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Delivery of multiple PspA fragments by CyaA toxoid induces broad protection against *Streptococcus pneumoniae*

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Abstract

Streptococcus pneumoniae is a leading cause of lower respiratory tract (LRT) infections and invasive pneumococcal diseases (IPD). Conjugate vaccines are effective against IPD and nasal colonization but offer coverage limited to serotypes included in the formulations. Pneumococcal surface protein A (PspA) stands out as a promising vaccine antigen. We had previously shown that recombinant adenylate cyclase toxoid from *Bordetella pertussis* (CyaA) carrying PspA fragments induces humoral responses and protection against pneumococcal infection in mice. Here, we present an optimized construct combining fragments derived from four PspA variants (CyaA-A2-A4/A1-A3). Immunization of mice with CyaA-A2-A4/A1-A3 conferred protection against IPD and promoted a rapid control of LRT infection. Antibody reactivity and protection were observed across strains expressing distinct PspAs and capsular serotypes. Importantly, CyaA-A2-A4/A1-A3 protected mice against serotype 3 isolates from clonal complex 180, a major clone causing disease worldwide. CyaA-A2-A4/A1-A3 may be proposed as a complementary vaccine to induce broad protection against pneumococcal infections.

Introduction

Streptococcus pneumoniae (pneumococcus) is a bacterial pathogen responsible for the highest number of lower respiratory tract (LRT) infections and a common cause of otitis media and severe diseases, such as meningitis and sepsis (invasive pneumococcal diseases, IPD)[1]. It affects mainly children under five years of age and the elderly, accounting for over 800,000 deaths annually[1, 2]. A major determinant of pneumococcal virulence is its polysaccharide capsule, which contributes to the evasion of host immune defense mechanisms[3]. Its antigenic variability drives the classification of pneumococci into over 100 serotypes.

Pneumococcal conjugate vaccines (PCVs) were introduced in the market in 2000. These vaccines are composed of capsular polysaccharides conjugated to carrier proteins, varying in valency according to the number of included serotypes. The 7-valent formulation (PCV7, Wyeth/Pfizer) was the first to be licensed and was later replaced by 10-valent (PCV10, GSK) and 13-valent (PCV13, Wyeth/Pfizer) formulations, which included additional serotypes. They were introduced into children immunization programs in most countries and significantly reduced the overall burden of IPD and associated mortality, as well as nasal colonization by vaccine serotypes[4, 5, 6]. However, the widespread use of these vaccines was followed by reports of serotype replacement, with non-vaccine serotypes becoming prevalent in the disease burden[6, 7]. In response, PCVs containing additional serotypes, 15-valent (PCV15, Merck) and 20-valent (PCV20, Pfizer), were recently licensed, while other PCVs are currently under evaluation in clinical trials[8]. Although these formulations offer expanded coverage of isolates, their long-term effectiveness remains uncertain due to the potential emergence of non-vaccine serotypes. Moreover, the high cost of high-valency PCVs is a challenge for their adoption in immunization programs, especially in low-income countries, which are more affected by pneumococcal diseases[1]. These limitations highlight the need for complementary strategies.

One alternative is the development of serotype-independent, protein-based vaccines[9]. The Pneumococcal surface protein A (PspA) is an important virulence factor, abundantly expressed on bacterial surface. It inhibits complement deposition, reducing the efficacy of clearance by opsonophagocytosis[10]; inhibits the bactericidal activity of apolactoferrin[11]; and interacts with host metabolic enzymes exposed on the surface of dying lung cells, contributing to bacterial persistence and virulence during lung infections[12, 13]. Numerous studies have demonstrated the potential of PspA as a vaccine candidate, with antibody-mediated responses playing a central role to confer protection in animal models of IPD[14, 15]. A challenge for PspA-based formulations is the antigenic variability observed in the surface-exposed N-terminal α-helical domain, especially within its final 100 amino acids, known as the clade-defining region (CDR). Similarity in the CDR amino acid sequence is the basis for PspA classification into 6 clades, grouped into 3 families[16]. PspAs from family 1 (clades 1 and 2) and family 2 (clades 3, 4 and 5) are expressed by the great majority of pneumococcal isolates, whereas family 3 (clade 6) are rarely observed[17, 18, 19, 20]. As antibodies present cross-reactivity among PspA variants of the same clade and, generally, heterologous clades from the same family[21, 22], combining variants from the predominant PspA families, 1 and 2[23], may be a good strategy to obtain a broad-coverage vaccine.

Previously, we demonstrated the efficacy of using a recombinant adenylate cyclase toxin from *Bordetella pertussis* (CyaA), which targets $CD11b^+$ antigen-presenting cells, for the delivery of PspA fragments to the immune system. We had evaluated recombinant proteins containing fragments comprising the CDR and part of the proline-rich domain (referred to as “A” fragments) derived from PspA variants from clade 2 (A2) or clade 4 (A4), grafted into the AC domain of a detoxified version of CyaA. Immunization of mice with a mixture of CyaA-A2 and CyaA-A4, or with a single protein CyaA-A2-A4, in the absence of adjuvants, induced high levels of circulating anti-PspA antibodies and protection against IPD with serotype 3 pneumococci expressing PspAs from clades 2, 4 or 5[23]. Here, we show that the CyaA-A2-A4 antigen confers protection comparable to the PCV13 vaccine in a murine model of IPD, while promoting an earlier control of pneumococcal infection in the LRT. We also designed an optimized CyaA construction containing additional PspA fragments from clades 1 (A1) and 3 (A3), CyaA-A2-A4/A1-A3, capable of inducing broader reactive antibody responses and protection against strains expressing distinct capsular serotypes and PspA variants, including clades 1 and 3. CyaA-A2-A4/A1-A3 immunization also protected mice against human carriage isolates from serotype 3, a serotype that poses a challenge for currently used vaccination strategies. Finally, we show that the combination of CyaA-A2-A4/A1-A3 with an oil-in-water adjuvant promotes long-lasting antibody responses and effective protection using a reduced-dose protocol.

Results

Immunization with CyaA-A2-A4 promotes early control of pneumococcal LRT infection

In our previous study, we showed that immunization with the CyaA-A2-A4 protein confers protection to mice against IPD by serotype 3 strains[23]. Here, we evaluated whether it could also confer protection against non-invasive pneumonia. Additionally, we aimed to compare the efficacy of the CyaA-A2-A4 antigen with the PCV13 vaccine, which contains serotype 3 capsular polysaccharides (PS3). Mice (n = 12 per group) were immunized with three subcutaneous (s.c.) doses of CyaA-A2-A4, or 1/10 the volume of the PCV13 human dose; as controls, animals received the unrelated CyaA-OVA21 antigen or aluminum hydroxide (Alum). ELISA analysis of sera collected after the last dose confirmed the induction of high levels of specific anti-PspA2 and anti-PspA4 IgG in CyaA-A2-A4 immunized mice (Fig. 1A). In parallel, PCV13 immunized mice presented high levels of anti-PS3 IgG (Fig. 1B). Three weeks after immunization, animals were intranasally inoculated with the 3JYP2670 strain (serotype 3, PspA4). For half of the animals of each group, the lungs and the bronchoalveolar lavage fluid (BALF) were collected 12 h after challenge and plated on blood agar for quantification of colony forming units (CFU). CyaA-A2-A4 immunized mice presented significantly lower numbers of bacteria in the lung tissue and BALF compared to all other groups (~ 100-fold reduction) (Fig. 1C), as well as significantly higher levels of anti-PspA4 IgG in the LRT after challenge compared with control animals inoculated with CyaA-OVA21 (Fig. 1D). No significant reduction in bacterial load was observed in the PCV13 group in comparison to control groups (Fig. 1C) and anti-PS3 IgG was not detected in the LRT at the analyzed time point (Fig. 1E). IgA titers against PspA4 or PS3 were not observed in BALF samples. Instead, a slight increase in the absorbances of anti-PspA4 IgA, measured in 1:2 dilutions of BALFs from CyaA-A2-A4 immunized mice was detected (Supplementary Fig. 1). Flow cytometry analyses of BALF samples showed the presence of phagocytic cells (gating strategy is shown in Supplementary Fig. 2). Similar numbers of alveolar and interstitial macrophages were detected in BALF samples of all groups, while significantly lower levels of neutrophils were detected for CyaA-A2-A4 immunized mice, at 12 h after the challenge, compared to the other groups (Fig. 1F). Additionally, lower levels of TNF-α and IL-6 were observed in BALF samples of CyaA-A2-A4 immunized mice, indicating a reduced inflammatory response at 12 h after challenge (Fig. 1G). As for the other half of animals from each group, protection was evaluated in the IPD model, analyzing survival in a period of 10 days. While control mice, immunized with Alum or CyaA-OVA21, succumbed within three days after challenge, PCV13 and CyaA-A2-A4 immunized animals were significantly protected, with 100% survival in both groups (Fig. 1H). These results indicate that CyaA-A2-A4 may confer protection against IPD comparable to that provided by PCV13 and an earlier control of LRT infection.

New CyaA-PspA candidate promotes robust humoral responses against multiple PspA fragments

Aiming to optimize the CyaA-PspA vaccine by expanding its spectrum of reactivity and coverage of pneumococcal isolates, we added the A fragment of PspA from clades 1 and 3 (A1 and A3, respectively) (Supplementary Fig. 3A, B) to new constructions. The A1-A3 fusion was grafted into distinct sites of CyaA AC domain, either alone (Supplementary Fig. 3C, D) generating antigens to be used in a mixture with CyaA-A2-A4, or combined with A2-A4 in the same CyaA molecule (Supplementary Fig. 3E, F). All

constructions were expressed and purified to near homogeneity (Supplementary Fig. 4A), with low endotoxin contamination. A preliminary comparative analysis, in which mice were immunized with three s.c. doses of the new antigens, showed that all of them induced significant levels of antibodies against PspA clades grafted in the recombinant CyaAs (Supplementary Fig. 5). For subsequent studies, we selected the CyaA-A2-A4/A1-A3 construction as it induced high and more homogeneous levels of antibodies across PspA clades 1 to 4 (Supplementary Fig. 5), while presenting the benefit of constituting a single vaccine antigen. This protein contains the A2-A4 fusion between amino acids 224 and 225 and the A1-A3 fusion between amino acids 319 and 320 of CyaA (Supplementary Fig. 3E), presenting a predicted molecular mass of 237 kDa. Polyclonal sera of mice immunized with A1, A2, A3 or A4, recognized the CyaA-A2-A4/A1-A3 protein by western-blot (Supplementary Fig. 4B-E).

In order to evaluate the induction of antibodies against the CyaA vector, we have performed ELISA assays against the CyaA-OVA21 protein using sera of mice immunized with CyaA-A2-A4/A1-A3. High levels of IgG reacting with CyaA-OVA21 were observed in mice after two doses, followed by a non-significant increase after the third dose (Supplementary Fig. 6A). When the same samples were evaluated for antibodies against the A fragments, high levels were already observed after the second dose and a significant increase was observed after the third dose (Supplementary Fig. 6B). These results suggest that the induction of antibodies against the CyaA vector seems not to interfere with the induction of anti-PspA antibodies.

CyaA-A2-A4/A1-A3 was then compared to our previous antigen CyaA-A2-A4[23] through immunization experiments in mice. Animals (n = 5 or 6 per group) were immunized with three s.c. doses of CyaA-A2-A4/A1-A3, or CyaA-A2-A4; control animals received CyaA-OVA21. Sera collected after immunization were analyzed for the levels of IgG reacting specifically against PspAs from clades 1, 2, 3 or 4, or against a mixture of PspA fragments A1 to A4 (anti-A IgG). Mice immunized with CyaA-A2-A4/A1-A3 presented high titers of IgG reacting with each PspA variant, with mean titers between 10^3 and 10^4 (Fig. 2A-D). Significantly higher titers of anti-PspA1 IgG (Fig. 2A) and similar levels of anti-PspA2 (Fig. 2B) and anti-PspA4 (Fig. 2D) IgG were observed for animals immunized with CyaA-A2-A4/A1-A3, in comparison with CyaA-A2-A4. In the analysis of anti-PspA3 IgG, sera from CyaA-A2-A4 immunized mice produced positive titers (significant in comparison to the control group) (Fig. 2C) indicating cross-reactivity of antibodies produced against A2- A4 with PspA3. Nevertheless, the levels of anti-PspA3 IgG detected in the sera of CyaA-A2-A4/A1-A3 immunized animals were significantly higher than those observed for CyaA-A2-A4 (Fig. 2C). Accordingly, CyaA-A2-A4/A1-A3 sera presented significantly higher levels of total anti-A IgG (mean titers ~ 10^5) in comparison with CyaA-A2-A4 (mean titers ~ 10^4) (Fig. 2E). These results indicate that the combination of PspA fragments in CyaA-A2-A4/A1-A3 did not compromise the immunogenicity of A2 and A4, while promoting additional humoral responses against A1 and A3. Characterization of anti-A IgG in the sera of CyaA-A2-A4/A1-A3 immunized mice showed the effective induction of IgG1 and IgG2a (mean titers ~ 10^5 and between 10^4 and 10^5, respectively) (Fig. 2F). The low IgG1/IgG2a ratio indicates a balanced Th1/Th2 immune response, the same pattern observed for CyaA-A2-A4 (Fig. 2F) and in our previous studies with CyaA-PspA proteins[23]. Finally, significant levels of anti-A IgA were

observed in the sera of mice immunized with CyaA-A2-A4 and CyaA-A2-A4/A1-A3 (Supplementary Fig. 7).

Antibodies induced by CyaA-A2-A4/A1-A3 are broadly reactive with pneumococcal strains

We had previously shown that antibodies induced by CyaA proteins containing A2 and A4 presented high reactivity with pneumococcal strains expressing PspAs from clades 2, 4 and 5, but low or no capacity to bind to pneumococci expressing PspA1 and variable binding efficacy for strains expressing PspA3[23]. Here, to evaluate if the inclusion of A1 and A3 would contribute to broaden the spectrum of antibody reactivity, we performed *in vitro* assays using pooled sera from animals immunized with CyaA-A2-A4 or CyaA-A2-A4/A1-A3, or CyaA-OVA21 as a negative control. Pneumococcal strains representing different serotypes, expressing PspA variants from clades 1 to 5, were incubated with the sera and antibody binding to bacterial surface was detected by flow cytometry using FITC-conjugated anti-mouse IgG. The FITC-median fluoresce intensity (MFI) obtained for each sample was used for comparison among groups. For all tested strains, antibodies present in the CyaA-A2-A4/A1-A3 sera effectively bound to bacterial surface, producing MFI values 10 to 100-fold higher than those from the control group (Fig. 3). For strains expressing PspA2, PspA4 or PspA5, both CyaA-A2-A4 and CyaA-A2-A4/A1-A3 sera presented high MFIs (Fig. 3D, E, I-L). On the other hand, reactivity induced by CyaA-A2-A4/A1-A3 was stronger than CyaA-A2-A4 for bacteria expressing PspA1 (10 to 40-fold higher MFIs) (Fig. 3A-C) and PspA3 (4 to 10-fold higher MFIs) (Fig. 3F-H), indicating that antibodies generated against A1 and A3 increased the reactivity with these strains. To further explore the improvement in reactivity against pneumococcal strains expressing PspA1 and PspA3, individual sera were tested against selected strains expressing PspA variants 1 to 5. The results showed significant higher binding of IgG against the pneumococcal strains EF3030 (serotype 19F, PspA1) and M10 (serotype 11A, PspA3). Similar levels of IgG binding were observed for the pneumococcal strains A66.1 (serotype 3, PspA2), 3JYP2670 (serotype 3, PspA4) and ATCC6303 (serotype 3, PspA5) (Supplementary Fig. 8).

One of the proposed mechanisms for the protection conferred by anti-PspA antibodies is their ability to revert the inhibition of complement deposition[10]. In our previous work, we showed that pneumococci pre-incubated with sera from CyaA-A2 + CyaA-A4 immunized mice, followed by exposure to a complement source, presented increased deposition of complement component 3 (C3) on their surface compared to those pre-incubated with control CyaA-OVA21 sera; this effect was observed with strains expressing PspAs from clades 2, 4, 5 and, in some cases, clade 3, but not in strains expressing PspA1[23]. Here, the same assay was performed pre-incubating bacteria with sera from CyaA-A2-A4/A1-A3 immunized animals. After the addition of complement source, samples were incubated with FITC-conjugated anti-mouse C3 and analyzed by flow cytometry. We selected representative strains expressing PspAs from clades 1 to 5, and in all cases, bacteria pre-incubated with CyaA-A2-A4/A1-A3 sera exhibited significantly higher levels of surface bound C3 when compared to the control sera (Supplementary Fig. 9).

Together, these results confirm that antibodies induced by CyaA-A2-A4/A1-A3 effectively bind to native PspA expressed by pneumococci and are able to promote complement deposition onto bacterial surface, while presenting better reactivity with PspA1 and PspA3 expressing strains than the previous CyaA-PspA constructions. Notably, antibody reactivity was observed across pneumococci from distinct serotypes, expressing PspA variants from all predominant clades, indicating the potential of CyaA-A2-A4/A1-A3 as a broad-coverage vaccine candidate.

Immunization with CyaA-A2-A4/A1-A3 confers protection against pneumococci from different serotypes and expressing different PspAs

To evaluate protection, CyaA-A2-A4/A1-A3 immunized mice were challenged with the pneumococcal strains EF3030 (serotype 19F, PspA1), TIGR4 (serotype 4, PspA3) or ATCC6303 (serotype 3, PspA5) and lungs and BALF were collected 12 h or 24 h post-challenge for bacterial quantification. CyaA-OVA21 immunized mice were used as controls. Challenge with the EF3030 strain was performed with two independent sets of animals (n = 5 per group) due to the variability in the colonization by this strain. Although we did not observe a significant reduction in CFU in BALF at 24 h post-challenge, significantly lower numbers of bacteria were observed in the lungs of CyaA-A2-A4/A1-A3 immunized animals in comparison to the control (~ 10-fold reduction), with some mice presenting undetectable CFU (Fig. 4A). Significant levels of anti-PspA1 IgG were also detected in BALF after the challenge (Fig. 4B). In the challenge with the TIGR4 strain (n = 6 mice per group), CyaA-A2-A4/A1-A3 immunized animals presented significantly lower numbers of bacteria at 24 h post-challenge in both lungs and BALF compared to the control (~ 10-fold reduction) (Fig. 4C), along with significantly higher levels of anti-PspA3 IgG in the LRT (Fig. 4D). Mice challenged with the ATCC6303 strain were analyzed at 12 h or 24 h after inoculation (n = 5 per group, per time point). For CyaA-A2-A4/A1-A3 immunized animals, a significant reduction in bacterial numbers in lungs and BALF was already observed at 12 h post-challenge with further decrease at 24 h post challenge (~ 10-fold and ~ 100-fold reduction in comparison to the control groups, respectively) (Fig. 4E). Significant levels of anti-PspA5 IgG were already detectable at the LRT of CyaA-A2-A4/A1-A3 immunized mice before challenge (0 h, n = 5 per group) and further increased after infection (Fig. 4F). Anti-PspA IgA was also evaluated in LRT of these mice, but a weak increase in absorbances was observed only at 12h or 24h- post-challenge with the different pneumococcal strains, at 1:2 dilutions of the BALF samples (Supplementary Fig. 10). Finally, we confirmed the protective potential of CyaA-A2-A4/A1-A3 against IPD by analyzing survival for 10 days after ATCC6303 challenge: while control animals succumbed within 3 days, the immunized group presented 100% protection (n = 6 mice per group) (Fig. 4G).

CyaA-A2-A4/A1-A3 also protects mice against serotype 3 strains circulating in the post-PCV period

So far, we have evaluated antibody reactivity and protection against laboratory pneumococcal strains or clinical isolates obtained in Brazil before the introduction of PCVs. To assess the potential protective effect of CyaA-A2-A4/A1-A3 against pneumococci circulating after the introduction of PCV13, we analyzed serotype 3 isolates from human carriage samples, collected in the United Kingdom between 2012 and 2021. Isolates from clonal complex 180 (CC180) clades I-a and II, highly associated with currently circulating serotype 3 pneumococci, were included in the analysis. *In vitro* antibody binding assays were performed using pooled sera from animals immunized with CyaA-A2-A4/A1-A3 or CyaA-OVA21 as control. For all isolates tested, CyaA-A2-A4/A1-A3 sera exhibited high binding activity, producing MFIs 30-fold to 100-fold higher than those observed for the control group (Fig. 5A). Next, immunized animals were challenged with the LIV012-S3 isolate (serotype 3, sequence type 1377, PspA1) (n = 5 for CyaA-OVA21 or 6 for CyaA-A2-A4/A1-A3), or the LIV006-S3 isolate (serotype 3, CC180 clade I-a, PspA3) (n = 6 per group) to analyze survival following IPD. While CyaA-OVA21 control groups presented low rates of survival (below 20%), CyaA-A2-A4/A1-A3 immunized mice were significantly protected in both challenges, with 100% and 83% survival for LIV012 and LIV006, respectively (Fig. 5B, C). In accordance with the results of the challenge, CyaA-A2-A4/A1-A3 sera produced significant levels of IgG binding and C3 deposition onto the surface of both strains (Supplementary Fig. 11).

Immunization with CyaA-A2-A4/A1-A3 combined with AddaS03 induces long lasting antibody responses and protection against IPD

To optimize the vaccination protocol, we evaluated whether the inclusion of the AddaS03 adjuvant (InvivoGen), an oil-in-water emulsion, could enhance immunogenicity of CyaA-A2-A4/A1-A3, allowing the reduction of the number of doses while maintaining a high level of protection. Mice received one s.c. dose of CyaA-A2-A4/A1-A3 alone or adjuvanted with AddaS03; control animals received CyaA-OVA21 combined with AddaS03. Two weeks after immunization, total anti-PspA IgG levels were quantified. As expected, a single dose of non-adjuvanted CyaA-A2-A4/A1-A4 did not induce significant antibody response (Fig. 6A-E). In contrast, mice immunized with one dose of CyaA-A2-A4/A1-A3 + AddaS03 showed significant levels of specific antibodies to PspA1, PspA2, PspA3 and PspA4 (mean titers between 10^2 and 10^3) and anti-A IgG (mean titers ~ 10^3) (Fig. 6A-E), with balanced IgG1/IgG2a induction (ratio ~ 1 log) (Fig. 6F). One dose of CyaA-A2-A4/A1-A3 + AddaS03 also induced significant levels of circulating IgA, although some variability among the animals were observed (Supplementary Fig. 12). Three weeks after immunization, mice were intranasally challenged with the invasive strain 3JYP2670 (serotype 3, PspA4) and survival was monitored for 10 days. In line with the absence of anti-PspA antibody induction, mice immunized with one dose of non-adjuvanted CyaA-A2-A4/A1-A3 were not effectively protected in the challenge, with only 33% survival in the group (comparable to the survival of the CyaA-OVA21 + AddaS03 group, 17%) (Fig. 6G). On the other hand, the group immunized with one dose of CyaA-A2-A4/A1-A3 + AddaS03 was fully protected, with 100% survival (significantly superior to the two other groups) (Fig. 6G). In a second independent experiment, animals immunized with CyaA-A2-A4/A1-A3 + AddaS03 were challenged with the same strain and scored for symptoms of disease, from 1

(healthy) to 4 (critical), according to previously described criteria[24]. Within three days post-challenge, all CyaA-OVA21 inoculated animals presented progressive disease symptoms and were euthanized when reached score 4 (Supplementary Fig. 13A, C). In the meanwhile, CyaA-A2-A4/A1-A3 + AddaS03 immunized animals were asymptomatic or presented mild symptoms within the first 48 hours but fully recovered after this period (Supplementary Fig. 13B, C). As in the 5th day post-challenge, all immunized animals were healthy and active, lungs were collected for bacterial and antibody quantification. Bacteria were not detected (Supplementary Fig. 13D), while high titers of anti-PspA4 IgG were found in the LRT of mice immunized with CyaA-A2-A4/A1-A3 + AddaS03 (Supplementary Fig. 13E).

Next, to evaluate the longevity of antibody responses and protection, mice were immunized with one or two doses of CyaA-A2-A4/A1-A3 + AddaS03, or received CyaA-OVA21 + AddaS03 as control. Sera were collected monthly for 6 months post-immunization (mpi) to evaluate anti-A IgG levels. Both protocols of one or two doses of CyaA-A2-A4/A1-A3 + AddaS03 induced significantly high levels of antibodies which were sustained with no significant variation throughout the analyzed period (mean titers ~ 10^4 and ~ 10^5, respectively) (Fig. 7A). Notably, the two-dose protocol induced significantly higher anti-A IgG levels than the single-dose schedule. Sera collected at 1 and 6 mpi were analyzed for the levels of specific antibody reactivity with PspAs from clades 1 to 4, anti-A IgG1/IgG2a ratios and anti-A IgA, and presented similar results (Supplementary Fig. 14). At 6 mpi, mice were challenged with the 3JYP2670 strain. As observed for control groups, mice immunized with a single dose of CyaA-A2-A4/A1-A3 + AddaS03 presented progressive symptoms of disease (Supplementary Fig. 15A-C) and succumbed within 6 days after challenge (Fig. 7B). On the other hand, animals immunized with CyaA-A2-A4/A1-A3 + AddaS03 in the two-dose schedule were significantly protected (Fig. 7B), with 66% of mice presenting transitory disease symptoms (Supplementary Fig. 15D**)**, and low or no detectable bacterial numbers in the lungs at the 12th day after challenge (Fig. 7C). The *in vitro* activity of sera collected at 1 or 6 months after immunization with CyaA-A2-A4/A1-A3 + AddaS03 were compared. No reduction in antibody binding and complement deposition on the surface of the 3JYP2670 pneumococcal strain, used in the challenge, was observed in the 6 mpi sera (Supplementary Fig. 16).

Discussion

Here, we have studied the protective potential of a pneumococcal vaccine candidate based on the PspA antigen, using the CyaA toxoid as an antigen delivery system. CyaA has been explored as a vaccine vector due to its capacity to target $CD11b^+$ antigen presenting cells[25]. As CyaA is able to deliver its N-terminal AC domain into the cytosol of these cells, grafted antigens are internalized and degraded by the proteasome into peptides that are presented via MHC class I pathway to $CD8^+$ T cells[26]. CyaA can also be internalized by endocytosis after binding to CD11b[27], which leads to proteolytic processing within lysosomes and, therefore, antigen-derived peptides are presented through the MHC class II pathway to $CD4^+$ T cells[28]. The system has been tested for the presentation of antigens or derived epitopes from different pathogens, such as the lymphocytic choriomeningitis virus nucleoprotein, *Escherichia coli* MalE protein, the E7 oncoprotein from HPV and the Tat protein from HIV, and has been shown to effectively

induce antigen-specific T cellular responses and antibodies, as reviewed by Chenal and Ladant[28]. In our previous study, immunization with PspA derived A fragments alone failed to elicit significant levels of antibodies, likely due to their small molecular size (12 to 18 kDa); on the other hand, immunization with the CyaA-PspA proteins induced high levels of anti-PspA antibodies in the absence of adjuvants[23], confirming the system's efficacy to improve antigen-specific humoral responses. Here, we further show that a single CyaA antigen is able to induce robust humoral responses against multiple PspA fragments.

Most studies with PspA-based vaccines have targeted the α-helical domain of the protein, as it contains key protective epitopes[29, 30]. Since this region presents antigenic variability, especially within the CDR[16], one approach that has been explored is targeting conserved sequences within the α-helical domain. In fact, Roche *et al*[30] and Vadesilho *et al*.[31] showed that immunization with recombinant fragments comprising the beginning of the α-helical domain confer protection against pneumococcal challenges, when formulated with adjuvants. Although targeting more cross-reactive regions is a good strategy, our preliminary experiments using CyaA constructs containing such regions did not yield optimal immunogenicity or protection (unpublished data). Instead, our best results were obtained with the A fragment, which encompasses the CDR and the beginning of the proline-rich domain. In our previous work, we showed that immunization with the CyaA proteins containing the A fragment of PspAs from clades 2 and 4 conferred effective protection against pneumococci expressing homologous PspAs and, yet, against a strain expressing PspA5. However, we identified a limitation regarding the potential coverage of pneumococcal isolates, as anti-A2 and anti-A4 antibodies were not cross-reactive with strains expressing PspA1 and levels of reactivity against pneumococci expressing PspA3 varied depending on the strain tested[23]. Here, we designed new CyaA-PspA antigens aiming to improve the spectrum of antibody reactivity by including the A fragment of PspAs from clades 1 and 3. From the new candidates, we selected CyaA-A2-A4/A1-A3, a single recombinant protein able to induce robust antibody responses against the four grafted PspA fragments. We have shown that the inclusion of A1 and A3 was a successful approach, as antibodies induced by CyaA-A2-A4/A1-A3 presented high levels of reactivity with multiple strains expressing PspA variants from clades 1 to 5. We have further demonstrated that CyaA-A2-A4/A1-A3 immunized mice were protected in challenges performed with pneumococcal strains from different serotypes, expressing PspA variants from clades 1, 3, 4 and 5, indicating the potential of CyaA-A2-A4/A1-A3 as a broad-coverage vaccine candidate.

We had previously reported that immunization with CyaA-A2-A4 conferred effective protection to mice against IPD[23]. Here, we have shown that both CyaA-A2-A4 and CyaA-A2-A4/A1-A3 also promote protective responses in the LRT. We observed that immunized animals present reduced bacterial loads in the lungs and BALF as early as 12 h and 24 h post-challenge, demonstrating a rapid control of infection. In CyaA-A2-A4/A1-A3 immunized mice, anti-PspA IgG were observed in the LRT even in the absence of infection. As parenteral immunization does not typically induce effective humoral responses at mucosal surfaces, we speculate that the high levels of circulating IgG elicited by immunization may extravasate to LRT surface. Conversely, no significant levels of anti-PspA IgA were observed in the LRT. We have also found that CyaA-A2-A4 immunized animals present reduced LRT local inflammation at 12 h after

challenge in comparison to control groups, as observed by the lower levels of neutrophils and pro-inflammatory cytokines TNF-α and IL-6. As these parameters were assessed only at 12 h post-challenge, it was not possible to evaluate whether CyaA-A2-A4 immunized mice presented an overall reduction in inflammation from the initial stages of infection, or experienced an earlier inflammatory peak followed by rapid resolution in comparison to the other groups. Either way, it is possible to speculate that early control of bacterial numbers may limit the signaling for production of pro-inflammatory cytokines, as well as the availability of pneumococcal components that sustain inflammation. Future studies will explore this aspect.

When comparing the protective potential of CyaA-A2-A4 with the PCV13 vaccine, the formulations conferred equivalent protection to mice against IPD. Given the well-documented efficacy of PCVs against IPD in humans, this result highlights the potential of the CyaA-PspA formulations. Notably, in the LRT infection model, CyaA-A2-A4 showed superior efficacy by promoting early control of bacterial numbers. The lack of protection afforded by PCV13 in this context may be attributed to distinct factors. PCV-induced immunity relies on antibodies targeting capsular polysaccharides[32], which were undetectable in the LRT 12 h post-challenge. It is possible that a reduction in bacterial numbers might be observed at later time points following the presence of antibodies in the LRT. On the other hand, some studies have indicated that the efficacy of PCVs against LRT infection may be lower compared to that observed for IPD and nasal colonization, as reviewed by Loo *et al*.[33]. Another key factor is that the challenge was performed with the 3JYP2670 strain, which expresses a serotype 3 capsule. Despite its inclusion in the vaccine, it has been proposed that serotype 3 evades PCV13 immunity[33]. One of the mechanisms proposed by Luck and colleagues to explain this vaccine escape is the ability of serotype 3 strains to shed the capsule into the extracellular environment; this activity removes anti-capsular antibodies from the bacterial surface, impairing opsonophagocytosis and promoting bacterial persistence[34]. These findings reinforce the need for complementary vaccine strategies.

Pneumococcal serotype 3 draws considerable concern as its incidence in diseases and deaths remains high in the post-PCV13 period[35]. Besides the hypothesis of antibody displacement through capsule shedding, it has been proposed that PCV13-immunity may not be sufficient to reduce serotype 3 nasopharyngeal carriage, sustaining the circulation of this serotype in the population and its dissemination to individuals more susceptible to pneumococcal diseases[36]. Genetic diversification has also emerged as an important factor driving its persistence in carriage and disease. The major clonal complex currently associated with serotype 3 is CC180, which was classified into clade I (subclades I-a and I-b) and clade II[35]. A global genomic analysis of serotype 3 isolates conducted by Azarian *et al*. revealed a growing prevalence in carriage of CC180 clade II isolates after PCV13 introduction. It was also reported that clade II strains show higher antibiotic resistance, likely driving its expansion[35]. Here, we have shown that antibodies from CyaA-A2-A4/A1-A3 immunized mice presented high levels of reactivity with serotype 3 pneumococci isolated from human carriage, collected in the United Kingdom after the introduction of PCV13. Importantly, reactivity was observed with CC180 clade Ia and clade II isolates. We have established an IPD challenge model using two carriage isolates (including one from

CC180 clade I-a) and showed that CyaA-A2-A4/A1-A3 protected mice in both challenges. Together, these findings support the CyaA-A2-A4/A1-A3 potential for targeting serotype 3 pneumococci circulating after the introduction of PCVs.

Furthermore, we have analyzed the protective potential of CyaA-A2-A4/A1-A3 combined with AddaS03 as adjuvant using an immunization protocol with reduced number of doses. Using two independent sets of animals, we observed that a single immunization dose of this combination conferred full protection against IPD to mice challenged three weeks after immunization. We have also shown that mice immunized with either one or two doses of CyaA-A2-A4/A1-A3 + AddaS03 presented robust levels of anti-PspA antibodies, which were sustained with no significant variation for 6 months. However, when mice were challenged at 6 mpi, the single dose schedule was not sufficient to protect mice against IPD. We did not detect any significant differences in circulating IgG and IgA titers, the balance of specific anti-PspA antibodies, in the balance of IgG subclasses or in the efficacy of antibody binding and complement deposition to the pneumococcal surface between sera collected at 1 mpi and 6 mpi that could be attributed to the lack of protection. T-cell mediated immunity, including Th17 and Th1 responses, as well as memory cell populations have been implicated in naturally acquired and/or vaccine-induced protection against IPD or carriage[37, 38, 39]. A limitation of our work is that we did not evaluate adaptative cellular responses and induction of immunological memory, but it is possible that the single dose schedule failed to elicit effective T cell immunity and memory responses, which may have contributed to the lack of protection at 6 mpi. An additional factor to be considered is the age of mice by the time they were challenged. In most experiments, in which short-term protection was evaluated, mice were challenged with 3 months of age whereas in the long-term protection experiment mice were challenged with 9 months of age. As reported by Boyd and collaborators, differences in innate immune responses that lead to age-enhanced susceptibility of mice to pneumococcal pneumonia begins in midlife[40]. Regardless, mice that received two doses of CyaA-A2-A4/A1-A3 + AddaS03 were significantly protected in the 6 mpi challenge, indicating that the two-dose immunization schedule may be a better strategy to induce long-lasting effective protection.

In summary, our results support the proposition of the CyaA-A2-A4/A1-A3 antigen as a candidate for a complementary vaccine to confer broad-coverage and improved protection against pneumococcal disease. Notably, a single-antigen protein-based vaccine offers advantages in terms of manufacturing and potentially reduced costs, facilitating its implementation in low-income countries. Ou study focused on the humoral immune responses elicited by the CyaA-A2-A4-/A1-A3 vaccine, as anti-PspA antibodies were previously shown to be determinant for protection against IPD in animal models [15, 41]. However, a direct role of these antibodies in protection induced by the CyaA-A2-A4-/A1-A3 vaccine remains to be determined. The CyaA vector has been previously evaluated in clinical trials – for the study of CyaA proteins containing HPV E7 epitopes in a therapeutic approach for tumor regression in HPV-infected woman (GTL0001) – demonstrating safety and immunogenicity, with no interference from pre-existing immunity to CyaA[42]. Our results showed that despite the induction of anti-CyaA-OVA21 antibodies in mice, increasing levels of anti-A antibodies were observed. However, a possible interference of

antibodies against *B. pertussis* adenylate cyclase toxin, induced by pertussis infection or previous pertussis vaccination, in the efficacy of CyaA-PspA vaccines, will have to be evaluated. Another drawback of CyaA-based vaccines is the need to store proteins in 8 M urea. On-site dilution right before application in humans was used for GTL0001 vaccine and this will have to be considered in the potential development of CyaA-PspA vaccines. Nevertheless, our results support further studies on CyaA-PspA vaccines. These formulations could be used in combination with current PCV schedules in children, as well as be used as a stand-alone strategy for adults and the elderly.

Methods

Ethical statement

Animal experimentation was carried out in accordance with the guidelines established by the Brazilian National Council for the Control of Animal Experimentation (CONCEA), which adheres to international standards for animal welfare (ARRIVE Guidelines) and the principles of 3Rs. Experimental protocols were reviewed and approved by the Ethics Committee on Animal Use of Instituto Butantan (protocols 5370090919 and 5319280224). Mice were maintained in a biosafety level 2 animal housing facility, in individually ventilated microisolators (maximum of 6 animals per cage), under controlled temperature and light cycles. Animals had unrestricted access to food and water and were monitored daily.

Bacterial strains and growth conditions

Escherichia coli strains XL1-Blue and DH5-α were used for DNA manipulation. Expression of recombinant proteins was performed with *E. coli* strains BLR and BL21(DE3). Bacteria were cultured at 30 or 37°C in Luria-Bertani broth (Difco, Beckton Dickinson, USA) under agitation or grown in solid LB-agar. Media were supplemented with 100 µg/mL ampicillin or 30 µg/mL chloramphenicol. *S. pneumoniae* strains (described in Table 1) were cultured on Triptic soy agar containing 5% defibrinated sheep blood (blood agar, Laborclin, Brazil) at 37°C, under microaerophilic conditions, for 18 h. For *in vitro* assays, bacteria were transferred from blood agar to liquid Todd-Hewitt media (Difco) supplemented with 0.5% yeast extract (THY) and cultured at 37°C, without agitation. Stocks for animal challenge were cultured in the same way until exponential phase (OD_{600nm} = 0.4), centrifuged at 3800 rpm for 20 minutes, and suspended in 1/10 of the initial volume in THY containing 20% glycerol. Stocks were maintained at -80°C and quantified by plating on blood agar.

Recombinant PspAs and derived A fragments

PspA recombinant proteins and derived A fragments were used for ELISA and western blot assays. Recombinant PspAs from clades 1, 2, 3, 4 and 5 comprise the α-helical domain and the beginning of the proline rich domain of each molecule (N-terminal region); their expression and purification have been described before[21]. The A fragments comprise the CDR and the beginning of the proline-rich domain of PspA molecules as follows: A1 is composed of amino acids 184 to 309 of PspA1 (pneumococcal strain

435/96, GenBank accession number AY082387); A2 comprises amino acids 229 to 328 of PspA2 (pneumococcal strain Rx1, GenBank accession number M74122.1); A3 is composed of amino acids 203 to 353 of PspA3 (pneumococcal strain 259/98 strain, GenBank accession number AY082389); A4 contains amino acids 205 to 395 of PspA4 (pneumococcal strain 255/00, GenBank accession number EF649969.1). The expression and purification of A2 and A4 were previously described[23]. For expression of A1 and A3, the corresponding nucleotide sequences were amplified by PCR using the pAE-*pspA1*[21] and pAE-*pspA3*[43] plasmids, respectively, as templates and specific primers described in Supplementary Table 1. Fragments were cloned into the Bam HI and Hind III sites of the pAE vector[44], generating pAE-*a1* and pAE-*a3*, used for transformation of BL21 Star pLyS bacteria. A1 and A3 were expressed and purified through affinity chromatography using Ni^{2+} loaded columns, as previously described for A2 and A4[23].

Construction of plasmids for production of recombinant proteins CyaA-A1-A3, CyaA-OVA/A1-A3 and CyaA-A2-A4-A1-A3

PspA fragments A1 and A3 (Supplementary Fig. 2 A, B) were grafted in fusion between amino acids 224 and 225 of CyaA AC domain, generating CyaA-A1-A3 (Supplementary Fig. 2C), or between amino acids 319 and 320 of CyaA-OVA21 AC domain, producing CyaA-OVA/A1-A3 (Supplementary Fig. 2D). PspA fragments A2, A4, A1 and A3 were combined in fusion between amino acids 224 and 225 of CyaA AC domain to generate CyaA-A2-A4-A1-A3 (Supplementary Fig. 2F). Plasmids for the expression of the recombinant proteins were obtained using the synthetic DNAs (Twist Bioscience) described in Supplementary Table 2, designed for cloning of *a1* and *a3* into the desired positions of the parental plasmids by Gibson assembly[45]. The pCACTE5-OVA21 vector[46] was digested with Nhe I and Kpn I and combined to synthetic DNA III, resulting in the removal of the OVA sequence and insertion of *a1*, yielding the intermediate construct pCACTE5-*a1*. This plasmid was subsequently digested with Acc65I and combined to synthetic DNA IV for cloning of *a3* downstream of *a1*, resulting in the pCACT-*a1-a3* plasmid, used for expression of CyaA-A1-A3. Alternatively, the *a1* sequence was cloned into the BshTI restriction site of pCACTE5-OVA21 using synthetic DNA I, producing the intermediate construct pCACTE5-OVA/*a1*. This plasmid was then digested at the BcuI site and combined with synthetic DNA II, resulting in the insertion of *a3* downstream of *a1* and production of the pCACTE5-OVA/*a1-a3* plasmid (used for expression of CyaA-OVA/A1-A3). Finally, for production of CyaA-A2-A4-A1-A3, we constructed the pCACTE5-*a2-a4-a1-a3* plasmid. The *a1-a3* sequence was amplified by PCR using the pCACTE5-OVA/*a1-a3* plasmid as template and the primers 5'-AAAAACCAGCTCCAGCTCCAGGTACCCTGAAAGAAATCGACGAATCTGACTCT-3' (Acc65I restriction site underlined) and 5'-TGGCCTCGCTGGCGGCGCGGTGCCGGAGCAGGTTGCTCGGGCTTT-3'. The PCR product was cloned downstream of *a4* in the pCACTE5-*a2-a4* vector[23] digested with Acc65I, yielding the pCACTE5-A2-A4-A1-A3 construct. All enzymes used for digestion reactions were obtained from Thermo Fisher (USA). Gibson assembly reactions were performed using the NEBuilder HiFi DNA Assembly Kit (New England Biolabs, USA), according to manufacturer's instructions. For all plasmid constructions, the appropriate insertion of *a1* or *a3* was confirmed by nucleotide sequencing (Eurofins Scientific, France).

CyaA-PspA recombinant proteins

CyaA-PspA antigens were produced by genetically grafting the A fragments in the AC domain of a detoxified CyaA variant (produced by the insertion of a dipeptide in the adenylate cyclase catalytic site)[47]. The construction of the pCACTE5-*a2-a4* plasmid for expression of CyaA-A2-A4 was previously described[23]. For production of CyaA-A2-A4/A1-A3 (containing the A2-A4 fusion between amino acids 224 and 225 and the A1-A3 fusion between amino acids 319 and 320 of the AC domain), the pCACTE5-*a2-a4/a1-a3* plasmid was constructed using pCACTE5-*a2-a4* as the parental plasmid and synthetic DNAs (obtained from Twist Bioscience, USA) (Supplementary Table 2) designed for *a1* or *a3* cloning by Gibson assembly[45]. First, the a1 sequence was cloned into the BshT I restriction site of pCACTE5-*a2-a4* (using synthetic DNA I) resulting in the intermediate construct pCACTE5-*a2-a4/a1*. Next, the *a3* sequence was cloned into the Bcu I restriction site of pCACTE5-*a2-a4/a1* (using synthetic DNA II). Cloning reactions were performed using the HiFi DNA assembly kit (NEBuilder, USA), according to manufacturer's instructions. For both plasmid constructions, the appropriate insertion of *a1* or *a3* was confirmed by nucleotide sequencing (Eurofins Scientific, France). The construction of plasmids for expression of additional CyaA proteins containing A1 and A3 tested in this work is described in the Supplementary Material. CyaA-PspA proteins were expressed in BLR bacteria and purified as previously described for the CyaA-A2-A4 protein[23]. Briefly, recombinant proteins were purified from inclusion bodies solubilized in 8 M urea HEPES-buffer using anion exchange chromatography on a Q-Sepharose resin (Cytiva, USA), followed by hydrophobic interaction chromatography on a Phenyl-Sepharose resin (Cytiva, USA) with an additional wash with isopropanol to remove endotoxins[23, 46]. The CyaA-OVA21 protein, which contains an ovalbumin derived peptide, was obtained under the same conditions using BLR cells transformed with the pCACTE5-CyaA-OVA21 plasmid[46]. The predicted molecular mass and molecular extinction coefficient of each protein were calculated from its amino acid sequence using the ProtParam tool (http://www.expasy.org/tools/protparam.html). Concentration of purified proteins was determined by measuring absorbance at 278 nm using a DS-7 spectrophotometer (DeNovix, USA) and applying the molecular extinction coefficient of each protein. Proteins were maintained in 8 M urea HEPES-buffer until use. The presence of residual endotoxin in the protein preparations was measured using the Quantitative Chromogenic Limulus Amebocyte Lysate assay (QCL-1000 kit, Lonza, USA), following the manufacturer's protocol. Endotoxin levels in the final formulations administered to the animals were confirmed to be below 1 EU/mL. Purified proteins were analyzed by SDS-PAGE and Coomassie Blue staining. CyaA-A2-A4/A1-A3 was analyzed by western blot using mice polyclonal anti-A1, anti-A2, anti-A3 or anti-A4 sera (produced in the laboratory; 1:1000 dilution) and Horseradish Peroxidase (HRP)-conjugated anti-mouse IgG (Sigma-Aldrich, USA). The A fragments and recombinant proteins from clades 1 to 4 were used as positive controls and CyaA-OVA21 was used as a negative control. Reactions were developed using Amersham ECL Prime (Cytiva, USA) and visualized using the Amersham ImageQuant 800 system (Cytiva, USA).

Immunization of mice

Female BALB/c mice, aged 6 to 8 weeks, were obtained from animal facilities of the Medical School of University of São Paulo or the University of Campinas (Brazil). Mice (n = 5 to 12 per group, depending on the experiment) were immunized subcutaneously with formulations prepared by diluting the antigens in endotoxin-free saline to a final volume of 100 µL per dose. CyaA-PspA proteins were administered in doses of 10 µg; control animals received 10 µg of CyaA-OVA21. In the non-adjuvanted immunization schedule, animals received three doses administered at 15-day intervals. When CyaA-A2-A4/A1-A3 or CyaA-OVA21 was combined with AddaS03 (InvivoGen, USA) as adjuvant, animals received either 1 dose, or 2 doses at a 15-day interval. The proportion of antigen: adjuvant was 1:1 (V/V), as recommended by the manufacturer. For immunization with PCV13 (PREVNAR 13, Pfizer, USA), mice received 1/10 of the human dose administered in 3 s.c. doses, 15 days apart; as control, a group of animals received 50 µg of aluminum hydroxide (Alum).

Sera collection and analysis of antibody induction

Blood samples were collected via the retro-orbital plexus under local anesthesia with proxymetacaine chloride (Alcon, USA), either 14 days after the last immunization dose or, to analyze the longevity of antibody responses, every 30 days for 6 months after immunization. Sera were analyzed by ELISA. High-binding 96-well polystyrene plates (Costar) were coated with either 1 µg/mL of an equimolar mixture of PspA fragments A1, A2, A3, and A4 or with 1 µg/mL of recombinant PspA1, PspA2, PspA3, PspA4 or PspA5 proteins to evaluate specific IgG and IgA reactivity. Sera were added in serial dilutions starting at 1:20. HRP-conjugated anti-mouse IgG (Sigma-Aldrich, USA) or goat anti-mouse IgA, goat-anti-mouse IgG1 and IgG2a, followed by HRP-conjugated anti-goat IgG (Southern Biotech, USA), were used as secondary antibodies, as previously described[23]. Alternatively, ELISA was performed to evaluate antibody responses against the purified capsular polysaccharides from a serotype 3 pneumococcal strain (PS3) (American Type Culture Collection, USA), following previously established protocols[48]. Sera were serially diluted, starting at 1:20, and pre-incubated with 5 µg/mL purified PS from a serotype 22F pneumococcal strain (American Type Culture Collection, USA) to minimize non-specific binding. Samples were then applied to 96-well polystyrene MICROLON 200 medium-binding microplates (Greiner Bio-One) pre-coated with 5 µg/mL of PS3. HRP-conjugated anti-mouse IgG (Sigma-Aldrich, USA) was used as secondary antibody. For all assays, the enzymatic reaction was developed using o-phenylenediamine dihydrochloride as the chromogenic substrate and absorbance was measured at 492 nm using the EZ Read 400 microplate reader (Biochrom, USA). Antibody titers were determined as the reciprocal of the highest serum dilution that produced an absorbance value ≥ 0.1.

In vitro assays of antibody binding and complement deposition to pneumococcal surface

Different pneumococcal isolates (Table 1) were used in these experiments and *in vitro* assays were performed as previously described[14]. For the binding assays, sera from immunized animals were heat-inactivated, and incubated (5% v/v) with bacterial suspensions (10^8 CFU/mL). Detection was performed using FITC-conjugated anti-mouse IgG (MP Biomedicals, USA). To assess C3 deposition onto pneumococcal surface, heat-inactivated sera (10% v/v) were incubated with bacteria (10^8 CFU/mL).

Naive mouse serum (10% v/v) was used as complement source. Complement deposition was detected using FITC-conjugated anti-mouse C3 (MP Biomedicals, USA). Samples were analyzed by flow cytometry using a FACS Canto II system (BD Biosciences, USA). Forward scatter (FSC), side scatter (SSC), and FITC channel voltages were adjusted based on unstained bacterial controls. A total of 15,000 events were acquired per sample. Data were processed using FlowJo software version 10.1 (Tree Star). FITC-median fluorescence intensity (MFI) values were used to compare experimental groups.

Pneumococcal challenges

Challenges were performed three weeks after the end of the immunization protocol or, alternatively to evaluate long-term protection, six months after the last immunization. Mice were anesthetized through the intraperitoneal (i.p.) route with 100 mg/Kg of ketamine chloride and 20mg/Kg of xylazine chloride (Ceva, Brazil). Pneumococcal strains 3JYP2670 ($3x10^5$ CFU), ATCC6303 ($3x10^5$ CFU), EF3030 ($1x10^6$ CFU) or TIGR4 ($1x10^6$ CFU) were diluted in saline for a final volume of 50 µL and inoculated intranasally for delivery to the LRT. Bacterial concentration used for each strain in the challenge was based on prior studies conducted by our group[49, 50]. Pneumococcal isolates from human carriage LIV006-S3[51] and LIV012-S3[52], collected in the Liverpool School of Tropical Medicine in 2021 and 2017, respectively, were inoculated in the concentration of $3x10^5$ CFU using the same procedure. In the IPD model (sepsis), survival was analyzed for 10 days; animals were monitored twice a day and scored for clinical signs of disease according to previously described criteria[24]. Mice exhibiting critical signs of disease (score 4), such as reduced activity, hunched posture and pronounced piloerection, were immediately euthanized (i.p. injection of a lethal dose of anesthetics, 60 mg/kg xylazine chloride and 300 mg/kg ketamine chloride). Mice that survived were healthy and active (score 1) by the end of the analyzed period. In some experiments, lungs were collected at the 5th or 12th day post challenge and macerates (100 µL) were plated on blood agar plates supplemented with 4 µg/mL gentamicin for CFU counting (limit of detection ≥ 10 CFU). In the LRT infection model, mice were euthanized 12 h or 24 h after challenge for the collection of lungs and BALF, as previously described (14). Bacterial loads were analyzed by plating 10 µL of the samples and their serial dilutions in blood agar plates supplemented gentamicin for CFU counting (limit of detection ≥ 100 CFU).

Quantification of macrophages, neutrophils, antibodies and cytokines in BALF

BALF samples were centrifuged at 1200 rpm, at 8°C, for 10 minutes. Pelleted cells were incubated with a mixture of anti-mouse F4/80-PE (clone BM8), anti-mouse CD11c-PerCP-Cy5.5 (clone N418), anti-CD11b-BV421 (clone M1/70) and anti-Ly6G-APC-Cy7 (clone 1A8) antibodies (BD Biosciences, USA), each at 1% (v/v), for 30 minutes on ice. Samples were fixed with BD Cytofix solution (BD Biosciences) and analyzed by flow cytometry in a FACS Canto II equipment (BD Biosciences), with 20,000 events recorded, excluding debris, bacteria and cell aggregates. Unstained BALF cells were used to define FSC, SSC and fluorescence channel voltages. Data were analyzed in FlowJo V10.1 software (Tree Star) using Fluoresce-Minus-One controls to define positive and negative populations to each cell marker. Alveolar macrophages were evaluated as the $F4/80^+CD11b^-CD11c^+$ population, interstitial macrophages as

$F4/80^+CD11b^+CD11c^-$ cells, and neutrophils as $F4/80^-CD11b^+Ly6G^+$ cells (gating strategy is shown in Supplementary Fig. 2). The supernatant from BALF samples was analyzed for anti-PspA or anti-PS3 IgG and IgA by ELISA. Plate coating and blocking were performed as described above; BALF samples were serially diluted starting at 1:4 (IgG) or at 1:2 (IgA) and alkaline phosphatase-conjugated anti-mouse IgG or anti-mouse IgA (Southern Biotech, USA) were used as secondary antibodies. Enzymatic reaction was developed using p-nitrophenyl phosphate (Sigma-Aldrich, USA) as substrate and absorbance was measured at 405 nm using an EZ Read 400 microplate reader (Biochrom, USA). Antibody titers were defined as the reciprocal of the highest BALF dilution yielding an absorbance value ≥ 0.1. TNF-α and IL-6 concentrations in the supernatant from BALF samples were measured by ELISA using Mini TMB EDK commercial kit (PeproTech, USA), following manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 6.0 software. For antibody titers, CFU and cell counts, statistical comparisons between two groups were performed using Student’s T-test, while One-way ANOVA or Two-way ANOVA with Tukey’s post-test were applied for multiple groups comparisons. Animal survival in the IPD challenge was analyzed using the Log-rank survival curve and the Mantel-Cox test for comparison between groups. In all cases, $P \leq 0.05$ was considered significant.

Declarations

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Competing Interests

GBC, MD, ENM, DL and MLSO have filed a patent application covering the use of CyaA-PspA recombinant proteins as vaccine formulations against pneumococcal infections, under the Patent Cooperation Treaty via the World Intellectual Property Organization (application number: PCT/BR2024/050175).

Author Contribution

MLSO and DL conceptualized the study. MLSO, DL and GBC designed the study and interpreted the data with assistance from ENM. ENM and MLSO designed the PspA fragments. DL produced the parental plasmid for expression of enzymatically inactive CyaA proteins. GBC and MD produced CyaA-PspA recombinant proteins, under the supervision of DL. GBC, GSO, KC and MLSO performed animal experiments. AH, DES and DMF were responsible for the isolation and characterization of serotype 3 pneumococci from human carriage samples of adult volunteers in the United Kingdom. GBC performed all in vitro experiments. GBC and MLSO were responsible for data formal analysis and curation. MLSO and DL supervised the study and were responsible for project administration and funding acquisition. GBC and MLSO wrote the manuscript, with suggestions and assistance from all co-authors. All the authors read and accepted the final contents of the manuscript.

Data Availability

All data generated or analyzed during this study are included in this article and its supplementary information files. Raw data used to produce the figures are provided in the Dataset 1 and Dataset 2.

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Tables

Table 1

Pneumococcal isolates used in this study

Strain	Capsular serotype	PspA	Reference
ATCC6301	1	clade 1 (family 2)	49
EF3030	19F	clade 1 (family 2)	53
0603	6B	clade 1 (family 2)	49
A66.1	3	clade 2 (family 2)	49
D39	2	clade 2 (family 2)	21
M10	11A	clade 3 (family 2)	24
TIGR4	4	clade 3 (family 2)	54
HU473/06	9V	clade 3 (family 2)	54
3JYP2670	3	clade 4 (family 2)	23
255/00	14	clade 4 (family 2)	43
ATCC6303	3	clade 5 (family 2)	24
122/02	23F	clade 5 (family 2)	43
LIV004-S3*	3[a]	clade 3 (family 2)	55
LIV006-S3*	3[a]	clade 3 (family 2)	51
LIV008-S3*	3[a]	clade 3 (family 2)	51
LIV009-S3*	3[b]	clade 5 (family 2)	*
LIV011-S3*	3[b]	clade 5 (family 2)	56
LIV012-S3*	3[c]	clade 1 (family 1)	52

*Isolates from human carriage studies performed at Liverpool School of Tropical Medicine, United Kingdom (collected between 2014 and 2021). (a) Strains from clonal complex 180 (CC180) clade Ia. (b) Strains from CC180 clade II. (c) Strain from sequence type 1377

Figures

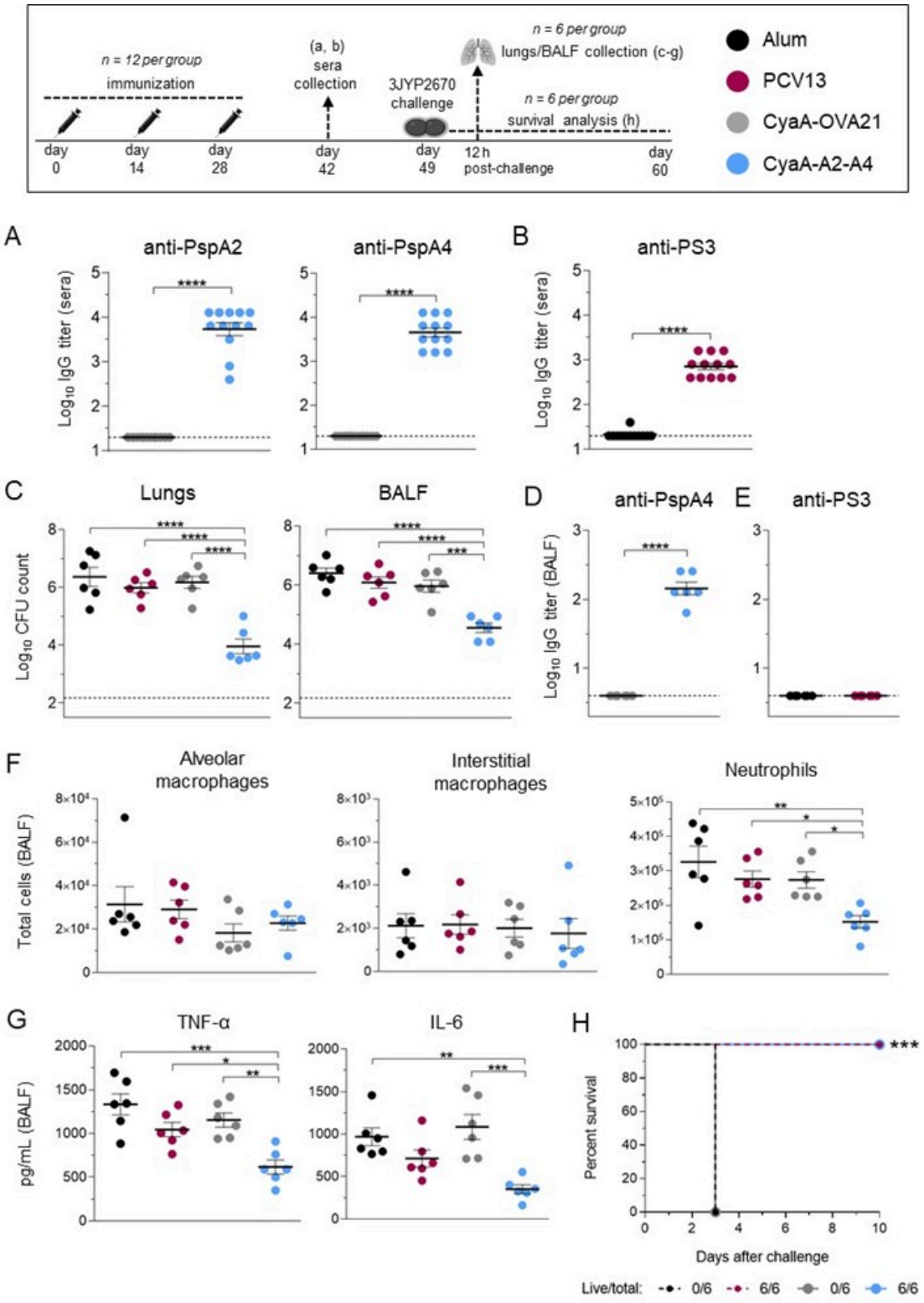


Figure 1

Mice immunized with CyaA-A2-A4 present reduced bacterial loads in the LRT at 12h after challenge.

Mice (n = 12 per group) were immunized with three s.c. doses of PCV13 or CyaA-A2-A4; control groups received Alum or CyaA-OVA21; schematic representation of immunization protocol is shown at the top of the figure. Sera collected two weeks after the third dose were analyzed for (**A**) anti-PspA2 and anti-

PspA4 or (**B**) anti-PS3 IgG titers by ELISA. Three weeks after immunization, mice were challenged with the pneumococcal strain 3JYP2670 (serotype 3, PspA4). Lungs and BALF from 6 animals from each group were collected 12 h after challenge. (**C**) Samples were plated on blood-agar for CFU counting. (**D**) Anti-PspA4 or (**E**) anti-PS3 IgG titers in BALF samples were evaluated by ELISA. (**F**) BALF cells were stained and analyzed by flow cytometry to assess populations of alveolar macrophages ($F4/80^{+}/CD11b^{-}/CD11c^{+}$), interstitial macrophages ($F4/80^{+}/CD11b^{+}/CD11c^{-}$) and neutrophils ($F4/80^{-}/CD11b^{+}/Ly6G^{+}$), with 20,000 events recorded. Cell populations were quantified using the FlowJo V10 software. (**G**) TNF-α and IL-6 concentrations in BALF were determined by ELISA. Circles represent individual data; lines represent the mean for each group ± SEM and dashed lines indicate the limit of detection. Student's T test was used to compare two groups and One-way ANOVA with Tukey's post-test was used to compare multiple groups: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. (**H**) For 6 animals from each immunization group, survival was monitored for 10 days after challenge. Protection was analyzed using the Log-Rank survival curve with Mantel-Cox post-test: ***P<0.001 (comparison with control groups).

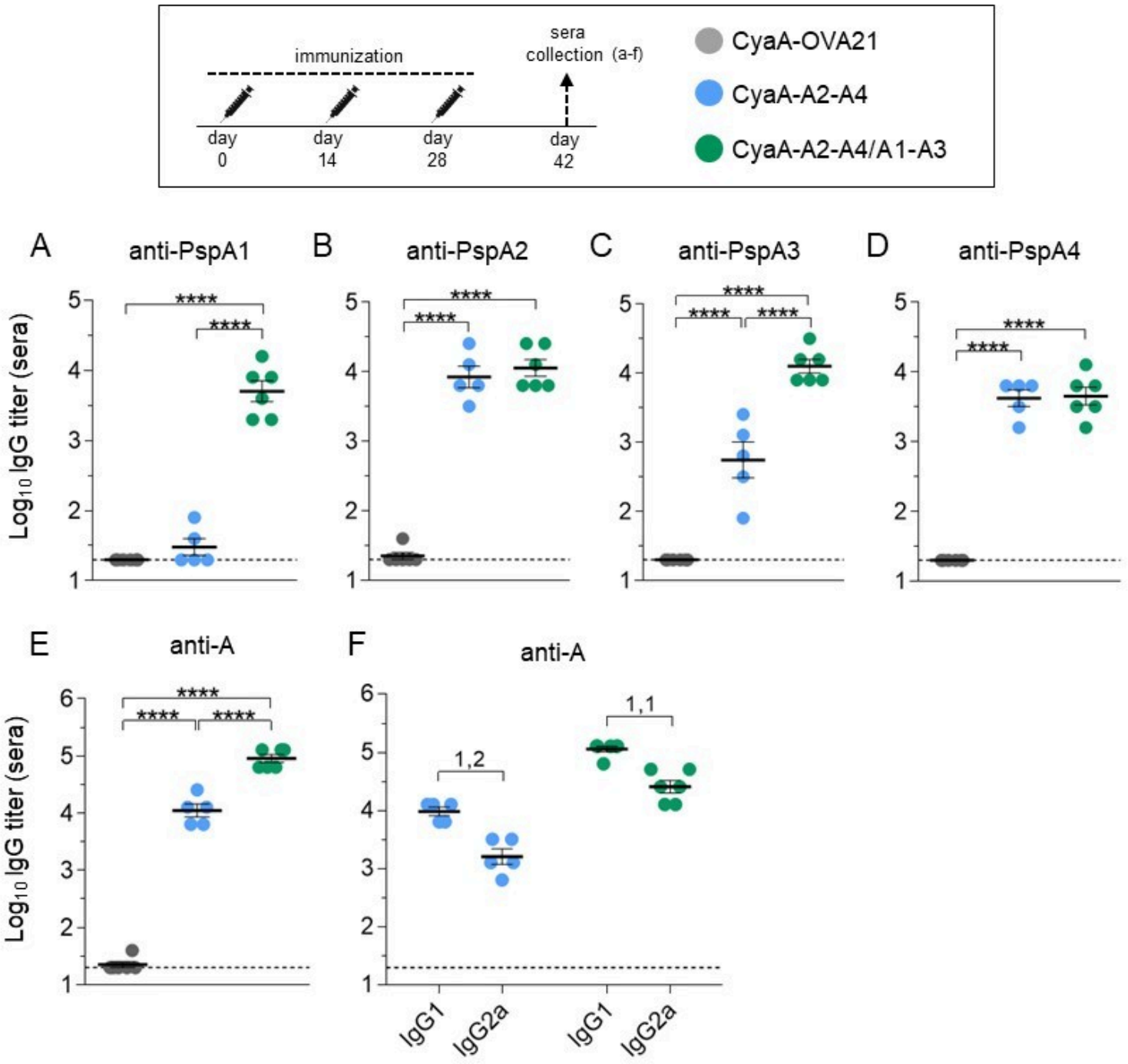


Figure 2

Immunization with CyaA-A2-A4/A1-A3 induces high levels of circulating anti-PspA IgG.

Mice (n = 5 or 6 per group) were immunized with three s.c. doses of CyaA-OVA21, CyaA-A2-A4 or CyaA-A2-A4/A1-A3; schematic representation of immunization protocol is shown at the top of the figure. Sera collected 2 weeks after the third dose were analyzed by ELISA for the levels of specific IgG against the N-terminal region of (**A**) PspA1, (**B**) PspA2, (**C**) PspA3 or (**D**) PspA4 or (**E**) for the levels of anti-A IgG. Results were analyzed by One-way ANOVA with Tukey's post-test: ****P<0.0001. (**F**) Sera were analyzed for anti-A IgG1 and IgG2a; the IgG1/IgG2a ratio is indicated in the graph. Circles represent individual

data; lines represent the mean for each group ± SEM and dashed lines indicate the limit of detection. Results are representative of four independent immunization experiments.

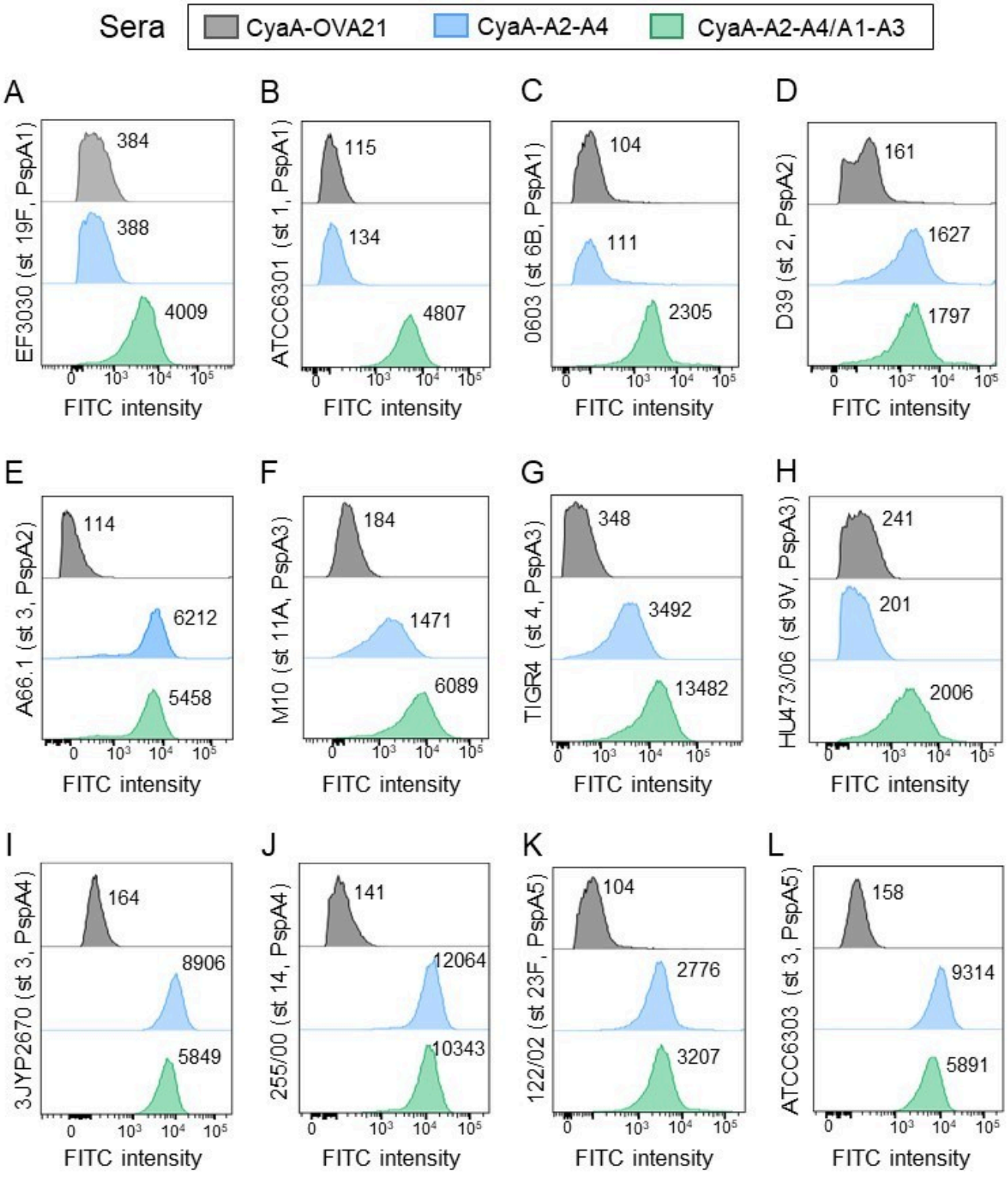
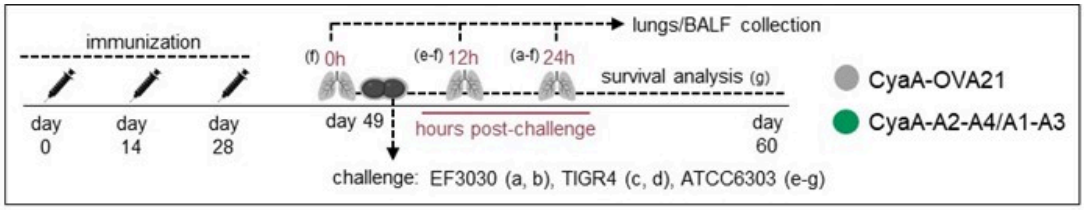


Figure 3

Sera from CyaA-A2-A4/A1-A3 immunized mice present high levels of reactivity with different pneumococcal strains.

Mice were immunized with three s.c. doses of CyaA-OVA21, CyaA-A2-A4 or CyaA-A2-A4/A1-A3 (n = 5 or 6 per group). (**A-L**) Sera collected after the third dose were pooled and incubated with the indicated pneumococcal strains. Antibody binding was detected by incubation with anti-mouse IgG-FITC. Samples were analyzed by flow cytometry, with 15,000 events recorded. Results were analyzed using the FlowJo V10 software. Numbers indicate the MFI for each histogram of FITC-fluorescence. Serotype (st) and PspA clade are indicated in each graph. Results are representative of two assays using sera from two independent immunization experiments.



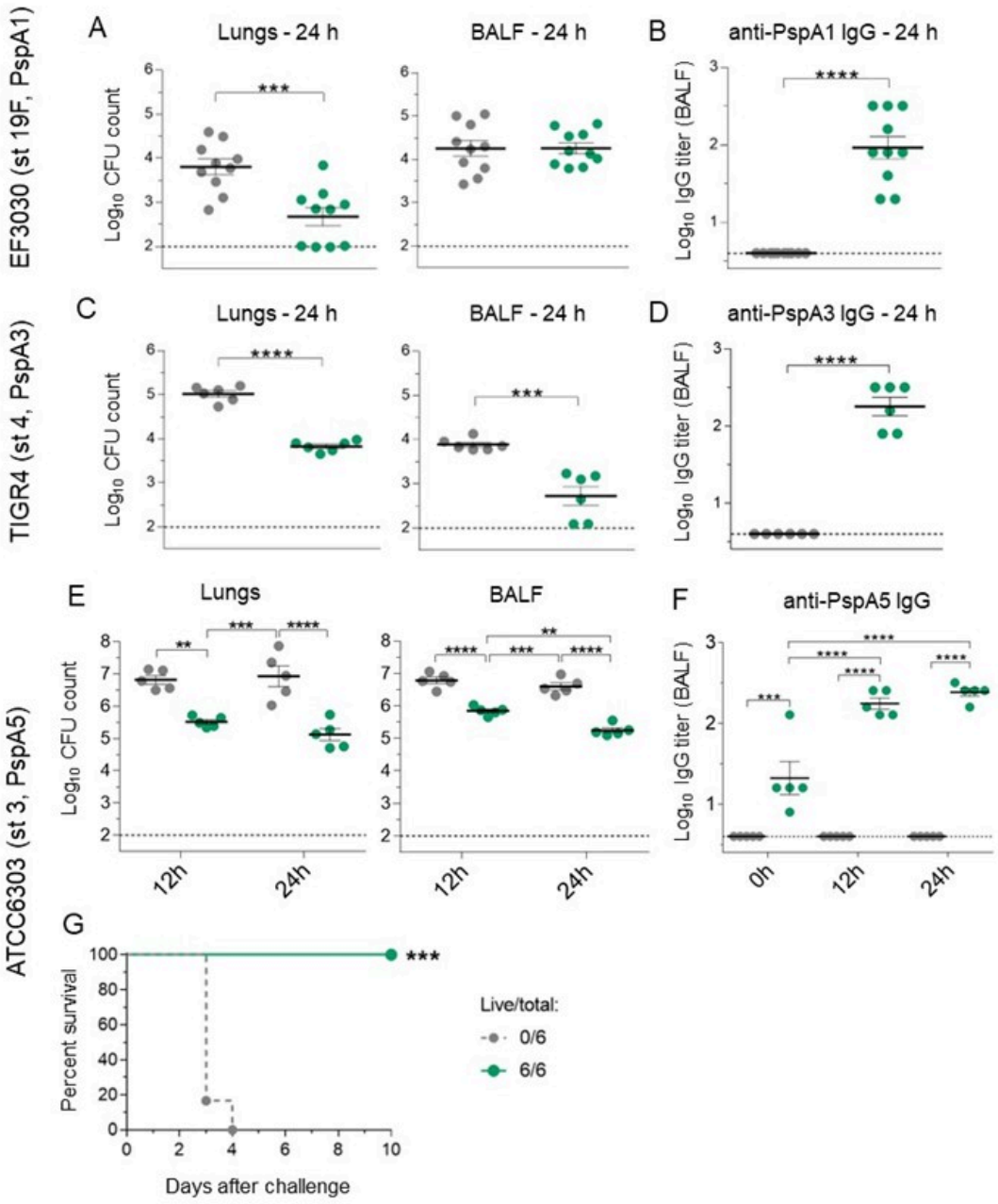


Figure 4

Mice immunized with CyaA-A2-A4/A1-A3 present reduced bacterial loads in the LRT after challenge with different pneumococcal serotypes.

Mice were immunized with three s.c. doses of CyaA-A2-A4/A1-A3, while control animals received CyaA-OVA21; schematic representation of immunization protocol is shown at the top of the figure. Three weeks after the third dose, animals were intranasally challenged with pneumococcal strains (**A, B**) EF3030 (serotype 19F, PspA1), (**C, D**) TIGR4 (serotype 4, PspA3) or (**E-G**) ATCC6303 (serotype 3, PspA5). Lungs and BALF from mice challenged with (**A**) EF3030 (n = 5 per group; graphs are composed of data from two independent experiments) or (**C**) TIGR4 (n = 6 per group) were collected 24 h after challenge and plated on blood-agar for CFU counting. BALF samples were evaluated for (**B**) anti-PspA1 or (**D**) anti-PspA3 IgG titers by ELISA. Results were analyzed using Student's T test. (**E**) Lungs and BALF from mice inoculated with ATCC6303 were collected at 12 h or 24 h after challenge (n = 5 per group, per time point) and plated on blood-agar for CFU counting. (**F**) BALF samples from challenged mice or non-infected mice (0 h, n = 5 per group) were analyzed by ELISA for anti-PspA5 IgG levels. Two-way ANOVA with Tukey's post-test was used to analyze results among time points and immunization groups. Circles represent individual data; lines represent the mean for each group ± SEM and dashed lines indicate the limit of detection. (**G**) Survival of ATCC6303 challenged mice (n = 6 per group) was monitored for 10 days. Protection was analyzed using the Log-Rank survival curve with Mantel-Cox post-test (comparison with the control group). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

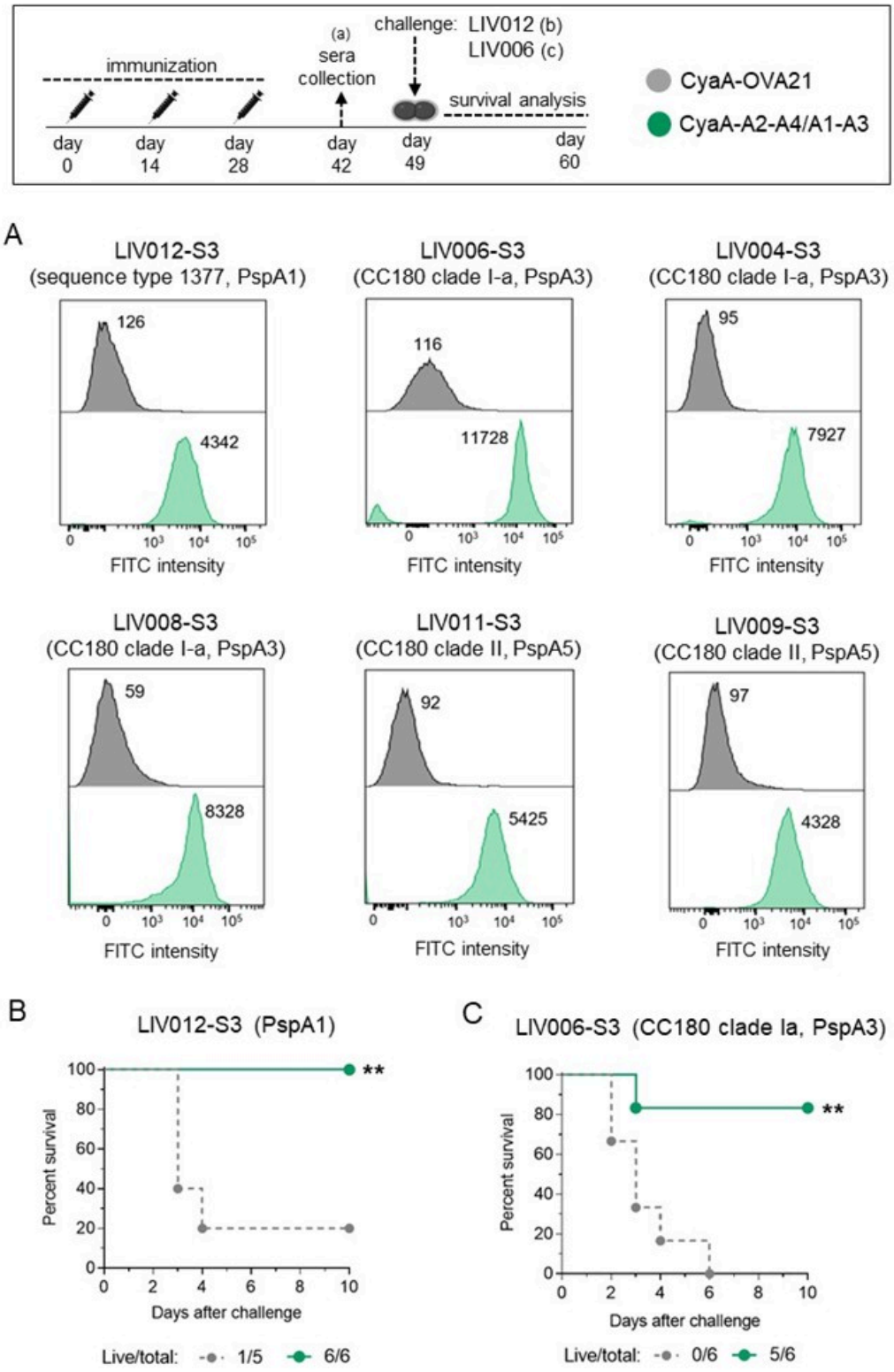


Figure 5

CyaA-A2-A4/A1-A3 induces antibody reactivity and protection against serotype 3 human carriage isolates.

Mice were immunized with three s.c. doses of CyaA-OVA21 or CyaA-A2-A4/A1-A3, schematic representation of immunization protocol is shown at the top of the figure. (**A**) Sera collected after the

third dose (n = 6 per group) were pooled and incubated with the indicated serotype 3 human carriage isolates. Antibody binding was detected by incubation with anti-mouse IgG-FITC and analyzed by flow cytometry, with 15,000 events recorded. Results were analyzed using the FlowJo V10 software. Numbers indicate the MFI for each histogram of FITC-fluorescence. Sequence type, clonal complex and PspA clade are indicated for each graph. Three weeks after the third dose, mice were challenged with human carriage isolates (**B**) LIV012-S3 (n = 5 for CyaA-OVA21 or 6 for CyaA-A2-A4/A1-A3) or (**c**) LIV006-S3 (n = 6 per group) and survival was monitored for 10 days. Protection was analyzed using the Log-Rank survival curve with Mantel-Cox post-test: **P<0.01.

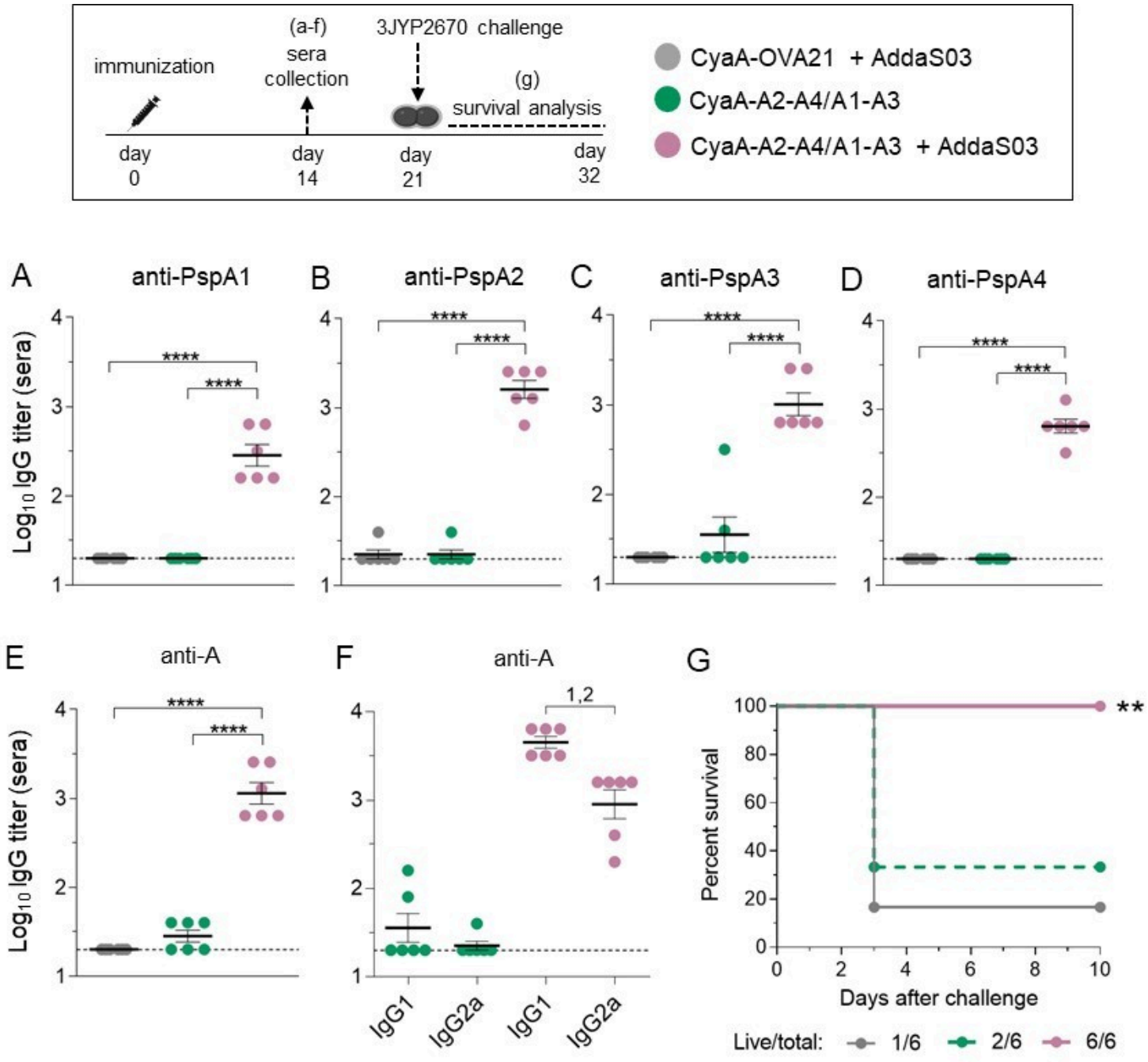


Figure 6

Immunization with a single dose of CyaA-A2-A4/A1-A3 + AddaS03 confers full protection against IPD.

Mice (n = 6 per group) were immunized subcutaneously with one dose of CyaA-A2-A4/A1-A3 or CyaA-A2-A4/A1-A3 + AddaS03; the control group received CyaA-OVA21 + AddaS03. Schematic representation of immunization protocol is shown at the top of the figure. Sera collected after the immunization were analyzed by ELISA for the levels of specific IgG against the N-terminal region of (**A**) PspA1, (**B**) PspA2, (**C**) PspA3 or (**D**) PspA4 or (**E**) for the levels of anti-A IgG. Results were analyzed with One-way ANOVA with Tukey's post-test: ****P<0.001. (**F**) Sera were analyzed for anti-A IgG1 and IgG2a (the IgG1/IgG2a ratio is indicated in the graph). Circles represent individual data; lines represent the mean for each group ± SEM and dashed lines indicate the limit of detection. (**G**) Three weeks after immunization, mice were challenged with pneumococcal strain 3JYP2670 (serotype 3, PspA4) and survival was monitored for 10 days. Protection was analyzed using the Log-Rank survival curve with Mantel-Cox post-test: **P<0.01 (comparison to the other groups).

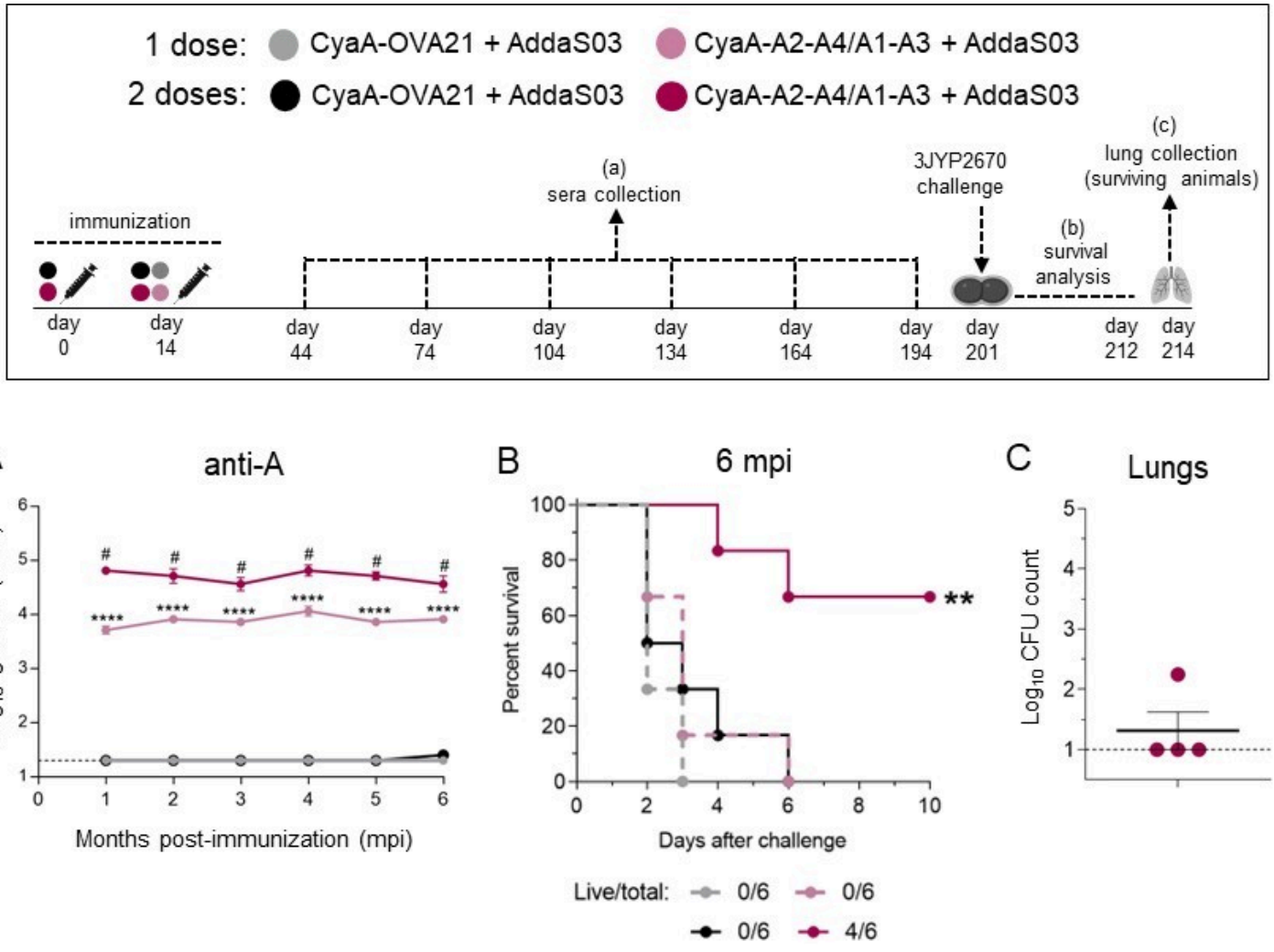


Figure 7

Immunization with CyaA-A2-A4/A1-A3 + AddaS03 promotes long-lasting antibody responses, but two doses are required for long-term protection.

Mice were immunized subcutaneously with one or two doses of CyaA-A2-A4/A1-A3 + AddaS03 or received CyaA-OVA21 + AddaS03 as control (n = 6 per group). Schematic representation of immunization protocol is shown at the top of the figure. (**A**) Sera collected monthly were analyzed by ELISA for anti-A IgG levels for 6 months post-immunization (mpi). Results at each time point are shown as the mean ± SEM. Data were analyzed by Two-way ANOVA with Tukey's post-test: ****P<0.0001 (comparison with control groups); # indicates significant difference (P<0.0001) in comparison to control groups and the group immunized with 1 dose of CyaA-A2-A4/A1-A3 + AddaS03; no significant differences were detected within the same group among sera collected at different time points. (**B**) At 6 mpi, mice were challenged with 3JYP2670 (serotype 3, PspA4) and survival was monitored for 10 days. Protection was analyzed using the Log-Rank survival curve with Mantel-Cox post-test: **P<0.01 (comparison to the other groups). (**C**) At the 12^{th} day after challenge, lungs from surviving animals were collected and macerates were plated on blood-agar for CFU counting. Circles represent individual data; lines represent the mean for each group ± SEM and dashed lines indicate the limit of detection.

Supplementary Files

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- Dataset1.xlsx
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