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

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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.

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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 $\mu\text{g ml}^{-1}$; erythromycin MIC = 0.25 $\mu\text{g ml}^{-1}$, levofloxacin MIC = 1 $\mu\text{g ml}^{-1}$, tetracycline MIC = 0.5 $\mu\text{g ml}^{-1}$, chloramphenicol MIC = 4 $\mu\text{g ml}^{-1}$) and strain 48 (serotype 23F; amoxicillin MIC = 16 $\mu\text{g ml}^{-1}$; erythromycin MIC > 128 $\mu\text{g ml}^{-1}$, levofloxacin MIC = 1 $\mu\text{g ml}^{-1}$, tetracycline MIC = 64 $\mu\text{g ml}^{-1}$, chloramphenicol MIC = 4 $\mu\text{g ml}^{-1}$). Pneumococcal isolates were cultured on blood agar plates at 37 °C in 5% CO₂ or in Todd-Hewitt broth supplemented with 0.5% yeast extract to an optical density at 580 nm (OD₅₈₀) of 0.4 (approximately 10⁸ 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

experiments performed in this work were approved by the Animal Care and Use Committee of ISCIII (CBA PA 52_2011-v2 and PROEX 218/15).

The pneumococcal LytB protein used for immunization studies was purified as previously described [9, 10] and was prepared for animal inoculations at 20–40 µg in Alum (Alhydrogel; aluminum hydroxide, InvivoGen) as the adjuvant in a 1:1 proportion. Groups of 5 mice were immunized by intraperitoneal (IP) inoculation of 200 µl of Alum alone or 200 µl of LytB protein preparation in Alum adjuvant on days 0, 7, and 14 as previously described [15]. Animals were euthanized on day 21 and blood was collected from cardiac puncture and conserved as pooled for further *in vitro* assays. For protection experiments against sepsis, groups of 10 mice were immunized as previously described, followed by IP challenge on day 21 with 10^3 CFU/ per mouse of serotype 3 strain or 10^7 CFU per mouse of serotype 23F strain representing at least the lethal dose 50 (LD₅₀) of each strain for sepsis infection. The immunization schedule for protection against pneumonia was the same as before although the bacterial challenge was performed by the intranasal route with 50 µl of 10^4 CFU/mouse of serotype 3 strain or 5×10^7 CFU/mouse of serotype 23F strain representing at least the LD₅₀ of each strain for pneumonia. Bacterial counts were determined during the first 24–48 h from blood samples (6 µl per mouse) obtained from the tail vein of infected animals as previously described [16]. The development of disease was monitored daily, and mice were sacrificed when they exhibited severe signs of disease.

2.3. Enzyme linked immunosorbent assays to detect Ig subclasses

Specific antibody titers in pooled sera from five mice of each group were measured by enzyme-linked immunosorbent assays (ELISA) using 96-well polystyrene Maxisorp 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 OD₄₉₂ 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 μ l 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-

SE; Molecular Probes) and human HL-60 cells (CCL-240; ATCC) differentiated to granulocytes. The general conditions of the assay were based on those described previously except that clinical isolates of serotype 3 and 23F were incubated with pooled sera from mice immunized with Alum alone or immunized with the mixture LytB–Alum [16, 21]. Infection assays were performed with a ratio of 10 bacteria per cell. A minimum of 6,000 cells were analyzed using a FACS Calibur flow cytometer. Results were expressed as a RFI 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.

2.5. T cell response

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

2.6. Gene lytB sequence accession numbers

The LytB nucleotide sequences of clinical isolates 48 (serotype 23F) and 957 (serotype 3) have been deposited in the GenBank databases. They have been assigned 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).

237 3.5. *Vaccination with LytB did not modify the T cell response.*

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

245 3.6. *Immunization with LytB protects mice against S. pneumoniae infection*

246 IPD is a devastating infectious process associated to high mortality rates that mainly
247 affects the elderly and children. The protective activity of LytB against IPD was
248 investigated in a sepsis model of infection caused by clinical isolates of *S. pneumoniae*
249 belonging to serotypes 3 and 23F (Fig. 7). Vaccination with 20 µg of LytB decreased
250 bacterial counts in blood at 48 h in comparison to Alum group and increased survival
251 rates against sepsis caused by the serotype 3 strain (Fig. 7A and B). Mice immunized
252 with 40 µg of LytB during three weeks (three doses) and infected with the serotype 3
253 strain, showed increased protection against sepsis caused by this clinical isolate with
254 protective rates of 70% in the LytB-vaccinated group vs 10% in the Alum group (P
255 <0.01) (Fig. 7C). In addition, a significant reduction in the bacterial load at 24 and 48 h
256 was observed in the blood of mice immunized with LytB (Fig. 7D). Protection against
257 sepsis mediated by LytB was also tested using a serotype 23F strain. Vaccination with
258 20 µg of LytB increased survival and decreased bacterial counts in blood (Fig. 7E and
259 F) confirming that LytB might be a promising vaccine candidate against sepsis caused
260 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

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

References

- [1] van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet*. 2009;374:1543–56.
- [2] Koedel U, Scheld WM, Pfister HW. Pathogenesis and pathophysiology of pneumococcal meningitis. *Lancet Infect Dis*. 2002;2:721–36.
- [3] O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet*. 2009;374:893–902.
- [4] O'Brien KL, Levine OS. Effectiveness of pneumococcal conjugate vaccine. *Lancet*. 2006;368:1469–70.
- [5] Park IH, Geno KA, Yu J, Oliver MB, Kim KH, Nahm MH. Genetic, biochemical, and serological characterization of a new pneumococcal serotype, 6H, and generation of a pneumococcal strain producing three different capsular repeat units. *Clin Vaccine Immunol* 2015;22:313–8.

- [6] Brueggemann AB, Pai R, Crook DW, Beall B. Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. *PLoS Pathog.* 2007;3:e168.
- [7] Golubchik T, Brueggemann AB, Street T, Gertz RE, Jr., Spencer CC, Ho T, et al. Pneumococcal genome sequencing tracks a vaccine escape variant formed through a multi-fragment recombination event. *Nature Genet.* 2012;44:352–5.
- [8] Aguinagalde L, Corsini B, Domenech A, Domenech M, Camara J, Ardanuy C, et al. Emergence of amoxicillin-resistant variants of Spain9V-ST156 pneumococci expressing serotype 11A correlates with their ability to evade the host immune response. *PLoS One.* 2015;10:e0137565.
- [9] De Las Rivas B, García JL, López R, García P. Purification and polar localization of pneumococcal LytB, a putative endo-beta-N-acetylglucosaminidase: the chain-dispersing murein hydrolase. *J Bacteriol.* 2002;184:4988–5000.
- [10] García P, González MP, García E, López R, García JL. LytB, a novel pneumococcal murein hydrolase essential for cell separation. *Mol Microbiol.* 1999;31:1275–81.
- [11] Ramos-Sevillano E, Moscoso M, García P, García E, Yuste J. Nasopharyngeal colonization and invasive disease are enhanced by the cell wall hydrolases LytB and LytC of *Streptococcus pneumoniae*. *PLoS One.* 2011;6:e23626.
- [12] Moscoso M, García E, López R. Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *J Bacteriol.* 2006;188:7785–95.
- [13] Bai XH, Chen HJ, Jiang YL, Wen Z, Huang Y, Cheng W, et al. Structure of pneumococcal peptidoglycan hydrolase LytB reveals insights into the bacterial cell wall remodeling and pathogenesis. *J Biol Chem.* 2014;289:23403–16.

- [14] Wizemann TM, Heinrichs JH, Adamou JE, Erwin AL, Kunsch C, Choi GH, et al. Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection. *Infect Immun*. 2001;69:1593–8.
- [15] Jomaa M, Yuste J, Paton JC, Jones C, Dougan G, Brown JS. Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of *Streptococcus pneumoniae*. *Infect Immun*. 2005;73:6852–9.
- [16] Cafini F, Yuste J, Giménez MJ, Sevillano D, Aguilar L, Alou L, et al. Enhanced in vivo activity of cefditoren in pre-immunized mice against penicillin-resistant *S. pneumoniae* (serotypes 6B, 19F and 23F) in a sepsis model. *PLoS One*. 2010;5:e12041.
- [17] Ramos-Sevillano E, Urzainqui A, Campuzano S, Moscoso M, González-Camacho F, Domenech M, et al. Pleiotropic effects of cell wall amidase LytA on *Streptococcus pneumoniae* sensitivity to the host immune response. *Infect Immun*. 2015;83:591–603.
- [18] Yuste J, Sen A, Truedsson L, Jönsson G, Tay LS, Hyams C, et al. Impaired opsonization with C3b and phagocytosis of *Streptococcus pneumoniae* in sera from subjects with defects in the classical complement pathway. *Infect Immun*. 2008;76:3761-70.
- [19] Ramos-Sevillano E, Rodríguez-Sosa C, Cafini F, Giménez MJ, Navarro A, Sevillano D, et al. Cefditoren and ceftriaxone enhance complement-mediated immunity in the presence of specific antibodies against antibiotic-resistant pneumococcal strains. *PLoS One*. 2012;7:e44135.
- [20] Exley RM, Shaw J, Mowe E, Sun YH, West NP, Williamson M, et al. Available carbon source influences the resistance of *Neisseria meningitidis* against complement. *J Exp Med*. 2005;201:1637–45.

- [21] Romero-Steiner S, Libutti D, Pais LB, Dykes J, Anderson P, Whitin JC, et al. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against *Streptococcus pneumoniae* using differentiated HL-60 cells. Clin Diagn Lab Immunol. 1997;4:415–22.
- [22] Brown JS, Hussell T, Gilliland SM, Holden DW, Paton JC, Ehrenstein MR, et al. The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. Proc Natl Acad Sci U S A. 2002;99:16969–74.
- [23] Wardlaw T, Salama P, Johansson EW, Mason E. Pneumonia: the leading killer of children. Lancet. 2006;368:1048–50.
- [24] Feldman C, Anderson R. Review: current and new generation pneumococcal vaccines. J Infect. 2014;69:309–25.
- [25] Briles DE, Tart RC, Wu HY, Ralph BA, Russell MW, McDaniel LS. Systemic and mucosal protective immunity to pneumococcal surface protein A. Ann N Y Acad Sci. 1996;797:118–26.
- [26] Brooks-Walter A, Briles DE, Hollingshead SK. The *pspC* gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. Infect Immun. 1999;67:6533–42.
- [27] Glover DT, Hollingshead SK, Briles DE. *Streptococcus pneumoniae* surface protein PcpA elicits protection against lung infection and fatal sepsis. Infect Immun. 2008;76:2767–76.
- [28] Yuan ZQ, Lv ZY, Gan HQ, Xian M, Zhang KX, Mai JY, et al. Intranasal immunization with autolysin (LytA) in mice model induced protection against five

prevalent *Streptococcus pneumoniae* serotypes in China. Immunol Res. 2011;51:108–15.

[29] Moscoso M, Obregón V, López R, García JL, García E. Allelic variation of polymorphic locus *lytB*, encoding a choline-binding protein, from streptococci of the mitis group. Appl Environ Microbiol. 2005;71:8706–13.

[30] Bologa M, Kamtchoua T, Hopfer R, Sheng X, Hicks B, Bixler G, et al. Safety and immunogenicity of pneumococcal protein vaccine candidates: monovalent choline-binding protein A (PcpA) vaccine and bivalent PcpA-pneumococcal histidine triad protein D vaccine. Vaccine. 2012;30:7461–8.

[31] Kamtchoua T, Bologa M, Hopfer R, Neveu D, Hu B, Sheng X, et al. Safety and immunogenicity of the pneumococcal pneumolysin derivative PlyD1 in a single-antigen protein vaccine candidate in adults. Vaccine. 2013;31:327–33.

[32] Uddin S, Borrow R, Haeney MR, Moran A, Warrington R, Balmer P, et al. Total and serotype-specific pneumococcal antibody titres in children with normal and abnormal humoral immunity. Vaccine. 2006;24:5637–44.

[33] Lottenbach KR, Mink CM, Barenkamp SJ, Anderson EL, Homan SM, Powers DC. Age-associated differences in immunoglobulin G1 (IgG1) and IgG2 subclass antibodies to pneumococcal polysaccharides following vaccination. Infect Immun. 1999;67:4935–8.

[34] Kamboj KK, Kirchner HL, Kimmel R, Greenspan NS, Schreiber JR. Significant variation in serotype-specific immunogenicity of the seven-valent *Streptococcus pneumoniae* capsular polysaccharide-CRM197 conjugate vaccine occurs despite vigorous T cell help induced by the carrier protein. J Infect Dis. 2003;187:1629–38.

[35] Shelly MA, Jacoby H, Riley GJ, Graves BT, Pichichero M, Treanor JJ. Comparison of pneumococcal polysaccharide and CRM197-conjugated

pneumococcal oligosaccharide vaccines in young and elderly adults. *Infect Immun.* 1997;65:242–7.

[36] Wuorimaa T, Kayhty H, Leroy O, Eskola J. Tolerability and immunogenicity of an 11-valent pneumococcal conjugate vaccine in adults. *Vaccine.* 2001;19:1863–9.

[37] Soininen A, Seppala I, Nieminen T, Eskola J, Kayhty H. IgG subclass distribution of antibodies after vaccination of adults with pneumococcal conjugate vaccines. *Vaccine.* 1999;17:1889–97.

[38] Bredius RG, Driedijk PC, Schouten MF, Weening RS, Out TA. Complement activation by polyclonal immunoglobulin G1 and G2 antibodies against *Staphylococcus aureus*, *Haemophilus influenzae* type b, and tetanus toxoid. *Infect Immun.* 1992;60:4838–47.

[39] Amir J, Scott MG, Nahm MH, Granoff DM. Bactericidal and opsonic activity of IgG1 and IgG2 anticapsular antibodies to *Haemophilus influenzae* type b. *J Infect Dis.* 1990;162:163–71.

[40] Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol.* 2004;172:2731–8.

[41] Standish AJ, Weiser JN. Human neutrophils kill *Streptococcus pneumoniae* via serine proteases. *J Immunol.* 2009;183:2602–9.

[42] Johansson PJ, Sternby E, Ursing B. Septicemia in granulocytopenic patients: a shift in bacterial etiology. *Scand J Infect Dis.* 1992;24:357–60.

[43] Zuluaga AF, Salazar BE, Rodriguez CA, Zapata AX, Agudelo M, Vesga O. Neutropenia induced in outbred mice by a simplified low-dose cyclophosphamide regimen: characterization and applicability to diverse experimental models of infectious diseases. *BMC Infect Dis.* 2006;6:55.

1981;294:88–90.

[45] Dalia AB, Weiser JN. Minimization of bacterial size allows for complement evasion and is overcome by the agglutinating effect of antibody. *Cell Host Microbe*. 2011;10:486–96.

[46] Rodriguez JL, Dalia AB, Weiser JN. Increased chain length promotes pneumococcal adherence and colonization. *Infect Immun*. 2012;80:3454–9.

[47] Fenoll A, Granizo JJ, Giménez MJ, Yuste J, Aguilar L. Secular trends (1990-2013) in serotypes and associated non-susceptibility of *S. pneumoniae* isolates causing invasive disease in the pre-/post-era of pneumococcal conjugate vaccines in Spanish regions without universal paediatric pneumococcal vaccination. *Vaccine*. 2015;33:5691–9.

[48] Poolman J, Kriz P, Feron C, Di-Paolo E, Henckaerts I, Miseur A, et al. Pneumococcal serotype 3 otitis media, limited effect of polysaccharide conjugate immunisation and strain characteristics. *Vaccine*. 2009;27:3213–22.

[49] Choi EH, Zhang F, Lu YJ, Malley R. Capsular Polysaccharide Release by Serotype 3 Pneumococcal Strains Reduces the Protective Effect of Anti-Type 3 Capsular Antibodies. *Clin Vaccine Immunol* 2016; 23:162–7.

[50] Kaur R, Surendran N, Ochs M, Pichichero ME. Human antibodies to PhtD, PcpA, and Ply reduce adherence to human lung epithelial cells and murine nasopharyngeal colonization by *Streptococcus pneumoniae*. *Infect Immun*. 2014;82:5069–75.

[51] Ogunniyi AD, Grabowicz M, Briles DE, Cook J, Paton JC. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. *Infect Immun*. 2007;75:350–7.

530 vaccine in Gambian children aged 2-4 years: a phase II randomized study. Hum
531 Vaccine Immunother. 2016, 12:393–402.
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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

expressed as a relative % fluorescence index. 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. 4. Opsonophagocytosis (OP) assays using the HL-60 neutrophil cell line and *S. pneumoniae* strains incubated with HBSS (open bars), or pooled sera from mice immunized with Alum (gray bars) or with LytB mixed with Alum (black bars). (A) OP of a serotype 3 clinical isolate. (B) Example of a flow cytometry histogram for OP of serotype 3 strain. (C) OP of serotype 23F clinical isolate. (D) Example of a flow cytometry histogram for OP of serotype 23F strain. 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. 5. Phase-contrast microscopy images of the pneumococcal clinical isolate of serotype 23F exposed for 4 h at 37°C to different murine sera. (A) Normal mouse serum from naïve mice. (B) Pooled sera from mice immunized with Alum. (C) Pooled sera from mice immunized with 10 µg of LytB mixed with Alum. Bars, 25 µm.

Fig. 6. T cell response represented as proportion of CD3, CD4 and CD8 in non-immunized mice (white bars), mice immunized with Alum (gray bars) or mice immunized with LytB mixed with Alum (black bars). Error bars represent the SDs. Experiments were repeated three times.

the clinical isolate 957 of serotype 3 in mice immunized with Alum or with 20 µg of
 LytB. (B) Bacterial counts in blood at 24 h and 48 h from mice immunized with Alum
 or 20 µg of LytB and infected with strain 957 of serotype 3. (C) Survival after sepsis
 caused by clinical isolate 957 of serotype 3 in mice immunized with Alum or LytB (40
 µg). For differences in survival between Alum group and LytB group (** $P < 0.01$, Log-
 rank test) (D) Bacterial counts in blood at 24 h and 48 h from mice immunized with
 Alum or LytB (40 µg) and infected with strain 957 of serotype 3. (E) Survival after
 sepsis caused by clinical isolate 48 of serotype 23F in mice immunized with Alum or
 with LytB (20 µg). (F) Bacterial counts in blood at 24 h and 48 h from mice immunized
 with Alum or LytB (20 µg) and infected with strain 48 of serotype 23F. Error bars
 represent the SDs and asterisks indicate statistical significance of LytB immunized
 group compared to the Alum group. * $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 8. Protection against pneumococcal pneumonia after vaccination with Alum (black
 dots) or with LytB (20 µg) mixed with Alum (black triangle). (A) Survival after
 pneumonia caused by a clinical isolate of serotype 3. (B) Bacterial counts in blood at 24
 h and 48 h from mice infected with serotype 3 strain. (C) Survival after pneumonia
 caused by a clinical isolate of serotype 23F. (D) Bacterial counts in blood at 24 h and 48
 h from mice infected with serotype 23F strain. Error bars represent the SDs and
 asterisks indicate statistical significance of LytB immunized group compared to the
 Alum group. * $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 1

Figure 2
Figure 2

Figure 3
Figure 3

Figure 4

A

B

C

D

Figure 5
Figure 5

zoom

Figure 6

Figure 7

Figure 8