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Platelet-activating Factor Receptor Initiates Contact of *Acinetobacter baumannii* Expressing Phosphorylcholine with Host Cells[S]

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Background: The mechanism of interaction between *Acinetobacter baumannii* and host cells remains unclear.
Results: The presence of ChoP in *A. baumannii* increased bacterial adherence/invasion in A549 cells. Inhibitions of PAFR, G protein, and vacuole formation decreased this bacterial adherence/invasion. PAFR inhibition decreased lung bacterial loads *in vivo*.
Conclusion: We define a role for PAFR and ChoP in *A. baumannii* interaction with host cells.
Significance: This will aid in studying how *A. baumannii* invades host cells.

Adhesion is an initial and important step in *Acinetobacter baumannii* causing infections. However, the exact molecular mechanism of such a step between *A. baumannii* and the host cells remains unclear. Here, we demonstrated that the phosphorylcholine (ChoP)-containing outer membrane protein of *A. baumannii* binds to A549 cells through platelet-activating factor receptor (PAFR), resulting in activation of G protein and intracellular calcium. Upon *A. baumannii* expressing ChoP binding to PAFR, clathrin and β-arrestins, proteins involved in the direction of the vacuolar movement, are activated during invasion of *A. baumannii*. PAFR antagonism restricts the dissemination of *A. baumannii* in the pneumonia model. These results define a role for PAFR in *A. baumannii* interaction with host cells and suggest a mechanism for the entry of *A. baumannii* into the cytoplasm of host cells.

Acinetobacter baumannii is a significant human pathogen. This Gram-negative bacterium causes hospital and potentially severe infections such as ventilator-associated pneumonia, bacteremia, skin and soft tissue infections, surgical site infections, urinary tract infections, sepsis, and meningitis (1, 2).

The virulence of *A. baumannii* is based on a multitude of secreted and surface-associated components. An important group of virulence factors is the outer membrane proteins (OMPs).[2] In this group, the outer membrane protein A (OmpA) that interacts with host cells and induces biofilm formation on abiotic surfaces (3, 4) is responsible for the ability of *A. baumannii* to adhere and invade epithelial cells (5). Several other *A. baumannii* surface proteins and intracellular proteins have been identified and characterized. For many of them, isogenic mutants are less virulent *in vitro* and *in vivo*. Mutants deficient in OmpA, lipopolysaccharide (LPS), K1 capsular polysaccharide, penicillin-binding protein, and phospholipase D are less virulent *in vitro* and *in vivo* (4, 5–9).

In other Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Haemophilus influenzae*, mutants lacking phosphorylcholine (ChoP) expression in outer membrane show reduced interaction with host cells and virulence (10, 11). The location of ChoP is different depending on bacterial species. Thus, it has been observed with LPS, fimbriae, capsules, and teichoic and lipoteichoic acids (12–15) and can contribute to bacterial pathogenicity via a direct contact with host cells. Recently, it was demonstrated that ChoP plays an important role in *H. influenzae* biofilm maturation (16). In addition, binding of bacteria positive for ChoP to platelet-activating factor receptor (PAFR) mediates its adhesion to and invasion into human cells (10, 11, 17, 18). This binding has been studied extensively *in vitro*. Typical of G protein-coupled receptors, PAFR activation by ChoP results in an initiation of a two-host cell signal cascade. Firstly, after the coupling of bacteria with PAFR, the G protein and phospholipase C (PLC) recruitment to the PAFR site is activated, and intracellular Ca^{2+} is released after induction of inositol triphosphate (19). The intracellular Ca^{2+} increase was shown to be involved in cell death induced by various pathogens (20, 21). Secondly, the trafficking of vacuole has been shown to recycle the bacterial coupling of PAFR to the apical surface with further transmigration to the basolateral surface of the cell, with delivery of viable bacteria across endothelia and epithelia (22). In this second pathway, adapter

[S] This article contains supplemental Materials and Methods, Tables S1 and S2, and Figs. S1 and S2.

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[2] The abbreviations used are: OMP, outer membrane protein; PAFR, platelet-activating factor receptor(s); ChoP, phosphorylcholine; PLC, phospholipase C; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CPZ, chlorpromazine; MDC, monodansylcadaverine.

proteins such as clathrin and β-arrestin-1 and β-arrestin-2 play a role in the direction of the vacuolar movement because they tether the bacteria-occupied PAFR to the vesicular trafficking system (23). Once β-arrestins target PAFR to endocytosis and/or transcytosis, they recruit and activate extracellular signal-regulated kinase 1 (ERK-1)/ERK-2 and mitogen-activated protein (MAP) kinase (23). In the case of *A. baumannii*, the adhesion to and invasion of this bacterium into eukaryotic cells is mediated by pili and fimbrial-like structure (24, 25). A zipper-like mechanism (receptor-mediated entry) was suggested to be induced during the *A. baumannii* adherence to epithelial cells (5). Lee *et al.* (26) have shown that the *A. baumannii* surface protein activated the MAPKs including ERK, c-Jun N-terminal kinases, and p38 MAPK. Thus, circumstantial evidence suggests that *A. baumannii* uptake has characteristics of a PAFR-mediated event. In this work, we sought to study whether *A. baumannii* induced the PAFR-mediated uptake pathway *in vitro* and in a pneumonia model. Moreover, we aimed to determine the involvement of clathrins and β-arrestins in this pathway.

EXPERIMENTAL PROCEDURES

Expanded details of all methods are given in the supplemental Materials and Methods.

Bacterial Strains and Growth Conditions—*A. baumannii* clinical isolate 77wt, collected in Spain in 2000 (27), its derivative ciprofloxacin-resistant strain 77R, and six other *A. baumannii* clinical strains were used (28, 29). ATCC 17978wt and its derivative porinD mutant were also used (supplemental Table S1) (30). All strains were grown in a Mueller Hinton Broth or Luria Bertoni (LB).

DNA Procedures—Plasmid DNA was isolated and amplified using specific primers of *porinD* gene (supplemental Table S2). Isolated DNAs were separated by agarose gel electrophoresis and visualized by SYBR Safe DNA gel stain (Invitrogen).

Generation of porinD Knock-out from A. baumannii Strain ATCC 17978wt—To construct a *porinD* knock-out from *A. baumannii* ATCC 17978wt strain, an internal *porinD* 559-bp fragment obtained by PCR amplification with primers porinD IntUp and porinD IntLw (supplemental Table S2) was cloned into pGEM-T (31) to give plasmid porinD-pGEM-T. The resulting construct incorporated in *Escherichia coli* DH5α strain was purified and electroporated into *A. baumannii* ATCC 17978wt strain to knock out its *porinD* gene by allelic replacement. Transformants were selected on LB agar plates containing 80 μg/ml ticarcillin. *porinD* gene disruption within the resulting strain, designated PSAB03, was verified by PCR using a combination of primers matching the upstream region of *porinD* gene and the pGEM-T Easy vector.

Human Cell Culture and Infection—Type II pneumocyte cell line A549 derived from human lung carcinoma was grown in supplemented DMEM medium. The cells were seeded 24 h in 96-well plates for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) prior to infection with *A. baumannii* 77wt and 77R strains at a multiplicity of infection of 100.

Cellular Viability—A549 cells were pretreated with PAFR antagonist 1-O-hexadecyl-2-acetyl-*sn*-glycerol-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine, mouse anti-human PAFR monoclonal antibody, U73343, and U73122 and infected with *A. baumannii* 77wt and 77R strains (10^8 cfu/ml) for 24 h. *A. baumannii* cytotoxicity was assessed quantitatively by monitoring mitochondrial reduction activity using the MTT assay as described previously (32).

Intracellular Ca^{2+} Measurement—Dual-excitation imaging with fura-2/AM was used to measure intracellular Ca^{2+} changes in A549 cells pretreated or not with U73122 and infected with *A. baumannii* 77wt as shown previously (33).

siRNA Transfection—Chemically synthesized, double-stranded siRNAs for β-arrestin-1, β-arrestin-2, and control were transfected in 40–50% confluent A549 cells.

Immunofluorescence—A549 cells plated on coverslips, transfected or not with siRNA of β-arrestin-1, β-arrestin-2, and control, and infected with *A. baumannii* 77wt and 77R at 37 °C for 2 h were removed and washed five times with cold PBS.

ChoP Immunoblotting—Outer membrane fractions from ATCC 17978 wt, PSAB03, 77wt, 77R, and six other *A. baumannii* clinical strains were isolated as described previously (29). Western blot was performed using 10% SDS-PAGE with anti-ChoP TEPC-15 mAb as primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG antibody as secondary antibody. For peptide mass fingerprinting, a SimplyBlue™ SafeStain-stained band representing ChoP-containing OMP was excised from SDS-PAGE gel and analyzed by MALDI-TOF-TOF (MS-MS/MS).

β-Arrestin Immunoblotting—Proteins removed from transfected A549 cells were collected. Western blot was performed using 10% SDS-PAGE with rabbit anti-human β-arrestin-1/2 as primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibody.

Adhesion and Internalization Assays—A549 cells were pretreated with anti-ChoP TEPC-15 antibody, PAFR antagonist, anti-human PAFR monoclonal antibody, U73343, U73122, monodansylcadaverine, or chlorpromazine and infected with 77wt or 77R *A. baumannii* strains (10^8 cfu/ml).

Flow Cytometry—FACS analysis was used to determine ChoP expression in 77wt and 77R strains. A single color containing only FITC-labeled ChoP of bacteria was analyzed, and the percentage of FITC-labeled ChoP of bacteria was quantified. A minimum of 10,000 events were analyzed in each experiment.

Serum Bactericidal Assay—Complement-mediated serum bactericidal activity was determined in normal human serum as described previously (34).

Pneumonia Infection Model—A pneumonia model was used as described previously by our group (35). Two groups of nine mice were anesthetized intraperitoneally with 5% (w/v) sodium thiopental and were inoculated with 50 μl of 77wt (7.67 log cfu/ml), in the presence or absence of PAFR antagonist (1 μg/mouse).

Histopathological Study—Lung histopathology studies were performed as described previously by our group (35).

Statistical Analysis—The group data are presented as means ± S.D. A Student's *t* test was used to determine the differences between means. The difference was considered significant at $p < 0.05$. The SPSS (version 15.0) statistical package was used (SPSS Inc., Chicago, IL).

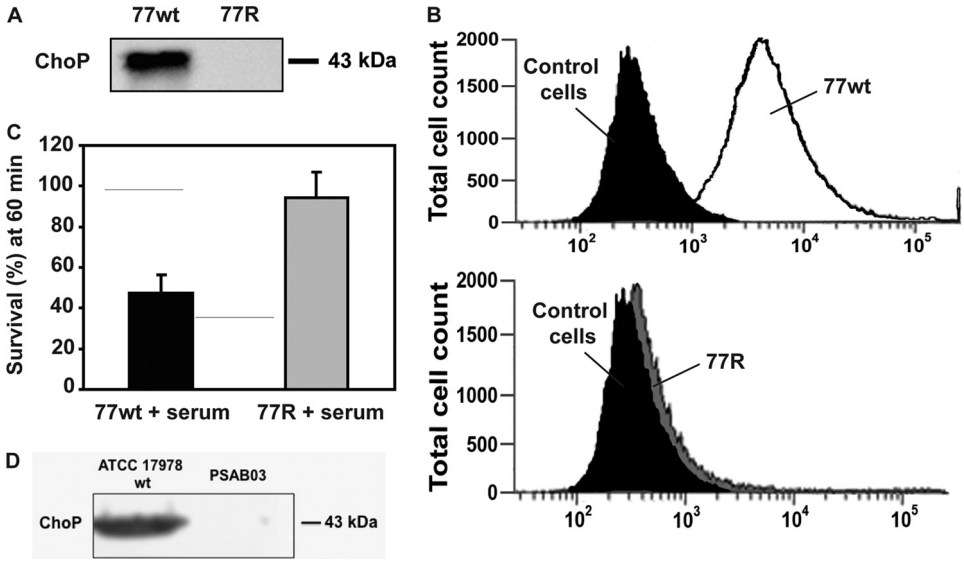

FIGURE 1. **Expression of ChoP on *A. baumannii*.** *A* and *D*, Western blot analysis of ChoP in *A. baumannii* 77wt, 77R, ATCC 17978wt, and PSAB03 strains. *B*, ChoP expression by *A. baumannii* was then quantified by FACS. Histograms illustrate the fluorescence intensities of control cells (*A. baumannii* without TEPC-15 antibody and with the FITC-tagged secondary antibody) when compared with those incubated with TEPC-15 and FITC-tagged secondary antibody. *C*, serum bactericidal assay on *A. baumannii* 77wt and 77R strains, grown to mid-log phase and treated for 60 min in 10% normal human serum. The percentage of survival is the number of cfus remaining when compared with controls in which complement activity was inactivated. Data are representative of three independent experiments.

RESULTS

ChoP-containing OMP Expression on A. baumannii—The Western blot analysis of OMPs isolated from seven *A. baumannii* clinical strains, with ChoP-specific mAb TEPEC-15, demonstrated the presence of a 43-kDa band that showed ChoP-containing OMP expression by these strains (supplemental Fig. S1). In the derivative ciprofloxacin-resistant strain (77R) of one of these strains (77wt), no detectable ChoP-containing OMP expression was apparent (Fig. 1*A*). The corresponding band of ChoP-containing OMP of 77wt was excised from a parallel SDS-PAGE and subjected to MS-MS/MS analysis and was found to correspond to porinD of *A. baumannii* (data not shown). To confirm that porinD contains ChoP, we used the allelic exchange strategy to delete the *porinD* gene from *A. baumannii* ATCC 17978wt strain. The presence or absence of *porinD* gene in *A. baumannii* ATCC 17978wt and PSAB03 strains was confirmed by PCR, showing that the PSAB03 strain is deficient in *porinD* gene (supplemental Fig. S2). The Western blot analysis of OMPs isolated from ATCC 17978wt and PSAB03 strains demonstrated the presence and absence of ChoP in ATCC 17978wt and PS03 strains, respectively (Fig. 1*D*). Flow cytometry analysis of both intact *A. baumannii* strains incubated with anti-ChoP mAb TEPC-15 antibody showed that the 77wt strain was positive for ChoP in 55.27 ± 17.7% of all the cells analyzed. In contrast, 77R was only positive for ChoP in 6.83 ± 2.11% of all the cells analyzed (Fig. 1*B*). These Western blot and flow cytometry results were further confirmed by serum bactericidal activity experiments. Previously, it was demonstrated that ChoP contributes to *H. influenzae* susceptibility to serum bactericidal activity (34). *A. baumannii* 77wt and 77R strains differing in expression of ChoP were compared for their ability to survive human serum bactericidal effect. 77wt strain survival in human serum for 60 min was 47.52 ± 8.73%, whereas 77R strain survival was 94.22 ± 12.55% (Fig. 1*C*). For the rest of the results of this study, we will name the 77wt strain positive for ChoP as ChoP$^+$ and name the 77R strain negative for ChoP as ChoP$^-$.

ChoP Involvement in A. baumannii Adhesion to and Internalization by Lung Epithelial Cells—To evaluate the involvement of ChoP in *A. baumannii* adherence and invasion of A549 cells, we studied the adherence and invasion of ChoP$^+$ and ChoP$^-$ of A549 cells for 2 h. We showed that ChoP$^+$ adhered to and invaded A549 cells 95% more than ChoP$^-$ (Fig. 2*A*). Infected A549 cells immunostaining with ChoP$^+$ or ChoP$^-$ using the anti-*A. baumannii* OMPs antibody or the anti-ChoP mAb TEPC-15 antibody showed ChoP$^+$ attachment to A549 cells. In contrast, infected A549 cells with ChoP$^-$ showed an absence of ChoP$^-$ attachment to A549 cells (Fig. 2*B*). We then determined the relationship between ChoP expression and *A. baumannii* adherence to and internalization by A549 cells. Anti-ChoP mAb TEPC-15 antibody incubation with ChoP$^+$ for 1 h significantly reduced ChoP$^+$ adhesion to and internalization by A549 cells to 25.29 ± 7.68 and 23.06 ± 11.97%, respectively (Fig. 2*C*). In contrast, pretreatment of A549 cells with unspecific antibody to ChoP, a mouse IgG, did not significantly reduce the adherence of ChoP$^+$ to A549 cells (data not shown). As negative control, ChoP$^-$ incubation with anti-ChoP mAb TEPC-15 antibody did not significantly decrease ChoP$^-$ adhesion to and invasion of A549 cells (Fig. 2*D*). Altogether, we found that ChoP expression in *A. baumannii* was involved in *A. baumannii* adhesion to and invasion of lung epithelial cells.

PAFR Involvement in the Adherence/Internalization of A. baumannii and in the Cell Death of Lung Epithelial Cell Induced by A. baumannii—To evaluate the involvement of PAFR in *A. baumannii* ChoP$^+$ adherence to and invasion of A549 cells, we studied the PAFR antagonist and anti-PAFR antibody effect on ChoP$^+$ adherence to and invasion of A549

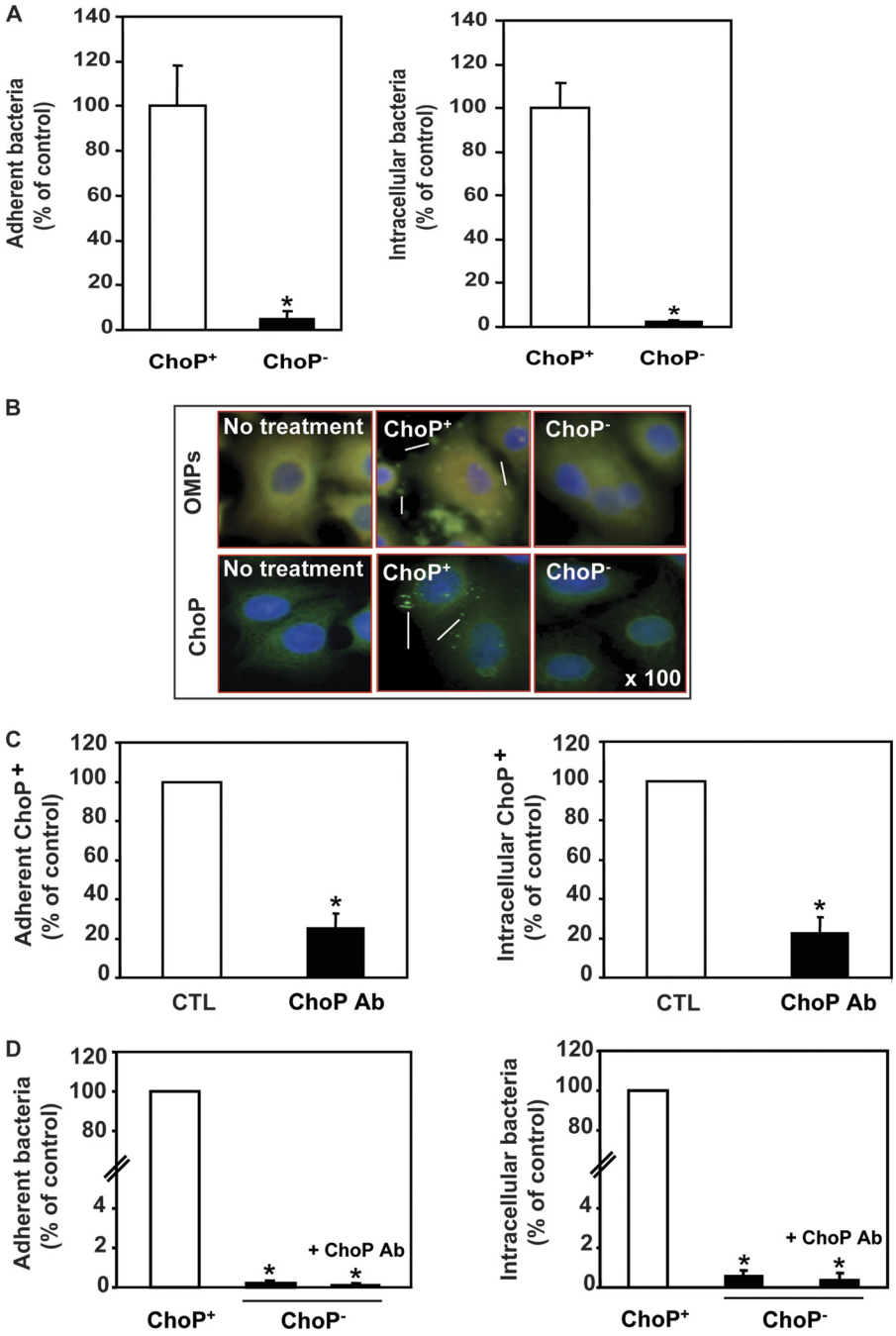

FIGURE 2. **Role of ChoP in the *A. baumannii* adhesion to and internalization by lung epithelial cells.** A549 cells were infected with 10^8 cfu/ml *A. baumannii* ChoP$^+$ or ChoP$^-$ for 2 h. *A*, an assay of adherence and invasion was performed as described under "Experimental Procedures." The adherence or invasion of *A. baumannii* ChoP$^-$ is expressed as the percentage of total *A. baumannii* ChoP$^+$ adhered to or internalized by A549 cells. *B*, immunostaining for A549 cells and *A. baumannii* OMPs and ChoP was performed and imaged by immunofluorescence microscopy. OMPs and ChoP (*white lines*) of *A. baumannii* strains were detected with mouse anti-*A. baumannii* OMPs and TEPC-15 antibodies and labeled with Alexa Fluor 488-tagged secondary antibodies (*green*). *Blue staining* shows the location of nuclei of A549 cells. *C* and *D*, adherence and invasion of ChoP$^+$ or ChoP$^-$, respectively, were determined in A549 cells pretreated with anti-ChoP TEPC-15 antibody. The effect of ChoP antibody on adherence and invasion of *A. baumannii* is expressed as the percentage of total nontreated *A. baumannii* ChoP$^+$ adhered to or internalized by A549 cells. Data are representative of three independent experiments. *, between ChoP$^+$ and ChoP$^-$, ChoP$^+$ + ChoP antibody, or ChoP$^-$ + ChoP antibody. *CTL*, control; *Ab*, antibody.

cells for 2 h. We showed that pretreatment of A549 cells with 50, 100, and 200 nM PAFR antagonist significantly reduced ChoP$^+$ adherence to and invasion of A549 cells to 73.53 ± 20.04, 64.17 ± 18.08, and 56.67 ± 5.2%, respectively (for adherence), and 89 ± 18.15, 55.66 ± 14.86, and 49.45 ± 8.93%, respectively (for invasion) (Fig. 3*A*). Similarly, pretreatment of A549 cells with 25 μg/ml anti-PAFR antibody significantly reduced ChoP$^+$ adherence to and invasion of A549 cells to 52.32 ± 14.8 and 56.26 ± 26.56%, respectively (Fig. 3*A*). Immunostaining of infected A549 cells with ChoP$^+$ and pretreating with 200 nM PAFR antagonist and 25 μg/ml anti-PAFR antibody showed reduction in ChoP$^+$ attachment to A549 cells from 53.92 ± 1.74% to 23.52 ± 8.08 and 27.08 ± 7.73%, respectively (Fig. 3*B*).

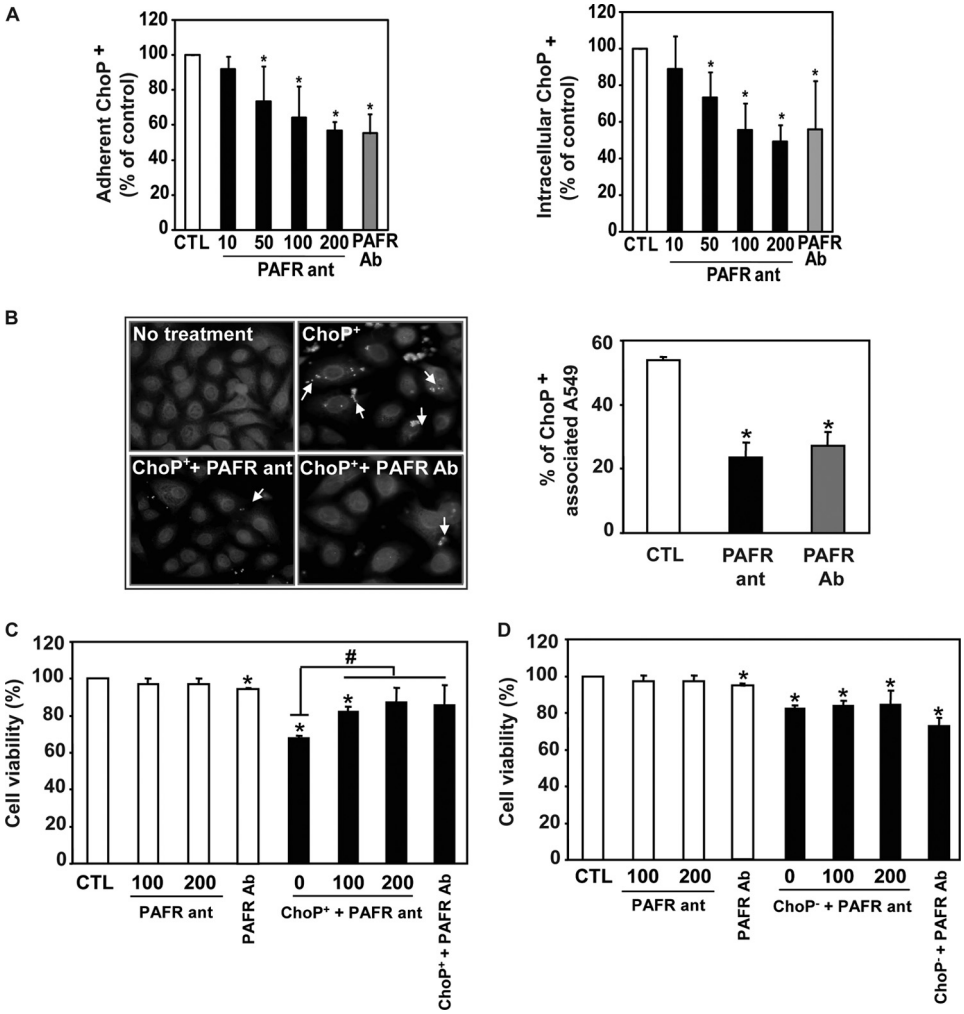

FIGURE 3. **Involvement of PAFR in the adherence/internalization of *A. baumannii* and in the cell death of lung epithelial cell induced by *A. baumannii*.** A549 cells were pretreated with PAFR antagonist (*PAFR ant*, 10, 50, 100 and 200 nM) or mouse anti-human PAFR antibody (*Ab*, 25 μg/ml) and infected with 10^8 cfu/ml *A. baumannii* ChoP$^+$ or ChoP$^-$. *A*, assay of adherence and invasion of *A. baumannii* ChoP$^+$ for 2 h was performed as described under "Experimental Procedures." The effect of PAFR antagonist and antibody on adherence or invasion of *A. baumannii* is expressed as the percentage of total nontreated *A. baumannii* ChoP$^+$ adhered to or internalized by A549 cells (*CTL*). *B*, immunostaining for *A. baumannii* OMPs in infected A549 cells for 2 h was performed and imaged by immunofluorescence microscopy. The *A. baumannii* ChoP$^+$ strain was detected by mouse anti-OMP antibody and labeled with Alexa Fluor 488-tagged secondary antibody (*green*). The percentage of ChoP$^+$ strain associated with A549 cells was calculated as ((number of A549 cells attached by ChoP$^+$ strain colonies/number of total A549 cells) $\times$ 100). *C* and *D*, the *A. baumannii* ChoP$^+$ or ChoP$^-$ strain cytotoxicity was determined by monitoring the mitochondrial reduction activity using the MTT assay for 24 h. Representative results of three independent experiments are shown, and data are the means $\pm$ S.D. $p < 0.05$: *, between nontreated and treated groups, #, between ChoP$^+$ treated groups.

In addition, we evaluated the involvement of PAFR in the cell death induced by *A. baumannii*. For this, we studied the PAFR antagonist and anti-PAFR antibody effect on cell death of A549 cells induced by ChoP$^+$ and ChoP$^-$ for 24 h by MTT assay analysis. We showed that pretreatment of A549 cells with 100 and 200 nM PAFR antagonist and 25 μg/ml anti-PAFR antibody significantly protects A549 cells from cell death induced by ChoP$^+$ from 68.11 $\pm$ 1.08% to 82.18 $\pm$ 2.69% and from 87.29 $\pm$ 7.97 to 87 $\pm$ 10.85%, respectively (Fig. 3*C*). We further confirmed these results by MTT assay analysis of ChoP$^-$-infected A549 cells and pretreated with PAFR antagonist and antibody. As shown in Fig. 3*D*, pretreatment of A549 cells with PAFR antagonist and antibody did not significantly increase cell viability in comparison with non-pretreated and ChoP$^-$-infected A549 cells. From these data, we found that PAFR play an important role in the adherence, internalization, and cell death induced by *A. baumannii* expressing ChoP.

Activation of G Protein Coupled to PLC during Invasion and Cell Death Induced by A. baumannii—To evaluate the involvement of G protein coupled to PLC in *A. baumannii* ChoP$^+$ adherence to and invasion of A549 cells, we studied the effect of G protein coupled to PLC inhibitor U73122 on ChoP$^+$ adherence to and invasion of A549 cells for 2 h. U73122 was found to be effective in blocking the ChoP$^+$ invasion of A549 cells to 16.06 $\pm$ 8.49%. However, the total of the cell-adhered bacteria did not differ between non-pretreated and U73122-pretreated A549 cells, indicating that the inhibition was not due to inefficient binding ChoP$^+$ to A549 cells. In contrast a control inactive analog compound, U73343, which did not show significant ability to block ChoP$^+$ invasion in A549 cells, also failed to show inhibition of bacteria adhered to A549 cells (Fig. 4*A*). To further confirm that inhibition of ChoP$^+$ invasion into U73122-pretreated A549 cells was the result of G protein coupled to PLC inhibition, intracellular Ca^{2+} mobilization in

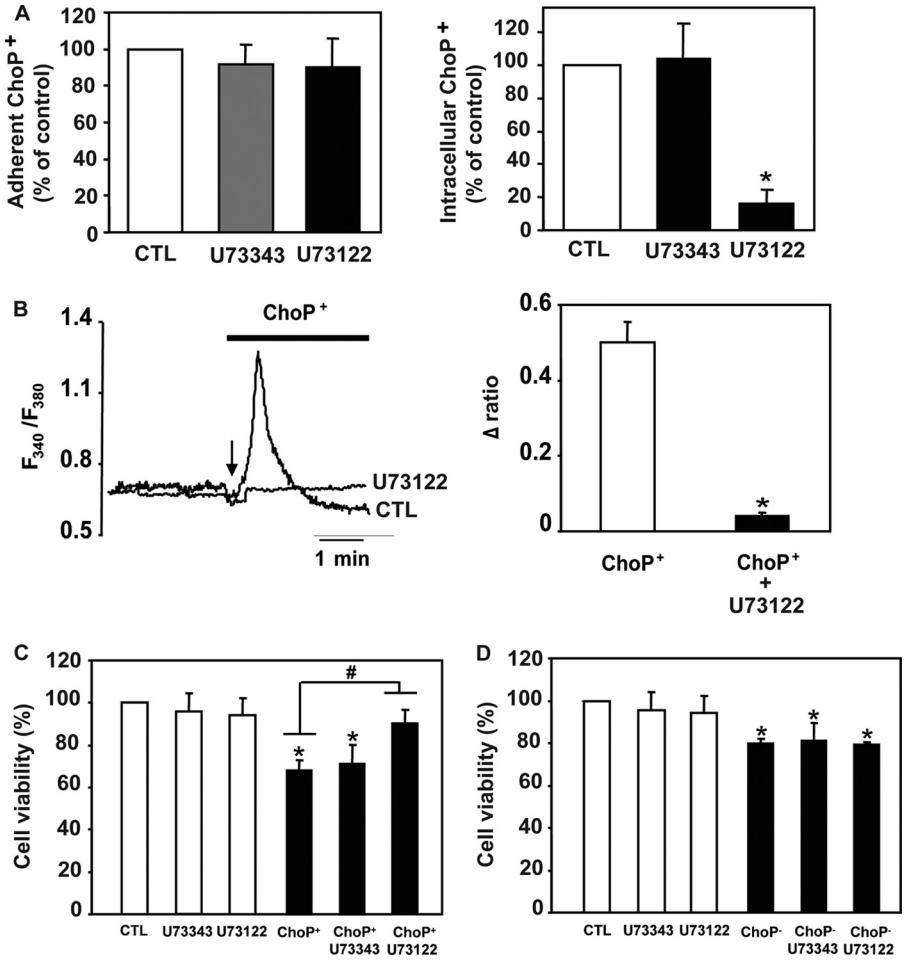

FIGURE 4. **Activation of G protein coupled to PLC during invasion and cell death induced by *A. baumannii*.** A549 cells were pretreated with U73122 (5 μM) or U73343 (5 μM) and infected with 10^8 cfu/ml *A. baumannii* ChoP$^+$ or ChoP$^-$. *A*, assay of adherence and invasion of *A. baumannii* ChoP$^+$ for 2 h was performed as described under "Experimental Procedures." The effect of U73122 or U73343 on adherence or invasion of *A. baumannii* is expressed as the percentage of total nontreated *A. baumannii* ChoP$^+$ adhered to or internalized by A549 cells. *CTL*, control. *B*, intracellular Ca^{2+} mobilization was monitored for 10 min in nontreated or pretreated A549 cells with U73122 and infected with ChoP$^+$ strain for 5 min. *C* and *D*, *A. baumannii* ChoP$^+$ or ChoP$^-$ strain cytotoxicity was determined by monitoring the mitochondrial reduction activity using the MTT assay for 24 h. Representative results of three independent experiments are shown, and data are the means ± S.D. $p < 0.05$: *, between nontreated and treated groups, #, between ChoP$^+$ treated groups.

ChoP$^+$-infected A549 cells pretreated with U73122 was monitored. We demonstrate that U73122 abolished almost completely the intracellular Ca^{2+} increase induced by ChoP$^+$ (Fig. 4*B*).

As mentioned in the Introduction, intracellular Ca^{2+} increase has been shown to be involved in cell death. Therefore, we studied the effect of U73122 and U73343 on cell death of A549 cells induced by ChoP$^+$ and ChoP$^-$ for 24 h. As shown in Fig. 4*C*, pretreatment of A549 cells with U73122 exhibited significant protection of A549 from cell death induced by ChoP$^+$ from 66.18 ± 5.87% to 90.38 ± 6.31%. In contrast, U73343-pretreated ChoP$^+$-infected A549 cells showed similar cell death to that observed with non-pretreated and ChoP$^+$-infected A549 cells. We further confirmed these results by MTT assay analysis of ChoP$^-$-infected A549 cells and A549 cells pretreated with U73122 and U73343. As shown in Fig. *4D*, pretreatment of A549 cells with U73122 and U73343 did not significantly increase the cell viability in comparison with non-pretreated ChoP$^-$-infected A549 cells. From these data, we found that G proteins coupled to PLC play a role in the adherence, internalization, and cell death induced by *A. baumannii* expressing ChoP.

Role of Clathrin and β-Arrestin in A. baumannii Internalization by Lung Epithelial Cells—After bacterial invasion by binding to PAFR, clathrin and β-arrestins would be expected to participate in bacterial uptake. We examined the effect of clathrin inhibitors on ChoP$^+$ adherence to and invasion of A549 cells for 2 h. Monodansylcadaverine (MDC) and chlorpromazine (CPZ), both specific inhibitors of assembly and recycling of clathrin-coated vesicles, were found to be effective in blocking ChoP$^+$ invasion to 57.46 ± 17.56 and 45.54 ± 16.49%, respectively. However, total cell-adhered bacteria did not differ between nontreated and monodansylcadaverine- or CPZ-treated A549 cells, indicating that the inhibition was not due to inefficient binding of ChoP$^+$ to A549 cells (Fig. 5*A*). Moreover, to determine the role of β-arrestins in *A. baumannii* internalization by A549 cells, we evaluated the effect of β-arrestin small interfering RNA (siRNA)-transfected A549 cells on ChoP$^+$ adherence to and invasion of A549 cells for 2 h. We first tested the ability of β-arrestin-1 or β-arrestin-2 siRNA to deplete the

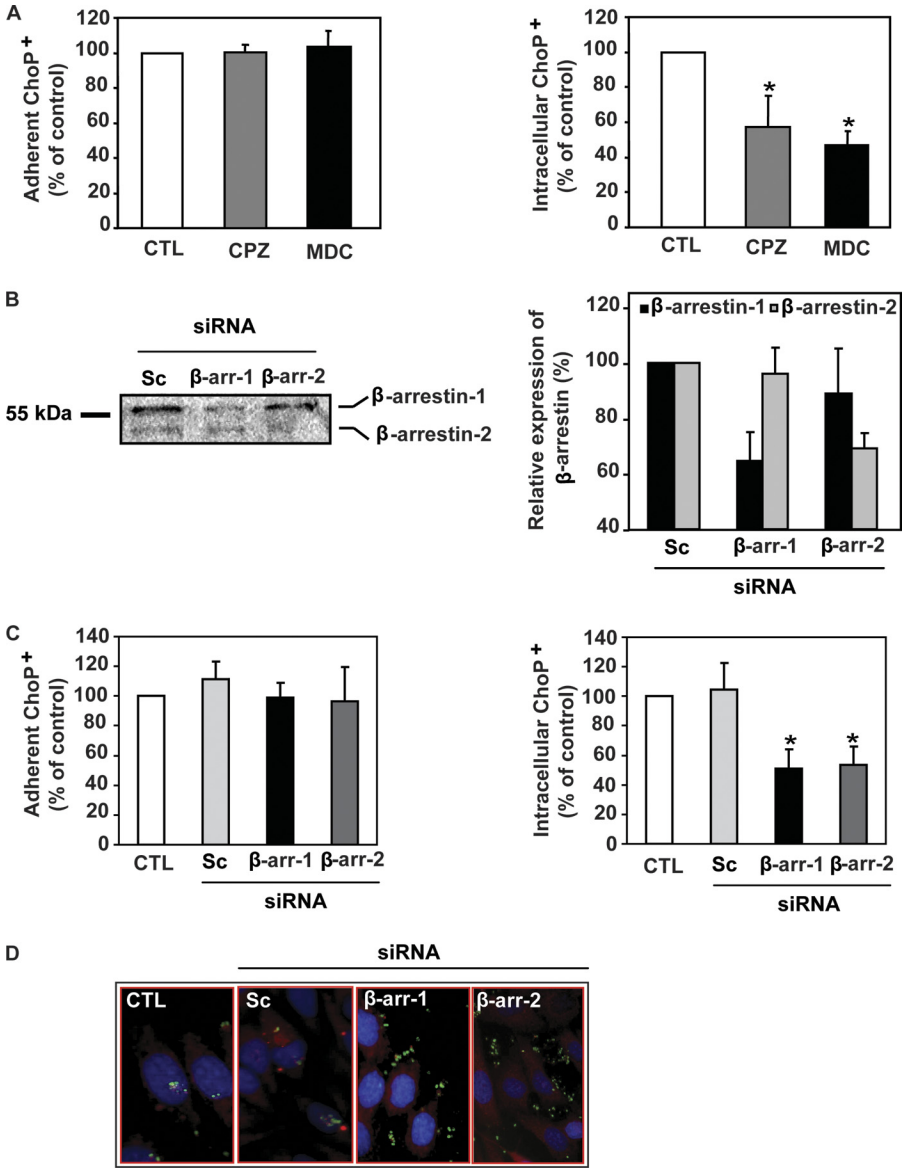

FIGURE 5. **Role of clathrin and β-arrestins in *A. baumannii* internalization by lung epithelial cells.** *A* and *C*, A549 cells were pretreated with monodansylcadaverine (*MDC*, 30 μM) or chlorpromazine (*CPZ*, 5 μM) or transfected with control, β-arrestin-1, and β-arrestin-2 siRNA and infected with 10^8 cfu/ml *A. baumannii* ChoP$^+$ for 2 h. An assay of adherence and invasion of *A. baumannii* ChoP$^+$ assay was performed as described under "Experimental Procedures." The effect of monodansylcadaverine-, CPZ-, or siRNA-mediated β-arrestin depletion on adherence or invasion of *A. baumannii* is expressed as the percentage of total nontreated *A. baumannii* ChoP$^+$ adhered to or internalized by A549 cells. *B*, immunoblot analysis of β-arrestin expression in control, β-arrestin-1 (*β-arr-1*), and β-arrestin-2 (*β-arr-2*) siRNA-transfected A549 cells. Values shown are expressed as the percentage of level of each siRNA of β-arrestin in control-transfected A549 cells. *D*, immunostaining for A549 cells and *A. baumannii* OMPs was performed in mock and control or β-arrestin-1 or β-arrestin-2 siRNA-transfected A549 cells and imaged by immunofluorescence microscopy. β-Arrestins of A549 cells and OMPs of *A. baumannii* strains were detected by rabbit anti-human β-arrestins and mouse anti-*A. baumannii* OMPs antibodies and labeled with Alexa Fluor 594- and Alexa Fluor 488-tagged the secondary antibodies (*red* and *green*), respectively. *Blue staining* shows the location of the nuclei of A549 cells. Representative results of three independent experiments are shown, and data are the means ± S.D. $p < 0.05$: *, between nontreated and treated groups. *Sc*: scramble.

β-arrestin levels in A549 cells. β-Arrestin-1 or β-arrestin-2 siRNA transfections reduce the expression of targeted β-arrestin by 35 and 31%, respectively, when compared with either nonsilencing or control siRNA-transfected A549 cells (Fig. 5*B*). Both β-arrestin-1 and β-arrestin-2 siRNA-transfected A549 cells were found to be effective in blocking the ChoP$^+$ invasion to 50.79 ± 13.21 and 53.68 ± 12.19%, respectively. However, total cell-adhered bacteria did not differ between control and β-arrestin-1 or β-arrestin-2 siRNA-transfected A549 cells, indicating that inhibition was not due to inefficient binding of ChoP$^+$ to A549 cells (Fig. 5*C*). In contrast, control siRNA-transfected A549 cells did not show significant blocking of the ChoP$^+$ invasion in A549 cells (Fig. 5*C*). Immunostaining studies showed that in A549 cells infected with ChoP$^+$, β-arrestin was colocalized with ChoP$^+$ in A549 cells (Fig. 5*D*). Similar results are observed with control siRNA-transfected A549 cells. Both β-arrestin-1 and β-arrestin-2 siRNA-transfected A549 cells prevent the colocalization between β-arrestin and ChoP$^+$ and, consequently, ChoP$^+$ invasion of A549 cells (Fig. 5*D*). Taken together, we demonstrated that clathrin and β-arrestins were involved in the invasion of lung epithelial cells by *A. baumannii* expressing ChoP.

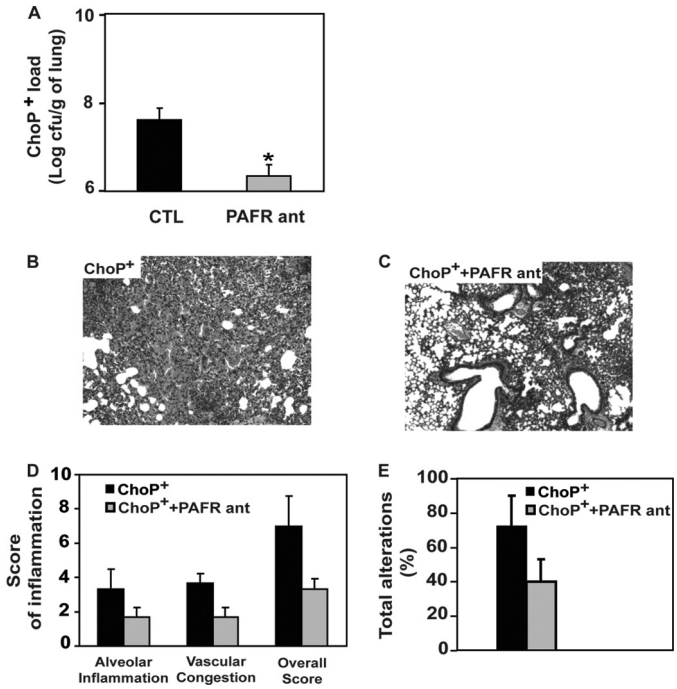

FIGURE 6. **Role of PAFR in the dissemination of *A. baumannii* in the pneumonia model and in the histological changes.** *A*, mice were intratracheally inoculated with the ChoP$^+$ strain in the presence or absence of PAFR antagonist (*PAFR ant*, 1 μg). *CTL*, control. *B* and *C*, bacterial loads were determined at 6 h of infection. *B* and *C*, representative lung slides of mice infected with ChoP$^+$ (*B*) or infected with ChoP$^+$ + PAFR antagonist (*C*) after 6 h of infection. *D* and *E*, histopathological score evaluated alveolar and vascular inflammation (*D*) and percentages of total alterations (*E*). See "supplemental Materials and Methods" for full description; sections are representative of at least three mice per group. Pictures were taken under 100× magnifications. Data are the means ± S.D. $p < 0.05$: *, between nontreated and treated groups.

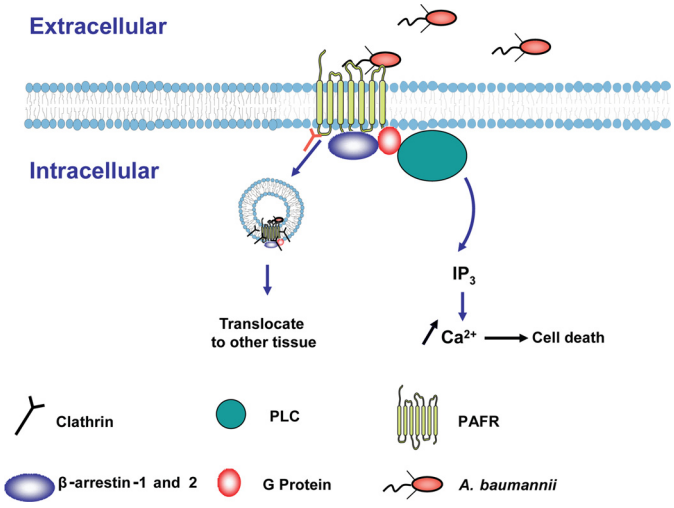

FIGURE 7. **A proposed scheme for the mechanism of *A. baumannii* adherence and invasion in human lung epithelial cells.** We propose that ChoP and PAFR enhance the adherence and invasion of *A. baumannii* in A549 cells. *A. baumannii* expressing ChoP adhere to A549 cells via PAFR, which thereafter activates a cascade of pathways composed of G protein-coupled PLC, clathrin, and β-arrestins, required for *A. baumannii* invasion. IP_3: inositol triphosphate.

Role of PAFR in the Dissemination of A. baumannii in Pneumonia Model—To study the role of PAFR on *A. baumannii* dissemination *in vivo*, we used the pneumonia model to infect mice with ChoP$^+$ and treated them with PAFR antagonist. Lung colonization and infection by ChoP$^+$ were attenuated to nearly 1.5 log cfu/ml by PAFR antagonist. Indeed, PAFR antagonist significantly reduced bacterial loads in infected lungs from 7.61 ± 0.28 to 6.34 ± 0.17 log cfu/ml (Fig. 6*A*). Furthermore, ChoP$^+$ induced a high level of alveolar inflammation and vascular congestion scores 3.33 ± 1.15 and 3.67 ± 0.85, respectively, whereas treatment with PAFR antagonist in ChoP$^+$-infected mice decreased these scores to 1.67 ± 0.58 and 1.67 ± 0.58 for both alveolar inflammation and vascular congestion (Fig. 6, *B–D*). Total lung alterations were reduced in ChoP$^+$-infected mice and in mice treated with PAFR antagonist in comparison with ChoP$^+$-infected mice (72.5 ± 17.68% *versus* 40 ± 13.23%) (Fig. 6*E*). From these data, we suggest that PAFR plays an important role in *A. baumannii* dissemination and pulmonary injury induction.

DISCUSSION

The present study provides new data highlighting the nature of the mechanism involved in the adherence to and invasion of host cells by *A. baumannii*. Here, we present the first evidences of the essential role played by PAFR in *A. baumannii* expressing ChoP adherence and invasion in human lung epithelial cells and dissemination of *A. baumannii* in the pneumonia model. We showed that *A. baumannii* expressing ChoP adhere to human lung epithelial cells via PAFR, which thereafter activate a cascade of pathways composed of G protein-coupled PLC, clathrin, and β-arrestins, which are required for invasion of *A. baumannii* (Fig. 7).

This study showed that ChoP-containing OMP is among the factors involved in the adherence to and invasion of epithelial cells by *A. baumannii*, consistent with other factors described in other pathogens (36, 37). Many results concerning the ability of pathogens expressing ChoP to invade human cells were related to different species and even different compartments from the same bacteria (10, 11, 38). Here, we showed that ChoP was present in OMPs of *A. baumannii*. Several studies have suggested that loss of ChoP might result in bacterial invasion dysfunction (10, 11, 19), which is not enough to avoid the human defense systems. We demonstrated that *A. baumannii* lacking ChoP, even if they expressed OMPA (data not shown), adhere to and invade epithelial cells much less strongly than *A. baumannii* expressing ChoP.

ChoP binding to PAFR is known to mediate the internalization of bacteria by human cells (10, 17). In our study, we focused on determining the role of PAFR in *A. baumannii* adherence and invasion of epithelial cells. Previously, we have shown that *A. baumannii* was able to adhere to and invade lung epithelial cells (39). Here, we showed that *A. baumannii* expressing ChoP acts through PAFR, which are functionally linked to the G protein signal pathway, and that the inhibition of G protein-coupled PLC prevented *A. baumannii* invasion of epithelial cells. Previous studies have determined that G protein coupled to PLC inhibition blocks the bacterial invasion of human endothelial and epithelial cells (40–42). Binding of bacteria to PAFR is known to increase inositol triphosphate and mobilization of intracellular Ca^{2+} (19), which is known to involve Ca^{2+} influx through ionic channels leading to cell death (20, 21). There is now considerable evidence that increase of intracellular Ca^{2+}

plays an important role in the cell death mediated by calpain activation, a substrate for Ca^{2+} (39, 43, 44). In this study, we demonstrated that *A. baumannii* increased intracellular Ca^{2+}, and we showed that inhibition of G protein coupled to PLC decreases Ca^{2+} influx and cell death induced by *A. baumannii*. Along the same lines, it has been shown that interaction of *E. coli* and *Listeria monocytogenes* with human endothelial and epithelial cells triggers PLC activation followed by increase of inositol triphosphate and intracellular Ca^{2+} (40, 45).

On the other hand, several studies have identified clathrin and β-arrestins as key signaling players in endocytosis and transcytosis (46, 47). However, only a few studies have reported that clathrin and β-arrestin-1 mediate bacterial invasion of epithelial cells (23, 48). To our knowledge, the present study provides the first evidence for clathrin and β-arrestin-1 and especially β-arrestin-2 involvement in *A. baumannii* invasion of epithelial cells. Firstly, we demonstrated that inhibition of clathrin prevented *A. baumannii* invasion of A549 cells. Secondly, we showed that β-arrestin-1 and β-arrestin-2 siRNA inhibited *A. baumannii* invasion of A549 cells.

Importantly, in the *in vivo* pneumonia model caused by *A. baumannii*, treatment with PAFR antagonist reduces bacterial loads in lungs, which indicates that PAFR participates in *A. baumannii* dissemination *in vivo*. Previous studies have determined that in PAFR knock-out and PAFR antagonist-treated mice, bacterial loads in blood, cerebrospinal fluid, and lung in *Streptococcus pneumoniae* and *P. aeruginosa* pneumonia and meningitis models were reduced (10, 23, 49, 50); meanwhile our data are the first to demonstrate functionally that PAFR is essential for *A. baumannii* dissemination *in vivo*. Moreover, we showed that PAFR antagonist pretreatment reduced but did not abolish *A. baumannii* dissemination *in vitro* and *in vivo*, indicating that other receptors and/or components of eukaryotic cells might mediate this residual *A. baumannii* invasion and dissemination. Alternatively, the residual *A. baumannii* invasion could be mediated by cytoskeletal rearrangement (5), fibronectin binding (51), and/or by laminin receptor (52); in addition, the last is known to initiate bacterial contact with the blood-brain barrier in experimental meningitis models. However, further investigations are needed to highlight these other specific mechanisms involved in *A. baumannii* dissemination.

In summary, we have demonstrated that PAFR play an important role in *A. baumannii* expressing ChoP adherence and in invasion of epithelial cells that requires G protein-coupled PLC, clathrin, and β-arrestin activation.

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