euthanized at different time points. Whole lungs were dissected, and the expression of mBDs was measured by RT-qPCR at 6, 12, and 24 h postinfection. At 6 h postinfection, levels of mBD1 and mBD4 in the lungs of infected and noninfected mice were similar (Fig. 3). In contrast, levels of mBD14 were higher in the lungs of infected mice than in those of noninfected mice (Fig. 3). No significant differences were found between the levels of mBD14 induced by Kp52145 versus 52145-ΔwcaK2. At 12 h postinfection, levels of mBD1 and mBD4 were higher in the lungs of infected mice with 52145-ΔwcaK2 than in those of mice infected with Kp52145 (Fig. 3). The levels of both defensins in the lungs of mice infected with Kp52145 were not significantly different from those in the lungs of noninfected mice (Fig. 3). At 12 h postinfection, levels of mBD14 were higher in the lungs of infected mice than in the lungs of noninfected mice, and no differences were found between the levels induced by the two strains (Fig. 3). At 24 h postinfection, levels of mBD4 and mBD14 were higher in the lungs of mice infected with 52145-ΔwcaK2 than in those of mice infected with Kp52145 (Fig. 3). Levels of mBD1 and mBD14 were also higher in the lungs of mice infected with Kp52145 than in the lungs of noninfected mice. Levels of mBD4 were not significantly different between noninfected mice and mice infected with Kp52145 (Fig. 3).

NF-κB and MAPKs are involved in the upregulation of human β-defensins by the K. pneumoniae CPS mutant. We sought to determine which intracellular pathways are involved in 52145-ΔwcaK2-induced hBD2 and hBD3 expression. Based on the key role of NF-κB in controlling the expression of genes involved in the host defense against infections, we asked whether NF-κB could be behind the mutant-induced increased expression of hBDs. Therefore, we analyzed whether CAPE, a chemical inhibitor used to block the NF-κB signaling pathway (53), would alter 52145-ΔwcaK2-induced hBD expression. As shown in Fig. 4, CAPE reduced 52145-ΔwcaK2-induced hBD2 expression (Fig. 4A), whereas the expression of hBD3 was not affected (Fig. 4B).

In addition to the NF-κB signaling cascade, many cellular stimuli also activate MAPK pathways (17). Therefore, we asked whether MAPKs are also involved in 52145-ΔwcaK2-induced hBD expression. To determine the contribution of MAPKs to hBD expression, infections were performed in the presence of SB203580, SP600125, or U0126, which are specific chemical inhibitors for the MAPKs p38, JNK, and p44/42, respectively. The MAPK p38 inhibitor did not affect the expression of either hBD2 or hBD3 (Fig. 4A and B). SP600125, the JNK inhibitor, significantly reduced 52145-ΔwcaK2-induced expression of hBD2 and hBD3, whereas U0126, the p44/42 inhibitor, reduced the expression of hBD2 only (Fig. 4A and B). Control experiments revealed that the addition of dimethyl sulfoxide (DMSO) (the vehicle solution for all inhibitors) to 52145-ΔwcaK2-infected cells did not affect the expression of either hBD2 or hBD3 (Fig. 4A and B).

Previously we demonstrated that a K. pneumoniae CPS mutant, strain 52K10, activates NF-κB by inducing the phosphorylation of IκBα, followed by degradation of the protein (59). To confirm that 52145-ΔwcaK2 activates NF-κB, we analyzed the expression levels of IκBα in cell extracts by immunoblot analysis. IκBα degradation was apparent in extracts from cells infected with 52145-ΔwcaK2 45 and 60 min postinfection (Fig. 4C). For the sake of comparison, the levels of IκBα in cell extracts from Kp52145-infected cells were also analyzed (Fig. 4C). IκBα degradation was apparent only at 30 min postinfection; at later time points, IκBα levels were similar to those in noninfected cells (Fig. 4C).

Activation of p38, p44/42, and JNK occurs through phosphorylation of serine and threonine residues (17). Therefore,
we determined whether 52145-ΔwcaK2 induced the phosphorylation of p38, p44/42, and JNK. Western blot analysis showed that infection with 52145-ΔwcaK2 induced the phosphorylation of the three MAPKs (Fig. 4D). 52145-ΔwcaK2-induced phosphorylation was apparent 15 min postinfection (Fig. 4D). p44/42 and JNK phosphorylation was still detected at 60 and 45 min postinfection, respectively (Fig. 4D). Kp52145 also induced the phosphorylation of the three MAPKs. However, Kp52145-induced phosphorylation occurred at later time points than phosphorylation in cells infected with 52145-ΔwcaK2. Thus, p38, p44/42, and JNK phosphorylation was apparent 45, 30, and 60 min postinfection, respectively (Fig. 4D).

In summary, these results suggest that NF-κB and MAPKs p44/42 and JNK are involved in hBD2 expression induced by 52145-ΔwcaK2, whereas activation of the MAPK JNK would be required for increased expression of hBD3.

**Dissection of host cell receptors involved in the induction of human β-defensin expression upon infection by the K. pneumoniae CPS mutant.** In most cases, the activation of NF-κB and MAPK signaling pathways is dependent on the activation of the so-called pattern recognition receptors (PRRs) (4, 9). The best-characterized PRRs belong to the family of Toll-like receptors (TLRs) (2). To explore the contribution of TLRs to hBD expression induced by 52145-ΔwcaK2 infection, RNA in-
terference technology was used to knock down gene expression. Given that almost all TLRs activate intracellular pathways through TIR domain-mediated interactions with the adaptor molecule MyD88 (4), the involvement of TLRs in \(52145^{\text{H9004}}\) \(wca\) \(K2\)-induced hBD expression can be examined by interrupting the function of the MyD88 adaptor molecule. After siRNA knockdown of MyD88, \(52145^{\text{H9004}}\) \(wca\) \(K2\) did not induce hBD2 expression (Fig. 5A). Previously, we determined that TLR2 and TLR4 are involved in \(K. pneumoniae\) recognition by airway epithelial cells (59, 60). Therefore, we asked whether TLR2 and TLR4 are involved in hBD2 upregulation by \(52145^{\text{H9004}}\) \(wca\) \(K2\). The results shown in Fig. 5A indicate that both receptors contribute to hBD2 activation during \(52145^{\text{H9004}}\) \(wca\) \(K2\) infection. In contrast, \(52145^{\text{H9004}}\) \(wca\) \(K2\) infection still activated the expression of hBD3 in MyD88 knockdown cells (Fig. 5B), thus suggesting that there is no role for MyD88-dependent TLR signaling in \(52145^{\text{H9004}}\) \(wca\) \(K2\)-induced expression of hBD3. Considering that TLR4 is also able to signal through the protein adaptor TRIF (4), we asked whether TLR4 participates in \(52145^{\text{H9004}}\) \(wca\) \(K2\)-mediated hBD3 upregulation. However, in TLR4 knockdown cells, \(52145^{\text{H9004}}\) \(wca\) \(K2\) induced hBD3 expression (Fig. 5B).

NOD1 belongs to the nucleotide binding and oligomerization domain (NOD)-like receptor (NLR) family of PRRs (9, 65), and its engagement also results in the activation of NF-\(\kappa\)B and MAPK signaling cascades (32). In addition, evidence indicates that NOD1 is constitutively expressed by epithelial cells (25, 30, 32, 68). We explored whether the protein adaptor TRIF (4), we asked whether TLR4 participates in \(52145^{\text{H9004}}\) \(wca\) \(K2\)-mediated hBD3 upregulation. However, in TLR4 knockdown cells, \(52145^{\text{H9004}}\) \(wca\) \(K2\) induced hBD3 expression (Fig. 5B).

NOD1 belongs to the nucleotide binding and oligomerization domain (NOD)-like receptor (NLR) family of PRRs (9, 65), and its engagement also results in the activation of NF-\(\kappa\)B and MAPK signaling cascades (32). In addition, evidence indicates that NOD1 is constitutively expressed by epithelial cells (25, 30, 32, 68). We explored whether NOD1 contributes to hBD3 expression induced by \(52145^{\text{H9004}}\) \(wca\) \(K2\) by using siRNA to knock down its expression. \(52145^{\text{H9004}}\) \(wca\) \(K2\) infection did not increase the expression of hBD3 in NOD1 knockdown cells (Fig. 5B).

Together, our data suggest that \(52145^{\text{H9004}}\) \(wca\) \(K2\)-increased expression of hBD2 is mediated by the TLR4-TLR2-MyD88 pathway. In contrast, NOD1 is involved in the increased expression of hBD3 induced by the CPS mutant.

**CYLD and MKP-1 act as negative regulators for \(K. pneumoniae\) CPS mutant-induced expression of human \(\beta\)-defensins.** In addition to their antimicrobial activity, hBDs facilitate and amplify innate responses (for a review, see reference 21). Therefore, excess hBD production may contribute to an overwhelming inflammatory response. For this reason, tight regulation of hBD expression could be critical for balancing the beneficial and detrimental effects of hBD production. Given that our data have shown that activation of NF-\(\kappa\)B is positively involved in hBD2 expression, we investigated whether CYLD, a key negative regulator of NF-\(\kappa\)B (37, 71, 79), is involved in regulating hBD2 expression induced by \(52145^{\text{H9004}}\) \(wca\) \(K2\) infection. The results shown in Fig. 6A revealed that \(52145^{\text{H9004}}\) \(wca\) \(K2\)-induced hBD2 expression was higher in CYLD knockdown cells than in cells treated with a control siRNA.

We have also demonstrated that MAPKs are involved in the expression of both hBD2 and hBD3. Negative regulation of MAPK activity is exerted primarily by MAPK phosphatases (MKP), a family of phosphatases that dephosphorylate the MAPKs on their threonine and serine residues (44). MKP-1 is the archetype of the family, and several immunomodulatory agents induce the expression of MKP-1 (44, 73). We asked whether MKP-1 is involved in regulating the expression of hBD2 and hBD3 upon infection with the CPS mutant by...
knocking down mkp-1 expression using siRNA. As shown in Fig. 6A, 52145-ΔwcaK2-induced expression of hBD2 and hBD3 was higher in MKP-1 knockdown cells than in cells treated with a control siRNA.

Having established that CYLD and MKP-1 are negatively involved in hBD2 and hBD3 expression induced by 52145-ΔwcaK2, we asked whether this strain activates the expression of CYLD and MKP-1. Figure 6B shows that 52145-ΔwcaK2 increased the expression of cyld and mkp-1 at the mRNA level. Western blot analysis revealed that 52145-ΔwcaK2 infection also increased the levels of the CYLD and MKP-1 proteins (Fig. 6C). The levels of both proteins in extracts from Kp52145-infected cells were analyzed. Of note, the wild-type strain also increased the levels of CYLD and MKP-1 (Fig. 6C).
However, Kp52145 did not induce hBD expression in either CYLD or MKP-1 knockout cells (Fig. 6D).

**Dissection of host cell receptors involved in the induction of CYLD and MKP-1 upon infection by the *K. pneumoniae* CPS mutant.** We investigated the requirement for PRRs in CYLD and MKP-1 activation by 52145-ΔwcaK2 siRNA was used to silence PRR gene expression, and Western blot analysis were carried out to determine the levels of CYLD and MKP-1 proteins. The results shown in Fig. 7A revealed that 52145-ΔwcaK2-induced CYLD expression was lower in TLR2 knockdown cells than in infected control cells. After the silencing of TLR4, 52145-ΔwcaK2-induced CYLD expression reached levels similar to those observed in infected cells treated with a control siRNA (Fig. 7A). Likewise 52145-ΔwcaK2-induced CYLD expression was similar in control and NOD1 knockdown cells (Fig. 7A). 52145-ΔwcaK2 increased the expression of MKP-1 in TLR2 knockdown cells, although the levels were lower than those in infected cells treated with a control siRNA (Fig. 7B). In TLR4 knockdown cells, infection did not increase the expression of MKP-1. After the silencing of NOD1, infection induced MKP-1 expression, although MKP-1 levels were lower than those found in infected cells treated with a control siRNA (Fig. 7B).

In summary, our data suggest that the induction of CYLD expression by 52145-ΔwcaK2 relies on the activation of TLR2. MKP-1 expression induced by 52145-ΔwcaK2 depends mainly on the activation of TLR4, whereas TLR2 and NOD1 make a minor contribution to 52145-ΔwcaK2 induction of MKP-1 expression.

**DISCUSSION**

In this work we present *in vitro* evidence (by using A549 and normal human bronchial cells) showing that, in contrast to wild-type *K. pneumoniae*, a *K. pneumoniae* CPS mutant induces the expression of hBDs. Furthermore, we present *in vivo* evidence indicating that a *K. pneumoniae* CPS mutant induces higher levels of mBDs than wild-type *K. pneumoniae*. The fact that an LPS O antigen mutant expressing wild-type levels of CPS does not induce the expression of hBDs suggests that the presence of CPS on the bacterial surface may affect the activation of cellular signaling pathways responsible for the up-regulation of BD expression. Consistent with this idea, our data demonstrate that the CPS mutant activates the expression of hBD2 via a positive IkB-dependent NF-κB pathway and MAPK p44/42 and JNK pathways, whereas hBD3 expression is dependent on the activation of the MAPK JNK. Interestingly, CYLD and MKP-1 negatively regulate *K. pneumoniae* CPS mutant-induced hBD2 and hBD3 expression. Moreover, the expression of CYLD and MKP-1 was dependent on the engagement of PRRs by the *K. pneumoniae* CPS mutant. Taking these findings together, this study provides novel insights not only into the interplay between *K. pneumoniae* and airway epithelial cells but also into the molecular mechanisms underlying the regulation of hBD expression induced by a bacterial pathogen.

All studies to date have shown that hBD1 is constitutively expressed by epithelial cells, including the airway epithelium, whereas the expression of hBD2 and hBD3 is inducible by pathogens and proinflammatory cytokines (27, 28, 47, 66). The promoter region of hBD2 contains NF-κB and AP-1 binding motifs (74). AP-1 is one of the transcription factors activated by different MAPK pathways. In good agreement, in this study we found that 52145-ΔwcaK2-induced expression of hBD2 was mediated by NF-κB and the MAPKs p44/42 and JNK. Interestingly, not every stimulus inducing the expression of hBD2 activates the same MAPK pathway. For example, in intestinal cells, JNK mediates hBD2 expression induced by LPS, peptidoglycan, or *Escherichia coli* Nissle 1917 (74, 75), whereas p38 and p44/42 are required for flagellin-dependent induction of hBD2 (55). p38 and JNK mediate *Fusobacterium nucleatum*-dependent hBD2 expression in human gingival epithelial cells (40), and the three MAPKs participate in *Helicobacter pylori*-induced hBD2 expression in gastric epithelial cells (72). Analysis of the promoter region of hBD3 reveals the presence of...
AP-1 binding motifs but not NF-κB binding sites (36). Most studies, including ours, support the involvement of MAPKs in hBD3 expression (7, 48, 67; also this study) and the lack of a role for NF-κB (7, 10, 48, 67; also this study). As with hBD2, different MAPKs mediate hBD3 expression depending on the stimulus and cell type. While the MAPK p38 is responsible for Staphylococcus aureus-triggered hBD3 expression in keratinocytes (48), p38, p44/42, and JNK contribute to hBD3 induction by H. pylori in gastric cells (7). Taken together, it is tempting to speculate that depending on the stimuli and/or cell type, different MAPKs contribute to hBD2 and hBD3 expression.

Activation of host defense responses, such as the expression of BDs, depends on the engagement of PRRs (4, 20), which, in turn, activate NF-κB and MAPK-dependent signaling cascades. The most extensively characterized PRRs belong to the family of TLRs. Several studies have shown that the expression of BDs can be activated by a plethora of purified TLR agonists (for a review, see reference 21). However, fewer studies have addressed the contribution of TLRs to BD expression induced by bacterial infections. TLR2 mediates hBD2 expression induced by F. nucleatum and S. aureus, whereas TLR2 and TLR4 are required for the induction of hBD2 by Propionibacterium acnes (34). Here we showed that 52145-ΔwcaK2 activated the expression of hBD2 via TLR2 and TLR4. In contrast, hBD3 expression was dependent on the activation of the intracellular receptor NOD1 (9, 65). NOD1 mediates hBD2 expression activated by H. pylori and Campylobacter jejuni (7, 80), yet, to the best of our knowledge, the current study is the first report implicating NOD1 in the expression of hBD3 induced by a pathogen.

Previous studies from our laboratory implicated TLR2 and TLR4 in the recognition of a K. pneumoniae CPS mutant, leading to the secretion of inflammatory mediators (59). In this work, we showed that a CPS mutant also engaged TLR2 and TLR4 to induce the expression of hBD2. Based on these data, we suggest that TLR-mediated recognition of K. pneumoniae is a key event for the induction of host defense responses to this pathogen. This hypothesis also implies that wild-type K. pneumoniae must somehow counteract TLR-mediated recognition. Our data showing that the presence of CPS on the surface of the wild-type cell prevented the induction of TLR-dependent responses (59; also this work) are consistent with this idea. Our findings implicate NOD1 in the recognition of the K. pneumoniae CPS mutant. Taking into account that we and others have shown that K. pneumoniae CPS mutants are internalized by epithelial cells (16, 59), NOD1-dependent recognition of the CPS mutant is consistent with the idea that this PRR participates in the recognition of internalized or obligately intracellular pathogens (24, 32, 38). In turn, the fact that CPS impedes the internalization of K. pneumoniae by cells can be taken as a strategy to avoid the activation of the NOD1-dependent host defense response. Altogether, we propose that evasion of PRR-mediated recognition may represent a feature of bacterial CPSs.

An increasing body of evidence indicates that BDs facilitate and amplify innate responses (for a review, see reference 21). Therefore, high levels of BDs would prompt the host to develop an overwhelming inflammatory response, leading to complications (23). Supporting this, BDs are upregulated in several inflammatory diseases (1, 11, 15, 19), highlighting the notion that the expression of BD-encoding genes should be tightly controlled. To the best of our knowledge, the current study is the first one describing negative mechanisms controlling the expression of BDs induced by bacteria. Having established the involvement of NF-κB and MAPK signaling pathways in 52145-ΔwcaK2-induced upregulation of BDs, we explored the possibility that inhibitors of these pathways might be involved in negative regulation of BD expression. We identified CYLD as a negative regulator of the expression of hBD2, whereas MKP-1 negatively controls hBD2 and hBD3 expression. CYLD has been identified as a negative regulator of NF-κB activation by deubiquitinating proteins of the TRAF family (14, 71). On the other hand, cells control the activation of MAPK-dependent signaling by MAPK phosphatases; MKP-1 is the archetype of this family (44, 73). Our data demonstrated that 52145-ΔwcaK2 induced the expression of CYLD and MKP-1 in a PRR-dependent manner. These results are consistent with the concept that to prevent uncontrolled innate responses, a number of signaling mechanisms are evoked, including the transcriptional induction of negative regulatory proteins (26, 43). Interestingly, wild-type K. pneumoniae also induced the expression of the negative regulators CYLD and MKP-1. A tantalizing hypothesis could be that K. pneumoniae could modulate the activation of inflammatory responses by, on the one hand, preventing TLR-dependent responses (59; also this work) and, on the other hand, targeting cellular functions controlling the expression of CYLD and MKP-1. Future studies will explore this hypothesis.

It is known that K. pneumoniae strains expressing small amounts of CPS are less virulent than strains expressing larger amounts of CPS (51, 52) and, furthermore, that CPS mutants are avirulent in a mouse model of pneumonia (13, 41, 42). A previous study from our laboratory showed that a CPS mutant is more susceptible to hBD1 than wild-type K. pneumoniae (8), and here we have shown that this is also true for hBD2. Moreover, in this work we showed that the CPS mutant induced the expression of hBD2 and hBD3. Thus, the avirulent strain not only activated host defense mechanisms but also was susceptible to them. Of particular interest in our study is the fact that hBD3 also killed wild-type K. pneumoniae. Therefore, our findings demonstrating that wild-type K. pneumoniae did not elicit the expression of BDs in vitro and elicited significantly lower levels of mBDs in vivo than the CPS mutant could be considered as a Klebsiella strategy for survival in the lung. Interestingly, results from our laboratory indicate that this strategy could be complemented by the expression of bacterial countermeasures against BDs, including an increase in CPS production and an alteration of LPS charge (8, 45; E. Llobet and J. A. Bengoechea, unpublished results). Collectively, these findings are consistent with a scenario in which K. pneumoniae facilitates the pathogenesis of pneumonia by, on the one hand, preventing the expression of BDs and, on the other hand, activating countermeasures against them.

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Klebsiella pneumoniae K1 and K2 with the presence of a plasmid encoding aerobactin. Infect. Immun. 54:603-608.


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ERRATUM

*Klebsiella pneumoniae* Capsule Polysaccharide Impedes the Expression of β-Defensins by Airway Epithelial Cells

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