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

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Running Title: Pneumococcal LytA avoids complement immunity

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ABSTRACT

The complement system is a key component of the host immune response for the recognition and clearance of *Streptococcus pneumoniae*. In this study, we have demonstrated that the amidase LytA, the main pneumococcal autolysin, inhibits complement-mediated immunity independent of effects on pneumolysin by a complex process of impaired complement activation, increased binding of complement regulators, and direct degradation of C3. The use of human sera depleted in either C1q or factor B confirmed that LytA prevented activation of both the classical and alternative pathways whereas pneumolysin only inhibited the classical pathway. LytA prevented binding of C1q and the acute phase protein CRP to *S. pneumoniae*, thereby reducing activation of the classical pathway on the bacterial surface. In addition, LytA increased recruitment of the complement down-regulators 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 double *lytA ply* mutant, confirming that a combination of LytA and Ply was essential for the establishment of pneumococcal pneumonia and sepsis in a murine model of infection. These data demonstrate LytA has pleiotropic effects on complement activation, which in combination with the effects of pneumolysin on complement to assist pneumococcal complement evasion confirm a major role of both proteins for the full virulence of the microorganism during septicemia.

INTRODUCTION

Streptococcus pneumoniae (also termed the pneumococcus) colonizes the human nasopharynx in a high percentage of the population and can be carried asymptomatically from the first days of life (1). *S. pneumoniae* is the most common etiologic agent of acute otitis media, 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 (CP), the alternative (AP) and the lectin pathways– leads to the formation of the key complement component C3b that 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 been recently reported for *S. pneumoniae* (7). A finely controlled set of specific surface-bound and fluid-phase regulators such as C4 binding protein (C4BP) and factor H (FH) protect host cells from complement activation and complement-mediated damage (13-18).

Although the expression of *S. pneumoniae* capsule is essential 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 down-regulators C4b-binding protein

(C4BP) and factor H (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 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, termed LytA, an amidase that cleaves the *N*-acetylmuramoyl-L-alanine bonds of pneumococcal peptidoglycan (23). Previously, LytA is thought to contribute towards 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. *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 in *lytA*, *ply*, *pspC*, or *lytB* were constructed by transformation with DNA prepared from mutants previously characterized and using standard protocols (18, 21, 27, 28). 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 CO_2 atmosphere, or in Todd-Hewitt broth supplemented with 0.5% yeast extract, to an optical density at 550 nm (OD_{550}) 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 human sera from five healthy male volunteers unvaccinated against *S. pneumoniae* (median age: 40 years) were obtained with informed consent according to institutional guidelines 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 in C1q and factor B were purchased from Calbiochem. C3b deposition was detected by incubating 5×10^6 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 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 FACS Calibur flow cytometer (BD Biosciences) or a Beckman-Coulter Cytomics FC500 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 that quantify 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

125 (Serotec). To detect CRP, FH and C4BP a secondary staining in PBS/0.1% Tween 20
126 containing FITC-conjugated polyclonal goat anti-rabbit or FITC/DYLIGHT 649 anti-
127 sheep antibodies (Serotec) was performed. Direct interaction between purified LytA and
128 purified C4BP or FH were performed by ELISA as previously described (10). Briefly,
129 NUNC maxysorp 96-well plates were coated with 10 µg/ml of purified LytA for 2 h at
130 37°C and blocked with a PBS-BSA 2% solution before 50 µl of different concentrations
131 of purified human C4BP or FH were added to each well. After 2h incubation at 37°C,
132 plates were incubated with 50 µl of sheep anti-human C4BP or FH (Serotec) diluted
133 1/2000 in PBS. Finally, plates were incubated with 50 µl of rabbit anti-sheep HRP
134 antibody (Santa Cruz) for 30 min and developed using *o*-phenylenediamine (Sigma
135 Aldrich) before determining the OD_{492} using a microtiter plate reader (Anthos 2020).

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

145 **C3b/iC3b degradation by LytA.** Purified LytA (displaying amidase activity), the
146 carboxy-terminal moiety or (choline-binding domain) of LytA (C-LytA) and the
147 enzymatically inactive $LytA_{H133A}$ protein —a mutated LytA amidase containing a
148 $His^{133} \rightarrow Ala$ substitution that inactivates the enzyme (29)— were obtained by
149 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 protein with or without treatment 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-glicine (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; ATCC) as AMs 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 7×10^5 cells per well) were infected in triplicate with 50 μl of a suspension of the pneumococcal strains at a ratio of 50 bacteria: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 previously 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 FAM-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, then washed six times with HBSS-0.2% BSA and stored in aliquots at −70°C in 10% glycerol for further assays. Infection assays were performed with 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 % phagocytosis index defined as the proportion of positive cells for fluorescent bacteria multiplied by the geometric mean of fluorescence intensity which correlates with the amount of bacteria phagocytosed per cell (8, 10, 27).

Confocal microscopy. *S. pneumoniae* strains expressing the GFP were obtained by transformation with pMV158GFP and were used for immunofluorescence microscopy. MH-S cells and HL-60 cells previously infected as described above were seeded on 12-mm circular coverslips for immunofluorescence staining. As HL-60 cells are in suspension, cells were centrifuged at 70 × *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 proteins 1 or 2 (LAMP1, LAMP2). Coverslips containing the infected cells were washed twice in PBS containing 0.1% saponin (in PBS) 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 a rat anti-mouse LAMP1 or LAMP2 (Southern Biotech) diluted 1/200,

200 and the DNA was stained with Hoechst (Invitrogen) diluted 1/2500. After 30 min
201 incubation with primary antibodies at room temperature, coverslips were washed twice
202 with PBS-saponin 0.1%, and once with PBS pH 7.0 before incubation during 30
203 minutes at room temperature with a dilution 1/200 of the secondary antibody goat anti-
204 rat TRITC (Serotec). Actin cytoskeleton was stained with rhodamine-phalloidin
205 (Invitrogen) diluted 1:200. Finally, coverslips were washed twice in PBS containing
206 0.1% saponin, once in PBS, and once in H_2O, mounted with Aqua Poly/Mount
207 (Polysciences), and analyzed with a Leica spectral SP5 confocal microscope using the
208 Leica software (LAS-AF).

209 **Experimental models of infection.** C57BL/6 mice were bred by CIB-CSIC and
210 ISCIII animal facilities. All mice used were 8–16 weeks old, and within each
211 experiment, groups of mice were matched for age and sex. Studies investigating the role
212 of Ply and LytA in the establishment of pneumococcal sepsis and pneumonia, groups of
213 5 mice were infected as previously described (10). Briefly, for the sepsis model of
214 infection, mice were challenged with 1×10^6 CFU/ml of each strain (in a volume of 200
215 µl) by the intraperitoneal route, whereas for pneumonia mice under anesthesia with
216 isofluorane were inoculated intranasally with 50 µl containing 10^7 CFU/mouse. At 24 h
217 after challenge, a lethal dose of pentobarbital was administered and bacterial counts
218 were determined from samples recovered from bronchoalveolar lavage (BAL), lung and
219 blood. Experiments were repeated twice using 5 mice in each group and results were
220 expressed as Log CFU/ml of bacteria recovered from the different sites. All animal
221 procedures were approved by the Animal Care and Use Committees of CIB-CSIC and
222 ISCIII (approval references CIB-FJD 06010017 and CBBA-PA 52_2011-v2).

223 **Quantification of capsular polysaccharide**. Serotype 2 pneumococcal capsular
224 polysaccharide (CPS) was either purchased from the American Type Culture Collection

(ATCC) or prepared as previously described (35). Glucuronic acid was determined with *m*-hydroxydiphenyl, as previously described (36) using type 2 CPS as standard and measuring the OD_{520} using a microtiter plate reader (Anthos 2020). Recognition of CPS of serotype 2 by specific antibodies was studied by a flow cytometry assay explained above using the wild-type D39 strain and the isogenic *lytA* mutant strain. The antibodies used for the detection were rabbit anti-serotype 2 (Statens Serum Institut) diluted 1/200 and a secondary goat anti-rabbit FITC conjugated antibody (Santa Cruz) diluted 1/300. Results were expressed as a fluorescence index explained above.

Statistical analysis. Data are representative of results obtained from repeated independent experiments, and each point represents the mean and standard deviations (SD) for 3 to 5 replicates. Statistical analysis was performed by using two-tailed Student's *t* test (for two groups), whereas analysis of variance (ANOVA) followed by a Dunnett's *post hoc* test were chosen for multiple comparisons. GraphPad InStat version 5.0 (GraphPad Software, San Diego, CA) was used for statistical analysis. Differences were considered statistically significant with $P < 0.05$ (*) and highly significant with $P < 0.01$ (**) and $P < 0.001$ (***).

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 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

249 bacterial surface compared to the wild-type strain. Higher levels of C3b were found on
250 the *lytA* mutant than on the *ply* defective strain, suggesting that LytA might avoid the
251 recognition by C3b using a Ply-independent mechanism (Fig. 1A and B). Binding to
252 C3b was even more pronounced for the double mutant *lytA ply* indicating that both
253 proteins might act in concert avoiding complement mediated immunity (Fig. 1A and B).
254 Incubation with human sera depleted in complement components C1q or factor B (CP
255 or AP activity abolished respectively) confirmed that the presence of Ply only reduced
256 the activation of the CP whereas the presence of LytA inhibited activation of both
257 complement pathways, providing additional evidence that LytA impairs complement
258 activation independently of Ply (Fig. 1C). Lack of enhanced C3b deposition when the
259 double mutant *lytA ply* was incubated in C1q- or factor B-depleted sera, confirmed that
260 the activity of both cascades was essential for the improved effect in complement
261 evasion mediated by both proteins (Fig. 1).

262 To exclude the possibility that cellular morphology (*i.e.,* chain formation) might
263 affect complement interaction, C3b deposition was analyzed on an isogenic *lytB* mutant
264 strain, which forms long chains of bacteria (Fig. 1D and E). There was no increase in
265 complement deposition on the *lytB* mutant, suggesting that at least, under our
266 experimental conditions the increased C3b levels on the *lytA* null strain were not due to
267 differences in cell separation (Fig 1). The capsule is known to inhibit complement
268 activity against the pneumococcus (19), but the content of glucuronic acid (a component
269 of the CPS of serotype 2) was actually higher for the *lytA* mutant strain (0.185 for wild-
270 type strain vs. 0.361 for the *lytA* mutant). In addition, the recognition of *S. pneumoniae*
271 by specific antibodies to serotype 2 increased in the absence of LytA (100 ± 17 for wild-
272 type strain vs. 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 the activation of the classical pathway on *S. pneumoniae*. Activation of the CP by binding of C1q or acute phase proteins such as CRP or SAP to the bacterium is essential for complement-mediated immunity against *S. pneumoniae* whereas bacterial components impairing activation are critical factors 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. *S. pneumoniae* 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 pneumococcal evasion of the CP activation using a Ply-independent strategy (Fig. 2A and B). CRP deposition was also increased in the absence of LytA but not 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. Lack of LytA was associated with increased detection of Pcho, indicating that LytA impaired recognition of pneumococci by the CP through effects on the availability of Pcho for binding to CRP (Figs. 2E–G).

LytA avoids complement immunity by recruiting fluid-phase down-regulators. To prevent damage of host cells by a constant low level of complement activation, a finely controlled set of soluble and membrane-bound regulators allow that any complement activation on host cells is either avoided or strongly inhibited (11).

298 Trapping fluid-phase down-regulators such as C4BP and FH by certain pathogens is a
299 successful strategy for avoiding the complement response (13). Binding of the CP
300 inhibitor protein C4BP was greatly reduced in the *lytA* null strain (Fig. 3A and B). As
301 PspC has been recently reported as a ligand for C4BP and FH, an isogenic *pspC* mutant
302 strain was investigated (15, 17, 18). Loss of PspC resulted in a reduced proportion of
303 C4BP binding with similar levels to those found in the absence of LytA, whereas loss of
304 both PspC and LytA showed greater reduction in C4BP binding, demonstrating that
305 both proteins play a key role in the recruitment of C4BP (Fig. 3A and B). Fluorescence
306 intensity values also confirmed that lack of either LytA or PspC was significantly
307 associated with impaired recruitment of C4BP (23 ± 5 for the wild-type strain, 17 ± 2
308 for the *ply* strain, 8 ± 2 for the *lytA* strain, 9 ± 1 for the *pspC* strain and 6 ± 1 for the
309 *pspC lytA* strain). To confirm the requirement of LytA for binding to C4BP, the C4BP
310 binding assays were performed using a serotype 23F isolate *lytA* mutant strain (termed
311 S3, the first described clinical isolate of *S. pneumoniae* deficient in LytA activity) and
312 the corresponding $lytA^+$ transformant (25) (termed S3C) (Fig. 3C and D). Loss of LytA
313 in strain S3 was again associated with reduced C4BP binding which was restored by
314 transformation with the $lytA^+$ allele (strain S3C), confirming that LytA is a novel ligand
315 of *S. pneumoniae* for C4BP (Fig. 3C and D). Direct binding of purified LytA with
316 different concentrations of purified human C4BP was observed, supporting a role for
317 LytA in recruiting C4BP to the bacterial cell surface (Fig. 3F).
318 Interaction with FH, the down-regulator of the AP, was also evaluated for the
319 different strains. As expected, lack of Ply did not affect FH binding, whereas loss of
320 LytA or PspC produced significantly lower levels of FH bound than those found with
321 the wild-type strain (Fig. 4A and B). In addition, binding of FH to the double mutant
322 *pspC lytA* was markedly impaired indicating that both proteins are important

323 pneumococcal ligands for FH binding (Fig. 4A and B). Fluorescence intensity values
324 confirmed that lack of either LytA or PspC on the D39 strain was significantly
325 associated with impaired binding to FH (72 ± 30 for the wild-type strain, 58 ± 10 for the
326 *ply* strain, 23 ± 7 for the *lytA* strain, 5 ± 1 for the *pspC* strain and 5 ± 1 for the *pspC lytA*
327 strain). Recruitment of FH was also evaluated for the strains S3 and S3C of serotype
328 23F (Fig. 4C–E). The proportion of bacteria binding to FH was similar between both
329 strains (Fig. 4C), but the intensity of the FH bound was significantly lower in the
330 absence of LytA (Fig. 4D and E) further supporting a role for LytA in pneumococcal
331 recruitment of high levels of FH. Furthermore, a direct interaction between purified
332 LytA autolysin and human FH proteins was observed (Fig. 4F). There were no
333 differences in the level of PspC measured between the wild-type and the *lytA*-deficient
334 strains (Fig. 5), confirming that the reduced levels of both C4BP and FH on the *lytA*-
335 deficient strain were not caused by effects of the *lytA* mutation on PspC expression (28).
336 **LytA impairs opsonization by degradation of C3b and iC3b.** Proteolytic enzymes
337 can counteract the effects of complement activation by bacterial pathogens (13). Hence,
338 the ability of LytA to cleave the C3b deposited on the bacterial surface and purified
339 C3b/iC3b components were investigated (Fig. 6). For these experiments we used a fully
340 active LytA protein (LytA), its C-terminal domain (C-LytA), and the enzymatically
341 inactive protein $LytA_{H133A}$ (see above). Degradation of C3b deposited was initially
342 investigated by adding the different LytA proteins to a previously opsonized *lytA*-
343 deficient strain (Fig. 6A). The results demonstrated full restoration of the wild-type
344 phenotype only when the fully active LytA amidase was used (Fig. 6A). This result was
345 confirmed by Western-blotting using an anti-C3 antibody. A C3 fragment of small size
346 was observed after addition of the LytA protein but not after addition of $LytA_{H133A}$, C-
347 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 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 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 opsonization of bacteria by the complement system (38, 39). Opsonization with Hanks balance salt solution (HBSS) or heat-killed human serum (HKS) did not support phagocytosis of *S. pneumoniae* by human neutrophils whereas normal human serum did, confirming the importance of complement-mediated immunity for this process (Figs. 7A–C). There was increased phagocytosis of the *lytA* and *ply* mutants compared to 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 resistance to phagocytosis by a mechanism that is independent of the release of Ply (Figs. 7A–C). Moreover, synergistic increases in phagocytosis were found for the double *lytA ply* mutant in comparison to the single mutants and the wild-type strain, demonstrating that both proteins contribute to evasion of phagocytosis by neutrophils (Figs. 7A–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

373 experiments were performed to evaluate the phagocytosis process within the
374 macrophage. At an early phase, phagocytosis of the *ply* and *lytA* single mutants was
375 increased (Fig. 7A). Phagocytosis of the double *ply* and *lytA* mutant strain was more
376 efficient in comparison to the single mutants confirming the additive effect of loss of
377 these two proteins on pneumococcal phagocytosis by AMs (Fig. 7E). Compared with 1
378 h, bacterial load was significantly reduced over time with more than 100-fold reduction
379 at 4 h suggesting that once the macrophage has recognized and phagocytosed
380 pneumococcal strains lacking Ply and LytA, the machinery of the macrophage
381 efficiently destroys the engulfed bacteria (Fig. 7F). To confirm this possibility, we
382 investigated maturation of the phagosome containing the *lytA ply* null strain using
383 immunofluorescence microscopy to analyze colocalization of phagocytosed GFP-
384 expressing bacteria with early and late endosomal markers. The double mutant was
385 observed in LAMP1- and LAMP2- positive compartments (Fig. 7G) suggesting that in
386 the absence of Ply and LytA, AMs efficiently process *S. pneumoniae* by the
387 conventional phagolysosomal pathway.
388
389 **LytA and Ply enhance the establishment of pneumococcal pneumonia and**
390 **invasive disease.** Mouse models of pneumonia and sepsis were used to characterize the
391 contribution of Ply and LytA to the pathogenesis of *S. pneumoniae*. Lack of either Ply
392 or LytA was associated with a significant attenuation in the sepsis model in comparison
393 to the wild-type strain (Fig. 8). In addition, virulence of the *lytA ply* double mutant was
394 greatly reduced compared to the single mutants and the wild-type strain, confirming that
395 both proteins contribute separately to the establishment of pneumococcal sepsis (Fig.
396 8A).

In the pneumonia model, the levels of *ply* or *lytA* single mutants recovered from BAL, 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 (Figs. 8B–D). Moreover, loss of both Ply and LytA had a caused further falls in CFU recovered from BAL, 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 (Figs. 8B–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 on virulence by LytA and Ply 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 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 that occurs when *S. pneumoniae* invades typically sterile sites causing bacteraemic 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 apparently not 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 these mutants in different models of infection suggesting that LytA is important for virulence (39, 44, 45). Activation of complement-

422 mediated immunity is an essential and critical component of the host immune response
423 against S. *pneumoniae* (5-7, 9), and Ply has previously been reported to reduce the CP
424 opsonic activity against *S. pneumoniae*. The effects of LytA on virulence has
425 traditionally been linked to the release of Ply and not to a direct effect of LytA (3, 46,
426 47). However, our results demonstrate that LytA plays a critical role in complement
427 evasion that is independent of the release of Ply. The *lytA* mutant had greater levels of
428 CRP, C1q and C3b binding to its surface than the *ply* mutant, and the double *ply lytA*
429 mutant had increased C3b deposition compared to the single mutant strains confirming
430 that both proteins confer complement resistance on *S. pneumoniae*. Our findings
431 confirm previous data suggesting that Ply impairs the activation of the CP through C1q
432 (21, 37, 46, 47) and this might be a possible explanation for the increased recognition
433 by C1q in the absence of both Ply and LytA. Using a *lytA* mutant strain, other authors
434 have reported increased sensitivity to complement-dependent clearance, and attributed
435 this attenuation to its increased bacterial chain length suggesting that chain length is an
436 important factor that increases the ability to fix complement C3b (39). Although a
437 certain deficit in promoting efficient separation of daughter cells in the absence of LytA
438 may increase the recognition by C3b, our results suggest that this is not the full
439 explanation for increased complement activity against the *lytA* mutant as formation of
440 longer chains by the *lytB* mutant strain does not affect complement deposition (27).
441 Instead, the *lytA* mutation affects complement activity by a variety of mechanisms
442 which led to increased CP and AP activity.

443 One mechanism was in the *lytA* mutant an increased amount or accessibility on the
444 cell surface of Pcho, the target for CRP, natural IgM and SAP binding to *S. pneumoniae*
445 and therefore of innate CP activation. Modification of Pcho levels did not affect the
446 expression of PspC, another important choline binding proteins involved in complement

447 evasion. Despite this, recruitment of the fluid-phase down-regulators C4BP and FH
448 (which are both known to bind to PspC) were decreased in the *lytA* mutant, and assays
449 using purified proteins demonstrated direct binding between LytA or C4BP and FH.
450 Hence, our findings demonstrate that LytA is an additional pneumococcal protein that
451 reduces complement-mediated immunity by recruiting C4BP and FH. Finally, we have
452 shown evidence that LytA mediates the direct degradation of C3 by *S. pneumoniae* that
453 has previously been described (48).

454 Our data suggests that LytA can inhibit complement activation against *S.*
455 *pneumoniae* by multiple mechanisms. The pneumococcal capsule also alters different
456 aspects of complement- and phagocyte-mediated immunity, resulting in a profound
457 inhibition of opsonophagocytosis (19), and effects on the thickness of the capsule layer
458 could potentially explain the pleiotropic effects of LytA on complement activity.
459 However, the content of glucuronic acid (a component of the serotype 2 CPS) and the
460 recognition by specific antibodies to CPS were actually slightly increased for the *lytA*
461 mutant. Hence LytA effects on the capsule are unlikely to explain the increased
462 complement deposition seen on the *lytA* mutant; in addition the direct interactions of
463 purified LytA with FH, C4BP, and C3 cannot be explained by effects of loss of LytA on
464 other aspects of *S. pneumoniae* biology. Location of bound C3b is important because
465 the accessibility of this component affects the recognition by phagocytic cells. In this
466 sense, the opsonic activity of C3b deposited on the bacterial cell wall may be less
467 efficient to induce phagocytosis. The presence of antibodies to the capsular
468 polysaccharide (CPS) and the cell wall can also influence complement deposition (49-
469 51). Human sera contain antibodies to multiple *S. pneumoniae* antigens but these would
470 not affect our results unless there was marked difference in expression of target antigens
471 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 non-physiological effect of reduced occupation of choline residues in the *lytA* mutant), direct binding of LytA to C4BP and FH (causing 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). Loss of Ply and LytA was associated with an 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 evidences showing synergistic inhibition of complement-dependent immunity and phagocytosis for *S. pneumoniae* proteins (21, 27, 54, 55). The efficiency of AMs phagolysosomal processing and bacterial killing within the macrophage was increased for the *ply* and *lytA* mutants demonstrating that lack of Ply and LytA increases the efficiency of AMs to clear the bacteria through their 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 as 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 in virulence of our *lytA* mutant was not significantly related to chain formation (27). Loss of LytA and Ply simultaneously 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

497 impaired levels of the mutants in the blood, as previously reported (21, 41, 43, 47),
498 suggest that pneumococcal strains lacking LytA and Ply have a reduced ability to
499 breach the epithelial barrier. This is in agreement with a previous study showing that
500 lack of both LytA and Ply had an additive effect in the median survival time in a murine
501 sepsis model of infection (43), and is a phenotype that has previously been shown to be
502 related to complement sensitivity for the double *ply pspA* mutant (21). Overall, our
503 results confirm that LytA plays an important role in bacteraemic pneumonia and sepsis
504 by a mechanism that is independent of Ply release, and is likely to reflect the additive
505 effects seen in vitro of Ply and LytA on complement inhibition and phagocytosis.
506

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513

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692 **FIGURE LEGENDS**

693

694 **FIG 1** LytA of *S. pneumoniae* avoids complement activation by a Ply independent
695 mechanism. (A) C3b deposition on the surface of the wild-type and isogenic defective
696 strains using normal human serum (NHS) and measured by flow cytometry assay. (B)
697 Example of a flow cytometry histogram for C3b deposition using NHS. (C) Deposition
698 of C3b via AP (white bars) or CP (grey bars) activity in C1q (white bars) or factor B
699 (grey bars) depleted human sera respectively. (D) Binding to C3b on the surface of the
700 wild-type D39 and *lytB* strains. (E) Phase-contrast microscopy images of pneumococcal
701 wild-type D39 and isogenic *lytA* and *lytB* strains. Error bars represent the standard
702 deviations (SDs) and asterisks indicate statistical significance compared to the wild-type
703 strain. $P < 0.01$ for the comparison of the C3b results for *lytA ply* versus the single
704 mutants using NHS. For the results for all defective strains compared to wild-type, $P <$
705 0.001 (one-way ANOVA with Dunnett's *post hoc* test).

706

707 **FIG 2** Ply and LytA divert classical pathway activation. (A) Deposition of C1q on the
708 surface of the different mutants compared to the wild-type strain. (B) Example of a flow
709 cytometry histogram for C1q deposition. (C) Recognition of the wild-type strain and the
710 different mutants by CRP. (D) Example of a flow cytometry histogram for CRP
711 deposition. (E) Pcho levels on the surface of wild-type strains D39 and 1515 of
712 serotypes (STs) 2 and 6B respectively and LytA-deficient mutants. (F) Example of a
713 flow cytometry histogram for Pcho level on the ST2 strain. (G) Example of a flow
714 cytometry histogram for Pcho level on the ST6B strain. Error bars represent the
715 standard deviations (SDs) and asterisks indicate statistical significance compared to the
716 wild-type strain. For the results for all defective strains compared to wild-type, $P <$
717 0.001 (one-way ANOVA with Dunnett's post hoc test).

718

719 **FIG 3** LytA recruits C4BP to reduce classical pathway activation. (A) Proportion of
720 bacteria positive for C4BP for the D39 wild-type strain and different mutants. (B)

721 Example of a flow cytometry histogram for C4BP binding for the D39 genetic
722 background strains. (C) Proportion of bacteria positive for C4BP for the S3 *lytA* mutant
723 strain and the complemented strain S3C (*lytA*$^{+}$) belonging to serotype 23F. (D) Example
724 of a flow cytometry histogram for C4BP binding for the ST23F strains. (E) Direct
725 binding of 10 μg/ml of LytA to different concentrations of C4BP by ELISA. Error bars
726 represent the SDs and asterisks indicate statistical significance of single mutants
727 compared to the wild-type strain or between different concentrations of C4BP compared
728 to the absence of protein. $P < 0.05$ for the comparison of C4BP results between *pspC*
729 *lytA* vs single mutants.
730
731 **FIG 4** LytA binds the down-regulator factor H to impair the activation of the alternative
732 pathway. (A) Proportion of bacteria positive for FH for the D39 wild-type strain and
733 different mutants. (B) Example of a flow cytometry histogram for FH binding for the
734 D39 genetic background strains. (C) Proportion of bacteria positive for FH for the S3
735 *lytA* mutant strain and the complemented strain S3C (*lytA*$^{+}$) belonging to serotype 23F.
736 (D) Mean Fluorescence Intensity of FH binding on the surface of S3 *lytA* strain and the
737 complement S3C (*lytA*$^{+}$) strain. (E) Example of a flow cytometry histogram for FH
738 binding of the ST23F strains. (F) Direct binding of 10 μg/ml of LytA to different
739 concentrations of FH by ELISA. Error bars represent the SDs and asterisks indicate
740 statistical significance compared to the wild-type strain or between different
741 concentrations of FH compared to the absence of protein. $P < 0.01$ for the comparison
742 of FH results between *pspC lytA* vs single mutants.
743
744 **FIG 5** PspC levels are similar in the wild-type and *lytA* strain. (A) PspC levels on the
745 surface of D39 wild-type strain and LytA-deficient mutant. (B) Example of a flow
746 cytometry histogram for PspC level. Error bars represent the SDs.
747
748 **FIG 6** LytA degrades C3b and iC3b to impair complement activation. (A) Flow
749 cytometry assay showing degradation of the C3b deposited on *lytA* strain previously

750 opsonized with serum in the presence of 0.3 μg of either LytA amidase, choline-binding
751 domain of LytA (C-LytA), or a mutated LytA protein without amidase activity
752 ($LytA_{H133A}$). Error bars represent the SDs and asterisks indicate statistical significance
753 compared to the wild-type strain. (B) Coomasie-stained polyacrilamide gel showing
754 C3b degradation by FH-FI and Western-Blot to detect C3 fragments using a *lytA* mutant
755 opsonized with serum (lanes 1-5) and exposed to 3 ng of LytA having amidase activity
756 (2), LytC lysozyme (3), C-LytA (4) and $LytA_{H133A}$ (5). (C–D) Western-blotting to
757 detect fragments of C3b and iC3b degradation respectively, after exposure of purified
758 C3b (C) and iC3b (D) to LytA or $LytA_{H133A}$. Black triangles mark typical bands of C3b
759 degradation by FH/FI (α65 and α43) and solid black arrows indicate C3b fragments
760 obtained after digestion with LytA. (E) Degradation of the C3b deposited on the R6
761 strain in the presence or absence of 0.3 μg of LytA.
762
763 **FIG 7.** Evasion of the phagocytosis process mediated by pneumolysin and LytA. (A)
764 Phagocytosis of FAM-SE labeled wild-type strain and the different mutant strains
765 incubated in 20% normal human serum using a flow cytometry assay. Results are
766 expressed as a percent fluorescent index relative to the results for the wild-type D39
767 strain. (B) Example of a flow cytometry histogram for phagocytosis by neutrophils. (C)
768 Opsonophagocytosis of the different strains expressing the GFP protein by human
769 neutrophils detected by confocal microscopy. DNA was stained by Hoechst and actin
770 cytoskeleton was visualized with Rhodamine-Phalloidin (RRX) staining. (D)
771 Attachment to AMs of the different mutant strains compared to the wild-type strain at 1
772 h post-infection. (E–F) Phagocytosis of the different strains by AMs at 1 h and 4 h post-
773 infection respectively. (G) Phagolysosomal maturation of AMs during infection with *S.*
774 *pneumoniae lytA ply* expressing the GFP. DNA was stained with Hoechst whereas late
775 endosomal markers were visualized using specific antibodies to recognize LAMP-1 and
776 LAMP-2. Error bars represent the SDs and asterisks indicate statistical significance
777 compared to the wild-type strain. $P < 0.01$ for the comparison of the phagocytosis results
778 for *lytA ply* versus the single mutants except for 4h phagocytosis of AMs between *lytA*

779 *ply* vs *lytA* (P =0.21). For the results for all defective strains compared to wild-type, P
780 <0.001 (one-way ANOVA with Dunnett's *post hoc* test).
781
782 **FIG 8.** Role of Ply and LytA in the establishment of sepsis (intraperitoneal inoculation)
783 and pneumococcal pneumonia (intranasal inoculation). (A) Bacterial levels recovered
784 from blood at 24 h after pneumococcal sepsis produced with the wild-type and mutant
785 strains. (B–D) Bacterial levels recovered at 24 h from BAL (B), lung homogenate (C)
786 and blood (D) after pneumonia infection with the wild-type and defective strains. Error
787 bars represent the SDs and asterisks indicate statistical significance of bacterial levels of
788 the different mutant strains compared to the wild-type strain. P <0.05 for the
789 comparison of bacterial levels for *lytA ply* versus the single mutants. For the results for
790 all defective strains compared to wild-type, P <0.01 (one-way ANOVA with Dunnett's
791 *post hoc* test).
792
793

Figure 1

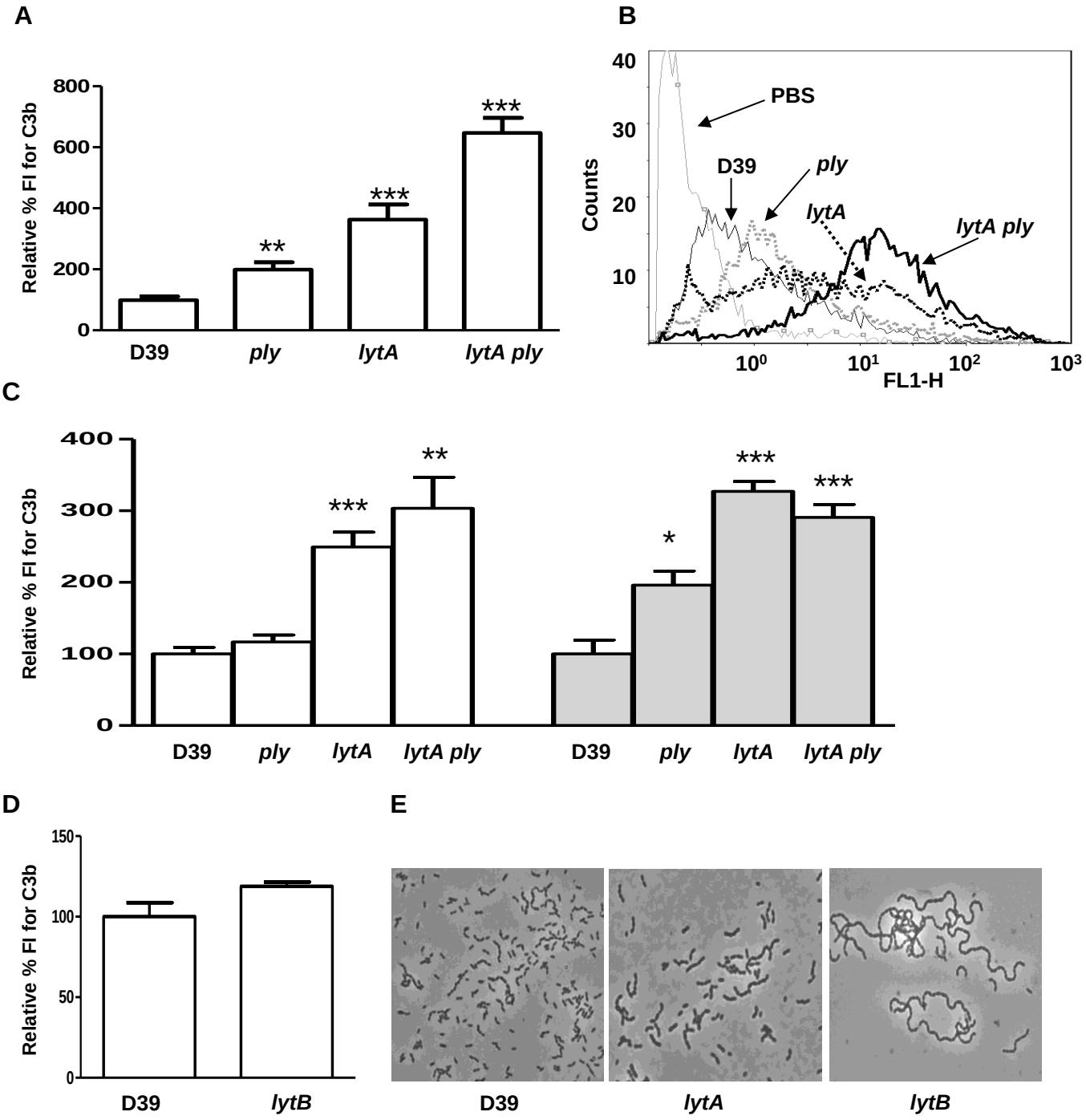

A
Relative % FI for C3b
800
600
400
200
0
**
***
***
D39
ply
lytA
lytA ply
B
Counts
40
30
20
10
PBS
D39
ply
lytA
lytA ply
10^0
10^1
10^2
10^3
FL1-H
C
Relative % FI for C3b
400
300
200
100
0
***
**
*
***
***
D39
ply
lytA
lytA ply
D39
ply
lytA
lytA ply
D
Relative % FI for C3b
150
100
50
0
D39
lytB
E
D39
lytA
lytB

Figure 2

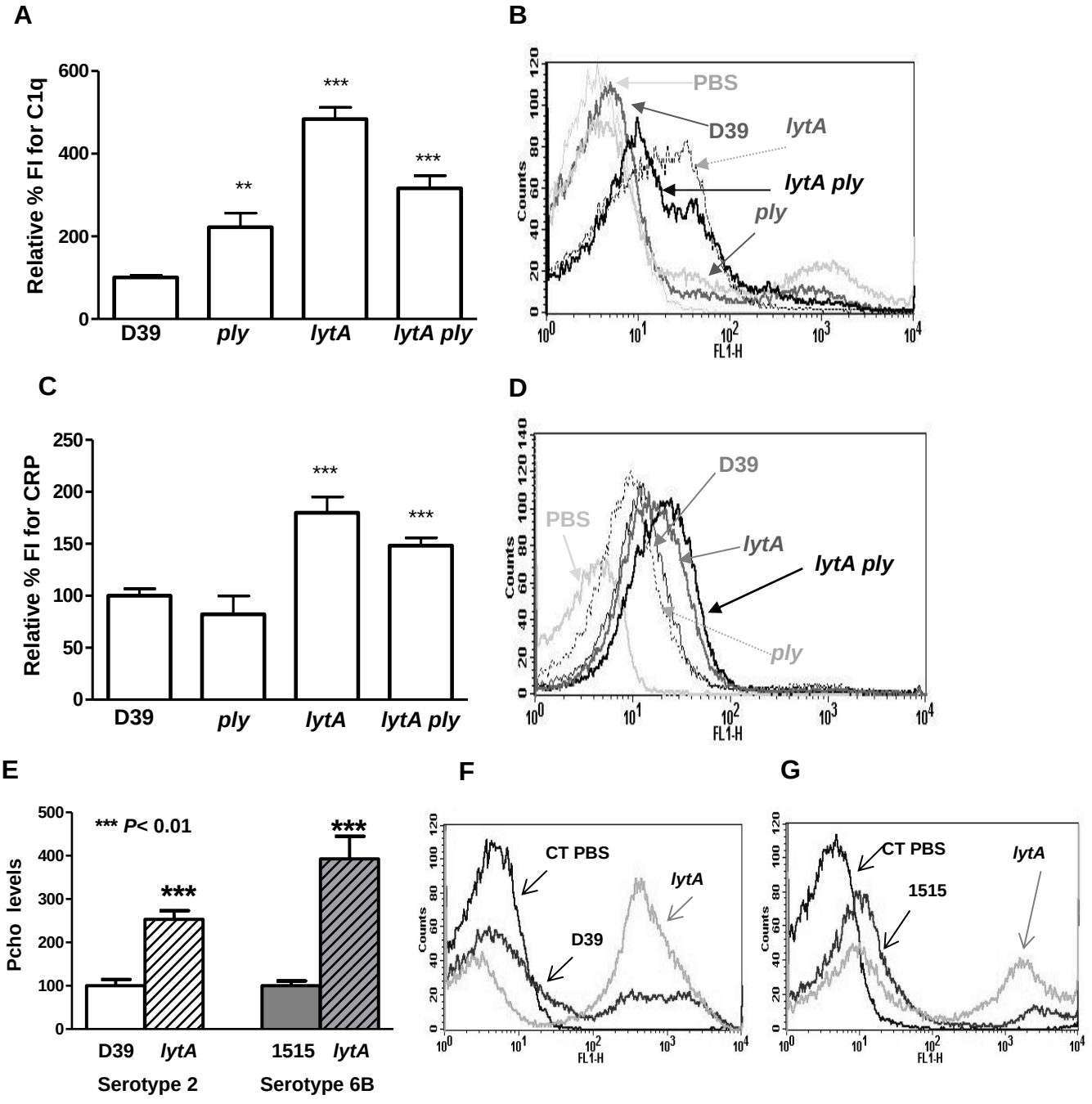
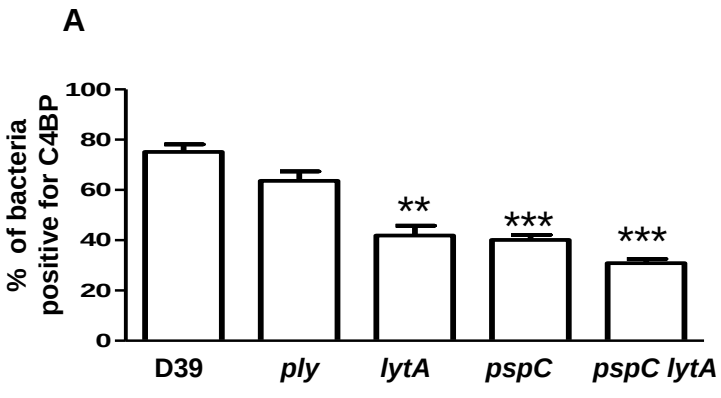
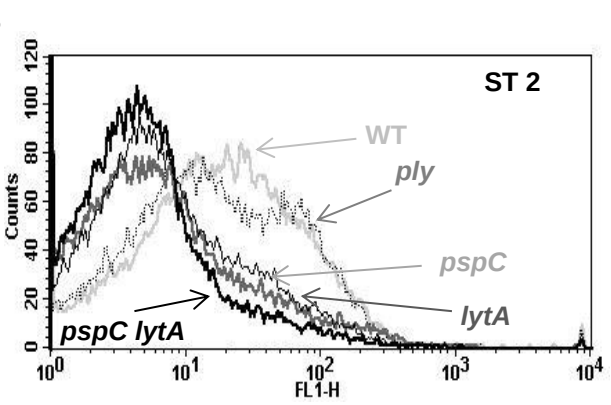
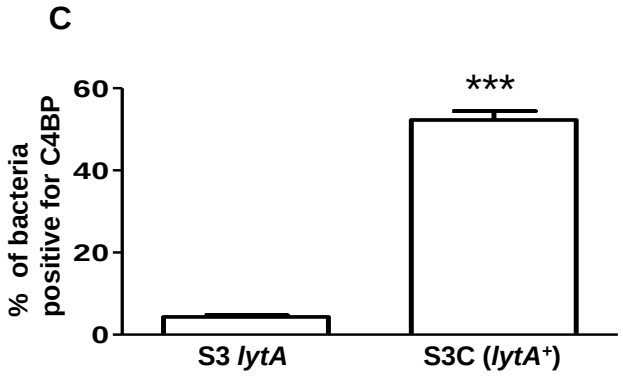
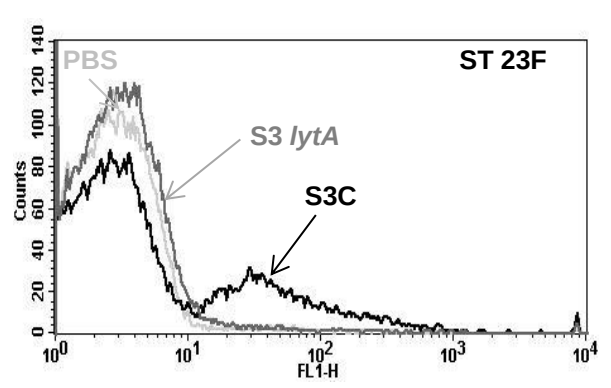

A
Relative % FI for C1q
600
400
200
0
**
***
***
D39
ply
lytA
lytA ply
B
PBS
D39
lytA
lytA ply
ply
Counts
FL1-H
C
Relative % FI for CRP
250
200
150
100
50
0
***
***
D39
ply
lytA
lytA ply
D
D39
PBS
lytA
lytA ply
ply
Counts
FL1-H
E
*** P< 0.01
***
***
Pcho levels
D39
lytA
1515
lytA
Serotype 2
Serotype 6B
F
CT PBS
lytA
D39
Counts
FL1-H
G
CT PBS
lytA
1515
Counts
FL1-H

Figure 3


A
% of bacteria positive for C4BP
100
80
60
40
20
0
**
***
***
D39
ply
lytA
pspC
pspC lytA


B
ST 2
WT
ply
pspC
lytA
pspC lytA
Counts
FL1-H


C
% of bacteria positive for C4BP
60
40
20
0
***
S3 lytA
S3C (lytA+)


D
PBS
ST 23F
S3 lytA
S3C
Counts
FL1-H

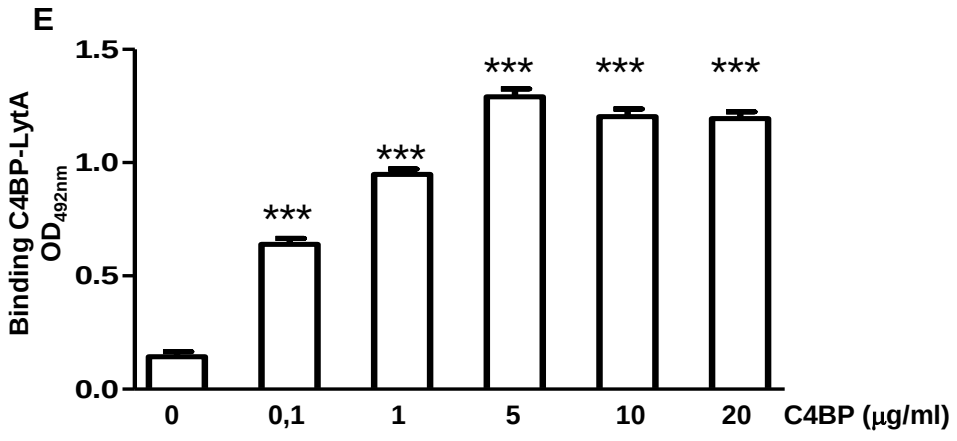

E
Binding C4BP-LytA
OD492nm
1.5
1.0
0.5
0.0
***
***
***
***
***
0
0,1
1
5
10
20
C4BP (μg/ml)

Figure 4

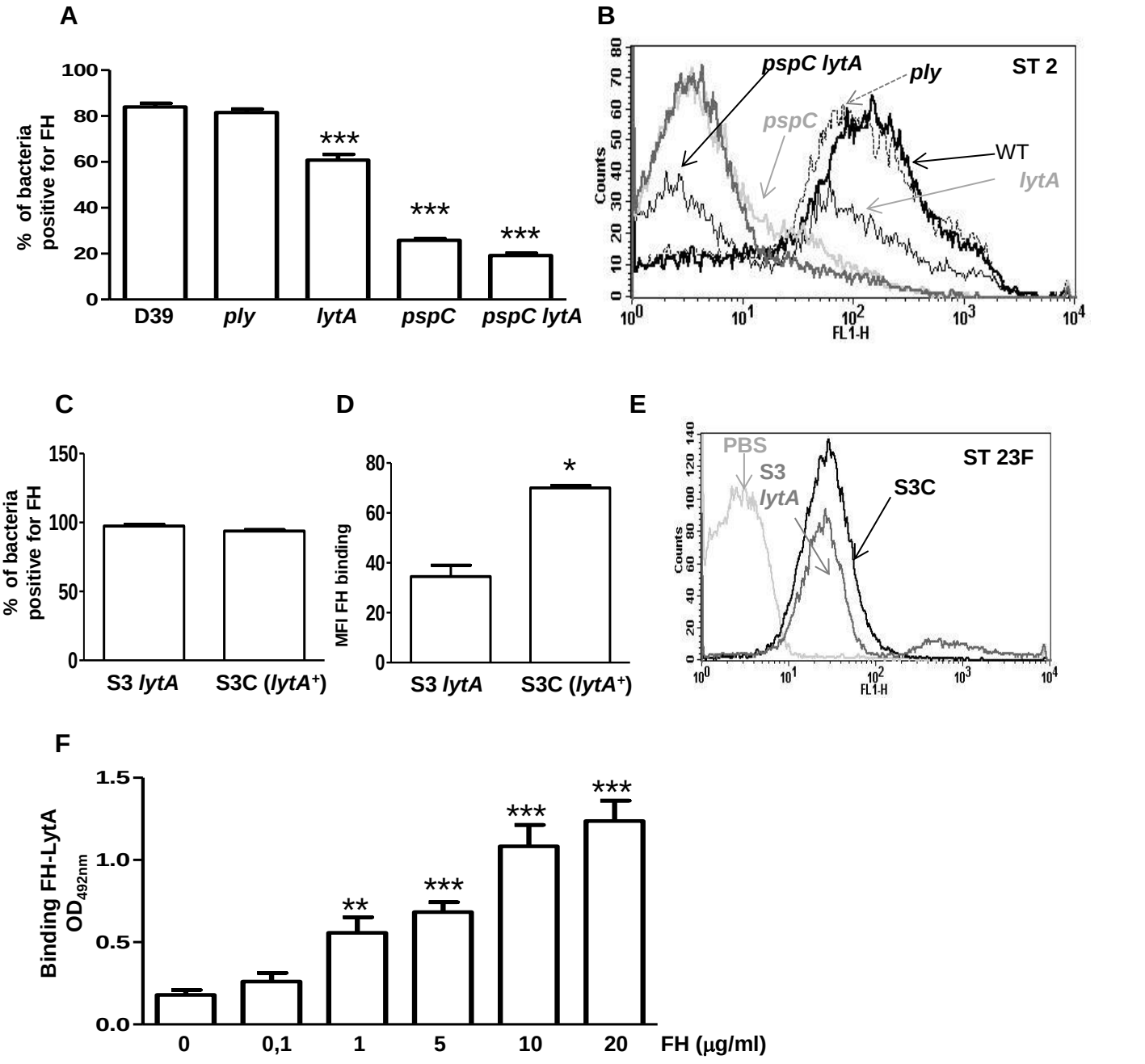
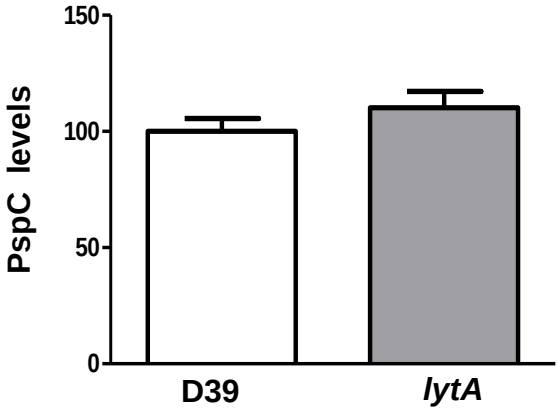

A
% of bacteria positive for FH
100
80
60
40
20
0
***
***
***
D39
ply
lytA
pspC
pspC lytA
B
Counts
pspC lytA
ply
ST 2
pspC
WT
lytA
FL1-H
C
% of bacteria positive for FH
150
100
50
0
S3 lytA
S3C (lytA+)
D
MFI FH binding
80
60
40
20
0
*
S3 lytA
S3C (lytA+)
E
Counts
PBS
S3
lytA
S3C
ST 23F
FL1-H
F
Binding FH-LytA
OD492nm
1.5
1.0
0.5
0.0
**
***
***
***
0
0,1
1
5
10
20
FH (µg/ml)

Figure 5

A


PspC levels
150
100
50
0
D39
lytA

B

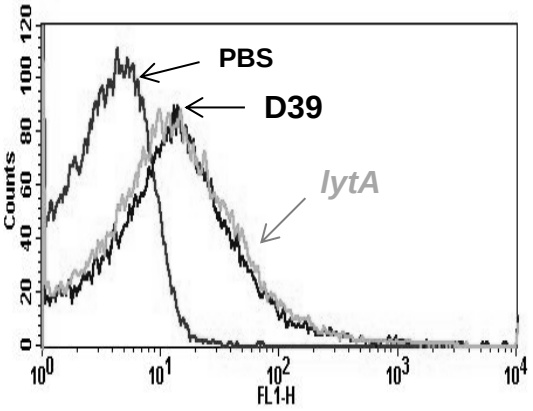

PBS
D39
lytA
Counts
0
20
40
60
80
100
120
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 6

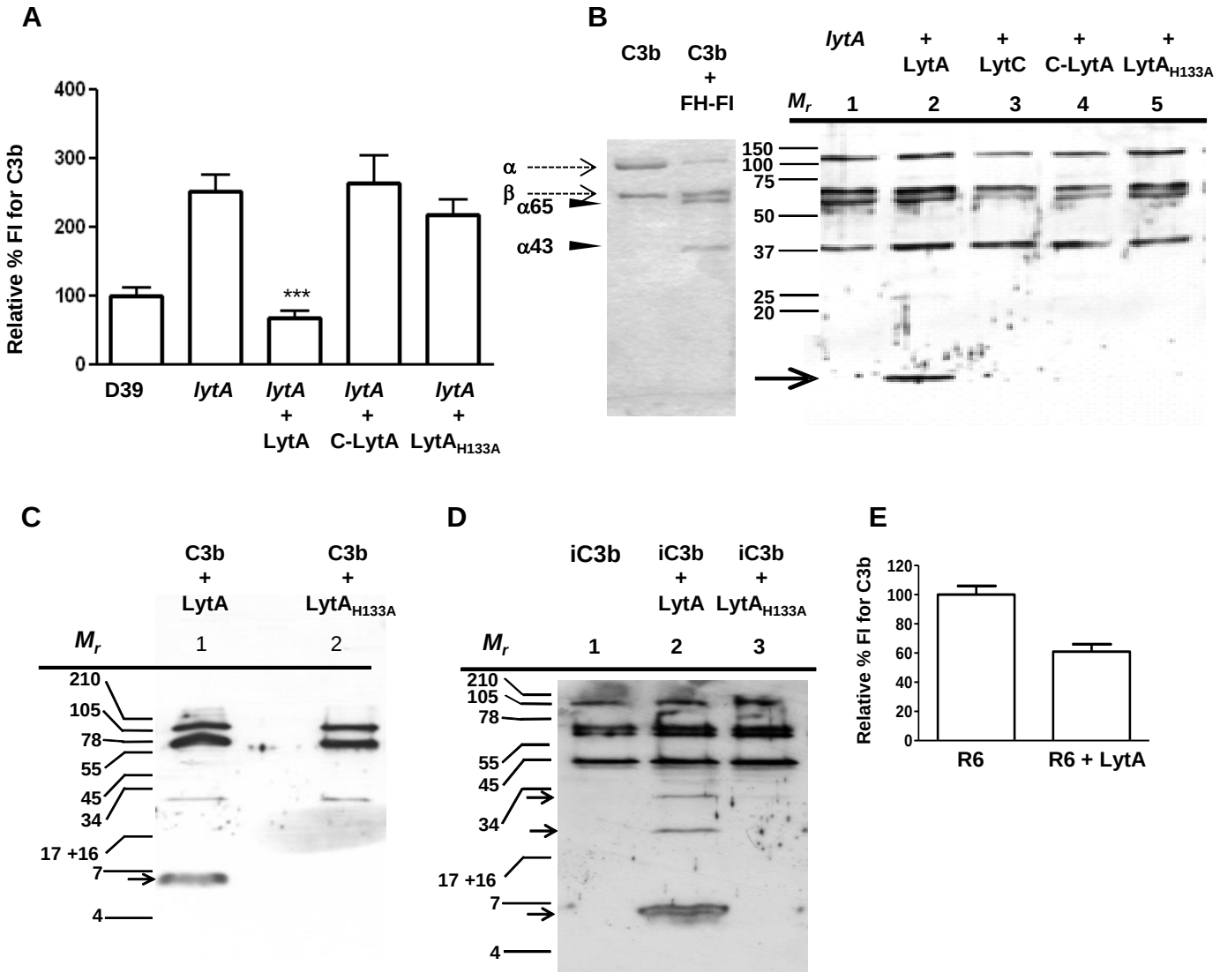

A
Relative % FI for C3b
400
300
200
100
0
***
D39
lytA
lytA + LytA
lytA + C-LytA
lytA + LytA$_{H133A}$
B
C3b
C3b + FH-FI
α
β
α65
α43
lytA
+ LytA
+ LytC
+ C-LytA
+ LytA$_{H133A}$
M_r
1
2
3
4
5
150
100
75
50
37
25
20
C
C3b + LytA
C3b + LytA$_{H133A}$
M_r
1
2
210
105
78
55
45
34
17 +16
7
4
D
iC3b
iC3b + LytA
iC3b + LytA$_{H133A}$
M_r
1
2
3
210
105
78
55
45
34
17 +16
7
4
E
Relative % FI for C3b
120
100
80
60
40
20
0
R6
R6 + LytA

Figure 7

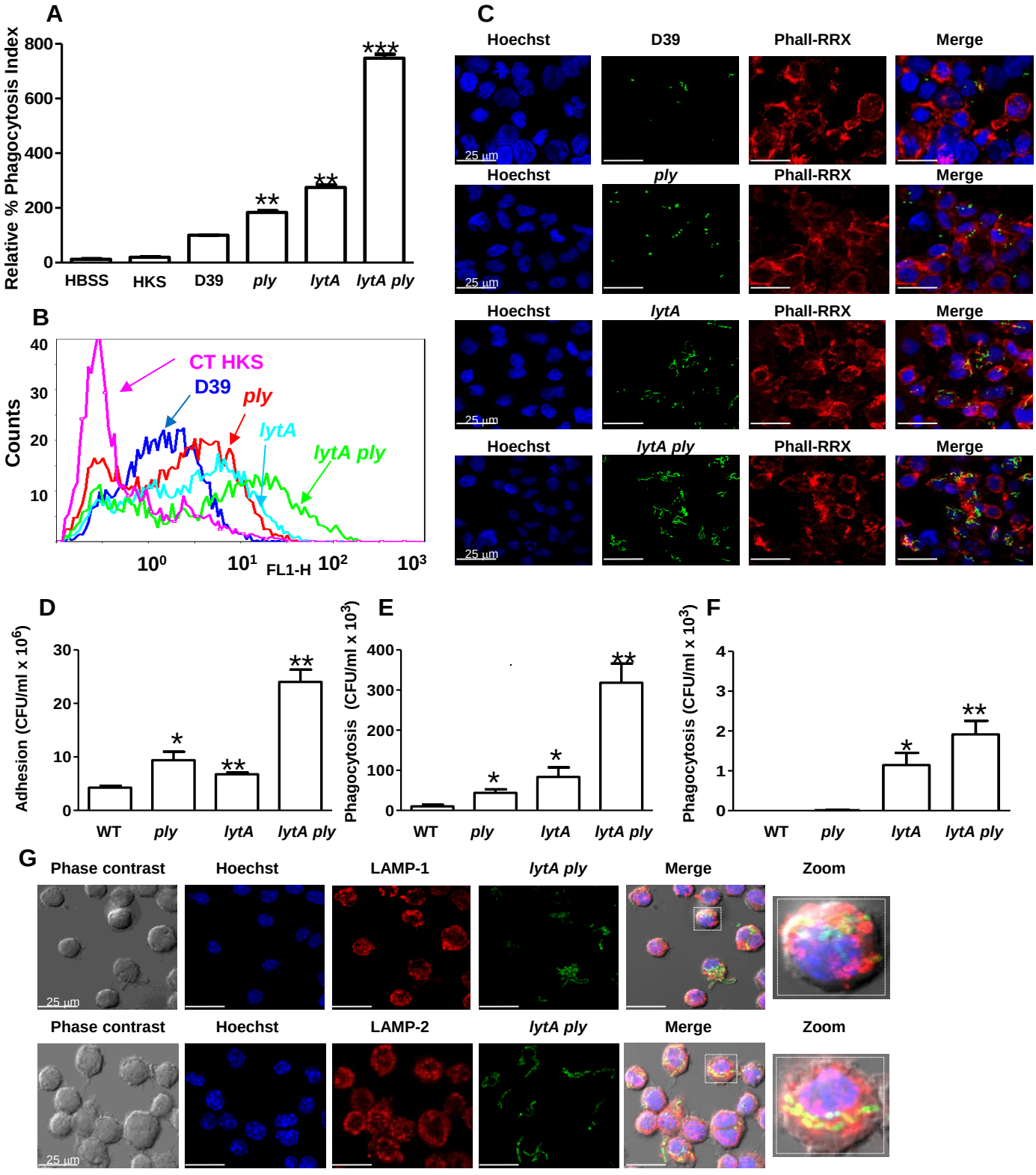
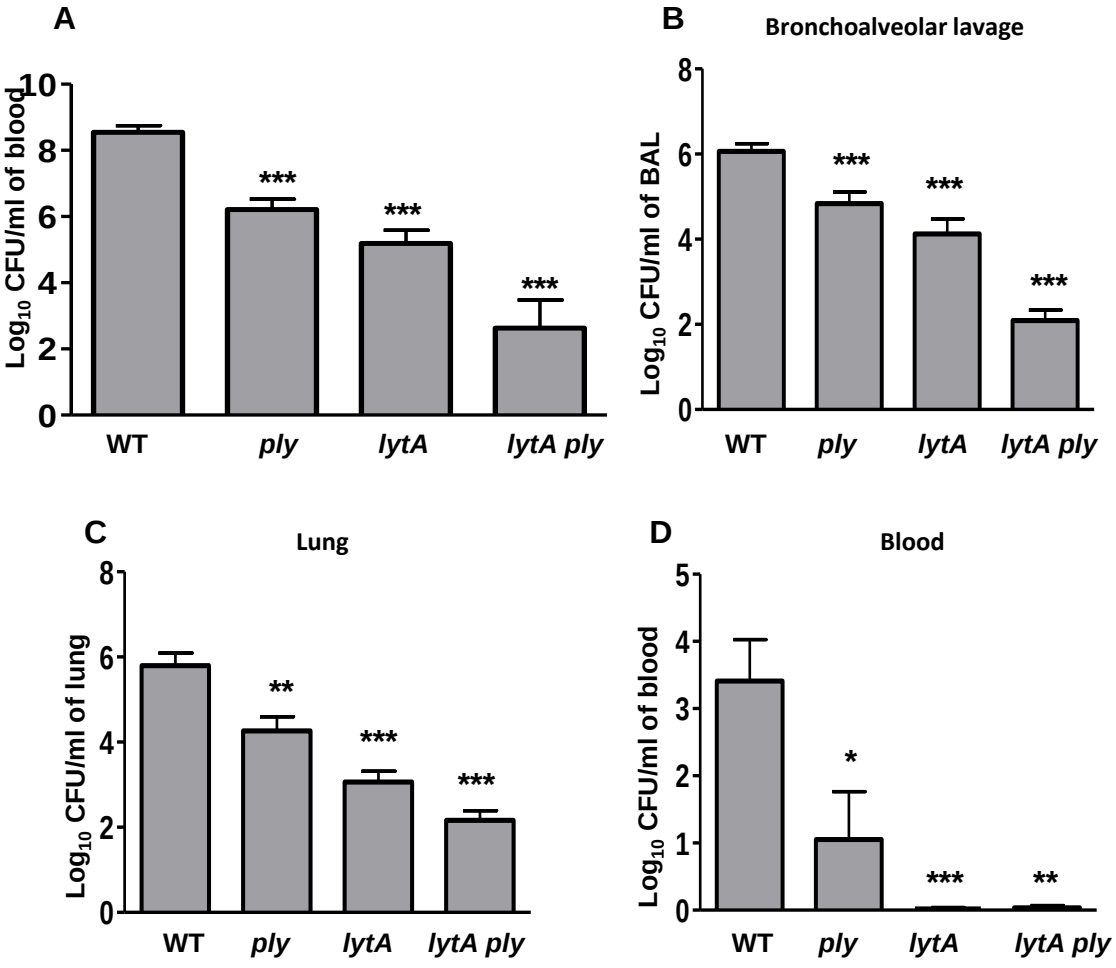

A
Relative % Phagocytosis Index
800
600
400
200
0
HBSS
HKS
D39
ply
lytA
lytA ply
B
Counts
40
30
20
10
CT HKS
D39
ply
lytA
lytA ply
10^0
10^1
FL1-H
10^2
10^3
C
Hoechst
D39
PhalI-RRX
Merge
ply
lytA
lytA ply
25 µm
D
Adhesion (CFU/ml x 10^6)
30
20
10
0
WT
ply
lytA
lytA ply
E
Phagocytosis (CFU/ml x 10^3)
400
300
200
100
0
F
4
3
2
1
0
G
Phase contrast
Hoechst
LAMP-1
LAMP-2
lytA ply
Merge
Zoom
25 µm

Figure 8


A
Log10 CFU/ml of blood
10
8
6
4
2
0
***
***
***
WT
ply
lytA
lytA ply
B
Bronchoalveolar lavage
Log10 CFU/ml of BAL
8
6
4
2
0
***
***
***
WT
ply
lytA
lytA ply
C
Lung
Log10 CFU/ml of lung
8
6
4
2
0
**
***
***
WT
ply
lytA
lytA ply
D
Blood
Log10 CFU/ml of blood
5
4
3
2
1
0
*
***
**
WT
ply
lytA
lytA ply