Immunization with LytB protein of *Streptococcus pneumoniae* activates complement-mediated phagocytosis and induces protection against pneumonia and sepsis

Bruno Corsini[a], Leire Aguinagalde[a], Susana Ruiz[b,c], Mirian Domenech[b,c], Marisa Antequera[a], Asunción Fenoll[a], Pedro García[b, c], Ernesto García[b, c], Jose Yuste[a,c*]

[a] *Centro Nacional de Microbiología, Instituto de Salud Carlos III (ISCIII), 28220 Madrid, Spain*

[b] *Centro de Investigaciones Biológicas (CIB-CSIC), 28040 Madrid, Spain*

[c] *CIBER de Enfermedades Respiratorias (CIBERES), 28029 Madrid, Spain*

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CFU, colony-forming units; CPS, capsular polysaccharide(s); ELISA, enzyme-linked immunosorbent assay(s); FAM-SE, 5, 6-carboxyfluorescein succinimidyl ester; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; IP, intraperitoneal; IPD, invasive pneumococcal disease; ISCIII, Instituto de Salud Carlos III; NMS, normal mouse serum; OD, optical density; OP, opsonophagocytosis; PCV, pneumococcal conjugate vaccine; PPSV, pneumococcal polysaccharide vaccine; RFI, relative percent fluorescence index; SD, standard deviation.

* Corresponding author at: Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra Majadahonda-Pozuelo km 2, 28220 Majadahonda, Madrid, Spain. Tel: +34918223620.

E-mail address: jyuste@isciii.es (J Yuste).

ABSTRACT

The cell wall glucosaminidase LytB of *Streptococcus pneumoniae* is a surface exposed protein involved in daughter cell separation, biofilm formation and contributes to different aspects of the pathogenesis process. In this study we have characterized the antibody responses after immunization of mice with LytB in the presence of alhydrogel as an adjuvant. Enzyme-linked immunosorbent assays measuring different subclasses of immunoglobulin G, demonstrated that the antibody responses to LytB were predominantly IgG1 and IgG2b, followed by IgG3 and IgG2a subclasses. Complement-mediated immunity against two different pneumococcal serotypes was investigated using sera from immunized mice. Immunization with LytB increased the recognition of *S. pneumoniae* by complement components C1q and C3b demonstrating that anti-LytB antibodies trigger activation of the classical pathway. Phagocytosis assays showed that serum containing antibodies to LytB stimulates neutrophil-mediated phagocytosis against *S. pneumoniae*. Animal models of infection including invasive pneumonia and sepsis were performed with two different clinical isolates. Vaccination with LytB increased bacterial clearance and induced protection demonstrating that LytB might be a good candidate to be considered in a future protein-based vaccine against *S. pneumoniae*.

Keywords: Streptococcus pneumoniae, cell wall hydrolase, LytB, vaccine protein, phagocytosis, complement immunity

1. Introduction

Streptococcus pneumoniae is one of the leading etiologic agents of community-acquired pneumonia, sepsis and bacterial meningitis particularly affecting children and elderly adults [1, 2]. Prevention of invasive pneumococcal disease (IPD) is one of the leading priorities in public health due to the high morbidity and mortality rates worldwide, especially in children in developing countries [3]. Current prophylactic measures against pneumococcal infection are based on polysaccharide-containing vaccines that may be conjugated to a carrier protein in order to elicit protection in children [4]. However, one of the major disadvantages of these polysaccharide vaccines is the extensive variability among the pneumococcal population with up to 96 different capsular polysaccharides (CPS) described to date [5]. An additional limitation is that capsular switching appeared after the introduction of pneumococcal conjugate vaccines (PCVs), which results in a serious concern because these strains may emerge by avoiding vaccine-induced immunity due to acquisition of capsular genes from non-vaccine serotypes [6-8]. To circumvent these problems, efforts are being made to investigate *S. pneumoniae* protein-based candidate vaccines that may protect against different serotypes. In this study we investigated the cell wall hydrolase LytB of *S. pneumoniae* as a potential vaccine candidate. LytB is located at sites close to the polar ends of the cell and is surface exposed. This enzyme has *N*-acetylglucosaminidase activity and plays an essential role in daughter cell separation [9, 10].

In terms of pathogenesis, LytB participates in biofilm formation, attachment to human epithelial cells and contributes to sepsis and pneumonia by increasing the ability of LytC to avoid complement-mediated immunity and phagocytosis [11-13]. From the prophylactic perspective, LytB might be a promising target for the development of a universal pneumococcal vaccine because an anti-LytB antiserum significantly protected

mice from a lethal challenge with different pneumococcal strains [14]. In this study we show that immunization with LytB is immunogenic and enhances complement-mediated immunity and phagocytosis of different serotypes of *S. pneumoniae*. As a consequence, vaccination using LytB increases bacterial clearance and induces protection against pneumococcal sepsis and invasive pneumonia.

2. Materials and methods

2.1. Bacterial strains

S. pneumoniae clinical isolates used in this study included strain 957 (serotype 3; amoxicillin MIC = 0.015 μg ml^{-1}; erythromycin MIC = 0.25 μg ml^{-1}, levofloxacin MIC = 1 μg ml^{-1}, tetracycline MIC = 0.5 μg ml^{-1}, chloramphenicol MIC = 4 μg ml^{-1}) and strain 48 (serotype 23F; amoxicillin MIC = 16 μg ml^{-1}; erythromycin MIC > 128 μg ml^{-1}, levofloxacin MIC = 1 μg ml^{-1}, tetracycline MIC = 64 μg ml^{-1}, chloramphenicol MIC = 4 μg ml^{-1}). Pneumococcal isolates were cultured on blood agar plates at 37 °C in 5% CO_2 or in Todd-Hewitt broth supplemented with 0.5% yeast extract to an optical density at 580 nm (OD_{580}) of 0.4 (approximately 10^8 colony-forming units (CFU) ml^{-1}) and stored at −70 °C in 10% glycerol as single-use aliquots.

2.2. Vaccination experiments in mice

BALB/c mice were bred by the Instituto de Salud Carlos III (ISCIII) animal facility. All mice used were 8–16 weeks old and, within each experiment, groups of mice were matched for age and sex. Animal experiments were performed at ISCIII in accordance with Spanish legislation (RD 1201/2005) and EU regulations (218/63/EU). The animal

97 experiments performed in this work were approved by the Animal Care and Use
98 Committee of ISCIII (CBA PA 52_2011-v2 and PROEX 218/15).
99 The pneumococcal LytB protein used for immunization studies was purified as
100 previously described [9, 10] and was prepared for animal inoculations at 20–40 μg in
101 Alum (Alhydrogel; aluminum hydroxide, InvivoGen) as the adjuvant in a 1:1
102 proportion. Groups of 5 mice were immunized by intraperitoneal (IP) inoculation of 200
103 μl of Alum alone or 200 μl of LytB protein preparation in Alum adjuvant on days 0, 7,
104 and 14 as previously described [15]. Animals were euthanized on day 21 and blood was
105 collected from cardiac puncture and conserved as pooled for further *in vitro* assays. For
106 protection experiments against sepsis, groups of 10 mice were immunized as previously
107 described, followed by IP challenge on day 21 with 10^3 CFU/ per mouse of serotype 3
108 strain or 10^7 CFU per mouse of serotype 23F strain representing at least the lethal dose
109 50 (LD_{50}) of each strain for sepsis infection. The immunization schedule for protection
110 against pneumonia was the same as before although the bacterial challenge was
111 performed by the intranasal route with 50 μl of 10^4 CFU/mouse of serotype 3 strain or 5
112 $\times 10^7$ CFU/mouse of serotype 23F strain representing at least the LD_{50} of each strain for
113 pneumonia. Bacterial counts were determined during the first 24–48 h from blood
114 samples (6 μl per mouse) obtained from the tail vein of infected animals as previously
115 described [16]. The development of disease was monitored daily, and mice were
116 sacrificed when they exhibited severe signs of disease.

117 *2.3. Enzyme linked immunosorbent assays to detect Ig subclasses*

118 Specific antibody titers in pooled sera from five mice of each group were measured
119 by enzyme-linked immunosorbent assays (ELISA) using 96-well polystyrene Maxisorp
120 plates (Nunc) coated with 0.5 μg of purified LytB protein for 2 h at 37 °C and blocked

with a PBS–2% bovine serum albumin (BSA) solution as previously described [17]. Bound antibodies were detected by using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 and IgA; Santa Cruz) for 30 min and developed using *o*-phenylenediamine (Sigma-Aldrich) before determining the OD_{492} using a microtiter plate reader (Anthos 2020).

2.4. Activation of complement immunity

Complement activation was assessed using flow cytometry assays, as described before [17-19]. Briefly, C3b deposition was analyzed by incubating 5×10^6 CFU of *S. pneumoniae* in 10 µl of serum (diluted to 50% in PBS) for 30 min at 37°C using pooled sera from mice immunized with Alum alone or immunized with LytB−Alum. Bacteria were then incubated for 30 min on ice with fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-mouse C3b antibody (ICN-Cappel) diluted 1/300 in PBS. Then, bacteria were fixed in 3% paraformaldehyde and analyzed on a FACS Calibur flow cytometer (BD Biosciences) using forward and side scatter parameters to gate on at least 25,000 cells. This assay was adapted for C1q binding incubating for 1 h with rabbit anti-mouse C1q antibody (Abcam) followed by an additional incubation with FITC-conjugated polyclonal goat anti-rabbit IgG. The results were expressed as a relative percent fluorescence index (RFI) 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 [19, 20].

2.4. Opsonophagocytosis

Phagocytosis was evaluated using a flow cytometry assay including *S. pneumoniae* strains described above labeled with 5, 6-carboxyfluorescein succinimidyl ester (FAM-

144 SE; Molecular Probes) and human HL-60 cells (CCL-240; ATCC) differentiated to
145 granulocytes. The general conditions of the assay were based on those described
146 previously except that clinical isolates of serotype 3 and 23F were incubated with
147 pooled sera from mice immunized with Alum alone or immunized with the mixture
148 LytB−Alum [16, 21]. Infection assays were performed with a ratio of 10 bacteria per
149 cell. A minimum of 6,000 cells were analyzed using a FACS Calibur flow cytometer.
150 Results were expressed as a RFI defined as the proportion of positive cells for
151 fluorescent bacteria multiplied by the geometric mean of fluorescence intensity, which
152 correlates with the amount of bacteria phagocytosed per cell.
153

154 *2.5. T cell response*

155 Experiments investigating cellular populations of lymphocytes were performed in
156 non-immunized mice and in mice immunized with Alum alone or vaccinated with LytB
157 mixed with Alum as explained above. Briefly, single-cell suspensions of the spleens of
158 each group, were prepared in staining buffer (2% fetal calf serum in PBS). Detection of
159 CD3, CD4 and CD8 was performed using standard protocols with the following
160 antibodies diluted 1/300 in staining buffer (Armenian hamster anti-mouse CD3-
161 phycoerythrin (PE), Tonbo; rat anti-mouse CD4-FITC, Biolegend; rat anti-mouse CD8-
162 PE, Tonbo). Cells were analyzed on a FACSCalibur flow cytometer.

163 *2.6. Gene* lytB *sequence accession numbers*

164 The LytB nucleotide sequences of clinical isolates 48 (serotype 23F) and 957
165 (serotype 3) have been deposited in the GenBank databases. They have been assigned
166 accession numbers KX151143 and KX151145, respectively. The multiple alignment of

the LytB glucosaminidases from these clinical isolates in comparison to the LytB of D39 (serotype 2) and TIGR4 (serotype 4) strains is shown in Supplemental Fig. 1.

2.7. Statistical analysis

Data are representative of results obtained from at least three independent experiments, and each data 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 was chosen for multiple comparisons. Survival was analyzed by the log-rank test. GraphPad InStat version 6.0 (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 $P < 0.001$ (***).

3. Results

3.1. Antibody response to LytB

The predominant IgG subclass responses after vaccination with LytB were assessed using ELISA specific for murine sera including total IgG, IgG1, IgG2a, IgG2b and IgG3. Antibody levels were investigated in normal mouse serum (NMS) from naïve (non-immunized) mice or sera from mice immunized with Alum alone or with LytB mixed with Alum. Sera from mice immunized with LytB elicited strong IgG levels compared to Alum alone and NMS groups (Fig. 1A). Vaccination with LytB induced consistent levels of IgGs predominantly of subclasses IgG1 and IgG2b followed by IgG3 and IgG2a (Fig. 1). Overall, these results indicated that pneumococcal LytB is an immunogenic protein that induces IgG antibodies of different subclasses that might be

functional in terms of host-immune response. Immunization with LytB by the IP route induced a certain level of IgA in blood compared to immunization with Alum alone and NMS (Fig. 1F). However, the importance of this immunoglobulin in host defense against pneumococcus, may be more relevant for mucosal immunity rather than systemic immunity.

3.2. Complement activation mediated by antibodies to LytB

The complement system is an important component of the host immune response against invading pathogens such as *S. pneumoniae*. Complement immunity is strongly initiated by IgG antibodies leading to the activation of the classical pathway which is the dominant cascade for complement activation against *S. pneumoniae* [18, 22]. Recognition by C1q, the first component of the classical pathway, was investigated using pneumococcal strains of two different serotypes and pooled sera from mice immunized with Alum alone or with LytB−Alum (Fig. 2). Incubation of pneumococcal clinical isolates with sera containing specific antibodies to LytB increased the recognition by C1q indicating that immunization with LytB triggers activation of the classical pathway against *S. pneumoniae* (Fig. 2). In addition, C3b deposition on the pneumococcal surface was significantly enhanced in the presence of anti-LytB antibodies (Fig. 3). Taken together, these results demonstrates that vaccination with LytB induces complement-mediated responses against *S. pneumoniae* (Fig. 2 and 3).

3.3. Immunization with LytB induces opsonophagocytosis of S. pneumoniae

Antibodies raised against LytB activate the opsonization process and may contribute to host defense by improving opsonophagocytosis (OP). We investigated this possibility by using a flow cytometry assay measuring the uptake of pneumococcal clinical isolates

mediated by HL-60 cells differentiated to granulocytes, which is a cell line widely used for measuring protection by current PCVs [21]. For these assays, FAM-SE-labeled *S. pneumoniae* strains were incubated with sera from mice immunized only with Alum or with LytB mixed with Alum. As negative controls, we included bacteria incubated with Hank's balanced salt solution (HBSS) to evaluate the phagocytosis level without serum components. In the absence of complement (HBSS control), phagocytosis was reduced compared to Alum group and markedly impaired compared to LytB immune sera, which confirmed the importance of complement components in phagocytosis mediated by antibodies to LytB (Fig. 4). The level of phagocytosis was significantly higher when antibodies to LytB were present in comparison to Alum group, confirming that immunization with LytB stimulates phagocytosis of *S. pneumoniae*.

3.4. Antibodies against LytB increases bacterial chain length

The impact of immunization with LytB on the morphology of *S. pneumoniae* was investigated. For this assay, the clinical isolate of serotype 23F was incubated for 4 h at 37°C with different sera including NMS, sera from mice immunized with Alum or sera from mice vaccinated with Alum in the presence of 20 μg of LytB, as previously described. Incubation of *S. pneumoniae* with NMS (diluted 1/200) or sera from mice immunized with Alum (diluted 1/200) showed the typical diplococcal morphology (Fig. 5A and B). However, incubation with sera containing specific antibodies to LytB (diluted 1/200) increased chain formation with pneumococcal cells displaying short and long chains compatible with an altered cell separation pattern (Fig. 5C). This phenotype of altered morphology mediated by LytB antibodies was also observed with a dilution of serum of 1/2000 (data not shown).

3.5. Vaccination with LytB did not modify the T cell response.

Activation of the cellular response after immunization with antigenic proteins might be protective against pneumococcal infection. For this purpose, groups of mice were immunized with Alum or with LytB mixed with Alum as described above, and after three immunizations, mice were culled and the spleen was obtained for detection of CD3, CD4 and CD8 lymphocytes. These results confirmed that vaccination with LytB did not affect the proportion of different T cell populations in comparison to immunization with Alum or with non-immunized mice (Fig. 6).

3.6. Immunization with LytB protects mice against S. pneumoniae *infection*

IPD is a devastating infectious process associated to high mortality rates that mainly affects the elderly and children. The protective activity of LytB against IPD was investigated in a sepsis model of infection caused by clinical isolates of *S. pneumoniae* belonging to serotypes 3 and 23F (Fig. 7). Vaccination with 20 μg of LytB decreased bacterial counts in blood at 48 h in comparison to Alum group and increased survival rates against sepsis caused by the serotype 3 strain (Fig. 7A and B). Mice immunized with 40 μg of LytB during three weeks (three doses) and infected with the serotype 3 strain, showed increased protection against sepsis caused by this clinical isolate with protective rates of 70% in the LytB-vaccinated group *vs* 10% in the Alum group ($P < 0.01$) (Fig. 7C). In addition, a significant reduction in the bacterial load at 24 and 48 h was observed in the blood of mice immunized with LytB (Fig. 7D). Protection against sepsis mediated by LytB was also tested using a serotype 23F strain. Vaccination with 20 μg of LytB increased survival and decreased bacterial counts in blood (Fig. 7E and F) confirming that LytB might be a promising vaccine candidate against sepsis caused by *S. pneumoniae*.

Pneumonia is a leading cause of mortality worldwide killing more children than AIDS, measles and malaria combined [3, 23]. Hence, protection against pneumococcal pneumonia induced by immunization with LytB was analyzed. Mice were vaccinated with 20 μg of LytB during three weeks (three doses) and infected by the intranasal route with a lethal dose of *S. pneumoniae* of serotypes 3 or 23F (Fig. 8). Increased survival rates were observed against pneumonia produced by serotype 3 strain ranging from 40% in the vaccinated group with LytB *vs* no protection in the Alum group (Fig. 8A). The higher survival conferred by vaccination with LytB was associated with a significant reduction in the bacterial levels in the blood in comparison to mice immunized only with Alum (Fig. 8B). These experiments were repeated using a serotype 23F strain confirming that immunization with LytB significantly increased the survival (P <0.01) showing a 70% protection in the group vaccinated with LytB *vs* 10% in the Alum group (Fig. 8C). Again, vaccination with LytB increased the bacterial clearance of *S. pneumoniae* from the systemic circulation (Fig. 8D) strongly suggesting that LytB might be a promising vaccine candidate against pneumococcal pneumonia and invasive disease.

4. Discussion

Several pneumococcal proteins are currently under investigation as alternative vaccine candidates that may overcome the limitations of vaccines based on CPS [24]. In this study we have characterized the immunological responses and the protective effect of the cell wall hydrolase LytB as a promising pneumococcal protein vaccine. LytB belongs to the choline-binding family of proteins in which several members including PspA, PspC, PcpA or LytA, have been proposed as vaccine candidates [25-28]. LytB, despite showing certain polymorphism in the number of choline-binding repeats, is a

285 well-conserved protein among *S. pneumoniae* clinical isolates [29]. Sequence analysis
286 of the LytB glucosaminidase from the two clinical isolates of this study were compared
287 to $LytB_{D39}$ and $LytB_{TIGR4}$ (Supplemental Fig. 1). Our results show that the LytB of the
288 clinical isolate of serotype 3 lacks two choline-binding repeats compared to the LytB of
289 strains 48 (serotype 23F) and D39 (serotype 2). However, the LytB of strains 48 and
290 D39 are very similar. Immunization was performed using alhydrogel as adjuvant or with
291 LytB mixed with alhydrogel since this adjuvant has been approved for human use in
292 studies evaluating pneumococcal proteins [30, 31]. Vaccination with LytB induced a
293 strong IgG response of different subclasses including IgG1, IgG2a, IgG2b, and IgG3.
294 This is important because some of these subtypes of IgGs are elicited by current human
295 vaccine formulations such as the 23-valent pneumococcal polysaccharide vaccine
296 (PPSV) and PCVs. In this regard, the antibody response conferred by PPSV is
297 predominantly of the IgG2 subclass [32, 33]. The magnitude of the serotype-specific
298 IgG response to PCVs varies by serotype, vaccine formulation and age [34]. In adults,
299 PCVs induce primarily an IgG2 response whereas in children the predominant response
300 is of the IgG1 subclass [33, 35, 36]. The specific IgG subclass generated is relevant
301 from the functional perspective as IgG1 and IgG3 antibodies fix complement and bind
302 Fcγ receptors more efficiently than IgG2 [37-39]. Immunization with LytB induced high
303 levels of these immunoglobulins suggesting that antibodies to this protein might activate
304 complement-mediated phagocytosis (22). In this sense, differences in the expression of
305 Ig isotypes between human and mice have been previously reported and direct
306 correlation between subtypes within classes in these species are difficult to make
307 because they are not directly equivalent [40]. However, despite the difficulty in
308 comparing the findings derived from studies of murine Ig responses to humans, these
309 studies can be useful in the characterization of novel protein antigens for vaccine

development by increasing the understanding of protective immune mechanisms against pneumococcal infection.

Experiments exploring complement activation on the surface of two different clinical isolates demonstrated that antibodies to LytB increased the recognition by C1q and C3b demonstrating that vaccination with LytB triggers classical pathway activation. This is critical in terms of pneumococcal infection as this pathway has been shown to be vital for complement activation to *S. pneumoniae* in humans and mice [18, 22]. Host defense against pneumococcus depends on opsonophagocytosis [1, 41] with neutrophils the important players in the immune response, as neutropenia is highly associated to increased susceptibility to pneumococcal infection [42, 43]. In this study, using a cell line differentiated into neutrophils, we show that immunization with LytB increased complement-mediated phagocytosis confirming that antibodies to LytB activate the phagocytosis process against *S. pneumoniae*. Generation of an IgG3 response by LytB immunization may be relevant against systemic infection as this immunoglobulin is highly protective against a fatal pneumococcal infection [44]. An additional explanation for the increased complement-dependent phagocytosis mediated by antibodies to LytB is due to morphological changes they induce in bacteria. This is important because LytB is essential for cellular separation at the end of cell division as pneumococcal strains lacking LytB form long chains instead of typical diplococci [9, 10]. This is important because chain formation in *S. pneumoniae* via antibody-mediated agglutination has been shown to be relevant to enhance complement-mediated recognition and phagocytosis [45]. Our results show that antibodies to LytB increased bacterial size inducing chaining, which is compatible with enhanced susceptibility to complement-mediated phagocytosis and impaired virulence [45, 46].

The protective role of LytB was explored in murine models of sepsis and pneumonia. Vaccination with LytB protected against pneumococcal sepsis and invasive pneumonia caused by clinical isolates of serotypes 3 or 23F. Hence, the presence of antibodies to LytB controlled bacterial replication in the bloodstream reducing the severity of the infection process in both serotypes. This is relevant from the prophylactic perspective of serotype 3 strains as IPD cases due to this serotype have not decreased after the introduction of the current PCVs [47]. Furthermore, lack of clinical efficacy against serotype 3 isolates following PCV administration has been associated to impaired immune response and the abundance of CPS produced by these strains [48]. In addition, the relative failure of protection induced by current PCV-13 against serotype 3 isolates has been recently linked to the release of type 3 CPS by these isolates interfering with antibody-mediated killing and protection by anti-CPS antibodies [49]. In this sense, our study shows that immunization with LytB increased complement-mediated phagocytosis and bacterial clearance in the systemic circulation, protecting against pneumococcal sepsis and pneumonia caused by a serotype 3 strain. The results of this study suggest that LytB is a promising vaccine candidate to be considered perhaps in a combined cocktail with other antigens, as combinations of pneumococcal proteins can provide additive or synergistic effects that may be beneficial [24, 50, 51]. Alternatively, the use of LytB as a carrier protein for type 3 CPS might be a prophylactic strategy to increase the immunogenicity against this serotype as combinations of pneumococcal proteins conjugated to CPS included in PCVs are being evaluated [52]. Overall, our study confirms the potential benefit of LytB as a protein vaccine antigen against pneumococcal pneumonia and invasive disease.

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Conflict of interest

The authors declare no competing financial interests.

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Figure Legends

Fig. 1. Antibody levels after immunization with Alum or LytB. ELISA was used to analyze the antibody levels including total IgG (A), IgG1 (B), IgG2a (C), IgG2b (D), IgG3 (E) and IgA (F). Specific antibodies were measured in normal mouse serum (gray circles), pooled sera from mice immunized with Alum as adjuvant (gray squares) and pooled sera from mice immunized with 20 μg of LytB mixed with Alum (black triangles). Error bars represent the SDs and asterisks indicate statistical significance of LytB immunization compared to the Alum group. * $P< 0.05$; **, $P<0.01$; ***, $P<0.001$.

Fig. 2. Classical pathway activation mediated by antibodies against LytB. (A) Deposition of mouse C1q on the surface of a serotype 3 clinical isolate using pooled sera from mice immunized with Alum (gray bar) or with LytB mixed with Alum (black bar). (B) Example of a flow cytometry histogram for C1q deposition on a serotype 3 strain. (C) Recognition of a clinical isolate of serotype 23F by mouse C1q using pooled sera from mice immunized with Alum (gray bar) or with LytB mixed with Alum (black bar). (D) Example of a flow cytometry histogram for C1q deposition on a serotype 23F strain. Results are expressed as a relative % fluorescence index (RFI). Error bars represent the SDs and asterisks indicate statistical significance of LytB immunization compared to the Alum group. * $P< 0.05$; **, $P<0.01$; ***, $P<0.001$.

Fig. 3. Recognition of *S. pneumoniae* by C3b using pooled sera from mice immunized with Alum (grey bars) or pooled sera from mice immunized with LytB mixed with Alum (black bars). (A) Deposition of mouse C3b on the surface of a serotype 3 clinical isolate. (B) Example of a flow cytometry histogram for C3b deposition on serotype 3

559 strain. (C) Recognition of a clinical isolate of serotype 23F by mouse C3b. (D) Example
560 of a flow cytometry histogram for C3b deposition on serotype 23F strain. Results are
561 expressed as a relative % fluorescence index. Error bars represent the SDs and asterisks
562 indicate statistical significance of LytB immunization compared to the Alum group. *
563 $P< 0.05$; **, $P<0.01$; ***, $P<0.001$.
564
565 **Fig. 4.** Opsonophagocytosis (OP) assays using the HL-60 neutrophil cell line and *S.*
566 *pneumoniae* strains incubated with HBSS (open bars), or pooled sera from mice
567 immunized with Alum (gray bars) or with LytB mixed with Alum (black bars). (A) OP
568 of a serotype 3 clinical isolate. (B) Example of a flow cytometry histogram for OP of
569 serotype 3 strain. (C) OP of serotype 23F clinical isolate. (D) Example of a flow
570 cytometry histogram for OP of serotype 23F strain. Error bars represent the SDs and
571 asterisks indicate statistical significance of LytB immunization compared to the Alum
572 group. * $P< 0.05$; **, $P<0.01$; ***, $P<0.001$.
573
574 **Fig. 5.** Phase-contrast microscopy images of the pneumococcal clinical isolate of
575 serotype 23F exposed for 4 h at 37°C to different murine sera. (A) Normal mouse serum
576 from naïve mice. (B) Pooled sera from mice immunized with Alum. (C) Pooled sera
577 from mice immunized with 10 μg of LytB mixed with Alum. Bars, 25 μm.
578
579 **Fig. 6.** T cell response represented as proportion of CD3, CD4 and CD8 in non-
580 immunized mice (white bars), mice immunized with Alum (gray bars) or mice
581 immunized with LytB mixed with Alum (black bars). Error bars represent the SDs.
582 Experiments were repeated three times.

583 **Fig. 7.** Protection against pneumococcal sepsis after vaccination with Alum (black dots)
584 or with LytB mixed with Alum (black triangle). (A) Survival against sepsis caused by
585 the clinical isolate 957 of serotype 3 in mice immunized with Alum or with 20 μg of
586 LytB. (B) Bacterial counts in blood at 24 h and 48 h from mice immunized with Alum
587 or 20 μg of LytB and infected with strain 957 of serotype 3. (C) Survival after sepsis
588 caused by clinical isolate 957 of serotype 3 in mice immunized with Alum or LytB (40
589 μg). For differences in survival between Alum group and LytB group (**P <0.01, Log-
590 rank test) (D) Bacterial counts in blood at 24 h and 48 h from mice immunized with
591 Alum or LytB (40 μg) and infected with strain 957 of serotype 3. (E) Survival after
592 sepsis caused by clinical isolate 48 of serotype 23F in mice immunized with Alum or
593 with LytB (20 μg). (F) Bacterial counts in blood at 24 h and 48 h from mice immunized
594 with Alum or LytB (20 μg) and infected with strain 48 of serotype 23F. Error bars
595 represent the SDs and asterisks indicate statistical significance of LytB immunized
596 group compared to the Alum group. * P< 0.05; **, P<0.01; ***, P<0.001.
597
598 **Fig. 8.** Protection against pneumococcal pneumonia after vaccination with Alum (black
599 dots) or with LytB (20 μg) mixed with Alum (black triangle). (A) Survival after
600 pneumonia caused by a clinical isolate of serotype 3. (B) Bacterial counts in blood at 24
601 h and 48 h from mice infected with serotype 3 strain. (C) Survival after pneumonia
602 caused by a clinical isolate of serotype 23F. (D) Bacterial counts in blood at 24 h and 48
603 h from mice infected with serotype 23F strain. Error bars represent the SDs and
604 asterisks indicate statistical significance of LytB immunized group compared to the
605 Alum group. * P< 0.05; **, P<0.01; ***, P<0.001.
606

Figure 1

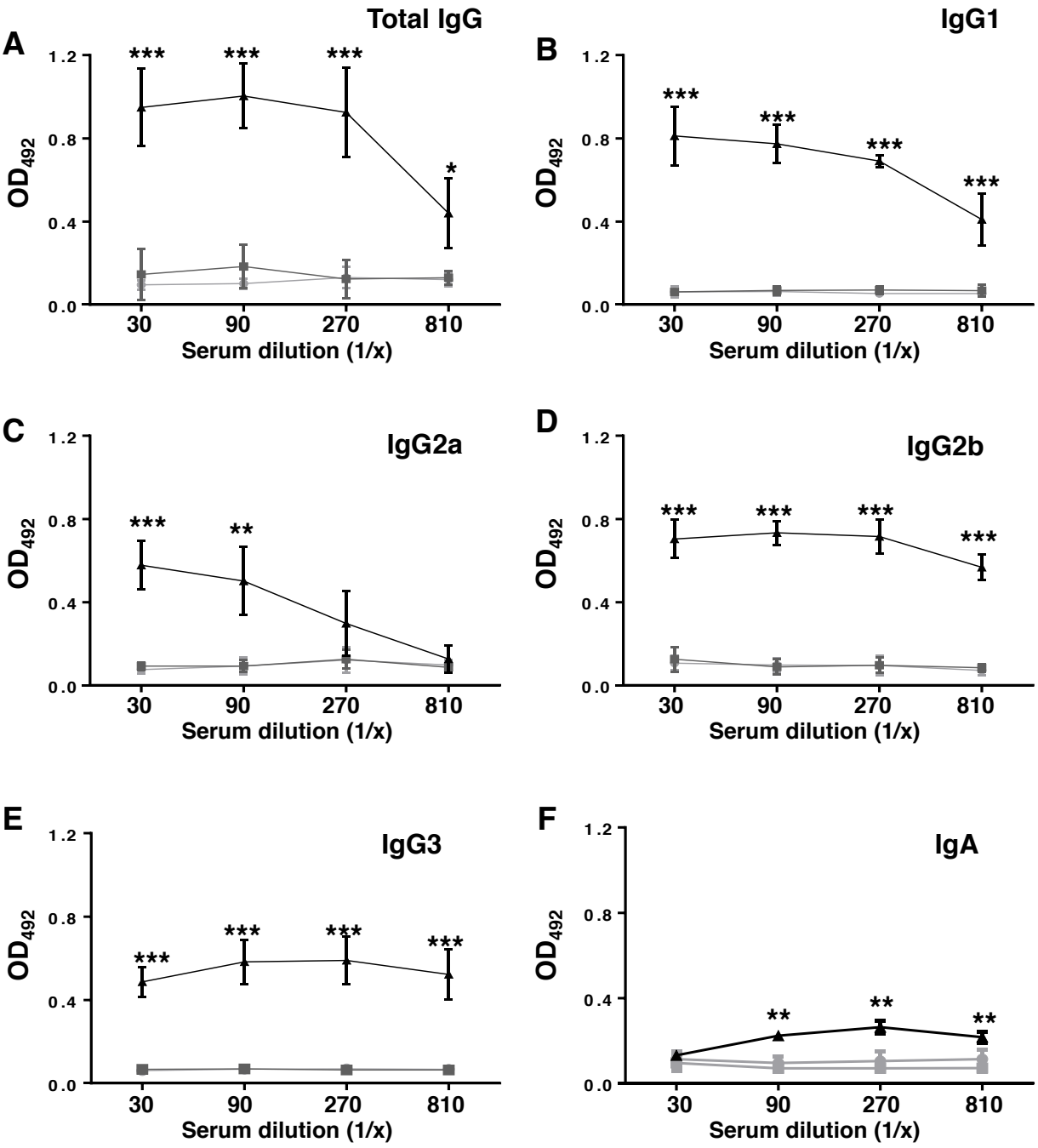

A
Total IgG
B
IgG1
C
IgG2a
D
IgG2b
E
IgG3
F
IgA
OD492
Serum dilution (1/x)
30
90
270
810
0.0
0.4
0.8
1.2
*
**
***

Figure 2

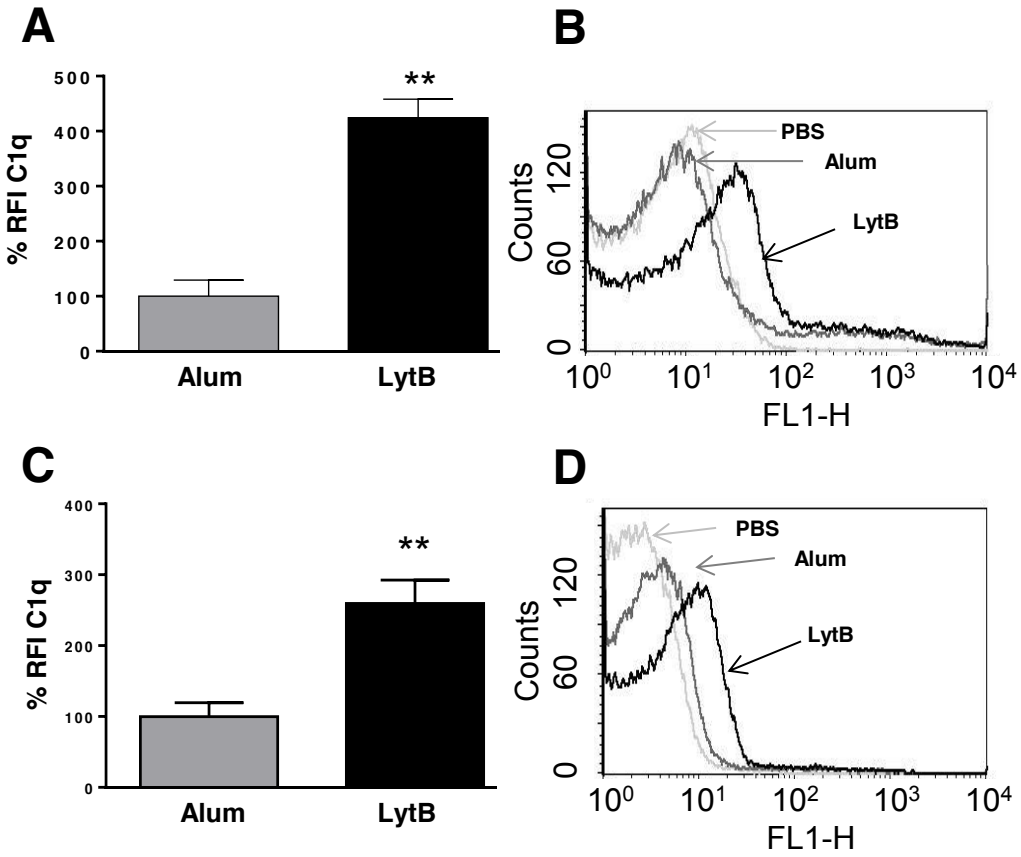

A
B
C
D
% RFI C1q
500
400
300
200
100
0
**
Alum
LytB
Counts
120
60
0
PBS
Alum
LytB
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 3

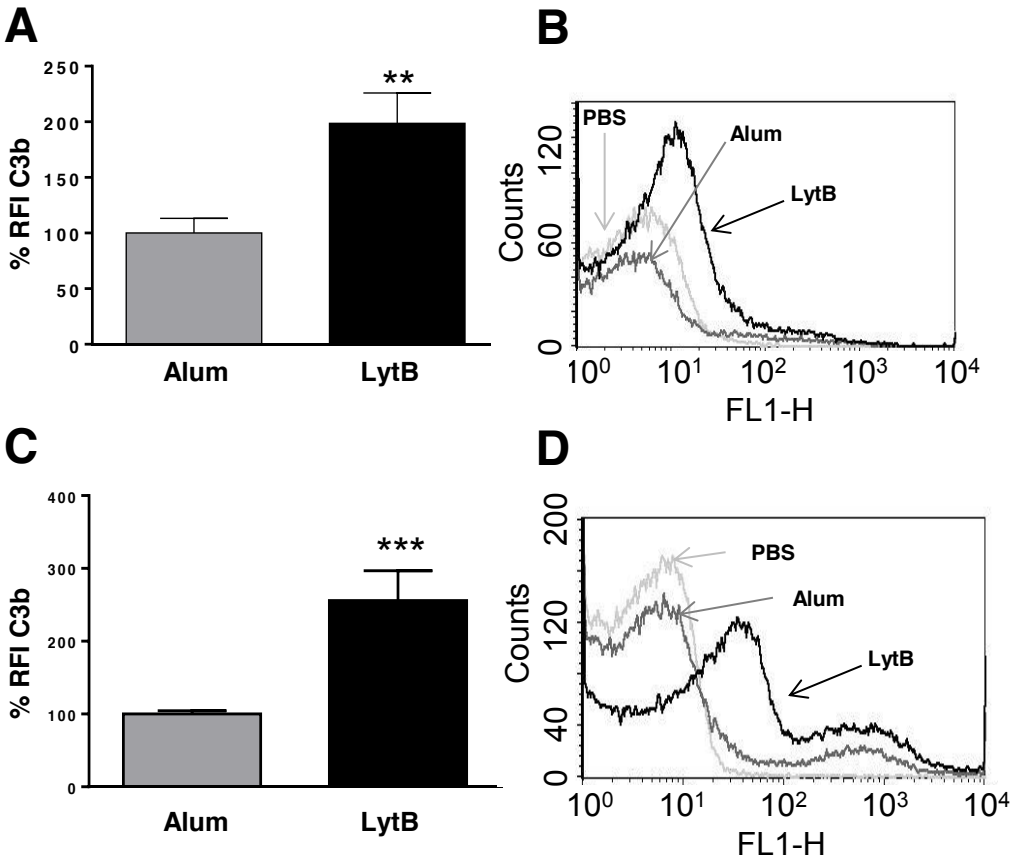

A
250
200
150
100
50
0
% RFI C3b
**
Alum
LytB
B
PBS
Alum
LytB
Counts
120
60
0
10^0
10^1
10^2
10^3
10^4
FL1-H
C
400
300
200
100
0
% RFI C3b
***
Alum
LytB
D
PBS
Alum
LytB
Counts
200
120
40
0
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 4

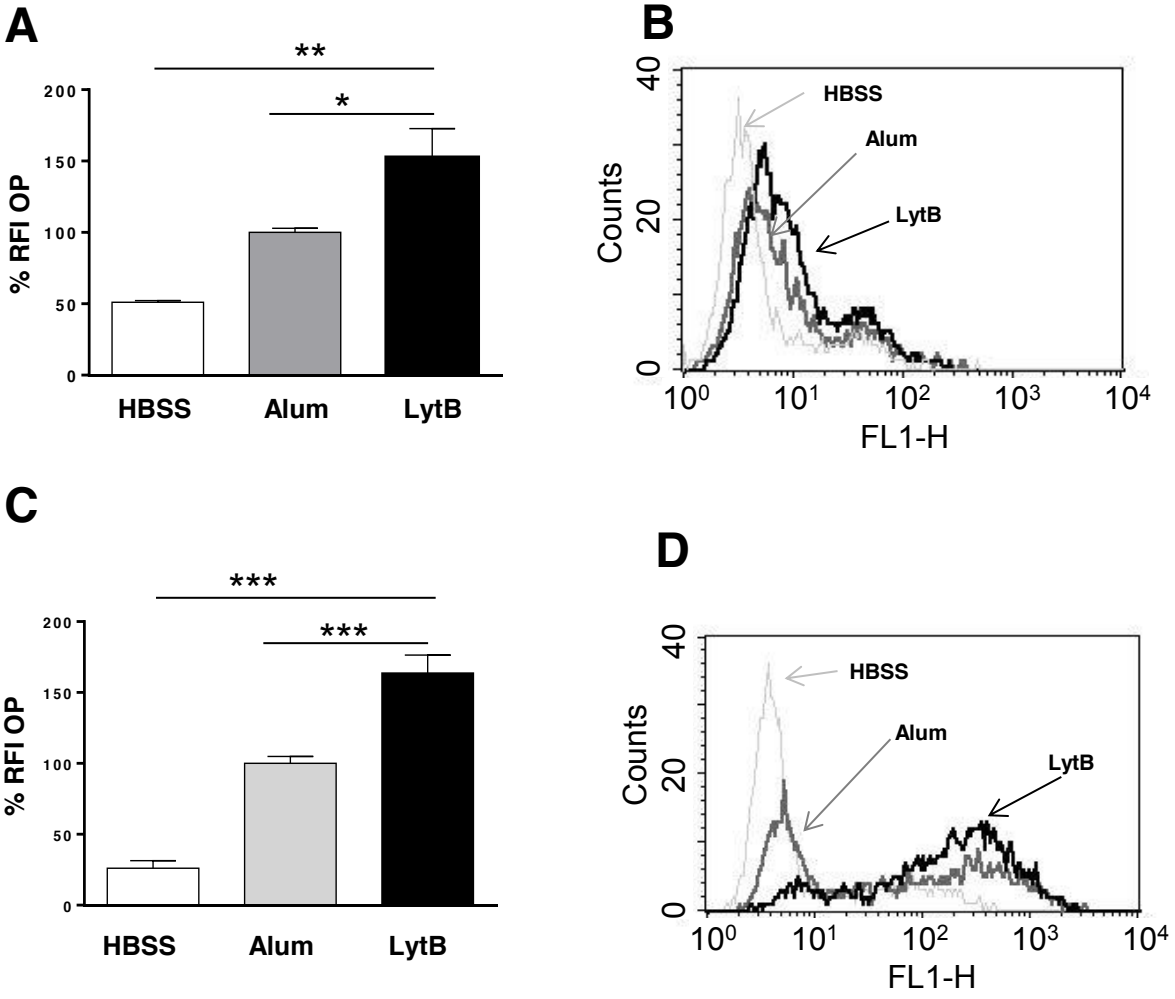

A
**
*
% RFI OP
200
150
100
50
0
HBSS
Alum
LytB
B
HBSS
Alum
LytB
Counts
40
20
0
10^0
10^1
10^2
10^3
10^4
FL1-H
C
***
***
% RFI OP
200
150
100
50
0
HBSS
Alum
LytB
D
HBSS
Alum
LytB
Counts
40
20
0
10^0
10^1
10^2
10^3
10^4
FL1-H

Figure 5

Figure 5

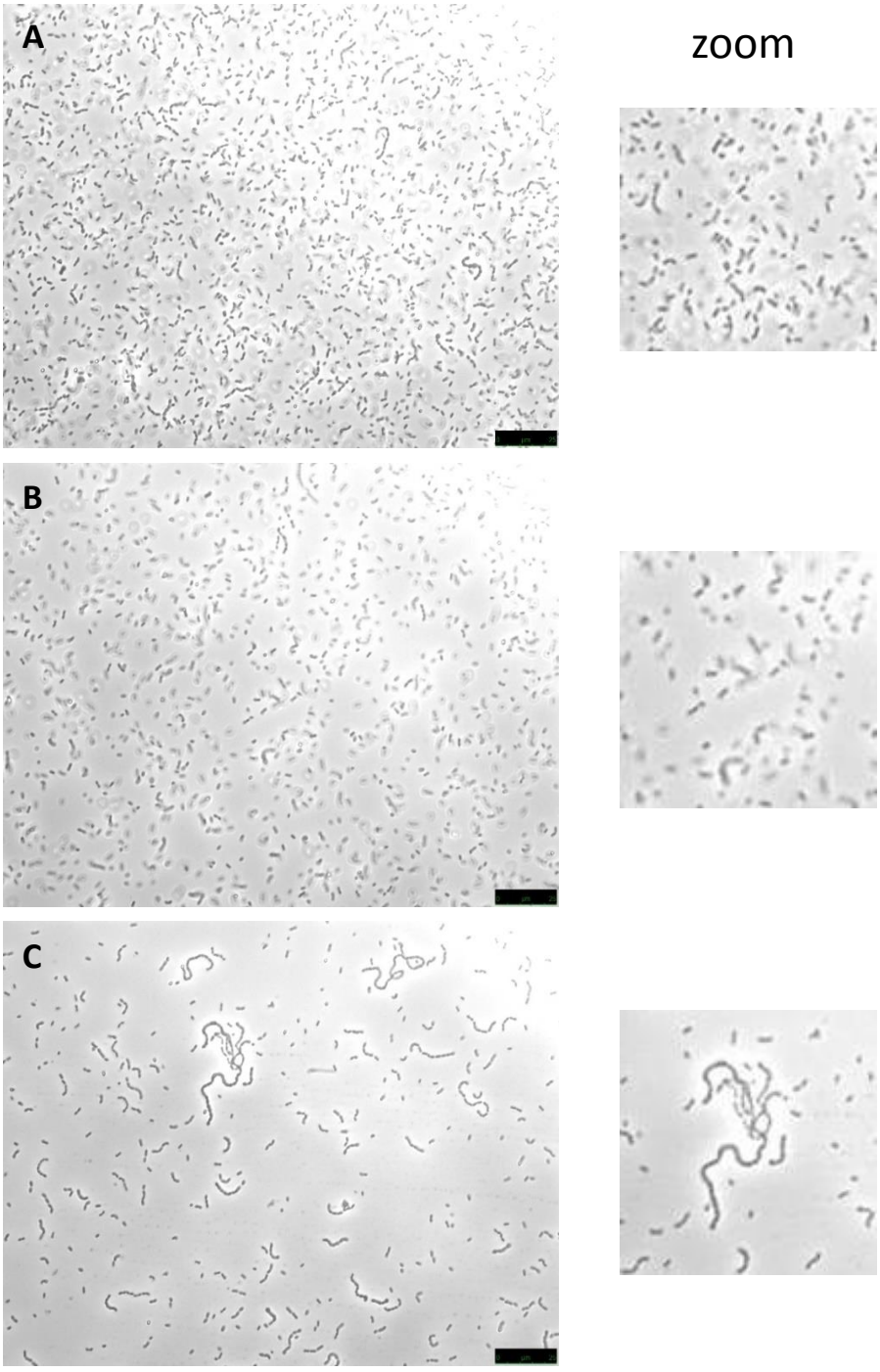


Figure 6

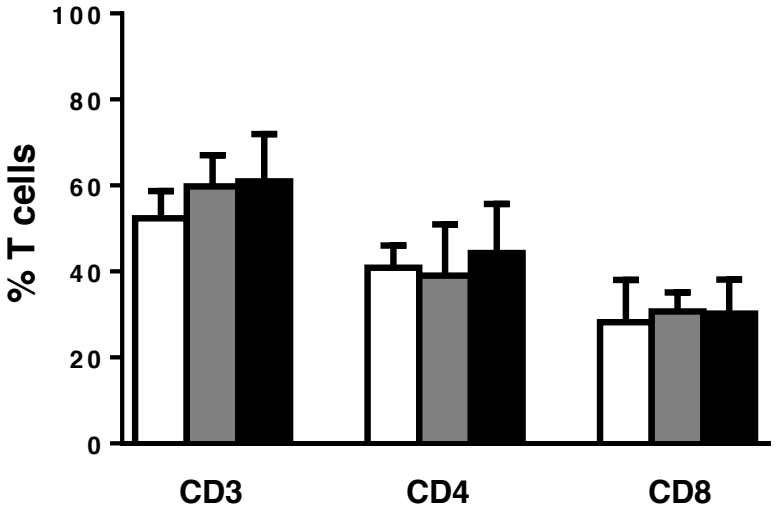


Figure 7

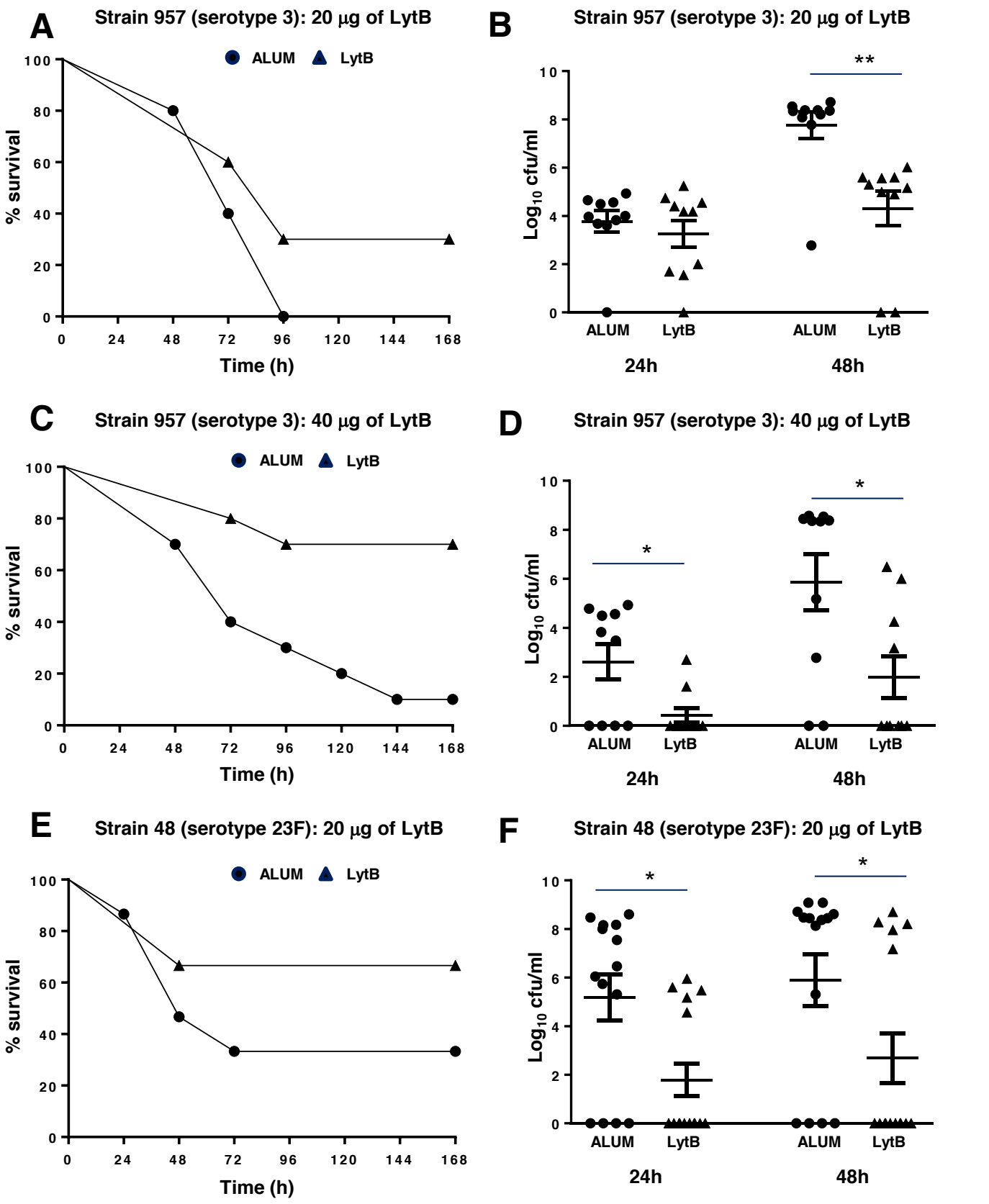
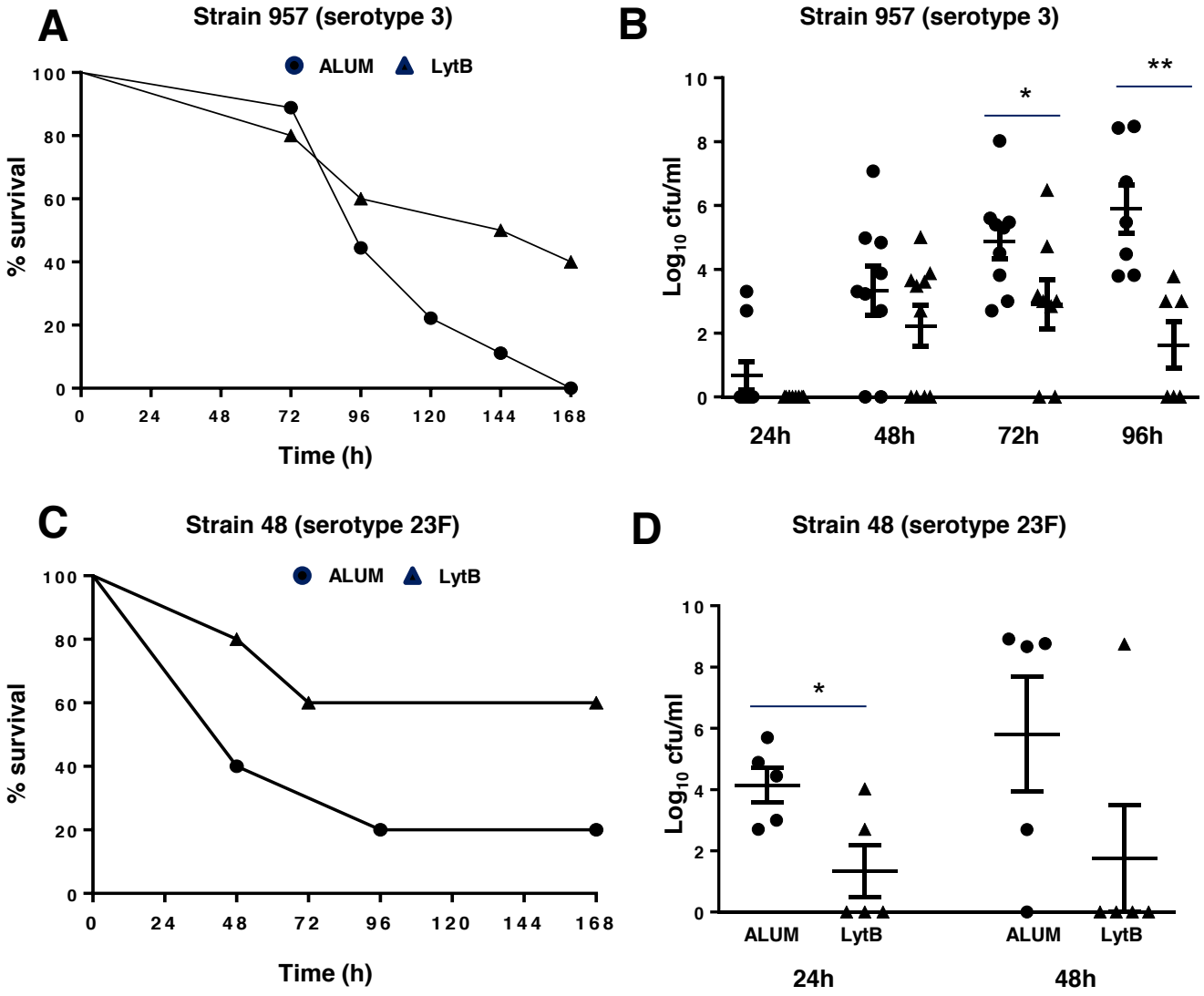

A
Strain 957 (serotype 3): 20 μg of LytB
ALUM
LytB
% survival
Time (h)
B
Strain 957 (serotype 3): 20 μg of LytB
Log10 cfu/ml
**
ALUM
LytB
24h
48h
C
Strain 957 (serotype 3): 40 μg of LytB
D
Strain 957 (serotype 3): 40 μg of LytB
*
E
Strain 48 (serotype 23F): 20 μg of LytB
F
Strain 48 (serotype 23F): 20 μg of LytB

Figure 8


A
Strain 957 (serotype 3)
ALUM
LytB
% survival
Time (h)
B
Strain 957 (serotype 3)
Log10 cfu/ml
24h
48h
72h
96h
*
**
C
Strain 48 (serotype 23F)
ALUM
LytB
% survival
Time (h)
D
Strain 48 (serotype 23F)
Log10 cfu/ml
ALUM
LytB
24h
48h
*

Supplemental Files

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