

Pleiotropic Effects of Cell Wall Amidase LytA on *Streptococcus pneumoniae* Sensitivity to the Host Immune Response

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The complement system is a key component of the host immune response for the recognition and clearance of *Streptococcus pneumoniae*. In this study, we demonstrate that the amidase LytA, the main pneumococcal autolysin, inhibits complement-mediated immunity independently of effects on pneumolysin by a complex process of impaired complement activation, increased binding of complement regulators, and direct degradation of complement C3. The use of human sera depleted of either C1q or factor B confirmed that LytA prevented activation of both the classical and alternative pathways, whereas pneumolysin inhibited only the classical pathway. LytA prevented binding of C1q and the acute-phase protein C-reactive protein to *S. pneumoniae*, thereby reducing activation of the classical pathway on the bacterial surface. In addition, LytA increased recruitment of the complement downregulators C4BP and factor H to the pneumococcal cell wall and directly cleaved C3b and iC3b to generate degradation products. As a consequence, C3b deposition and phagocytosis increased in the absence of LytA and were markedly enhanced for the *lytA ply* double mutant, confirming that a combination of LytA and Ply is essential for the establishment of pneumococcal pneumonia and sepsis in a murine model of infection. These data demonstrate that LytA has pleiotropic effects on complement activation, a finding which, in combination with the effects of pneumolysin on complement to assist with pneumococcal complement evasion, confirms a major role of both proteins for the full virulence of the microorganism during septicemia.

Streptococcus pneumoniae (also termed the pneumococcus) colonizes the human nasopharynx in a high percentage of the population and can be carried asymptotically from the first days of life (1). *S. pneumoniae* is the most common etiologic agent of acute otitis media and community-acquired pneumonia and a major cause of bacterial sepsis and meningitis, resulting in significant rates of morbidity and mortality worldwide (2). Prevention of pneumococcal disease requires efficient recognition and clearance of the invading pathogen by the complement system and professional phagocytes (3, 4). Activation of the three complement cascades—termed the classical pathway (CP), the alternative pathway (AP), and the lectin pathway—leads to the formation of the key complement component C3b, which plays a pivotal role in the host immune response, such as opsonization and clearance of invading pathogens (5–7). The CP is important for complement recognition of pneumococci and is generally activated by the recognition of antigen-antibody complexes on the bacterial surface (6, 8) as part of the adaptive immune response and by natural IgM, the lectin SIGN-R1, and acute-phase proteins as part of the innate immune response (6, 9, 10). In addition, the AP is activated by the spontaneous hydrolysis of the C3 component, triggering the amplification of C3 deposition (11, 12), and mannose binding lectin pathway activation has also recently been reported for *S. pneumoniae* (7). A finely controlled set of specific surface-bound and fluid-phase regulators, such as C4b-binding protein (C4BP) and factor H (FH), protects host cells from complement activation and complement-mediated damage (13–18).

Although the expression of the *S. pneumoniae* capsule is essen-

tial for the virulence of the microorganism, numerous pneumococcal proteins also contribute to pathogenesis, including by promoting complement evasion (3, 19). For example, the *S. pneumoniae* cell wall protein PspC can recruit the complement downregulators C4BP and FH to the bacterial cell surface, thereby inhibiting activation of the CP and AP, respectively (13–18). In addition, the cholesterol-dependent cytolysin pneumolysin (Ply) (20) prevents CP-mediated complement recognition of pneumococci through interactions with the CP component C1q (3, 21). However, export of Ply into extracellular fluid or for attachment

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to the cell wall seems to require lysis of the bacteria (22). The pneumococcal protein involved in lysis is the major autolytic enzyme of the bacterium and is termed LytA, an amidase that cleaves the *N*-acetylmuramoyl-L-alanine bonds of pneumococcal peptidoglycan (23). Previously, LytA was thought to contribute toward pneumococcal pathogenesis due to its importance for the release of Ply and inflammatory mediators, such as teichoic acids and peptidoglycan fragments from *S. pneumoniae* (23, 24), rather than direct effects on immune evasion independent of Ply.

In this study, we have investigated the contribution of Ply and LytA to the establishment of invasive pneumococcal disease (IPD), exploring their role in essential aspects of the pathogenesis process, including evasion of different components of the host immune response.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. pneumoniae* clinical isolates used were D39 (NCTC 07466, serotype 2 [ST2]); strain S3 *lytA* (ST23F) and its complemented mutant, S3C (*lytA*⁺) (25); and strain 1515/97 (ST6B) and its *lytA*-deficient strain (26). Isogenic D39 mutants with mutations in *lytA*, *ply*, *pspC*, or *lytB* were constructed by transformation, using standard protocols (18, 21, 27, 28), with DNA prepared from mutants previously characterized. Pneumococcal strains expressing the green fluorescent protein (GFP) were constructed by genetic transformation with pMV158GFP (tetracycline resistant) as previously described (28). Kanamycin (250 µg/ml), erythromycin (0.2 µg/ml), and tetracycline (0.5 µg/ml) were added to blood agar plates for isolation of bacterial transformants. *S. pneumoniae* strains were cultured on blood agar plates at 37°C in a CO₂ atmosphere or in Todd-Hewitt broth supplemented with 0.5% yeast extract to an optical density at 550 nm (OD₅₅₀) of 0.5 and stored at -70°C in 10% glycerol as single-use aliquots.

Binding of complement factors to *S. pneumoniae*. A pool of sera from five healthy male volunteers unvaccinated against *S. pneumoniae* (median age, 40 years) was obtained with informed consent according to institutional guidelines (LIB 14/2007, 3 July) and stored as single-use aliquots at -70°C as a source of complement and serum components. C1q, C3b, FH, C4BP, and C-reactive protein (CRP) were assessed using flow cytometry assays as previously described (10, 18, 28). Human sera depleted of C1q and factor B were purchased from Calbiochem. C3b deposition was detected by incubating 5 × 10⁶ CFU of the bacteria opsonized with 20% serum using a fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-human C3b antibody (ICN-Cappel) diluted 1/300 in phosphate-buffered saline (PBS)-0.1% Tween 20. After incubation, the bacteria were washed with PBS-Tween 20 (0.02%) to remove unbound components, fixed in 3% paraformaldehyde, and analyzed on a FAC-SCalibur flow cytometer (BD Biosciences) or a Beckman Coulter Cytomics FC500 flow cytometer using forward and side scatter parameters to gate on at least 25,000 bacteria. The results were expressed as a relative percent fluorescence index (FI) that measures not only the proportion of fluorescent bacteria positive for the host serum component investigated but also the intensity of fluorescence, which quantifies the immune component bound (8). This assay was adapted to assess the binding to C1q, CRP, FH, and C4BP using a conjugated polyclonal sheep anti-human C1q antibody (Serotec), a polyclonal rabbit anti-human CRP antibody (Calbiochem), a polyclonal sheep anti-human FH antibody (Serotec), and a polyclonal sheep anti-human C4BP antibody (Serotec). To detect CRP, FH, and C4BP, secondary staining in PBS-0.1% Tween 20 containing FITC-conjugated polyclonal goat anti-rabbit or FITC/DyLight 649 anti-sheep antibodies (Serotec) was performed. A direct interaction between purified LytA and purified C4BP or FH was determined by enzyme-linked immunosorbent assay (ELISA) as previously described (10). Briefly, Nunc MaxiSorp 96-well plates were coated with 10 µg/ml of purified LytA for 2 h at 37°C and blocked with a PBS-2% bovine serum albumin (BSA) solution before 50 µl of different concentrations of purified human C4BP or

FH was added to each well. After 2 h of incubation at 37°C, the plates were incubated with 50 µl of sheep anti-human C4BP or FH (Serotec) diluted 1/2,000 in PBS. Finally, the plates were incubated with 50 µl of rabbit anti-sheep HRP antibody (Santa Cruz) for 30 min and developed using *o*-phenylenediamine (Sigma-Aldrich) before determining the OD₄₉₂ using a microtiter plate reader (Anthos 2020).

Quantification of Pcho and PspC. The level of phosphorylcholine (Pcho) and PspC on the bacterial surface was detected by flow cytometry as previously described (28). The conditions of the assays were the same as those described above for complement components, except that the bacterial strains were incubated for 1 h at 37°C with TEPC-15 antibody (a monoclonal antibody specific for Pcho [Sigma-Aldrich]) diluted 1/25 or rabbit polyclonal antibody to PspC (a kind gift from Sven Hamerschmidt, University of Greifswald, Greifswald, Germany) diluted 1/300. The secondary antibodies used for the detection of Pcho and PspC were rabbit antimouse FITC (Serotec) and goat antirabbit FITC (Serotec), respectively.

C3b/iC3b degradation by LytA. Purified LytA (displaying amidase activity), the carboxy-terminal moiety or choline-binding domain of LytA (C-LytA), and the enzymatically inactive LytA_{H133A} protein (a mutated LytA amidase containing a His¹³³ → Ala substitution [LytA_{H133A}] that inactivates the enzyme [29]) were obtained by overexpression of previously described plasmids in *Escherichia coli* (30). A *lytA*-deficient strain was opsonized for 20 min with human serum, and after two washes with PBS-Tween 20, proteins were added and the C3b level was explored by flow cytometry. To detect C3 fragments by Western blotting, a *lytA*-null strain was opsonized with 50% human serum as a source of C3b in the absence or in the presence of different pneumococcal proteins (LytA, C-LytA, LytA_{H133A}, or the LytC lysozyme) for 2 h at 37°C. A sample of each supernatant was analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to a membrane, and fragments were revealed by immunoblotting using a goat anti-human C3b antibody. As controls, purified C3b proteins treated and not treated with FH and factor I were included. Additionally, purified C3b and iC3b proteins (3 µg) in sodium phosphate buffer (20 mM, pH 6.9) were treated with 3 ng of LytA or LytA_{H133A} and incubated for 2 h at 37°C. Samples were analyzed by SDS-PAGE using Tricine instead of Tris-glycine (31, 32).

Interaction of *S. pneumoniae* with phagocytes. Experiments investigating the recognition and phagocytosis by alveolar macrophages (AMs) were performed as previously described (27, 33). Briefly, murine MH-S cells (CRL-2019; American Type Culture Collection [ATCC]) were used as AMs and were grown in RPMI tissue culture medium supplemented with 10% heat-inactivated fetal calf serum and HEPES (10 mM). To test the recognition of the wild type and the different mutants by AMs, cells (seeded in 24-well plates containing 7 × 10⁵ cells per well) were infected in triplicate with 50 µl of a suspension of the pneumococcal strains at a ratio of 50 bacteria to 1 cell and incubated at 37°C. For adhesion assays, cells were infected for 1 h, washed five times with PBS, and lysed with 300 µl of a solution containing 0.025% saponin-PBS for 10 min. For phagocytosis assays, cells that had previously been infected for 1 h were washed five times with PBS and incubated for an additional hour in tissue culture medium containing penicillin (10 µg/ml) and gentamicin (200 µg/ml) to kill extracellular bacteria. Viable bacteria recovered from infected cells were obtained by plating serial dilutions on blood agar plates.

Phagocytosis by neutrophils was evaluated using HL-60 cells (CCL-240; ATCC) differentiated to granulocytes, and the general conditions of the assay were based on those described previously (10, 27, 34). Briefly, *S. pneumoniae* strains were fluorescently labeled by incubation with 6-carboxyfluorescein, succinimidyl ester (FAM-SE; Molecular Probes), solution (10 mg/ml in dimethyl sulfoxide; Sigma-Aldrich) in 0.1 M sodium bicarbonate buffer for 1 h at 37°C and then washed six times with Hanks balanced salt solution (HBSS)-0.2% BSA and stored in aliquots at -70°C in 10% glycerol for further assays. Infection assays were performed with bacteria at a ratio of 10 bacteria per cell. A minimum of 6,000 cells were

analyzed using a Cytomics flow cytometer. Results were expressed as a relative percent phagocytosis index, defined as the proportion of cells positive for fluorescent bacteria multiplied by the geometric mean of the fluorescence intensity, which correlates with the amount of bacteria phagocytosed per cell (8, 10, 27).

Confocal microscopy. *S. pneumoniae* strains expressing GFP were obtained by transformation with pMV158GFP and were used for immunofluorescence microscopy. MH-S cells and HL-60 cells that had previously been infected as described above were seeded on 12-mm circular coverslips for immunofluorescence staining. As the HL-60 cells were in suspension, the cells were centrifuged at $70 \times g$ for 2 min using a Cytospin centrifuge (Thermo Electron, Pittsburgh, PA). For the detection of late endosomal markers in AMs, we stained late antigen membrane protein 1 (LAMP1) or LAMP2. Coverslips containing the infected cells were washed twice in PBS containing 0.1% saponin and once in PBS and incubated for 30 min with primary antibodies. Staining was performed in PBS containing 10% horse serum, 0.1% saponin, and the primary antibodies using rat anti-mouse LAMP1 or LAMP2 (Southern Biotech) diluted 1/200, and the DNA was stained with Hoechst (Invitrogen) diluted 1/2,500. After 30 min incubation with primary antibodies at room temperature, the coverslips were washed twice with PBS–0.1% saponin and once with PBS (pH 7.0) before incubation for 30 min at room temperature with a 1/200 dilution of the secondary antibody goat antirat tetramethylrhodamine isocyanate (Serotec). The actin cytoskeleton was stained with rhodamine-phalloidin (Invitrogen) diluted 1:200. Finally, the coverslips were washed twice in PBS containing 0.1% saponin, once in PBS, and once in H₂O; mounted with Aqua Poly/Mount (Polysciences); and analyzed with a Leica spectral SP5 confocal microscope using Leica software (LAS-AF).

Experimental models of infection. C57BL/6 mice were bred by the CIB-CSIC and ISCIII animal facilities. All mice used were 8 to 16 weeks old, and within each experiment, groups of mice were matched for age and sex. In studies investigating the role of Ply and LytA in the establishment of pneumococcal sepsis and pneumonia, groups of 5 mice each were infected as previously described (10). Briefly, for the sepsis model of infection, mice were challenged with 1×10^6 CFU/ml of each strain (in a volume of 200 μ l) by the intraperitoneal route, whereas for pneumonia, mice, which were under anesthesia with isoflurane, were inoculated intranasally with 50 μ l containing 10^7 CFU/mouse. At 24 h after challenge, a lethal dose of pentobarbital was administered and bacterial counts were determined from samples recovered from bronchoalveolar lavage (BAL) fluid, lung, and blood. Experiments were repeated twice using 5 mice in each group, and results are expressed as the log number of CFU of bacteria/ml recovered from the different sites. All animal procedures were approved by the Animal Care and Use Committees of CIB-CSIC and ISCIII (approval reference numbers CIB-FJD 06010017 and CBBA-PA 52_2011-v2, respectively).

Quantification of capsular polysaccharide. Serotype 2 pneumococcal capsular polysaccharide (CPS) was either purchased from ATCC or prepared as previously described (35). Glucuronic acid was determined with *m*-hydroxydiphenyl, as previously described (36), using type 2 CPS as a standard and measuring the OD₅₂₀ using a microtiter plate reader (Anthos 2020). Recognition of the CPS of serotype 2 strains by specific antibodies was studied by the flow cytometry assay explained above using the wild-type D39 strain and the isogenic *lytA* mutant strain. The antibodies used for detection were rabbit anti-serotype 2 (Statens Serum Institut) diluted 1/200 and a secondary goat antirabbit FITC-conjugated antibody (Santa Cruz) diluted 1/300. Results are expressed as a fluorescence index, as explained above.

Statistical analysis. Data are representative of the results obtained from repeated independent experiments, and each point represents the mean and standard deviation (SD) from 3 to 5 replicates. Statistical analysis was performed by using a two-tailed Student's *t* test (for two groups), whereas analysis of variance (ANOVA) followed by Dunnett's *post hoc* test was chosen for use for multiple comparisons. GraphPad InStat (version 5.0) software (GraphPad Software, San Diego, CA) was used for statistical

analysis. Differences were considered statistically significant when *P* was <0.05 and highly significant when *P* was <0.01 and <0.001.

RESULTS

Ply and LytA divert C3b deposition following a cooperative strategy. The complement system is an efficient immune surveillance system detecting foreign intruders and is one of the first lines of the host immune defense against *S. pneumoniae* (11). To identify the role of Ply and LytA in the subversion of pneumococcal recognition by the key complement component C3b, strains defective in Ply and LytA were constructed. Strains lacking either LytA or Ply had increased C3b deposition on the bacterial surface compared to the wild-type strain. Higher levels of C3b were found on the *lytA* mutant than on the *ply*-defective strain, suggesting that LytA might avoid recognition by C3b using a Ply-independent mechanism (Fig. 1A and B). Binding to C3b was even more pronounced for the *lytA ply* double mutant, indicating that both proteins might act in concert to avoid complement-mediated immunity (Fig. 1A and B). Incubation with human serum depleted of complement component C1q or factor B (in which CP or AP activity, respectively, was abolished) confirmed that the presence of Ply reduced the activation of only the CP, whereas the presence of LytA inhibited the activation of both complement pathways, providing additional evidence that LytA impairs complement activation independently of Ply (Fig. 1C). A lack of enhanced C3b deposition when the *lytA ply* double mutant was incubated in C1q- or factor B-depleted sera confirmed that the activity of both cascades was essential for the improved effect in complement evasion mediated by both proteins (Fig. 1).

To exclude the possibility that cellular morphology (i.e., chain formation) might affect complement interaction, C3b deposition was analyzed on an isogenic *lytB* mutant strain, which forms long chains of bacteria (Fig. 1D and E). There was no increase in complement deposition on the *lytB* mutant, suggesting that, at least under our experimental conditions, the increased C3b levels on the *lytA*-null strain were not due to differences in cell separation (Fig. 1). The capsule is known to inhibit complement activity against the pneumococcus (19), but the content of glucuronic acid (a component of the CPS of serotype 2) was actually higher for the *lytA* mutant strain (0.185 for the wild-type strain versus 0.361 for the *lytA* mutant). In addition, the recognition of *S. pneumoniae* by antibodies specific to serotype 2 increased in the absence of LytA (100 ± 17 for the wild-type strain versus 201 ± 121 for the *lytA* mutant; *P* < 0.05). These results suggest that the increased C3b observed in the *lytA*-null strain cannot be due to reduced levels of CPS expression by the *lytA* mutant.

Ply and LytA prevent activation of the CP on *S. pneumoniae*. Activation of the CP by binding of C1q or acute-phase proteins, such as CRP or serum amyloid P component (SAP), to the bacterium is essential for complement-mediated immunity against *S. pneumoniae*, whereas bacterial components impairing activation are factors critical for immune evasion (6, 10, 37). Hence, we investigated the effect of deposition of C1q or CRP on the bacterial surface using pneumococcal strains lacking Ply, LytA, or both proteins simultaneously. An *S. pneumoniae* strain defective in either Ply or LytA showed higher levels of C1q binding, confirming that both proteins allow *S. pneumoniae* to impair the activation of the CP (Fig. 2A and B). This effect was more pronounced in the absence of LytA, suggesting that this amidase assists with pneumococcal evasion of CP activation using a Ply-independent strategy

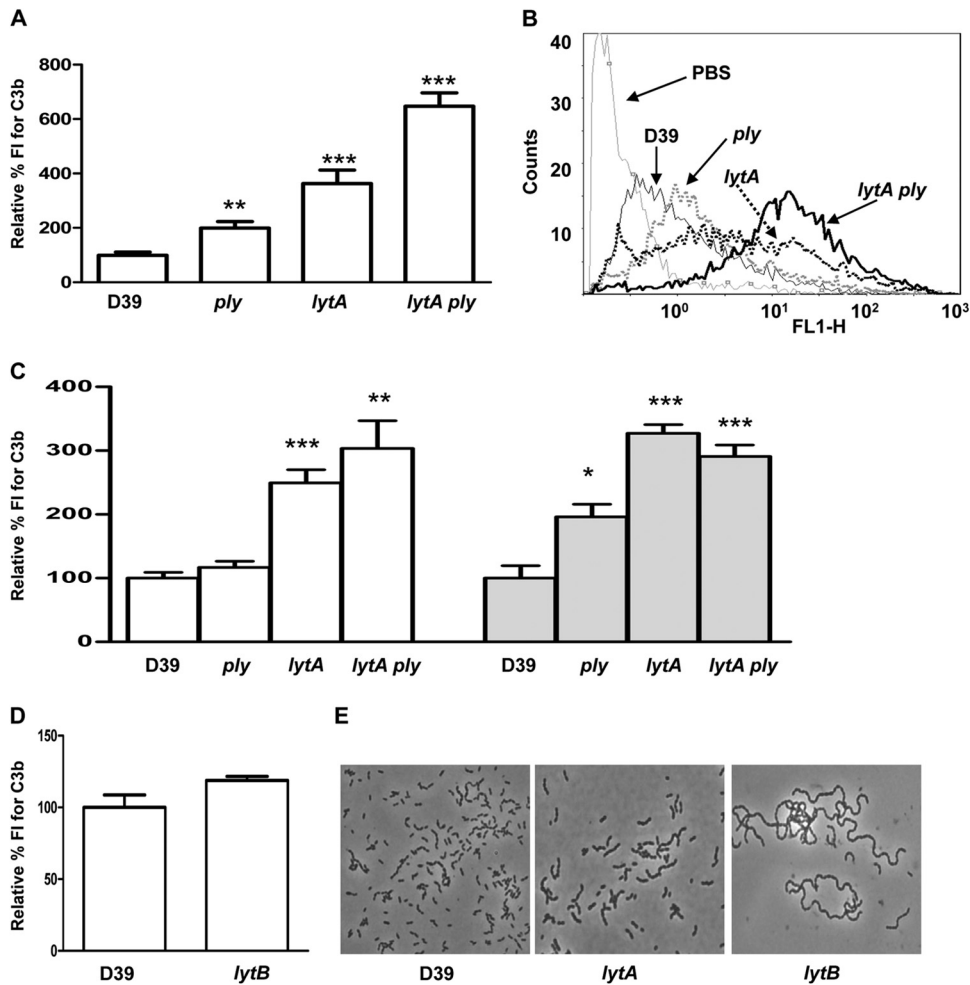


FIG 1 LytA of *S. pneumoniae* avoids complement activation by a Ply-independent mechanism. (A) C3b deposition on the surface of the wild-type and isogenic defective strains using NHS, as measured by a flow cytometry assay. (B) Example of a flow cytometry histogram for C3b deposition using NHS. (C) Deposition of C3b via AP (white bars) or CP (gray bars) activity in human serum in which C1q (white bars) or factor B (gray bars) was depleted, respectively. (D) Binding to C3b on the surface of the wild-type D39 strain and the *lytB* strain. (E) Phase-contrast microscopy images of pneumococcal wild-type D39 and isogenic *lytA* and *lytB* strains. Error bars represent SDs, and asterisks indicate statistically significant differences compared to the results for the wild-type strain: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. P was < 0.01 for the comparison of the C3b results for the *lytA ply* double mutant versus the single mutants using NHS. For the results for all defective strains compared to those for the wild type, P was < 0.001 (one-way ANOVA with Dunnett's *post hoc* test). FI, fluorescence intensity.

(Fig. 2A and B). CRP deposition was also increased in the absence of LytA but not in the absence of Ply (Fig. 2C and D). These results together demonstrated that LytA is more effective than Ply at impairing CP activation. As the increased levels of CRP on the *lytA* mutant might be due to differences in the amount of Pcho residues exposed on the bacterial cell wall, the level of Pcho was measured using pneumococcal strains of two different serotypes and the corresponding isogenic *lytA* mutants. A lack of LytA was associated with increased detection of Pcho, indicating that LytA impaired the recognition of pneumococci by the CP through effects on the availability of Pcho for binding to CRP (Fig. 2E to G).

LytA avoids complement immunity by recruiting fluid-phase downregulators. To prevent damage of host cells by a constant low level of complement activation, a finely controlled set of soluble and membrane-bound regulators allows any complement activation on host cells to be either avoided or strongly inhibited (11). Trapping of fluid-phase downregulators, such as C4BP and FH, by certain pathogens is a successful strategy for avoiding the

complement response (13). Binding of the CP inhibitor protein C4BP was greatly reduced in the *lytA*-null strain (Fig. 3A and B). As PspC has recently been reported to be a ligand for C4BP and FH, an isogenic *pspC* mutant strain was investigated (15, 17, 18). The loss of PspC resulted in a reduced proportion of C4BP binding, with the levels of binding being similar to those found in the absence of LytA, whereas the loss of both PspC and LytA resulted in a greater reduction in C4BP binding, demonstrating that both proteins play a key role in the recruitment of C4BP (Fig. 3A and B). Fluorescence intensity data also confirmed that a lack of either LytA or PspC was significantly associated with impaired recruitment of C4BP (23 ± 5 for the wild-type strain, 17 ± 2 for the *ply* strain, 8 ± 2 for the *lytA* strain, 9 ± 1 for the *pspC* strain, and 6 ± 1 for the *pspC lytA* strain). To confirm the requirement of LytA for binding to C4BP, the C4BP binding assays were performed using a serotype 23F *lytA* mutant strain (termed S3, the first described clinical isolate of *S. pneumoniae* deficient in LytA activity) and the corresponding *lytA*⁺ transformant (termed S3C) (25) (Fig. 3C

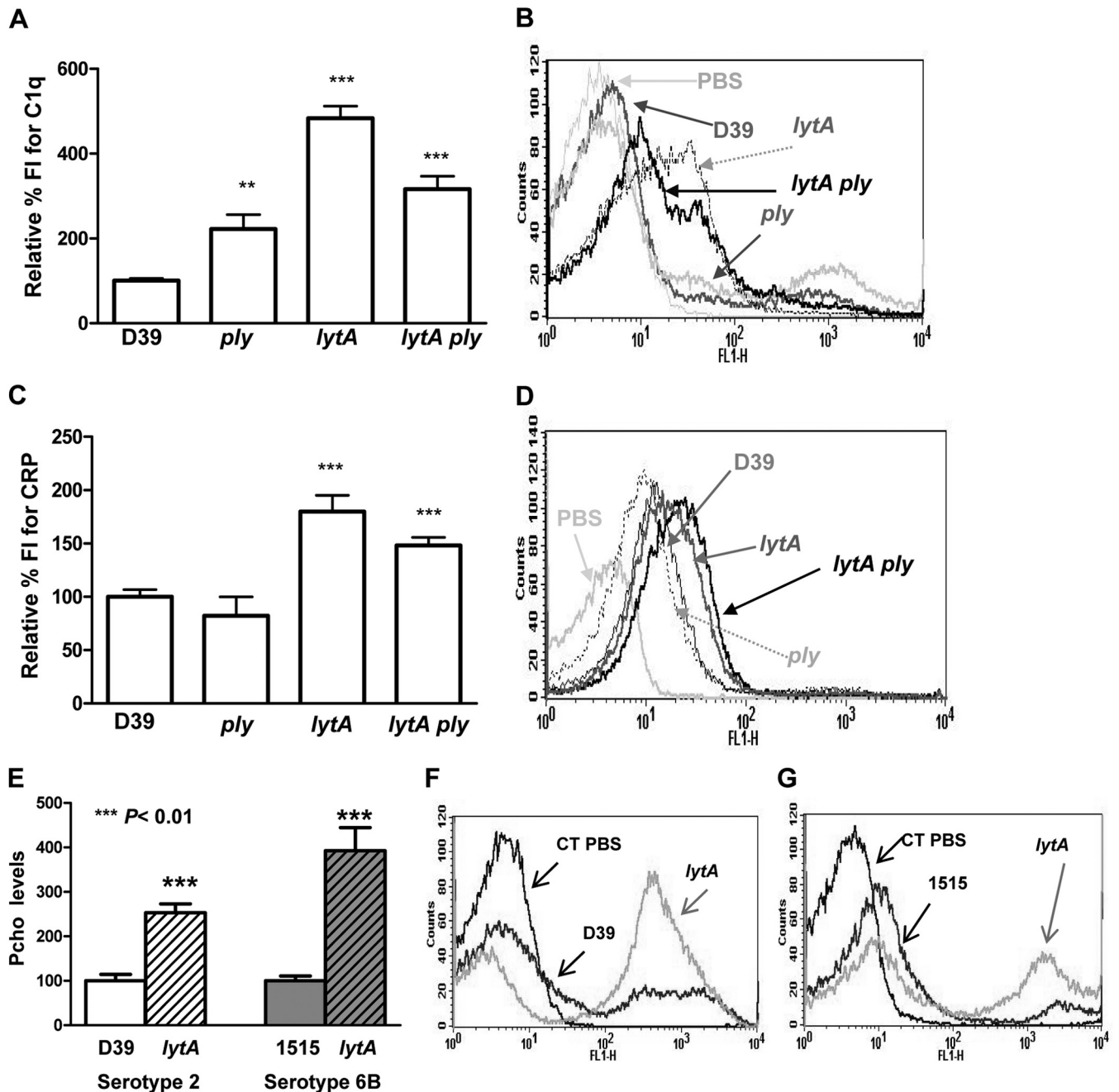


FIG 2 Ply and LytA divert classical pathway activation. (A) Deposition of C1q on the surface of the different mutants compared to that on the surface of the wild-type strain. (B) Example of a flow cytometry histogram for C1q deposition. (C) Recognition of the wild-type strain and the different mutants by CRP. (D) Example of a flow cytometry histogram for CRP deposition. (E) Pcho levels on the surface of wild-type strains D39 and 1515 of ST2 and ST6B, respectively, and LytA-deficient mutants. (F) Example of a flow cytometry histogram for the Pcho level on the ST2 strain. (G) Example of a flow cytometry histogram for the Pcho level on the ST6B strain. Error bars represent SDs, and asterisks indicate a statistically significant difference compared to the results for the wild-type strain: **, $P < 0.01$; ***, $P < 0.001$. For the results for all defective strains compared to the result for the wild type, P was < 0.001 (one-way ANOVA with Dunnett's *post hoc* test). CT, control (bacteria incubated in PBS).

and D). The loss of LytA in strain S3 was again associated with reduced C4BP binding, which was restored by transformation with the *lytA*⁺ allele (strain S3C), confirming that LytA is a novel ligand of *S. pneumoniae* for C4BP (Fig. 3C and D). Direct binding of purified LytA with different concentrations of purified human C4BP was observed, supporting a role for LytA in recruiting C4BP to the bacterial cell surface (Fig. 3F).

Interaction with FH, the downregulator of the AP, was also evaluated for the different strains. As expected, a lack of Ply did not affect FH binding, whereas a loss of LytA or PspC produced significantly lower levels of FH bound than those found with the wild-type strain (Fig. 4A and B). In addition, binding of FH to the *pspC lytA* double mutant was markedly impaired, indicating that both proteins are important pneumococcal ligands for FH bind-

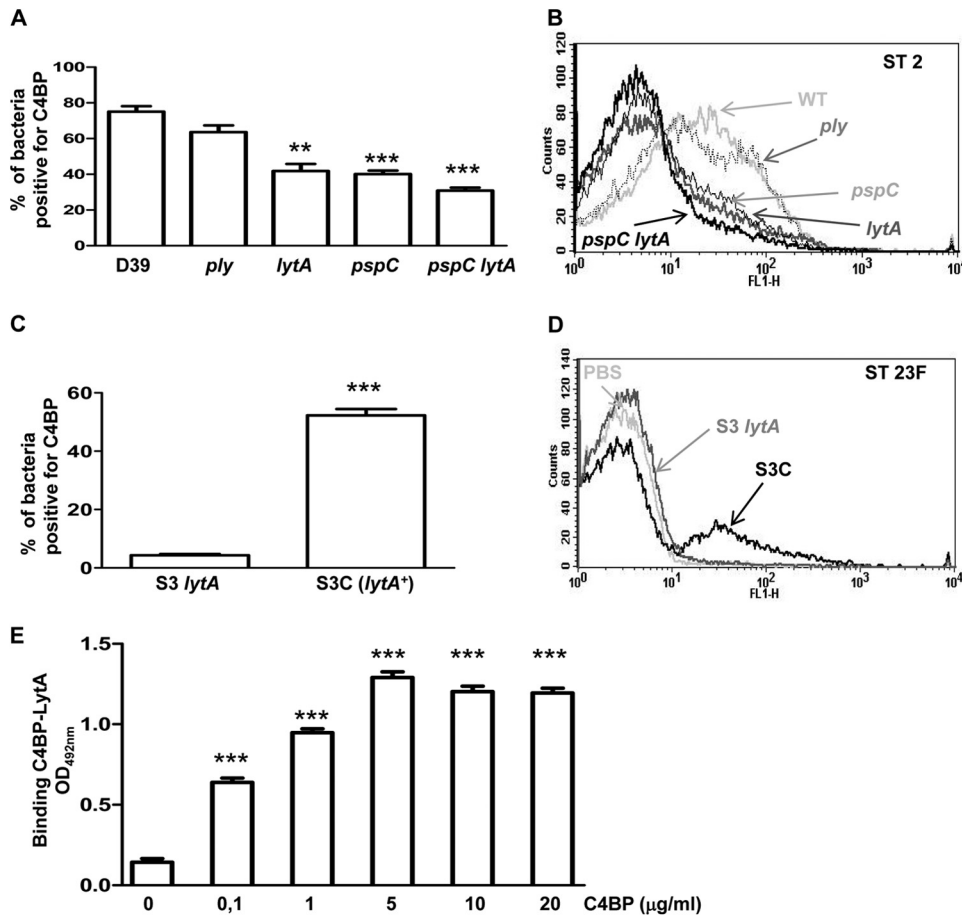


FIG 3 LytA recruits C4BP to reduce classical pathway activation. (A) Proportion of bacteria positive for C4BP for the D39 wild-type strain and the different mutants. (B) Example of a flow cytometry histogram for C4BP binding for strains with the D39 genetic background. (C) Proportion of bacteria positive for C4BP for the S3 *lytA* mutant strain and the complemented S3C strain (*lytA*⁺) belonging to ST23F. (D) Example of a flow cytometry histogram for C4BP binding for the ST23F strains. (E) Direct binding of 10 µg/ml of LytA to different concentrations of C4BP by ELISA. Error bars represent SDs, and asterisks indicate a statistically significant difference between the results for single mutants and the wild-type strain or between the results for different concentrations of C4BP and those in the absence of protein: **, $P < 0.01$; ***, $P < 0.001$. P was < 0.05 for the comparison of the C4BP results between the *pspC lytA* double mutant and the single mutants.

ing (Fig. 4A and B). Fluorescence intensity values confirmed that a lack of either LytA or PspC on the D39 strain was significantly associated with impaired binding to FH (72 ± 30 for the wild-type strain, 58 ± 10 for the *ply* strain, 23 ± 7 for the *lytA* strain, 5 ± 1 for the *pspC* strain, and 5 ± 1 for the *pspC lytA* strain). Recruitment of FH was also evaluated for strains S3 and S3C of serotype 23F (Fig. 4C to E). The proportion of bacteria binding to FH was similar between both strains (Fig. 4C), but the intensity of the FH bound was significantly lower in the absence of LytA (Fig. 4D and E), further supporting a role for LytA in pneumococcal recruitment of high levels of FH. Furthermore, a direct interaction between purified LytA autolysin and human FH proteins was observed (Fig. 4F). There were no differences in the level of PspC measured between the wild-type and the *lytA*-deficient strains (Fig. 5), confirming that the reduced levels of both C4BP and FH on the *lytA*-deficient strain were not caused by the effects of the *lytA* mutation on PspC expression (28).

LytA impairs opsonization by degradation of C3b and iC3b. Proteolytic enzymes can counteract the effects of complement activation by bacterial pathogens (13). Hence, the ability of LytA to

cleave the C3b deposited on the bacterial surface and purified C3b/iC3b components was investigated (Fig. 6). For these experiments, we used a fully active LytA protein (LytA), its C-terminal domain (C-LytA), and the enzymatically inactive protein LytA_{H133A} (see above). The degradation of the C3b deposited was initially investigated by adding the different LytA proteins to a previously opsonized *lytA*-deficient strain (Fig. 6A). The results demonstrated full restoration of the wild-type phenotype only when the fully active LytA amidase was used (Fig. 6A). This result was confirmed by Western blotting using an anti-C3 antibody. A small C3 fragment was observed after addition of the LytA protein but not after addition of LytA_{H133A}, C-LytA, or the LytC lysozyme (Fig. 6B). C3b degradation products were also found when LytA was incubated with either C3b (Fig. 6C) or iC3b (Fig. 6D), confirming the direct cleavage of C3b and iC3b components by LytA. Degradation of the C3b/iC3b deposited on the surface of the non-encapsulated R6 strain was also observed, indicating that LytA is involved in C3 degradation independently of capsule expression (Fig. 6E). These results demonstrated that the pneumococcal LytA autolysin can cause degradation of the complement components

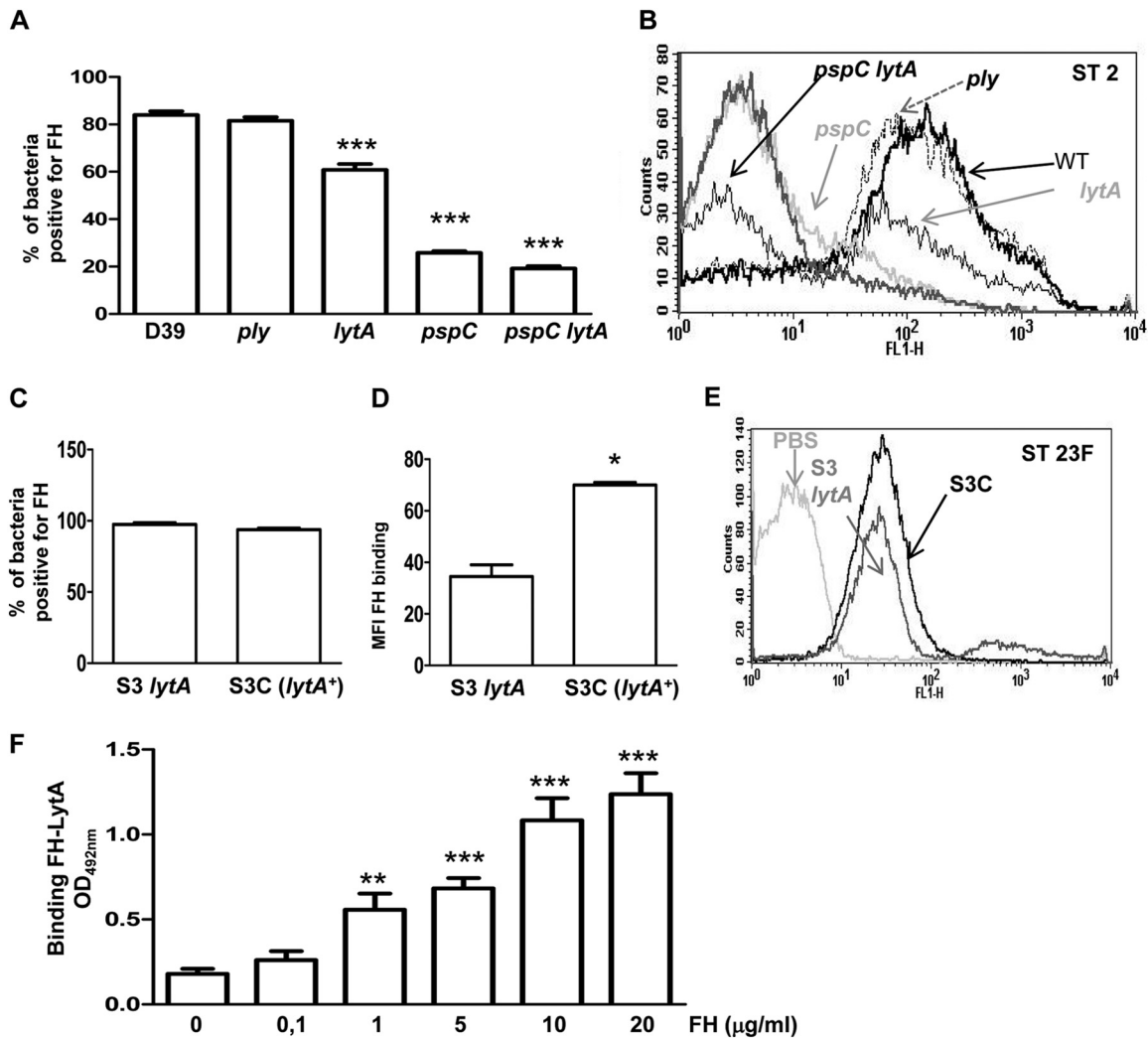


FIG 4 LytA binds the downregulator FH to impair the activation of the alternative pathway. (A) Proportion of bacteria positive for FH for the D39 wild-type strain and the different mutants. (B) Example of a flow cytometry histogram for FH binding for strains with the D39 genetic background. WT, wild type. (C) Proportion of bacteria positive for FH for the S3 *lytA* mutant strain and the complemented S3C strain (*lytA*⁺) belonging to ST23F. (D) Mean fluorescence intensity (MFI) of FH binding on the surface of the S3 *lytA* strain and the complemented S3C (*lytA*⁺) strain. (E) Example of a flow cytometry histogram for FH binding of the ST23F strains. (F) Direct binding of 10 µg/ml of LytA to different concentrations of FH by ELISA. Error bars represent the SDs, and asterisks indicate statistically significant differences compared to the results for the wild-type strain or between the results for different concentrations of FH compared to those in the absence of protein: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. P was < 0.01 for the comparison of the FH results between the *pspC lytA* double mutant and the single mutants.

C3b and iC3b, partially explaining why the *lytA*-deficient strain had higher levels of opsonization with C3b/iC3b.

LytA and Ply divert phagocytosis by professional phagocytes. Neutrophils control pneumococcal dissemination by phagocytosis, a process that requires the opsonization of bacteria by the complement system (38, 39). Opsonization with HBSS or heat-killed human serum (HKS) did not support the phagocytosis of *S. pneumoniae* by human neutrophils, whereas opsonization with normal human serum (NHS) did, confirming the importance of complement-mediated immunity for this process (Fig. 7A to C). There was increased phagocytosis of the *lytA* and *ply* mutants compared to that of the wild-type strain, showing that both proteins are important bacterial factors involved in evasion of phagocytosis. The *lytA* mutant exhibited higher phagocytosis levels than the *ply* mutant, suggesting that LytA participates in resis-

tance to phagocytosis by a mechanism that is independent of the release of Ply (Fig. 7A to C). Moreover, synergistic increases in phagocytosis were found for the *lytA ply* double mutant in comparison to the single mutants and the wild-type strain, demonstrating that both proteins contribute to evasion of phagocytosis by neutrophils (Fig. 7A to C).

Adhesion to a murine cell line of AMs was slightly increased in the absence of Ply or LytA and markedly increased in the double mutant, suggesting that these two proteins enable *S. pneumoniae* to divert the recognition by AMs (Fig. 7D). Time course experiments were performed to evaluate the phagocytosis process within the macrophage. At an early phase, phagocytosis of the *ply* and *lytA* single mutants was increased (Fig. 7A). Phagocytosis of the *ply lytA* double mutant strain was more efficient than that of the single mutants, confirming the additive effect of the loss of these

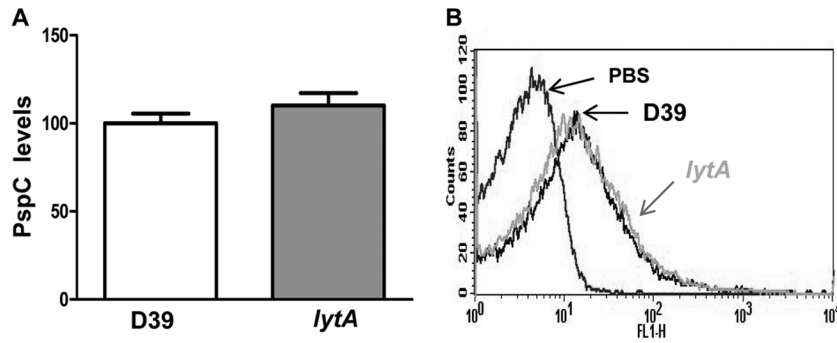


FIG 5 PspC levels are similar in the wild-type and *lytA* strains. (A) PspC levels on the surface of the D39 wild-type strain and the *LytA*-deficient mutant. (B) Example of a flow cytometry histogram for the PspC level. Error bars represent SDs.

two proteins on pneumococcal phagocytosis by AMs (Fig. 7E). Compared with the bacterial load at 1 h, the bacterial load was significantly reduced over time, with a more than 100-fold reduction being found at 4 h, suggesting that once the macrophage has recognized and phagocytosed pneumococcal strains lacking Ply

and *LytA*, the machinery of the macrophage efficiently destroys the engulfed bacteria (Fig. 7F). To confirm this possibility, we investigated maturation of the phagosome containing the *lytA* plynull strain using immunofluorescence microscopy to analyze the colocalization of phagocytosed GFP-expressing bacteria with

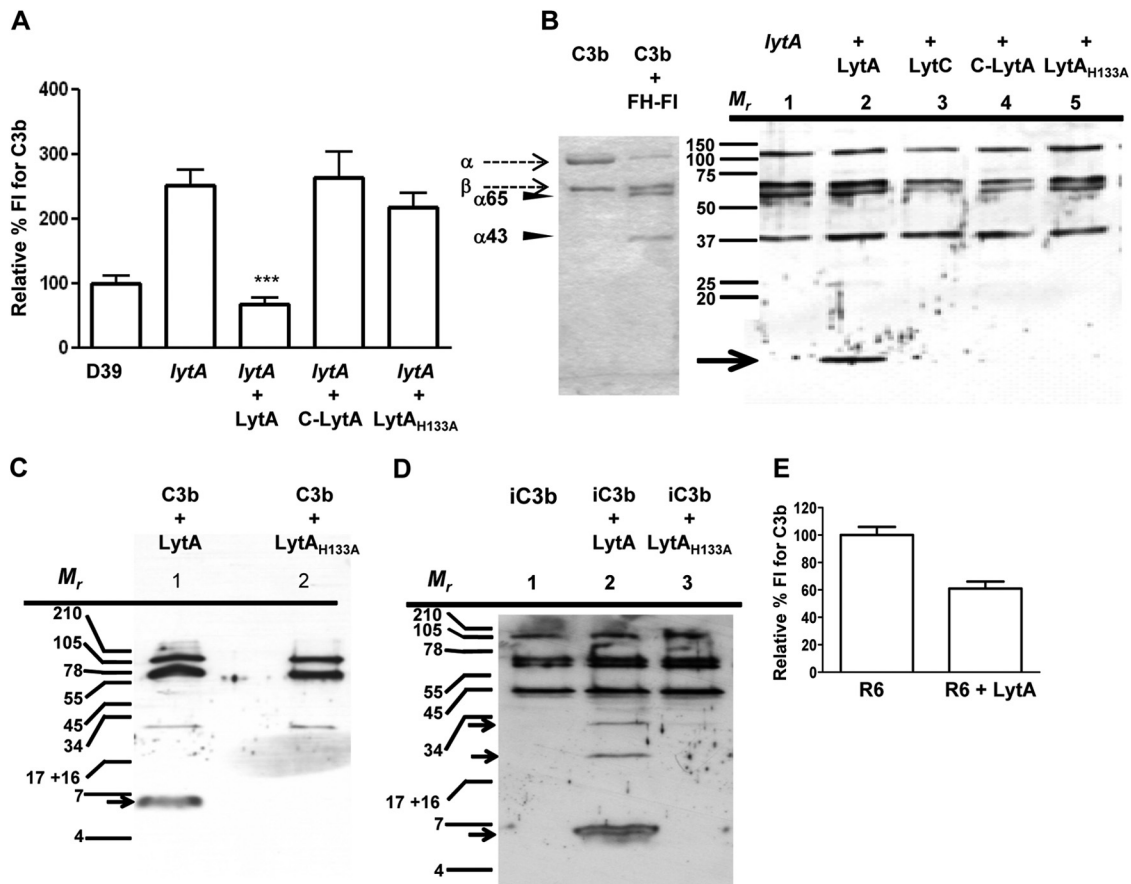


FIG 6 *LytA* degrades C3b and iC3b to impair complement activation. (A) Flow cytometry assay showing degradation of the C3b deposited on a *lytA* strain that had previously been opsonized with serum in the presence of 0.3 μ g of either *LytA* amidase, the choline-binding domain of *LytA* (C-*LytA*), or a mutated *LytA* protein without amidase activity (*LytA*_{H133A}). Error bars represent SDs, and asterisks indicate a statistically significant difference compared to the results for the wild-type strain: ***, $P < 0.001$. (B) Coomassie-stained polyacrylamide gel showing C3b degradation by FH-factor I (FI) and Western blotting to detect C3 fragments using a *lytA* mutant opsonized with serum (lanes 1 to 5) and exposed to 3 ng of *LytA* having amidase activity (lane 2), *LytC* lysozyme (lane 3), C-*LytA* (lane 4), and *LytA*_{H133A} (lane 5). (C and D) Western blotting to detect fragments of C3b and iC3b degradation, respectively, after exposure of purified C3b (C) and iC3b (D) to *LytA* or *LytA*_{H133A}. (B and D) Arrowheads, typical bands of C3b degradation by FH-FI (α 65 and α 43); arrows, C3b fragments obtained after digestion with *LytA*. (E) Degradation of the C3b deposited on the R6 strain in the presence or absence of 0.3 μ g of *LytA*.

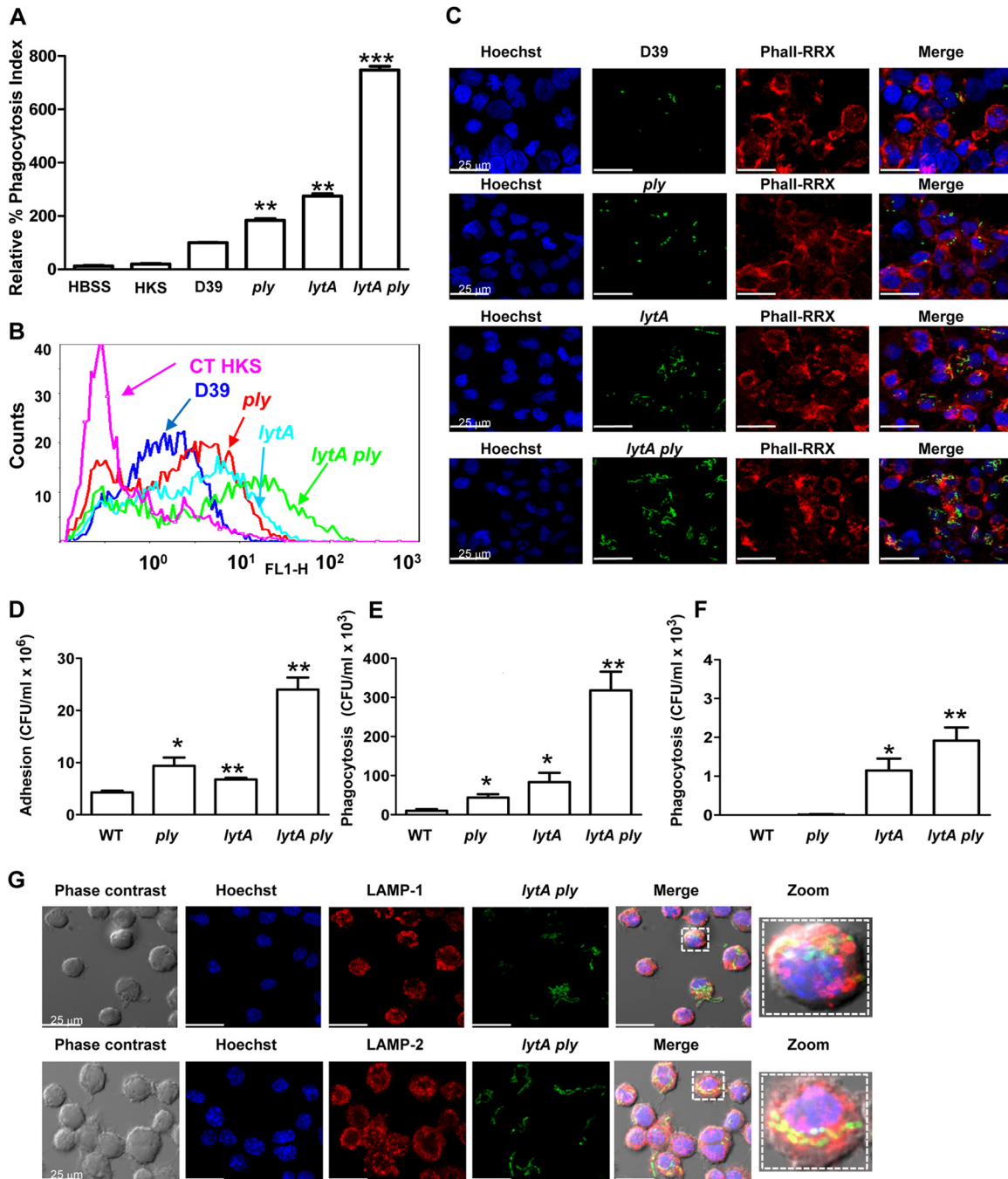


FIG 7 Evasion of the phagocytosis process mediated by pneumolysin and LytA. (A) Phagocytosis of the FAM-SE-labeled wild-type strain and the different mutant strains incubated in 20% normal human serum using a flow cytometry assay. Results are expressed as the fluorescent index (in percent) relative to the results for the wild-type D39 strain. (B) Example of a flow cytometry histogram for phagocytosis by neutrophils. (C) Opsonophagocytosis of the different strains expressing GFP by human neutrophils detected by confocal microscopy. DNA was stained by Hoechst, and the actin cytoskeleton was visualized with rhodamine-phalloidin (Phallo-RRX) staining. (D) Attachment to AMs of the different mutant strains compared to that of the wild-type strain at 1 h postinfection. (E and F) Phagocytosis of the different strains by AMs at 1 h (E) and 4 h (F) postinfection. (G) Phagolysosomal maturation of AMs during infection with *S. pneumoniae lytA ply* expressing GFP. DNA was stained with Hoechst, whereas late endosomal markers were visualized using specific antibodies to recognize LAMP1 (top) and LAMP2 (bottom). Error bars represent the SDs, and asterisks indicate statistically significant differences compared to the results for the wild-type strain: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. P was < 0.01 for the comparison of the phagocytosis results for the *lytA ply* double mutant and the single mutants for all times except 4 h of phagocytosis by AMs between the *lytA ply* double mutant and the *lytA* mutant, for which P was equal to 0.21. For the results for all defective strains compared to those for the wild type, P was < 0.001 (one-way ANOVA with Dunnett's *post hoc* test). CT, control (bacteria incubated in PBS).

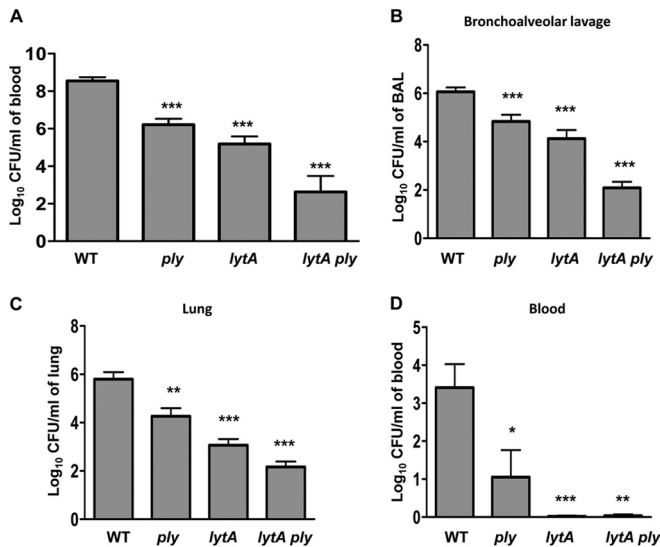


FIG 8 Role of Ply and LytA in the establishment of sepsis (intraperitoneal inoculation) and pneumococcal pneumonia (intranasal inoculation). (A) Bacterial levels recovered from blood at 24 h after pneumococcal sepsis produced with the wild-type and mutant strains. (B to D) Bacterial levels recovered at 24 h from BAL fluid (B), lung homogenate (C), and blood (D) after pneumonia caused by the wild-type and defective strains. Error bars represent SDs, and asterisks indicate statistically significant differences in the levels of the different mutant bacterial strains compared to the levels of the wild-type strain: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. P was < 0.05 for the comparison of the bacterial levels for the *lytA ply* double mutant and those for the single mutants. For the results for all defective strains compared to the wild type, P was < 0.01 (one-way ANOVA with Dunnett's *post hoc* test).

early and late endosomal markers. The double mutant was observed in LAMP1- and LAMP2-positive compartments (Fig. 7G), suggesting that in the absence of Ply and LytA, AMs efficiently process *S. pneumoniae* by the conventional phagolysosomal pathway.

LytA and Ply enhance the establishment of pneumococcal pneumonia and invasive disease. Mouse models of pneumonia and sepsis were used to characterize the contribution of Ply and LytA to the pathogenesis of *S. pneumoniae*. A lack of either Ply or LytA was associated with a significant attenuation of virulence in the sepsis model in comparison to the virulence of the wild-type strain (Fig. 8). In addition, the virulence of the *lytA ply* double mutant was greatly reduced compared to that of the single mutants and the wild-type strain, confirming that both proteins contribute separately to the establishment of pneumococcal sepsis (Fig. 8A).

In the pneumonia model, the levels of the *ply* or *lytA* single mutants recovered from BAL fluid, lung, and blood samples were significantly lower than those obtained with the parental strain, indicating that both proteins are involved in the pathogenesis of pneumococcal pneumonia (Fig. 8B to D). Moreover, the loss of both Ply and LytA caused further falls in the number of CFU recovered from BAL fluid, lung, and blood samples, confirming that the activity of both proteins is required for the full virulence of the bacterium in the respiratory tract and for spread from the lung to the blood (Fig. 8B to D). These results are compatible with the complement interaction data and confirm that inhibition of complement deposition on *S. pneumoniae* by the combination of LytA and Ply is essential for full virulence during systemic infection.

Collectively, these data suggest that the enhanced effect of LytA and Ply on virulence is mainly due to their combined inhibition of complement-dependent immunity and phagocytosis.

DISCUSSION

S. pneumoniae is the leading cause of community-acquired pneumonia and a major cause of sepsis and meningitis, which are associated with high morbidity and mortality rates worldwide (2, 40). As one of the most devastating human pathogens, *S. pneumoniae* has developed a wide arsenal of virulence factors to escape the well-balanced machinery of the immune system (3). Several proteins are involved in the establishment of IPD, which occurs when *S. pneumoniae* invades typically sterile sites, causing bacteremic pneumonia and sepsis, or when it crosses the blood-brain barrier, causing meningitis (3). Ply is a cytolytic protein with a significant role in pneumonia, sepsis, and meningitis but not, apparently, in carriage (3, 21, 24, 41–43). In contrast, the contribution of LytA to pneumococcal pathogenesis is poorly understood. The use of strains deficient in LytA has demonstrated attenuation of the virulence of these mutants in different models of infection, suggesting that LytA is important for virulence (39, 44, 45). Activation of complement-mediated immunity is an essential and critical component of the host immune response against *S. pneumoniae* (5–7, 9), and Ply has previously been reported to reduce the CP opsonic activity against *S. pneumoniae*. The effects of LytA on virulence have traditionally been linked to the release of Ply and not to a direct effect of LytA (3, 46, 47). However, our results demonstrate that LytA plays a critical role in complement evasion that is independent of the release of Ply. The *lytA* mutant had higher levels of CRP, C1q, and C3b binding to its surface than the *ply* mutant, and the *ply lytA* double mutant had increased levels of C3b deposition compared to those for the single mutant strains, confirming that both proteins confer complement resistance on *S. pneumoniae*. Our findings confirm previous data suggesting that Ply impairs the activation of the CP through C1q (21, 37, 46, 47), and this might be a possible explanation for the increased recognition by C1q in the absence of both Ply and LytA. Using a *lytA* mutant strain, other authors have reported increased sensitivity to complement-dependent clearance and attributed this attenuation to its increased bacterial chain length, suggesting that chain length is an important factor that increases the ability to fix complement C3b (39). Although a certain deficit in promoting the efficient separation of daughter cells in the absence of LytA may increase the level of recognition by C3b, our results suggest that this is not the full explanation for the increased complement activity against the *lytA* mutant, as the formation of longer chains by the *lytB* mutant strain does not affect complement deposition (27). Instead, the *lytA* mutation affects complement activity by a variety of mechanisms which lead to increased CP and AP activity.

One mechanism in the *lytA* mutant was an increased amount or accessibility to the cell surface of Pcho, the target for CRP, natural IgM, and SAP binding to *S. pneumoniae*, and therefore of innate CP activation. Modification of Pcho levels did not affect the expression of PspC, another important choline binding protein involved in complement evasion. Despite this, recruitment of the fluid-phase downregulators C4BP and FH (both of which are known to bind to PspC) was decreased in the *lytA* mutant, and assays using purified proteins demonstrated direct binding between LytA or C4BP and FH. Hence, our findings demonstrate that LytA is an additional pneumococcal protein that reduces

complement-mediated immunity by recruiting C4BP and FH. Finally, we have shown evidence that LytA mediates the direct degradation of C3 by *S. pneumoniae*, which has previously been described (48).

Our data suggest that LytA can inhibit complement activation against *S. pneumoniae* by multiple mechanisms. The pneumococcal capsule also alters different aspects of complement- and phagocyte-mediated immunity, resulting in a profound inhibition of opsonophagocytosis (19), and effects on the thickness of the capsule layer could potentially explain the pleiotropic effects of LytA on complement activity. However, the content of glucuronic acid (a component of the serotype 2 CPS) and the recognition by specific antibodies to CPS were actually slightly increased for the *lytA* mutant. Hence, the effects of LytA on the capsule are unlikely to explain the increased complement deposition seen on the *lytA* mutant; in addition, the direct interactions of purified LytA with FH, C4BP, and C3 cannot be explained by the effects of the loss of LytA on other aspects of *S. pneumoniae* biology. The location of bound C3b is important because the accessibility of this component affects the recognition by phagocytic cells. In this sense, the opsonic activity of C3b deposited on the bacterial cell wall may be less efficient for induction of phagocytosis. The presence of antibodies to CPS and the cell wall can also influence complement deposition (49–51). Human serum contains antibodies to multiple *S. pneumoniae* antigens, but these would not affect our results, unless there was a marked difference in the expression of target antigens between the strains investigated. Instead, our data indicate that increased complement activity against the *lytA* mutant was mediated by several separate mechanisms independent of antibodies, including increased Pcho availability (which could be a nonphysiological effect of the reduced occupation of choline residues in the *lytA* mutant), direct binding of LytA to C4BP and FH (causing the negative regulation of CP and AP activity, respectively), and enzymatic activity against C3.

AMs are one of the first barriers of the host immune defense system against pathogens invading the lungs, and neutrophils are key players controlling the dissemination of relevant microorganisms through the systemic circulation (52, 53). A loss of Ply and LytA was associated with the enhanced uptake of *S. pneumoniae* by AMs and neutrophils *in vitro*, confirming that the effects of these two proteins on complement are important for avoidance of the recognition and engulfment of *S. pneumoniae* by phagocytic cells. This is in agreement with previous evidence showing synergistic inhibition of complement-dependent immunity and phagocytosis for *S. pneumoniae* proteins (21, 27, 54, 55). The efficiency of AM phagolysosomal processing and bacterial killing within the macrophage was increased for the *ply* and *lytA* mutants, demonstrating that a lack of Ply and LytA increases the efficiency of AM clearance of the bacteria through the phagolysosomal route (56).

Data from the mouse models confirmed that Ply and LytA are critical proteins that cooperate in the establishment of IPD and pneumonia. Chain length formation has been identified to be a factor that might affect bacterial virulence (39). However, pneumococci growing as chains due to a *lytB* mutation did not show impaired virulence in our models, suggesting that the attenuation of the virulence of our *lytA* mutant was not significantly related to chain formation (27). The strain with the simultaneous loss of LytA and Ply showed a marked attenuation in virulence, indicating that both proteins cooperate in the replication of the bacterium in the respiratory tract and systemic circulation.

The impaired levels of the mutants in the blood, as previously reported (21, 41, 43, 47), suggest that pneumococcal strains lacking LytA and Ply have a reduced ability to breach the epithelial barrier. This is in agreement with the findings of a previous study showing that a lack of both LytA and Ply had an additive effect on the median survival time in a murine sepsis model of infection (43), and this phenotype has previously been shown to be related to complement sensitivity for the *ply pspA* double mutant (21). Overall, our results confirm that LytA plays an important role in bacteremic pneumonia and sepsis by a mechanism that is independent of Ply release and is likely to reflect the additive effects seen *in vitro* of Ply and LytA on complement inhibition and phagocytosis.

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