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Klebsiella pneumoniae Outer Membrane Protein A Is Required to Prevent the Activation of Airway Epithelial Cells*

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Outer membrane protein A (OmpA) is a class of proteins highly conserved among the *Enterobacteriaceae* family and throughout evolution. *Klebsiella pneumoniae* is a capsulated Gram-negative pathogen. It is an important cause of community-acquired and nosocomial pneumonia. Evidence indicates that *K. pneumoniae* infections are characterized by a lack of an early inflammatory response. Data from our laboratory indicate that *K. pneumoniae* CPS helps to suppress the host inflammatory response. However, it is unknown whether *K. pneumoniae* employs additional factors to modulate host inflammatory responses. Here, we report that *K. pneumoniae* OmpA is important for immune evasion *in vitro* and *in vivo*. Infection of A549 and normal human bronchial cells with 52OmpA2, an *ompA* mutant, increased the levels of IL-8. 52145-Δ*wca*$_{K2}$ompA, which does not express CPS and *ompA*, induced the highest levels of IL-8. Both mutants could be complemented. *In vivo*, 52OmpA2 induced higher levels of *tnfα*, *kc*, and *il6* than the wild type. *ompA* mutants activated NF-κB, and the phosphorylation of p38, p44/42, and JNK MAPKs and IL-8 induction was via NF-κB-dependent and p38- and p44/42-dependent pathways. 52OmpA2 engaged TLR2 and -4 to activate NF-κB, whereas 52145-Δ*wca*$_{K2}$ompA activated not only TLR2 and TLR4 but also NOD1. Finally, we demonstrate that the *ompA* mutant is attenuated in the pneumonia mouse model. The results of this study indicate that *K. pneumoniae* OmpA contributes to attenuate airway cell responses. This may facilitate pathogen survival in the hostile environment of the lung.

The OM[3] of Gram-negative bacteria is composed of phospholipids, LPS, and outer membrane proteins. These proteins are important for membrane integrity and transport of molecules across the membrane (for a review, see Ref. 1). OmpA (outer membrane protein A) is one of the best characterized OM proteins. Its crystal structure has been determined (at a resolution of 1. 65 Å), and it consists of an eight-stranded all-next-neighbor antiparallel β-barrel with short turns at the periplasmic barrel (2). A number of studies have highlighted the role of OmpA in pathogenesis. *Escherichia coli* OmpA mediates adhesion and/or invasion to epithelial cells and macrophages (3, 4). It also mediates serum resistance and may protect bacteria against the bactericidal action of lung collectins SP-D and SP-A (5, 6). However, OmpA is also targeted by the innate immune system. Neutrophil elastase, a protein included in the array of oxygen-independent weapons of the innate immune system, degrades OmpA, resulting in cell death (7). The acute-phase protein serum amyloid protein A binds to OmpA, and this is associated with an increased uptake of bacteria by neutrophils and macrophages (8, 9).

Airway epithelial cells play a pivotal role in lung defense against infections by detecting pathogens, thereby leading to the activation of signaling pathways resulting in the production of antimicrobial molecules, the expression of co-stimulatory molecules, and the release of cytokines and chemokines (10, 11). In particular, IL-8 is a major secreted product of infected cells independent of the infecting microorganism (12, 13). This chemokine is a potent chemoattractant for polymorphonuclear cells into the infected tissue (12, 13). To launch these responses, airway epithelial cells recognize conserved molecules expressed by pathogens, the so-called PAMPs, through a set of germ line-encoded receptors referred to as PRRs (14, 15). The best characterized PRRs belong to the families of Toll-like receptors (TLRs) and nucleotide binding and oligomerization domain-like receptors (NLRs) (16–18). Among TLRs, most of the studies focus on TLR4, mainly involved in the detection of LPS, and on TLR2, which responds to a variety of Gram-positive PAMPs (10, 14, 15). Among NLRs, NOD1 has received increasing attention. NOD1 is located intracellularly, and evidence indicates that it recognizes a peptidoglycan motif from Gram-negative bacteria (16, 17).

Klebsiella pneumoniae is a capsulated Gram-negative pathogen that causes a wide range of infections, from urinary tract infections to pneumonia, the latter being particularly devastating among immunocompromised patients with mortality rates between 25 and 60% (19). The best characterized virulence fac-

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[3] The abbreviations used are: OM, outer membrane; CPS, capsule polysaccharide; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; TLR, Toll-like receptor; NLR, nucleotide binding and oligomerization domain-like receptor; CAPE, caffeic acid phenethyl ester; cfu, colony-forming units; RT-qPCR, real-time quantitative PCR; ANOVA, analysis of variance.

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tor of this species is CPS, which is responsible for protection against complement and antimicrobial peptide-mediated killing (20–22). In addition, isogenic CPS mutant strains are avirulent, being unable to cause pneumonia and urinary tract infections (23–25).

A wealth of evidence indicates that activation of inflammatory responses is essential to clear *Klebsiella* infections (26–28) and that TLRs seem to play a major role in detecting *K. pneumoniae* (29, 30). Conversely, this suggests that *K. pneumoniae* may somehow try to counteract the induction of these host defense responses. Indeed, we and others (31, 32) have shown that in sharp contrast to wild-type strains, avirulent CPS mutants activate an inflammatory program through TLR-dependent pathways. In fact, it has been postulated that CPS helps to suppress the host inflammatory response, thereby allowing the bacteria to replicate in a more permissive niche. Whether *K. pneumoniae* employs additional factors to modulate host inflammatory responses is still unknown. In this context, several studies have shown that recombinant purified OmpA from *K. pneumoniae* induces the expression of inflammatory molecules in a TLR2-dependent manner in various cell types (33–35). Hence, it has been postulated that detection of *K. pneumoniae* OmpA may contribute to the activation of host responses leading to the clearance of *K. pneumoniae* (36). However, there might be differences between the cellular recognition of recombinant purified OmpA and OmpA expressed in the complex lipid environment of the bacterial OM, and hence the cellular responses could be different.

The purpose of this study was to analyze whether OmpA may contribute to the activation of inflammatory responses when expressed in the bacterial OM. To this end, we compared the host responses to wild-type *K. pneumoniae* and an isogenic *ompA* mutant. We report that *K. pneumoniae* OmpA is important for immune evasion of *K. pneumoniae in vitro* and *in vivo*. Mechanistically, we demonstrate that the *ompA* mutant induces the secretion of inflammatory mediators via activation of NF-κB and MAPKs p38 and p44/42. In addition, we show that *ompA* mutant induction of inflammatory responses is dependent on TLR activation. Finally, we demonstrate that the *K. pneumoniae ompA* mutant is attenuated in a mouse model of pneumonia.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and Reagents—*K. pneumoniae* 52145 is a clinical isolate (serotype O1:K2) described previously (24, 37). The isogenic mutants 52OmpA2, which does not express *ompA*; 52145-Δwca$_{K2}$, which does not express CPS; and 52145-Δ*wca*$_{K2}$ompA, which does not express CPS and *ompA*, have been described previously (38, 39). Analysis of OM proteins from these strains as well as the description of the complementing strains have been reported previously (39). Bacteria were grown in Luria-Bertani medium at 37 °C. When appropriate, antibiotics were added to the growth medium at the following concentrations: rifampicin (50 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (25 μg/ml). Caffeic acid phenethyl ester (CAPE), an NF-κB inhibitor, and SB203580, a p38 MAPK inhibitor, were purchased from Sigma, whereas U0126, a p44/42 MAPK inhibitor, and SP600125, a JNK inhibitor, were purchased from Calbiochem.

CPS Purification and Quantification—Cell-associated CPSs from *K. pneumoniae* strains, grown in 5 ml of LB, were obtained using the hot phenol-water method exactly as described previously (20). CPS was quantified by determining the concentration of uronic acid in the samples, using a modified carbazole assay (40), exactly as described by Rahn and Whitfield (41).

Cell Culture and Infection—Monolayers of A549 (ATCC CCL185) and NHBE (Lonza) cells were grown as described previously (32). For infections, A549 cells were seeded to 90% confluence (3×10^5 cells/well) in 24-well tissue culture plates. Cells were serum-starved for 16 h before infection. For NHBE cells, cells were seeded to 80% confluence (2×10^5 cells/well) in collagen-coated 24-well tissue culture plates using 1 ml of bronchial epithelial cell growth medium (Lonza) per well. Bacteria were prepared as described (32, 42), and infections were performed using a multiplicity of infection of 100 bacteria/cell, unless indicated otherwise. To synchronize infection, plates were centrifuged at 200 × *g* for 5 min. For incubation times longer than 120 min, bacteria were killed by the addition of gentamicin (100 μg/ml), which was not removed until the end of the experiment. For experiments reported in Fig. 2, infections were performed for 3 h. Cell viability was assessed by trypan blue dye exclusion, and it was >95% even after 9 h of infection.

Adhesion and Internalization Assays—To determine the adhesion of *Klebsiella* strains to A549 cells, after 2 h of infection, monolayers were washed five times with PBS, and cells were lysed with 300 μl of 0.5% saponin-PBS for 5 min at room temperature, and serial dilutions were plated on LB agar plates for viable counts of bacteria. The results are expressed as log cfu/well. For invasion assays, cells were infected for 2 h and washed three times with PBS and then incubated for an additional 2 h with fresh medium plus gentamicin (100 μg/ml) to kill extracellular bacteria. This treatment was long enough to kill all extracellular bacteria. Epithelial monolayers were washed three times with PBS and lysed as described before. The results are expressed as log cfu/well. Experiments were carried out in triplicate on at least two independent occasions.

IL-8 Stimulation Assay—Epithelial monolayers were infected for different time periods. Supernatants were removed from the wells, cell debris was removed by centrifugation, and samples were frozen at −80 °C. IL-8 in the supernatants was determined by a commercial ELISA (Bender MedSystems) with a sensitivity of <2 pg/ml. Experiments were run in duplicate and repeated at least three times.

Transfections and Luciferase Assays—A549 cells were seeded into 24-well tissue culture plates to obtain a 40–60% confluence 24 h later. Cells were washed twice with PBS before transfection. Transfection experiments were carried out in 500 μl of Opti-MEM reduced serum medium (Invitrogen) using Lipofectamine™ 2000 transfection reagent following the manufacturer's recommendations (Invitrogen).

For luciferase experiments, the PathDetect® NF-κB *cis*-reporting plasmid (250 ng; Stratagene) was co-transfected with the pRL-TK *Renilla* luciferase control reporter vector (20 ng; Promega). For luciferase assays, cells were lysed with passive

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lysis buffer (Promega). Luciferase activity was assayed using the dual luciferase assay kit according to the manufacturer's instructions (Promega). Firefly luciferase values were normalized to *Renilla* control values. Results were plotted as relative luciferase activity compared with activity measured in non-stimulated control cells. The luciferase assay was carried out in triplicate on at least three independent occasions.

Small Interfering RNA (siRNA)—Interference RNA for TLR4 (catalogue number HSS110818), TLR2 (catalogue number HSS110813), and CARD4/NOD1 (catalogue number HSS115906) were purchased from Invitrogen. Stealth™ RNAi negative control low GC was used as control interference RNA for TLR2 and TLR4, whereas Stealth™ RNAi negative control medium GC was used as control interference RNA for NOD1. RNA-mediated interference for down-regulating MyD88 was done by the transfection of MyD88 siRNA (5′-AACTGGAA-CAGACAAACTATC-3′) purchased from Qiagen. The AllStars negative control siRNA (Qiagen) was used as non-silencing control interference RNA. In all cases, 20 nM siRNA/well was used for transfection using Lipofectamine™ 2000 transfection reagent and following the manufacturer's recommendations (Invitrogen). Cells were infected 48 h post-transfection as described above.

Real-time Quantitative PCR (RT-qPCR)—Quantification of mRNA levels was conducted by RT-qPCR. RNA was purified using a Nucleospin RNAII kit (Macherey-Nagel) exactly as recommended by the manufacturer. cDNA was obtained by retrotranscription of 1.5–2 μg of total RNA using a commercial RT^2 first strand kit as recommended by the manufacturer (SABioscience). The reaction included one step to eliminate traces of genomic DNA. Real-time PCR (RT-PCR) analyses were performed with a Smart Cycler real-time PCR instrument (Cepheid, Sunnyvale, CA). To amplify human *TLR2*, *TLR4*, *MyD88*, and *NOD1*, 500 ng of cDNA were used as a template in a 25-μl reaction containing 1× QuantiTect SYBR Green PCR mix (Qiagen) and QuantiTect primer assays (Qiagen; catalogue numbers QT00236131 (*TLR2*) and QT00035238 (*TLR4*)) or the intron-spanning primers: 5′-GGCATCACCACACTTGAT-GAC-3′ (sense) and 5′-ATAGACCAGACACAGGTGCCAG-3′ (antisense) for *MyD88* and 5′-TCAAGTTGGGGATGAAG-GAG-3′ (sense) and 5′-GCCAAACTCTCTGCCACTTC-3′ (antisense) for *NOD1*. As an internal control, we amplified the human housekeeping gene *GAPDH* using 50 ng of cDNA and the following intron spanning primers: 5′-GAAGGTGAA-GGTCGGAGTC-3′ (sense); 5′-GAAGATGGTGATGGGAT-TTC-3′ (antisense). 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 *TLRs*, *MyD88*, and *NOD1* or 54 °C for *GADPH* for 30 s; and extension at 72 °C for 30 s. The SYBR Green dye was measured at 521 nm during the annealing phase. The threshold cycle (*Ct*) value reflects the cycle number at which the fluorescence generated within 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 mRNA amount in each sample was calculated based on its *Ct* in comparison with the *Ct* of *GAPDH*. Specificity of the PCR products was determined by melting curve analysis, and amplification products were resolved on a 1.5% agarose gel to confirm the correct size of the amplicons (92, 102, 185, 201, and 226 bp for *TLR2*, *TLR4*, *MyD88*, *NOD1*, and *GAPDH*, respectively).

Immunoblotting—Proteins from lysed cells were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose membrane, and blocked with 4% skimmed milk in PBS. Immunostainings for MKP-1, CYLD, and IκBα were performed using polyclonal rabbit antibodies anti-MKP-1 (1:1,000; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), anti-IκBα (1:1,000; Cell Signaling), and anti-CYLD (1:1,000) (Imgenex Corp.) Immunostainings to assess phosphorylation of NF-κB p65, p38, p44/42, and JNK MAPKs were performed with polyclonal rabbit anti-phospho-p65, anti-phospho-p38, anti-phospho-p44/42, and anti-phospho-JNK antibodies, respectively (all used at 1:1,000; Cell Signaling). Immunoreactive bands were visualized by incubation with swine anti-rabbit immunoglobulins conjugated to horseradish peroxidase (Dako P0217) using the Super-Signal West-dura system (Pierce). Blots were reprobed with polyclonal anti-human tubulin antibody (1:3,000; Santa Cruz Biotechnology, Inc.) to control that equal amounts of proteins were loaded in each lane.

NOD1 NF-κB Activation Assay—HEK293T, seeded in 24-well plates, were transfected overnight with pCI-Nod1 (0.3 ng; kindly donated by T. Kufer), PathDetect® NF-κB *cis*-reporting plasmid (75 ng), pRL-TK *Renilla* luciferase (7.5 ng), and 120 ng of pcDNA3 (120 ng) to balance the DNA concentration. Cells were transfected using calcium chloride. 24 h post-transfection, cells were washed once with PBS and infected for 2 h, washed once with PBS, and then incubated for an additional 6 h with fresh medium plus gentamicin (100 μg/ml) before performing luciferase measurements.

Intranasal Infection Model—5–7-week-old female C57BL/6JOlaHsd mice (Harlan) were anesthetized by intraperitoneal injection with 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 5×10^6 cfu/ml for analysis of cytokine expression and to 5×10^4 cfu/ml for determination of bacterial loads. 20 μl of the bacterial suspension were inoculated intranasally in four 5-μl aliquots. Non-infected 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 placed on a 45° incline while recovering from anesthesia. At the indicated times after infection, mice were euthanized by cervical dislocation, and lungs were either rapidly dissected for bacterial load determination or immediate frozen in liquid nitrogen and stored at −80 °C until purification of RNA was carried out. To this end, lungs were quickly weighted and homogenized with 1 ml of TRI reagent (Ambion) using an Ultra-Turrax TIO basic (IKA) on ice. Total RNA was purified first using a standard chloroform/isopropyl alcohol protocol, and the obtained RNA was further purified using a Nucleospin RNAII kit (Macherey-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. The following intron-spanning primers were used: 5′-CCACATC-

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TCCCTCCAGAAAA-3′ (sense) and 5′-AGGGTCTGGGCCATAGAACT-3′ (antisense) for *tnf-α*; 5′-CCGGAGAGGAGACTTCACAG-3′ (sense) and 5′-CAGAATTGCCATTGCACAAC-3′ (antisense) for *il-6*; and 5′-GACAGACTGCTCTGATGGCA-3′ (sense) and 5′-TGCACTTCTTTTCGCACAAC-3′ (antisense) for *kc*. As internal controls, we amplified the mouse housekeeping *actin* and *gapdh* (5′-TGTTACCAACTGGGACGACA-3′ (sense) and 5′-CTGGGTCATCTTTTCACGGT-3′ (antisense) for *actin* and 5′-CCCACTAACATCAAATGGGG-3′ (sense) and 5′-CCTTCCACAATGCCAAAGTT-3′ (antisense) for *gapdh*. The sizes of the amplicons are 259, 134, 292, 139, and 274 bp for *tnf-α*, *il-6*, *kc*, *actin*, and *gapdh*, respectively.

Dissected lungs from those animals infected to determine bacterial loads were homogenized in 500 μl of PBS using an Ultra-Turrax TIO basic disperser (IKA) on ice. Bacteria from the homogenates and serial dilutions thereof were recovered in LB agar plates containing rifampicin for the wild-type strain or rifampicin plus kanamicin for the *ompA* mutant. Results were reported as log cfu/g of tissue. 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 (Spain).

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

RESULTS

Infection of Airway Epithelial Cells by K. pneumoniae ompA Mutant Induces the Secretion of IL-8—Previous data (32), further confirmed here, had shown that wild-type *K. pneumoniae* strain 52145 (hereafter referred to as Kp52145) does not induce the secretion of IL-8 by A549 cells (Fig. 1*A*). We asked whether infection with strain 52OmpA2, an isogenic *ompA* mutant, would induce the secretion of IL-8. Time course experiments showed a correlation between the duration of the infection with 52OmpA2 and the levels of IL-8 secreted by A549 cells, the levels of IL-8 being higher at 8 than at 4 h postinfection (Fig. 1*A*). Moreover, at 6 and 8 h postinfection, 52OmpA2-infected cells secreted levels of IL-8 significantly higher than those of Kp52145-infected cells, which were similar to those of non-infected cells (Fig. 1*A*) ($p > 0.05$; one-tailed Student's *t* test).

Evidence indicates that *K. pneumoniae* CPS mutants induce the secretion of IL-8 by airway epithelial cells (32). Therefore, the induction of IL-8 by 52OmpA2 could be due to a reduction in the amount of CPS expressed by this mutant. However, Kp52145 and 52OmpA2 expressed similar amounts of cell-bound CPS (360 ± 15 $\mu g/10^6$ cells and 339 ± 23 $\mu g/10^6$ cells, respectively; $p > 0.05$; one-tailed Student's *t* test). We tested whether the absence of OmpA further increases the levels of IL-8 induced by 52145-Δwca_{K2}, an isogenic CPS mutant from Kp52145. Indeed, at 8 h postinfection, 52145-Δwca_{K2}ompA-infected cells secreted higher levels of IL-8 than 52145-Δwca_{K2}- or 52OmpA2-infected cells (Fig. 1*B*). IL-8 levels induced by 52OmpA2Com- and 52145-Δwca_{K2}ompACom-complemented strains of 52OmpA2 and 52145-Δwca_{K2}, respectively, were not significantly different from those triggered by Kp52145 and 52145-Δwca_{K2}, hence indicating that the absence of OmpA is responsible for the induction of IL-8 secretion by OmpA mutants. For the sake of comparison, IL-8 levels induced by 52OmpA2 and 52145-Δwca_{K2}ompA were compared in a time course experiment (Fig. 1*C*). At all time points, 52145-Δwca_{K2}ompA-infected cells secreted higher levels of IL-8 than 52OmpA2-infected ones. *ompA* mutants also induced higher levels of IL-8 than parental strains in normal human bronchial cells (Fig. 1*D*). At 8 h postinfection, *ompA* mutants induced higher levels of IL-8 than the parental strains, 52145-Δwca_{K2}ompA being the strain inducing the highest levels. Both mutants were complemented (Fig. 1*D*). Collectively, these data show that CPS and OmpA are bacterial factors required to reduce the secretion of IL-8 by airway epithelial cells upon *K. pneumoniae* infection.

Control experiments revealed that the adhesion and internalization to cells were not significantly different between each *ompA* mutant and its corresponding parental strain (Fig. 1*E*). As reported previously (43, 44), strains lacking CPS adhered and were internalized in higher numbers than CPS-expressing strains (for each comparison between CPS-expressing and CPS-negative strains, $p < 0.05$ (one-tailed Student's *t* test) (Fig. 1*E*).

ompA Mutant Attenuates IL-1β-induced IL-8 Secretion—Recently, we have shown that Kp52145 attenuates proinflammatory mediator-induced IL-8 secretion (45). This process requires bacteria-cell contact, and removal of bacteria by washing followed by 1-h gentamicin treatment rendered cells responsive to agonist-induced IL-8 secretion (45). To exert this anti-inflammatory effect, Kp52145 up-regulates the expression of the deubiquitinase CYLD and the phosphatase MKP-1 (DUSP1) in a NOD1-dependent manner (45). To explore whether OmpA could account for the Kp52145 anti-inflammatory effect, we determined the effect of 52OmpA2 on IL-1β-induced IL-8 secretion by A549 cells using the assay that we have described (45). Similarly to Kp52145, 52OmpA2 did attenuate IL-1β-induced IL-8 secretion (Fig. 2*A*). Furthermore, Western blot analysis revealed that 52OmpA2 induced the expression of CYLD and MKP-1 (Fig. 2*B*). This was dependent on NOD1 because 52OmpA2 did not increase the expression of CYLD and MKP-1 in NOD1 knockdown cells (Fig. 2*C*).

K. pneumoniae ompA Mutant Induces Higher Levels of Cytokines than Wild-type Strain in Vivo—We investigated whether 52OmpA2 induces higher levels of cytokines than Kp52145 in the lungs of infected mice. Mice were infected intranasally and euthanized at different time points. Whole lungs were dissected, and the expressions of *kc, tnfα*, and *il6* were measured by RT-qPCR at 6, 12, and 24 h postinfection (Fig. 3). Levels of *kc*, *tnfα*, and *il6* were higher in lungs of infected mice than in lungs of non-infected animals ($p < 0.05$ for all comparisons *versus* non-infected mice (one-tailed Student's *t* test)) (Fig. 3). However, levels of *kc* were higher in lungs of mice infected with 52OmpA2 than in those infected with Kp52145 at all time points analyzed. 52OmpA2Com induced similar levels of *kc* than Kp52145. Only at 12 h postinfection, levels of *tnfα* were

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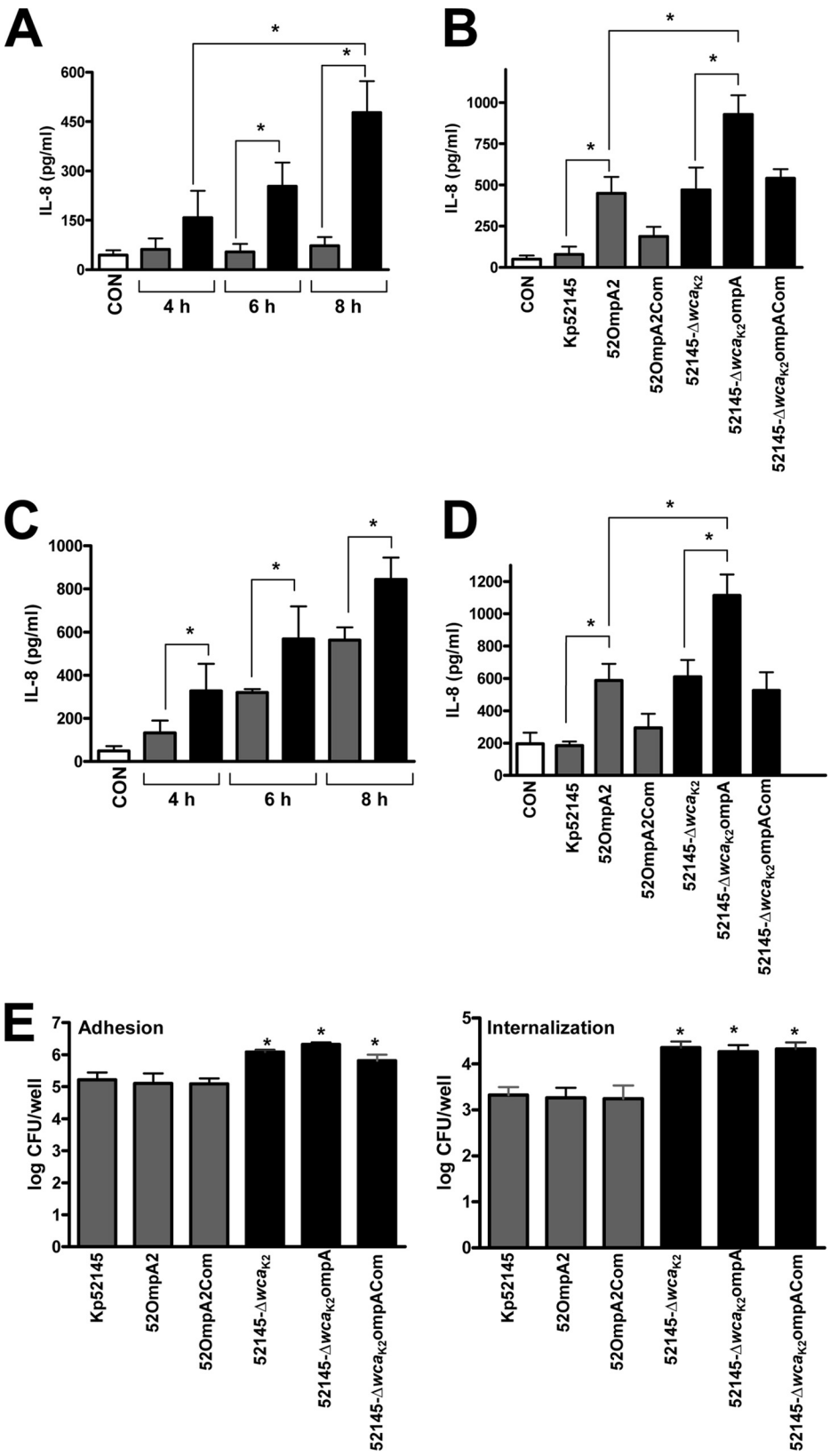

FIGURE 1. ***K. pneumoniae ompA* mutants induce higher levels of IL-8 than wild-type strains by airway epithelial cells.** *A*, ELISA of IL-8 released by A549 cells left untreated (*CON*) or infected for different time points with Kp52145 (*gray bars*) and 52OmpA2 (*black bars*) ($n = 3$). *B*, ELISA of IL-8 released by A549 cells left untreated (*CON*) or infected for 8 h with different *K. pneumoniae* strains ($n = 3$). *C*, ELISA of IL-8 released by A549 cells left untreated (*CON*) or infected for different time points with 52OmpA2 (*gray bars*) and 52145-Δwca_{K2}ompA (*black bars*) ($n = 3$). *D*, ELISA of IL-8 released by NHBE cells left untreated (*CON*) or infected for 8 h with different *K. pneumoniae* strains ($n = 3$). *E*, adhesion (*left*) and internalization (*right*) of *Klebsiella* strains to A549 cells ($n = 3$). *Gray bars*, CPS-expressing strains; *black bars*, CPS negative strains. The data (*A–E*) are means and S.E. (*error bars*). *A–D*, *, $p < 0.05$ (for the indicated comparisons; one-way ANOVA). *E*, *, $p < 0.05$ (results are significantly different from the results for Kp52145; one-tailed *t* test).

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significantly different between Kp52145- and 52OmpA2-infected mice. At 12 and 24 h postinfection, levels of *il6* were higher in lungs of mice infected with 52OmpA2 than in those infected with Kp52145. Levels of *il6* were not significantly different between mice infected with Kp52145 and animals infected with 52OmpA2Com at all time points. In summary, these data show that OmpA plays a role in *vivo* contributing to reduce host inflammatory responses.

Activation of NF-κB Is Required for K. pneumoniae ompA Mutant-induced IL-8 Expression—We next aimed to identify the signaling pathways activated by the *ompA* mutant to induce an inflammatory response. Several studies show that NF-κB participates in the inducible expression of genes involved in the inflammatory response, including IL-8 (46). Therefore, we analyzed the effect of 52OmpA2 on the NF-κB activation pathway by studying the activation of a reporter construct controlled by synthetic NF-κB response elements. A549 cells were transiently transfected with a NF-κB-dependent luciferase reporter followed by infection, and infection-induced NF-κB activation was measured as relative luciferase activity (Fig. 4*A*). In contrast to Kp52145, 52OmpA2 induced the activation of the reporter construct. Complemented strain 52OmpA2Com induced a NF-κB-dependent luciferase activity similar to Kp52145-infected cells. Supporting previous findings (22, 32), 52145-Δwca_{K2} induced the reporter construct, which was further induced by 52145-Δwca_{K2}ompA (Fig. 4*A*). Complementation of the mutant, strain 52145-Δwca_{K2}ompACom, restored NF-κB-dependent luciferase activity to 52145-Δwca_{K2}-induced levels (Fig. 4*A*).

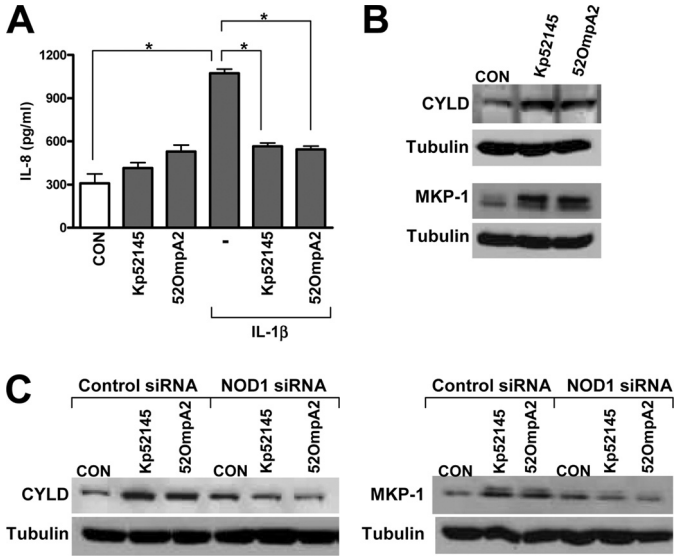

FIGURE 2. ***K. pneumoniae* ompA attenuates IL-1β-induced IL-8 expression.** *A*, ELISA of IL-8 released by A549 left untreated (*CON*) or infected for 1 h with Kp52145 or 52OmpA2 and then stimulated with IL-1β (50 ng/ml) for 2 h ($n = 3$). *, $p < 0.05$ (for the indicated comparisons; one-way ANOVA). *B*, immunoblot analysis of CYLD, MKP-1, and tubulin levels in A549 cells left uninfected (*CON*) or infected with Kp52145 or 52OmpA2 for 3 h. Data are representative of three independent experiments. *C*, immunoblot analysis of CYLD, MKP-1, and tubulin levels in A549 cells transfected with either control or NOD1 siRNA, which were left uninfected (*CON*) or infected with Kp52145 or 52OmpA2 for 3 h. Data are representative of three independent experiments.

Upon cellular stimulation, in the canonical pathway of NF-κB activation, IκBα, which is normally present in the cytosol complexed to NF-κB dimers preventing nuclear translocation, becomes phosphorylated, leading to its ubiquitination and subsequent degradation by the proteosome, thereby allowing nuclear translocation of NF-κB, a process linked to phosphorylation of the p65 subunit (47). We analyzed the levels of phosphorylated p65 by Western blot. At 60 min postinfection, phosphorylated p65 was detected in extracts from infected cells (Fig. 4*B*). However, these levels were higher in 52OmpA2-infected cells than in Kp52145-infected ones. Complementation of the mutant restored phoshorylated p65 levels to those of Kp52145-infected cells (Fig. 4*B*). IκBα levels in cytoplasmic extracts were analyzed by immunoblot. IκBα degradation was apparent in extracts from cells infected with 52OmpA2 already 15 min postinfection, and it was still apparent at 90 min postinfection. In contrast, IκBα degradation was only detected at 15 and 30 min postinfection in extracts from Kp52145-infected cells, and at later time points, IκBα levels were similar to those of non-infected cells. For the sake of comparison, the levels of IκBα in cell extracts infected with the CPS mutants were also analyzed. In 52145-Δwca_{K2}ompA-infected cells, IκBα degradation was clearly detected at 15 min postinfection, and it was almost total at 90 min postinfection. IκBα degradation in 52145-Δwca_{K2}-infected cells was apparent at 30 min postinfection and was still detected at 90 min postinfection.

To determine the contribution of NF-κB activation to *ompA* mutant-induced IL-8 expression, we asked whether CAPE, a chemical inhibitor used to block the NF-κB signaling pathway (48), alters infection-induced IL-8 expression. As shown in Fig. 4*D*, CAPE (15 μg/ml) reduced 52OmpA2- and 52145-

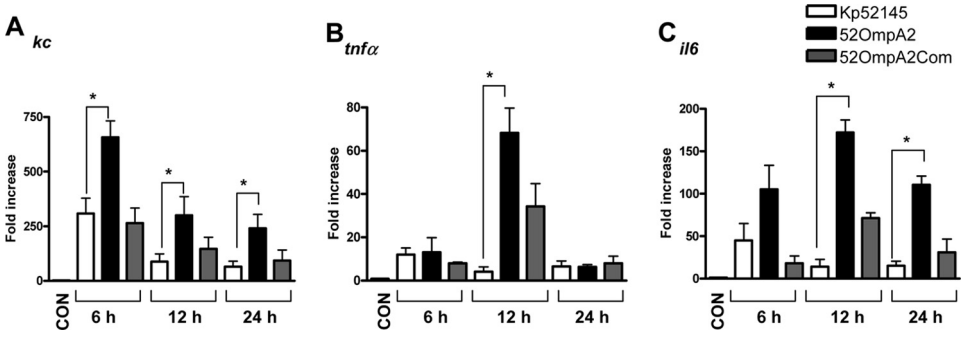

FIGURE 3. **Proinflammatory cytokine expressions in mice lungs after *K. pneumoniae* infections.** Mice were non-infected (*white bars*, $n = 5$) or infected with wild-type *K. pneumoniae* 52145 (*black bars*, $n = 15$), 52OmpA2 (*black bars*; $n = 15$), or 52OmpA2Com (*gray bars*; $n = 15$). *kc* (*A*), *tnfα* (*B*), and *il6* (*C*) mRNA expressions in whole lungs at the indicated time points postinfection were assessed by RT-qPCR (5 mice/time point). *Bars*, mean ± S.E. (*error bars*). *, $p < 0.05$ (for the indicated comparisons; one-way ANOVA).

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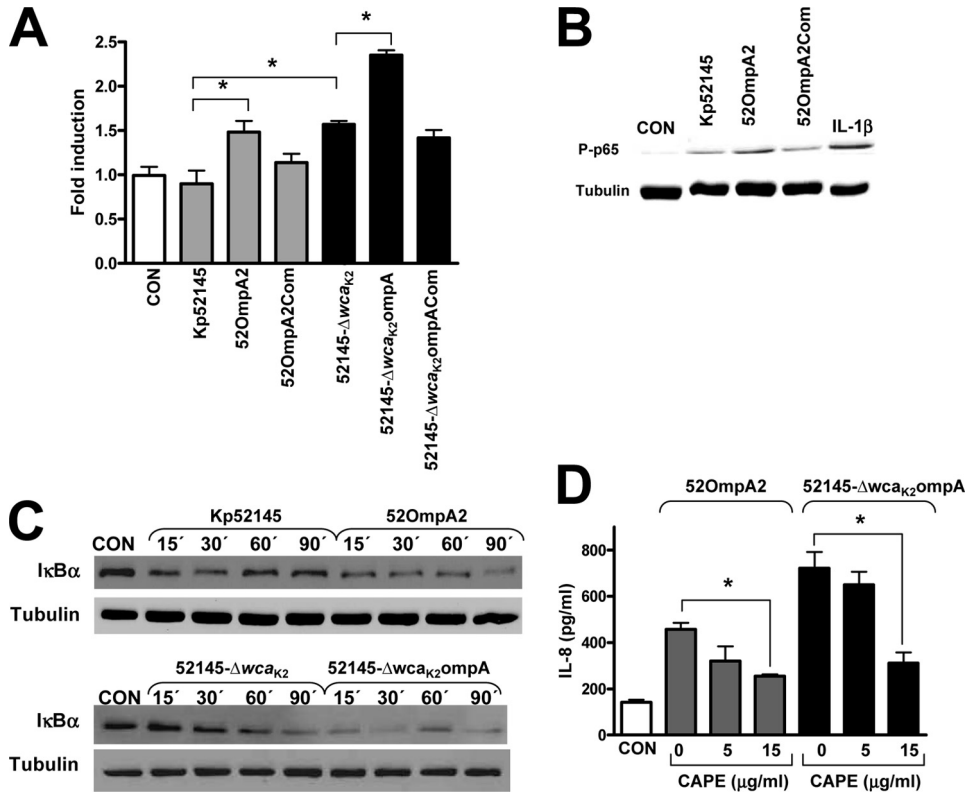

FIGURE 4. **Activation of NF-κB is required for *K. pneumoniae ompA* mutant-induced IL-8 expression.** *A*, activation of a NF-κB luciferase reporter plasmid in A549 cells left untreated (*CON*) or infected for 8 h with *K. pneumoniae* strains. Activity is normalized by correction of *Renilla* expression and is presented relative to the cells untreated ($n = 3$). *, $p < 0.05$ (for the indicated comparisons; one-way ANOVA). *B*, immunoblot analysis showing phospho-p65 and tubulin levels in lysates of A549 infected with *K. pneumoniae* strains for 90 min IL-1β (20 ng/ml for 30 min) was used as a positive control for NF-κB activation. The results are representative of three independent experiments. *C*, immunoblots of IκBα and tubulin levels in lysates of A549 cells left untreated (*CON*; time 0) or infected for different time periods with *K. pneumoniae* strains. Data are representative of three independent experiments. *D*, ELISA of IL-8 released by A549 cells left untreated (*CON*; *white bar*) or infected for 6 h with 52OmpA2 (*gray bars*) or 52145-Δ*wca*$_{K2}$ompA (*black bars*) in the absence or presence of different concentrations of CAPE, an inhibitor of NF-κB, which was added 1 h before infecting the cells and kept until the end of the experiment ($n = 3$). The data (*A* and *C*) are means and S.E. (*error bars*).

Δ*wca*$_{K2}$ompA-triggered IL-8 levels. Control experiments showed that addition of DMSO (the solvent used for CAPE) to either 52OmpA2- or 52145-Δ*wca*$_{K2}$ompA-infected cells did not affect IL-8 levels (in the absence of DMSO, 457 ± 28 and 721 ± 70 pg/ml, respectively; in the presence of DMSO, 490 ± 35 and 747 ± 64 pg/ml, respectively). CAPE-treated cells (15 μg/ml) expressed levels of IL-8 (110 ± 85 pg/ml) similar to those of non-treated cells (97 ± 50 pg/ml).

Collectively, these data demonstrated that IκBα-dependent activation of NF-κB is required for *ompA* induction of IL-8 expression in A549 cells. Moreover, CPS and OmpA contribute to limit the activation of the canonical NF-κB signaling pathway.

Activation of MAPKs Is Required for K. pneumoniae ompA Mutant-induced IL-8 Expression—In addition to the NF-κB signaling cascade, many cellular stimuli also activate MAPK pathways (49). The activation of the three MAPKs p38, JNK, and p44/42 occurs through phosphorylation of serine and threonine residues. We sought to determine the phosphorylation levels of the three MAPKs in 52OmpA2-A549-infected cells. Western blot analysis shown in Fig. 5*A* revealed that infection with 52OmpA2 triggered the phosphorylation of the three MAPKs. Phosphorylation of the three MAPKs occurred at earlier time points in 52OmpA2-infected cells than in Kp52145-infected ones. MAPKs phosphorylation levels were also studied in 52145-Δ*wca*$_{K2}$ompA- and 52145-Δ*wca*$_{K2}$-infected cells. In 52145-Δ*wca*$_{K2}$ompA-infected cells, phosphorylation of p38 was apparent at 30 min postinfection and maximum levels were observed at 120 min postinfection, whereas in 52145-Δ*wca*$_{K2}$-infected cells, p38 phosphorylation was apparent at 60 min postinfection, and maximum levels were observed at 120 min postinfection. No clear differences in the phosphorylation of p44/42 were observed between 52145-Δ*wca*$_{K2}$ompA- and 52145-Δ*wca*$_{K2}$-infected cells. In 52145-Δ*wca*$_{K2}$ompA-infected cells, maximum levels of phosphorylated JNK were observed at 30 min postinfection, whereas in 52145-Δ*wca*$_{K2}$-infected cells, phosphorylation of JNK was apparent at 30 min postinfection, and maximum levels were observed at 120 postinfection.

To explore whether the activation of MAPKs is involved in 52OmpA2-induced IL-8 expression, infections were carried out in the presence of chemical inhibitors for each MAPK. Fig. 5*B* shows that SB203580, a specific inhibitor of p38 MAPK, reduced 52OmpA2-induced IL-8 expression. The p44/42 inhibitor, U0126, also reduced 52OmpA2-dependent IL-8 expression but only at the highest dose tested (Fig. 5*C*). Finally, SP600125, a JNK inhibitor, did not alter 52OmpA2-induced IL-8 expression (Fig. 5*B*). We sought to determine whether activated MAPKs are involved in 52145-Δ*wca*$_{K2}$ompA-triggered IL-8 levels. SB203580 and U0126 at the highest dose tested also reduced 52145-Δ*wca*$_{K2}$ompA-induced IL-8 expression,

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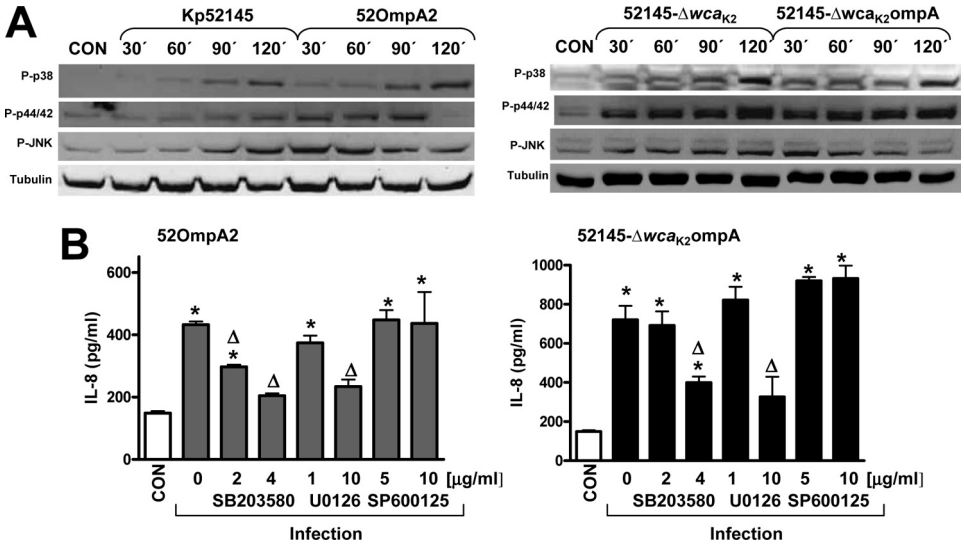

FIGURE 5. **Activation of MAPKs is required for *K. pneumoniae ompA* mutant-induced IL-8 expression.** *A*, immunoblots showing phospho-p38 (*P-p38*), phospho-p44/42 (*P-p44/42*), phospho-JNK (*P-JNK*), and tubulin levels in cell extracts of A549 cells left uninfected (*CON*; time 0) or infected with *K. pneumoniae* strains for different times. The results are representative of three independent experiments. *B*, ELISA of IL-8 released by A549 cells left untreated (*CON*; *white bar*) or infected for 6 h with either 52OmpA2 (*gray bars*) or 52145-Δ*wca*$_{K2}$ompA (*black bars*) in the absence or presence of different concentrations of SB203580 (2 and 4 μg/ml; p38 MAPK inhibitor), U0126 (1 and 10 μg/ml; p44/42 MAPK inhibitor), or SP600125 (5 and 10 μg/ml; JNK MAPK inhibitor), which were added 2 h before infecting the cells and kept until the end of the experiment ($n = 3$). The data (*B*) are means and S.E. (*error bars*). *, $p < 0.05$ (results are significantly different from the results for untreated cells; one-way ANOVA). Δ, $p < 0.05$ (results are significantly different from the results for infected cells in the absence of inhibitor).

whereas SP600125 did not reduce IL-8 levels (Fig. 5*B*). SB203580-, U0126-, and SP600125-treated cells secreted similar amounts of IL-8 (120 ± 95, 95 ± 67, and 135 ± 87 pg/ml, respectively) as non-treated cells (130 ± 75 pg/ml). Taken together, these findings indicate that the activation of MAPKs p38 and p44/42 is involved in *K. pneumoniae ompA* mutant-induced expression of IL-8 in A549 cells.

Dissection of Host Cell Receptors Required for Activation of Inflammatory Responses by K. pneumoniae ompA Mutant Strains—PRRs have a central role in pathogen recognition by airway epithelial cells leading to the activation of NF-κB- and MAPK-dependent signaling pathways; we therefore investigated the involvement of PRRs in recognition of *K. pneumoniae ompA* mutants by using as cellular read-outs the activation of NF-κB and the secretion of IL-8. Almost all TLRs activate cellular signaling pathways through TIR domain-mediated interactions with the adaptor molecule MyD88 (50). To explore the involvement of TLRs in *ompA* mutant-induced cell activation, the function of the MyD88 adaptor molecule was interrupted by siRNA. In MyD88 knocked down cells, 52OmpA2 induced neither the activation of the NF-κB reported construct nor the secretion of IL-8 (Fig. 6, *A* and *B*, respectively). 52145-Δ*wca*$_{K2}$ompA still activated the NF-κB reported construct and induced the secretion of IL-8, although the levels were significantly lower than those of infected cells transfected with control siRNA (Fig. 6, *A* and *B*). Similar results were obtained when cells were infected with 52145-Δ*wca*$_{K2}$ (Fig. 6, *A* and *B*). To further dissect the contribution of TLR-dependent signaling to *ompA* mutant-induced cell activation, TLR2 and TLR4 were knocked down by siRNA. Results shown in Fig. 6, *C–F*, indicate that both receptors contributed to 52OmpA2- and 52145-Δ*wca*$_{K2}$ompA-induced cell activation. However, 52145-Δ*wca*$_{K2}$ompA still triggered the activation of NF-κB and induced the secretion of IL-8 in TLR knocked down cells (Fig. 6, *E* and *F*, respectively). On the whole, these data suggest that 52OmpA2-induced NF-κB activation and IL-8 secretion is mediated by the TLR4-TLR2-MyD88 pathway and that 52145-Δ*wca*$_{K2}$ompA may also activate additional MyD88-independent signaling pathway(s).

Another PRR constitutively expressed by airway epithelial cells is NOD1 (51–54), and its engagement also results in the activation of NF-κB and MAPK signaling cascades (53). This led us to explore whether NOD1 contributes to 52145-Δ*wca*$_{K2}$ompA-induced cell activation by using siRNA to knock down its expression. As shown in Fig. 6, *G* and *H*, there was a reduction in 52145-Δ*wca*$_{K2}$ompA-triggered NF-κB activation and IL-8 secretion in NOD1 knockdown cells compared with those found in control siRNA-treated cells. Similar results were obtained when cells were infected with 52145-Δ*wca*$_{K2}$ (Fig. 6, *G* and *H*). 52OmpA2-induced NF-κB activation and IL-8 secretion in NOD1 knockdown cells were similar to those found in control siRNA-treated cells (Fig. 6, *G* and *H*). In contrast to Kp52145 and 52OmpA2, 52145-Δ*wca*$_{K2}$ompA and 52145-Δ*wca*$_{K2}$ did trigger NF-κB-dependent reporter activity in NOD1-expressing HEK293T cells (Fig. 6*I*). Altogether, these data indicate that the other signaling pathway engaged by 52145-Δ*wca*$_{K2}$ompA to activate NF-κB and induce IL-8 secretion is NOD1-dependent. The efficiency of siRNA-mediated down-regulation of target gene mRNA levels was confirmed by RT-qPCR (Fig. 6*J*).

Virulence of K. pneumoniae ompA Mutant—To determine the ability of 52OmpA2 to cause pneumonia, C57BL/6JOlaHsd mice were infected intranasally, and 24 and 96 h postinfection, bacterial loads in trachea and lung homogenates were determined. At 24 h postinfection, Kp52145 and 52OmpA2 colonized trachea and lungs, although bacterial loads of 52OmpA2

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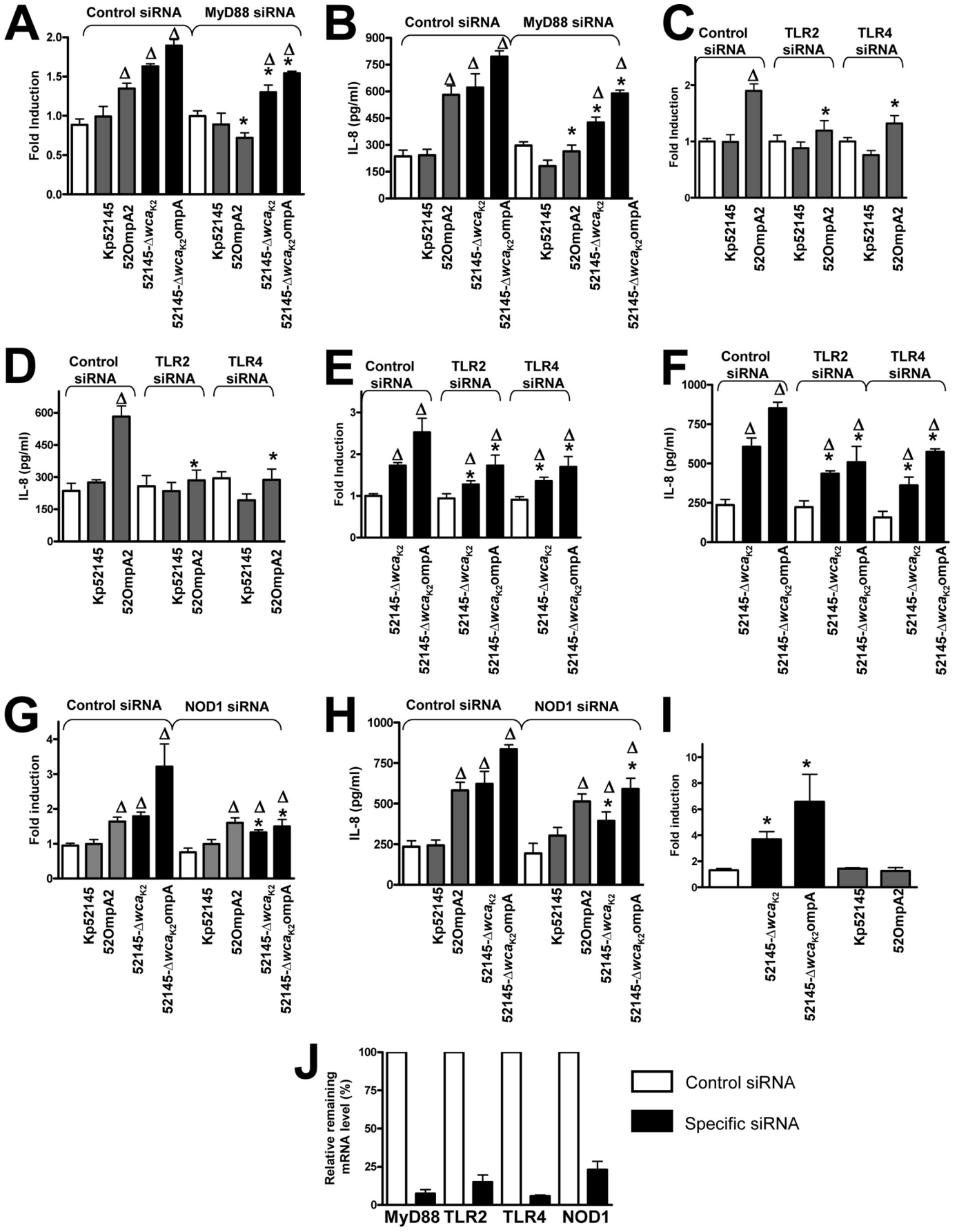

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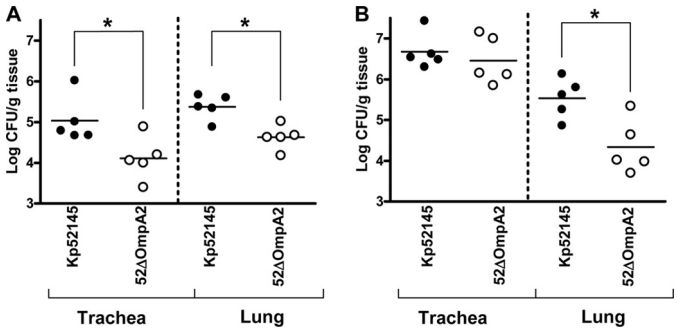

FIGURE 7. **Bacterial counts in mouse organs at 24 h postinfection (*A*) or 96 h postinfection (*B*).** Mice were infected intranasally with a bacterial mixture containing 5×10^4 bacteria of wild type (Kp52145) (●) or *ompA* mutant (52OmpA2) (○). Results were reported as log cfu/g of tissue. *, results are significantly different ($p < 0.05$; one-tailed *t* test) from the results for Kp52145.

were lower than those of the wild type (Fig. 7*A*). At 96 h postinfection, an increase of bacterial loads was found in tracheas (Fig. 7, compare *A* and *B*) that were not significantly different between both strains (Fig. 7*B*). In contrast, bacterial loads of 52OmpA2 in lungs were significantly lower than those of the wild-type strain (Fig. 7*B*).

DISCUSSION

In this study, we provide new insights into the role of OmpA as a virulence factor. Our findings revealed that *K. pneumoniae* OmpA plays a role in the progression of the infection and that it is important for immune evasion. Thus, we show that the *K. pneumoniae ompA* mutant, strain 52OmpA2, induces higher levels of inflammatory mediators than the wild-type strain *in vitro* and *in vivo*. Mechanistically, we present evidence showing that 52OmpA2 activates NF-κB-dependent and MAPK p38- and p44/42-dependent signaling pathways. In addition, *ompA* mutant induction of inflammatory responses is dependent on TLR2-TLR4-MyD88 activation. Finally, we demonstrate that the *ompA* mutant is attenuated in the pneumonia mouse model.

Innate immune responses, particularly inflammation, are essential for effective lung defense against pathogens. To activate these responses, the host recognizes conserved molecules uniquely expressed by pathogens, the so-called PAMPs, which are characterized by being expressed among entire classes of pathogens, are important for the survival/virulence of the pathogen, and are distinguishable from "self." These PAMPs are also targeted by weapons of the innate system with antibacterial properties, such as antimicrobial peptides and the complement system. In turn, many PAMPs play an important role in immune evasion by the pathogen. Our findings, together with others, give support to the notion that *K. pneumoniae* OmpA should be considered a *bona fide* PAMP. OmpA is targeted by neutrophil elastase and serum amyloid protein A, hence leading to cell death and increased bacterial phagocytosis, respectively (7–9). However, OmpA protects bacteria against the bactericidal action of lung collectins and antimicrobial peptides (6, 39), and here we have shown that it acts as immune evasin. On the whole, this evidence points out that an essential attribute of *K. pneumoniae* OmpA is to thwart the innate immune system.

Other studies have shown that recombinant purified *K. pneumoniae* OmpA induces the expression of proinflammatory molecules in various cell types, including airway epithelial cells (for a review, see Ref. 36). Hence, it has been postulated that recognition of OmpA contributes to the activation of host responses leading to the clearance of *K. pneumoniae* (36). Given that in this work we used a bacterial mutant instead of a recombinant protein, our data, far from challenging these findings, shed new light on OmpA functions in its physiological context, the OM. In fact, we emphasize that care should be taken to extrapolate findings obtained using purified products to the outcome of an infection process and that using whole live bacteria is a more likely *in vivo* scenario. It should be noted that the *ompA* mutant strain was shown to induce more inflammation *in vivo* and *in vitro* than the wild-type strain. Interestingly, and in agreement with our findings, *E. coli ompA* mutant strain induces higher expression of proinflammatory mediators than the wild type *in vitro* (55).

Recently, we have demonstrated that *K. pneumoniae* counteracts the activation of inflammatory responses by inducing the deubiquitinase CYLD and the phosphatase MKP-1 (DUSP1) in a NOD1-dependent manner (45). This action was strictly dependent on bacteria-cell contact (45). Furthermore, bacterial removal by gentamicin treatment rendered cells again responsive to proinflammatory mediators (45). Considering that CPS was necessary but not sufficient to attenuate inflammation (45), in this work we asked whether OmpA could mediate this attenuation. However, findings shown in Fig. 2 do not support this. These results do not contradict the other findings reported in this work showing that 52OmpA2 does activate inflammatory responses. It should be noted that in most experiments of this study, gentamicin is used to remove bacteria after 2 h of infection, and, for example, IL-8 in the supernatants is measured at 4 or 6 h postinfection. In turn, the fact that 52OmpA2-induced responses were dependent on TLR2-TLR4-MyD88 activation suggests that OmpA, together with CPS, perturbs TLR-dependent recognition of *K. pneumoniae*.

FIGURE 6. **Role of MyD88, TLR2, TLR4, and NOD1 in *K. pneumoniae ompA* mutant-induced cell activation.** *A*, *C*, *E*, and *G*, activation of an NF-κB luciferase reporter plasmid in A549 cells transfected with either control or the indicated siRNA for different pattern recognition receptors, which were left untreated (*white bars*) or infected for 8 h with different strains. Activity is normalized by correction of *Renilla* expression and is presented relative to the cells untreated ($n = 3$). *B*, *D*, *F*, and *H*, ELISA of IL-8 released by A549 cells transfected with either control or the indicated siRNA for different pattern recognition receptors, which were left untreated (*white bars*) or infected for 8 h with different strains ($n = 3$). *I*, activation of an NF-κB luciferase reporter plasmid in HEK293T cells transfected with HA-NOD1 plasmid, which were left untreated (*white bar*) or infected with the indicated strains for 8 h. Activity is normalized by correction of *Renilla* expression and is presented relative to untreated cells (data are means and S.E. (*error bars*); $n = 3$). *J*, siRNA efficiency was quantified by RT-qPCR in samples from the same experiment shown in *A–H*. mRNA level was normalized to GADPH, and then relative mRNA levels in cells transfected with control siRNA or specific siRNA were compared. mRNA levels in cells transfected with control siRNA were set to 100% (data are means and S.E.; $n = 3$). *Gray bars*, CPS-expressing strains; *black bars*, CPS-negative strains. *A–H*, Δ, $p < 0.05$ (results are significantly different from the results for untreated cells; one-way ANOVA); *, $p < 0.05$ (results are significantly different from those for transfected cells with control siRNA infected with the same strain). *I*, *, $p < 0.05$ (results are significantly different from the results for untreated cells; one-way ANOVA).

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On the whole, this evidence is consistent with a model in which the reduced inflammatory response characteristic of *K. pneumoniae* infections is the sum of prevention of TLR-dependent recognition of *Klebsiella* (mediated at least by CPS and OmpA) and the anti-inflammatory effect (*K. pneumoniae* effectors yet to be identified) recently described (45).

Our findings further sustain the notion that airway epithelial cells orchestrate a defense response upon pathogen challenge by activating NF-κB- and MAPK-dependent signaling pathways (56). In fact, activation of these pathways seems to be a central event of pathogen-exposed epithelial cells lining mucosal surfaces. Interestingly, this is true regardless of the tissue local environment, either constantly exposed to microbes, like the intestinal tract, or essentially sterile, like the urinary and respiratory tracts. Although p38 and p44/42 MAPKs were required for *ompA* mutant strain-induced IL-8, the mutants also activated JNK MAPK, hence suggesting that there may be additional host cell responses activated in a JNK MAPK-dependent manner. Future studies will aim to identify these responses.

The *ompA* mutant strains tested in this study engaged different PRRs, depending on the expression of CPS on their surfaces. Strain 52OmpA2, expressing CPS, engaged TLR2 and TLR4, whereas strain 52145-Δwca_{K2}ompA, lacking CPS, activated not only TLR2 and TLR4 but also NOD1. These and our previous findings implicating TLR2 and TLR4 in the recognition of a *K. pneumoniae* CPS mutant (22, 32) suggest that TLR-mediated recognition of *K. pneumoniae* is a key event for the induction of host defense responses against this pathogen. Supporting this, MyD88- and TLR4-dependent responses are essential for successful mouse defense against *K. pneumoniae* pneumonia (29, 30). Our results implicate NOD1 in the recognition of *K. pneumoniae* CPS mutants, strains 52145-Δwca_{K2}ompA (this work) and 52145-Δwca_{K2} (this work and Ref. 22). Taking into account that *K. pneumoniae* CPS mutants are internalized by epithelial cells (43, 44), a NOD1-dependent recognition is consistent with the idea that this PRR participates in the recognition of internalized pathogens (53, 57, 58). Intracellular 52145-Δwca_{K2}ompA and 52145-Δwca_{K2} reside in vacuoles displaying features of acidic late endosomes,[4] thereby raising the question of how bacteria that do not reach the cytosol could activate NOD1. However, recent studies indicate that there is a link between endosomal acidification/maturation and NOD-dependent responses (59–61). Furthermore, data indicate that bacterial products are actively transported from the phagosome to the cytosol to engage NOD receptors (59–61).

Here, we demonstrate for the first time, using a pneumonia mouse model, that OmpA is important for bacterial survival in the lung. Research over the last 20 years has demonstrated a correlation between activation of inflammatory responses and *Klebsiella* clearance from the airways (26–28). Hence, the higher inflammatory response induced by 52OmpA2 may contribute to the mutant's clearance. The *in vivo* scenario is complex, and the final outcome of pneumonia is the combination of the action of antimicrobial factors (among others complement and antimicrobial peptides) and several types of cells, including alveolar macrophages, epithelial cells, and neutrophils. It should be noted that cytokines and chemokines released by epithelial cells do increase the bactericidal activity of professional phagocytes. Studies are ongoing to determine whether OmpA plays any role in the interaction of *K. pneumoniae* with alveolar macrophages and neutrophils.

Finally, it is worthwhile commenting on the clinical implications of our findings. We put forward the idea that compounds directed to block OmpA may be new promising therapeutic molecules to treat infections caused by Gram-negative bacteria, at least those caused by *Klebsiella*. Data from our laboratory suggest that these compounds will render bacteria susceptible to the bactericidal elements of the innate immune system (39) and will also increase TLR-mediated defense responses (this work). Both actions should facilitate the clearance of the pathogen from the airways, which might be further enhanced with the aid of antibiotics.

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[4] C. March and J. A. Bengoechea, unpublished data.

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