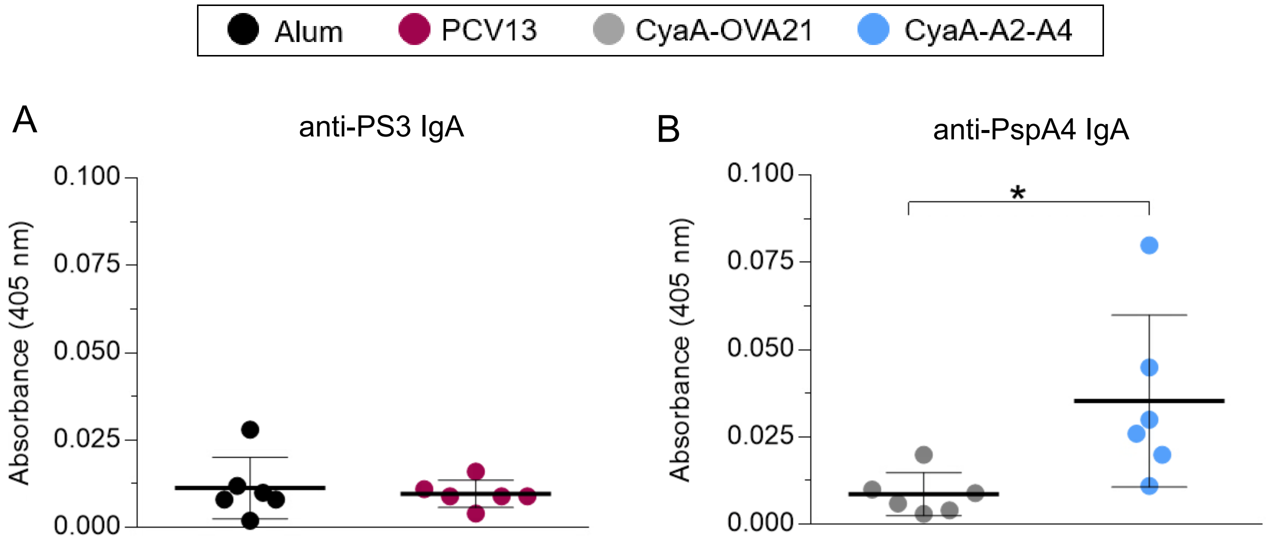
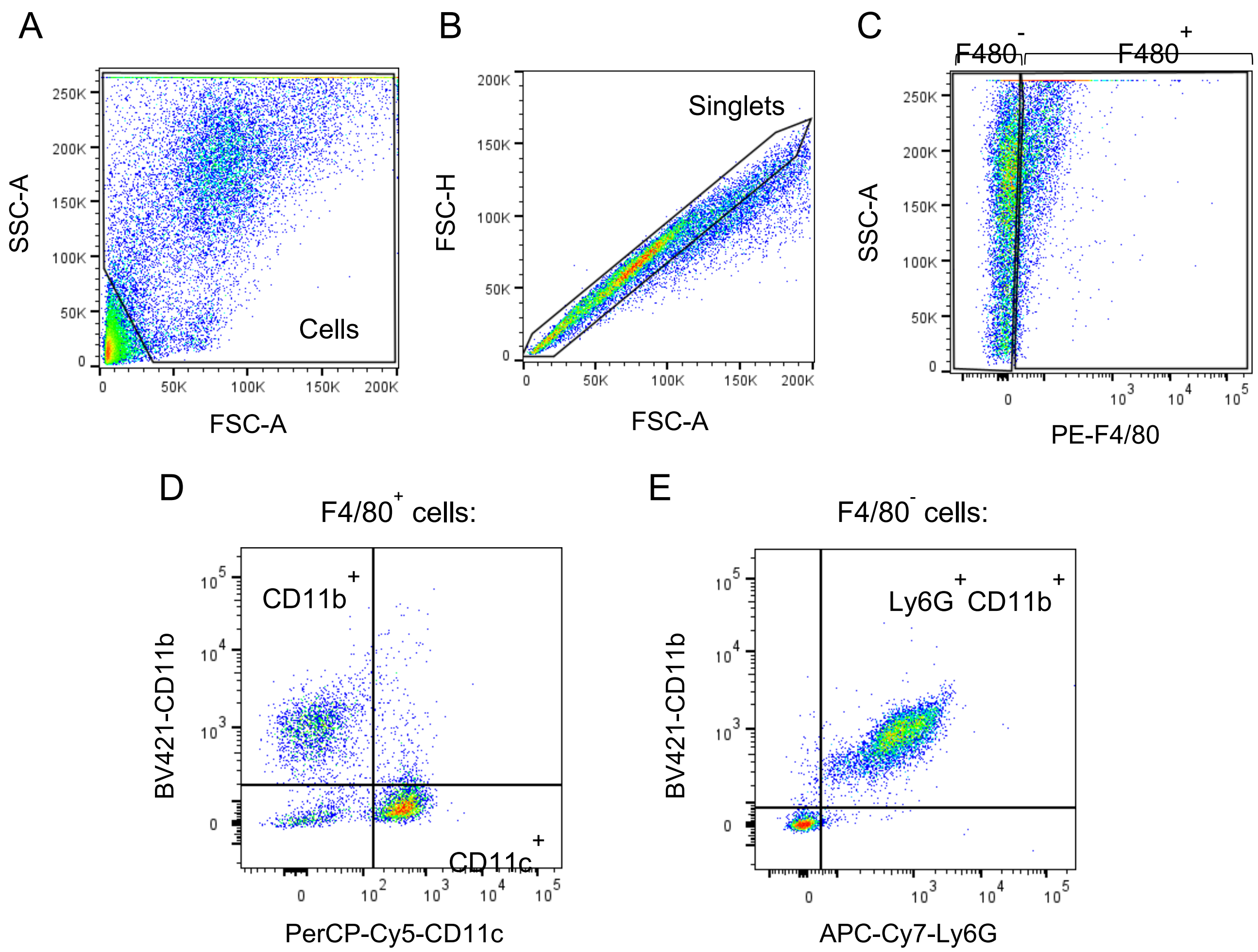
SUPPLEMENTARY FIGURES



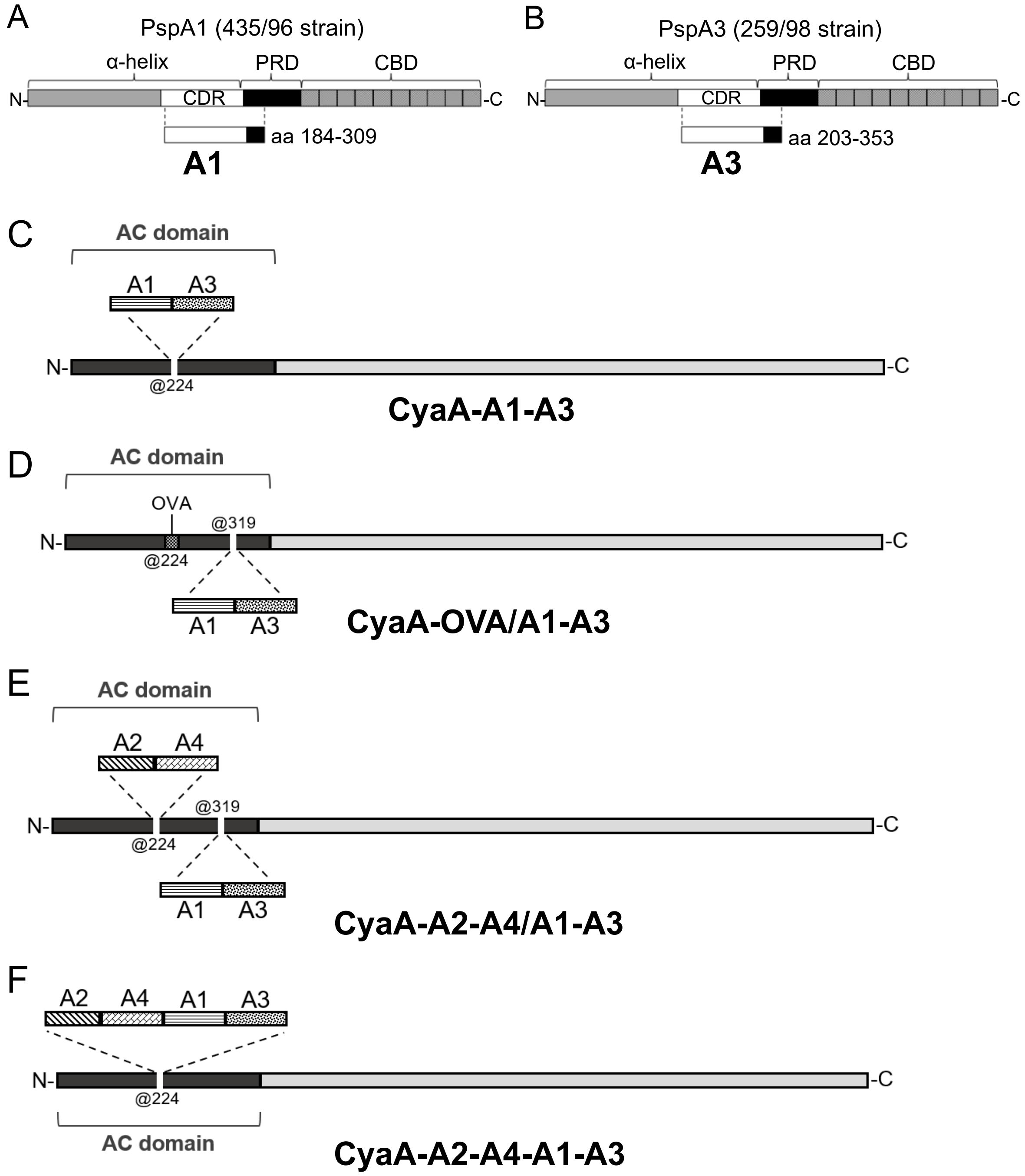
Supplementary Fig. 1. Immunization with CyaA-A2-A4 induces minimal levels of anti-PspA IgA in the LRT of mice.

Mice (n = 6 per group) were immunized with three s.c. doses of Alum, PCV13, CyaA-OVA21 or CyaA-A2-A4. Three weeks after the third dose, animals were intranasally challenged with the pneumococcal strain 3JYP2670 (serotype 3, PspA4). BALF samples, collected 12 h post-challenge, were analyzed by ELISA for (**A**) anti-PS3 or (**B**) anti-PspA4 IgA levels and the absorbance values at 1:2 dilutions are shown. Student's T teste was used to analyze results among groups: *$P<0.05$. Circles represent individual data; lines represent the mean for each group ± SEM.



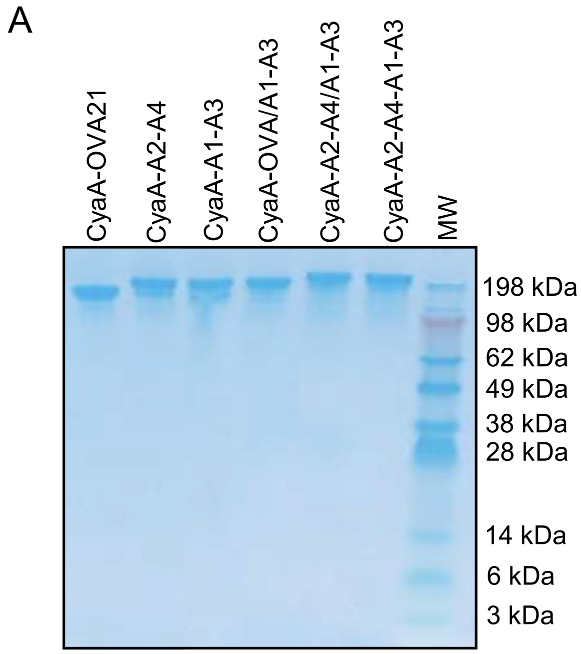
Supplementary Fig. 2. Flow cytometry gating strategy to analyze populations of macrophages and neutrophils in the bronchoalveolar lavage fluid.

Cells from BALF samples were stained with anti-mouse F4/80-PE, anti-mouse CD11c-PerCP-Cy5.5, anti-CD11b- BV421 and anti-Ly6G-APC-Cy7 antibodies and analyzed by flow cytometry. 20,000 events acquired in the cell gate **(A)** were analyzed using the FlowJo v10 software (Tree Star). (**A**) Cells were gated based on forward (FSC) and side (SSC) scatter excluding events with low size and granularity. (**B**) The cell population was evaluated using FSC-A and FSC-H parameters to discriminate singlets from aggregates. (**C**) Singlets were evaluated for PE fluorescence to define F4/80^{-} and F4/80^{+} populations. (**D**) F4/80^{+} cells were analyzed for PerCP-Cy5 and APC-Cy7 fluorescence to identify F4/80^{+}CD11b^{+}CD11c^{-} interstitial macrophages and F4/80^{+}CD11b^{-}CD11c^{+} alveolar macrophages. (**e**) F4/80^{-} cells were analyzed for BV421 and APC-Cy7 fluorescence to identify F4/80^{-}CD11b^{+}Ly6G^{+} neutrophils. Fluorescence gates were set according to Fluorescence-minus-one controls.

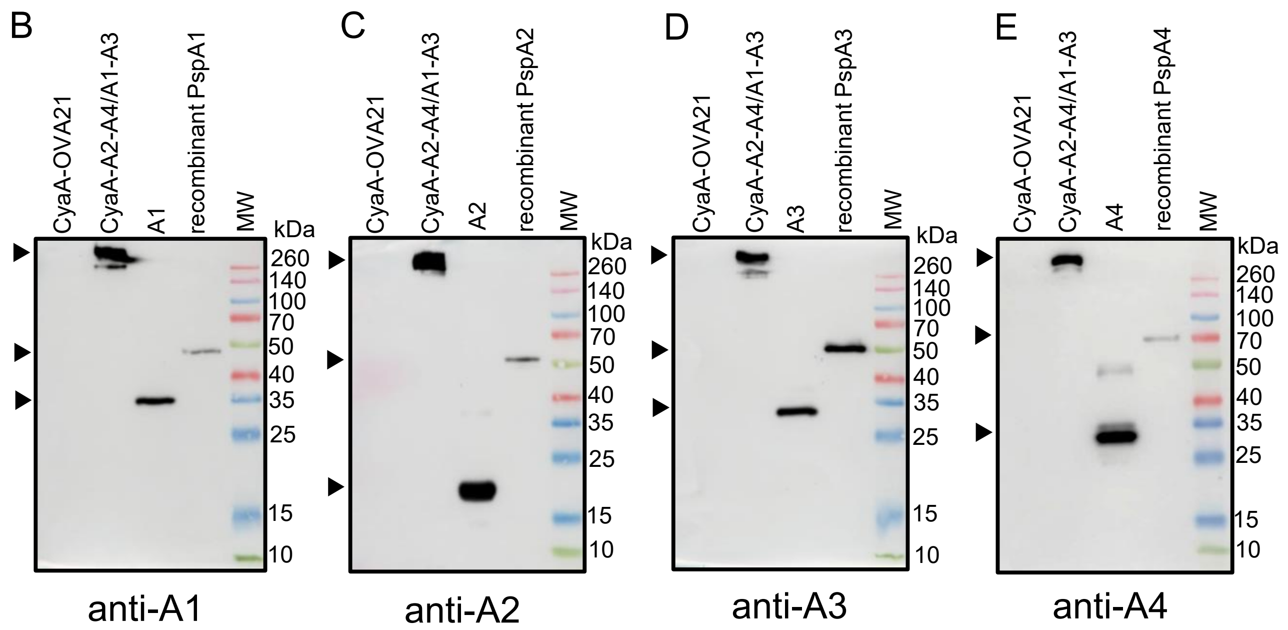


Supplementary Fig. 3. Construction of new CyaA-PspA antigens.

Schematic representation of (**A**) PspA1 and (**B**) PspA3 and their respective fragments, A1 and A3, comprising the clade definition region (CDR) and the beginning of the proline-rich domain (PRD) of each protein. CBD = choline binding domain. (**C**) The A1-A3 fusion was inserted between amino acids 224 and 225 of the AC domain of CyaA to generate CyaA-A1-A3. (**D**) The A1-A3 fusion was inserted between amino acids 319 and 320 of the AC domain of CyaA-OVA21, which contains the OVA peptide between amino acids 224 and 225, thus generating CyaA-OVA/A1-A3. (**E**) The A2-A4 fusion was grafted between amino acids 224 and 225, while the A1-A3 fusion was inserted between amino acids 319 and 320 of the AC domain, producing CyaA-A2-A4/A1-A3. (**F**) The A2-A4-A1-A3 fusion was grafted between amino acids 224 and 225 of the AC domain to generate CyaA-A2-A4-A1-A3.

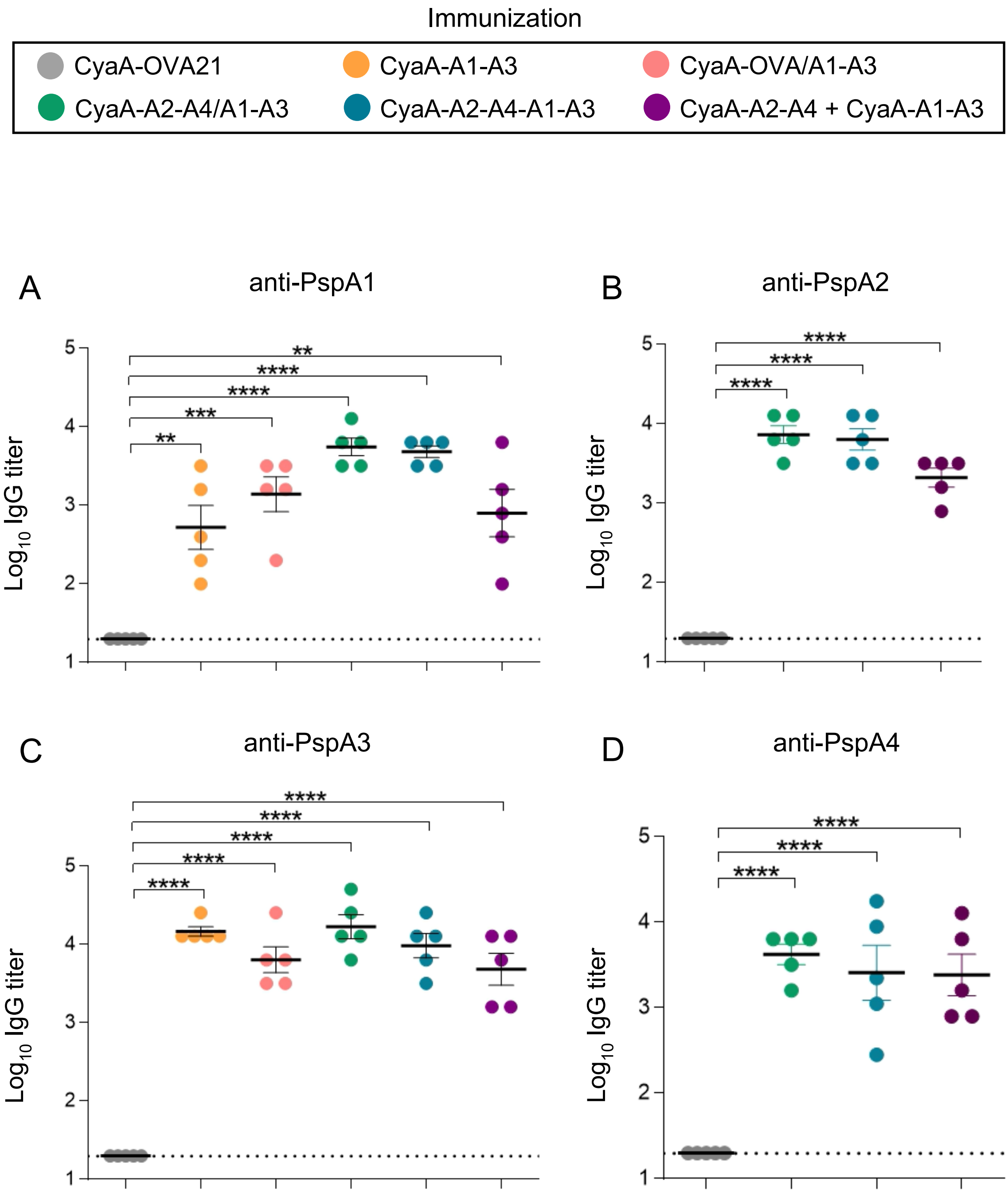


recombinant protein	molecular mass
CyaA-OVA21	179 kDa
CyaA-A2-A4	207 kDa
CyaA-A1-A3	208 kDa
CyaA-OVA/A1-A3	209 kDa
CyaA-A2-A4/A1-A3	237 kDa
CyaA-A2-A4-A1-A3	236 kDa



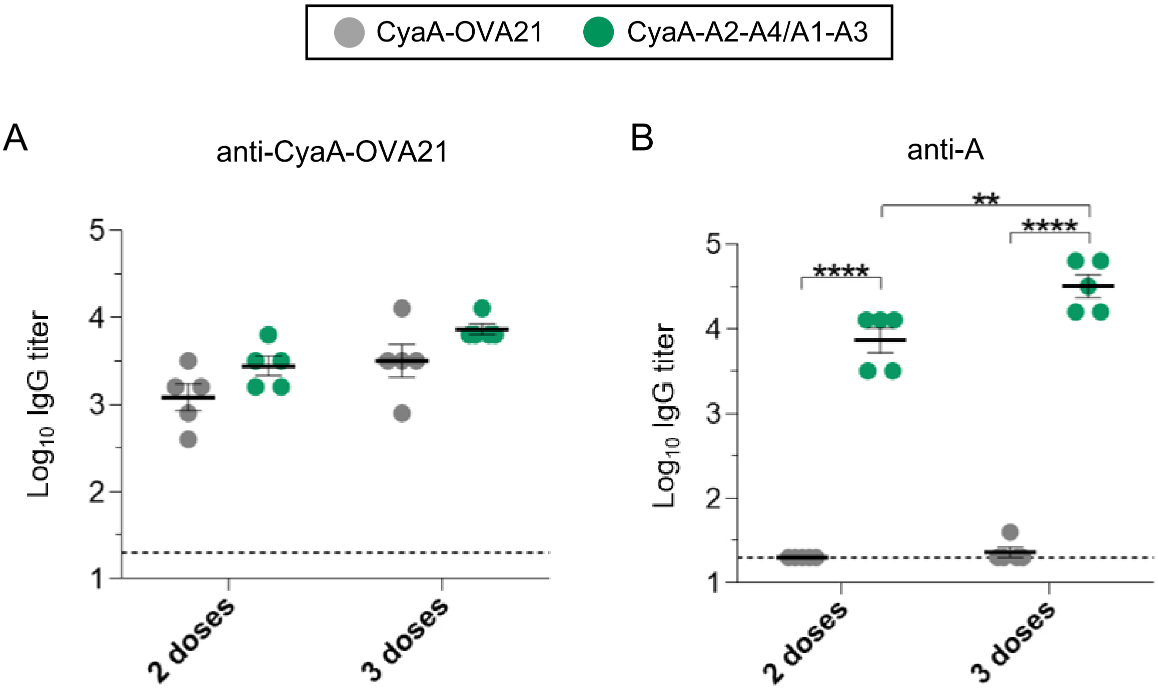
Supplementary Fig. 4. Purified CyaA-PspA recombinant proteins.

(**A**) Purified recombinant proteins were analyzed by SDS-PAGE using 8% polyacrylamide gel and Coomassie Blue staining. MW = SeeBlue Plus2 Pre-Stained Protein Standard, (Thermo Fischer Scientific, USA). The predicted molecular mass for each recombinant protein is indicated on the right side. CyaA-A2-A4/A1-A3 was analyzed by western blot using (**B**) anti-A1, (**C**) anti-A2, (**D**) anti-A3 and (**E**) anti-A4 polyclonal sera. The respective A fragments and recombinant proteins comprising the N-terminal region of PspAs from clades 1 to 4 were used as positive controls. CyaA-OVA21 was used as negative control. Arrows point bands of interest. MW: Spectra Multicolor Broad Range Protein Ladder Full range (Thermo Fischer Scientific, USA).



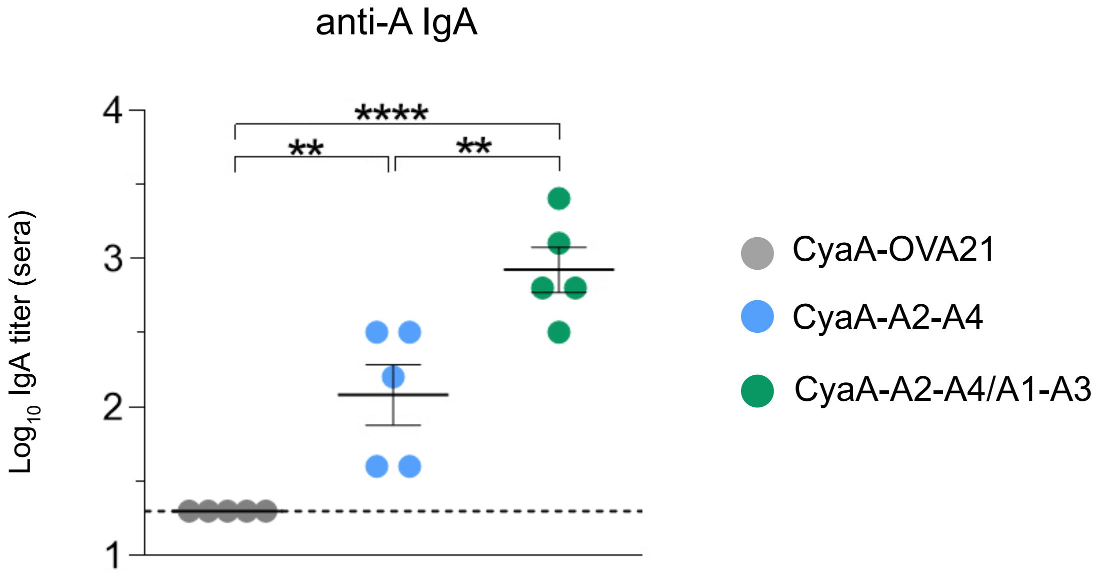
Supplementary Fig. 5. Immunogenicity of the new CyaA-PspA antigens.

Mice were immunized with three s.c. doses of the new CyaA-PspA antigens or with a combination of CyaA-A1-A3 with the CyaA-A2-A4 antigen; control group received CyaA-OVA21 (n = 5 animals per group). Sera collected two 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. Circles represent individual data; lines represent the mean for each group ± SEM and dashed lines indicate the limit of detection. Statistical analyses were performed by One-way ANOVA with Tukey's post-test: **P<0.01; ***P<0.001; ****P<0.0001.



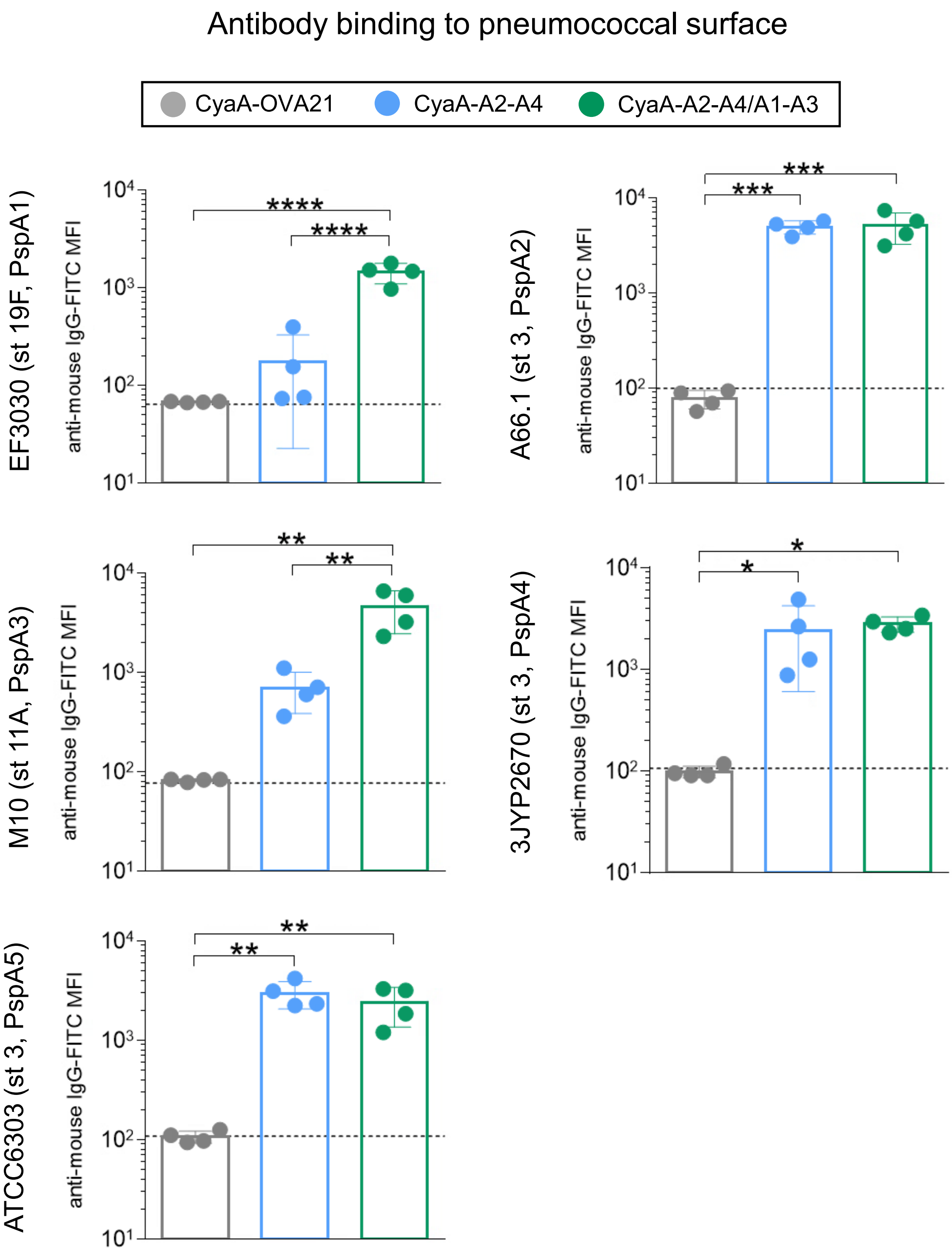
Supplementary Fig. 6. Immunization with CyaA-A2-A4/A1-A3 induces IgG antibodies against CyaA-OVA21 and A fragments.

Mice (n = 5 per group) were immunized with three s.c. doses of CyaA-OVA21 or CyaA-A2-A4/A1-A3. Sera collected 2 weeks after the second and the third dose were analyzed by ELISA for the levels of specific IgG against **(A)** CyaA-OVA21 or **(B)** a mixture of the A fragments (A1, A2, A3 and A4). Results were analyzed by Two-way ANOVA with Tukey's post-test: **P<0,01 and ****P<0.0001. Circles represent individual data; lines represent the mean for each group ± SEM and dashed lines indicate the limit of detection.



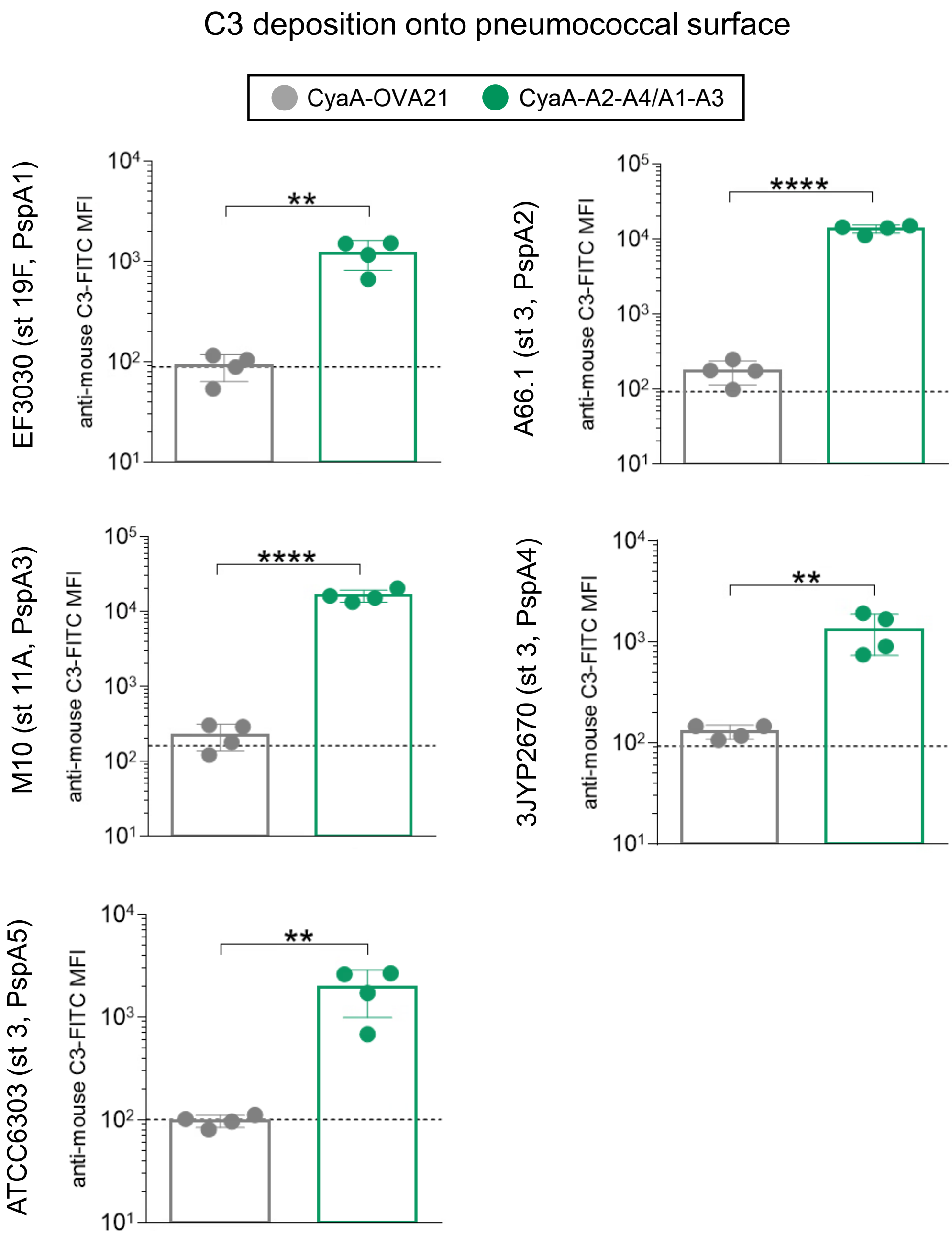
Supplementary Fig. 7. Immunization with CyaA-A2-A4/A1-A3 induces high levels of circulating anti-PspA IgA.

Mice (n = 5 per group) were immunized with three s.c. doses of CyaA-OVA21, CyaA-A2-A4 or CyaA-A2-A4/A1-A3. Sera collected 2 weeks after the third dose were analyzed by ELISA for the levels of specific IgA against a mixture of the A fragments (A1, A2, A3 and A4). Results were analyzed by One-way ANOVA with Tukey's post-test: **P<0,01 and ****P<0.0001. Circles represent individual data; lines represent the mean for each group ± SEM; dashed line indicates the limit of detection.



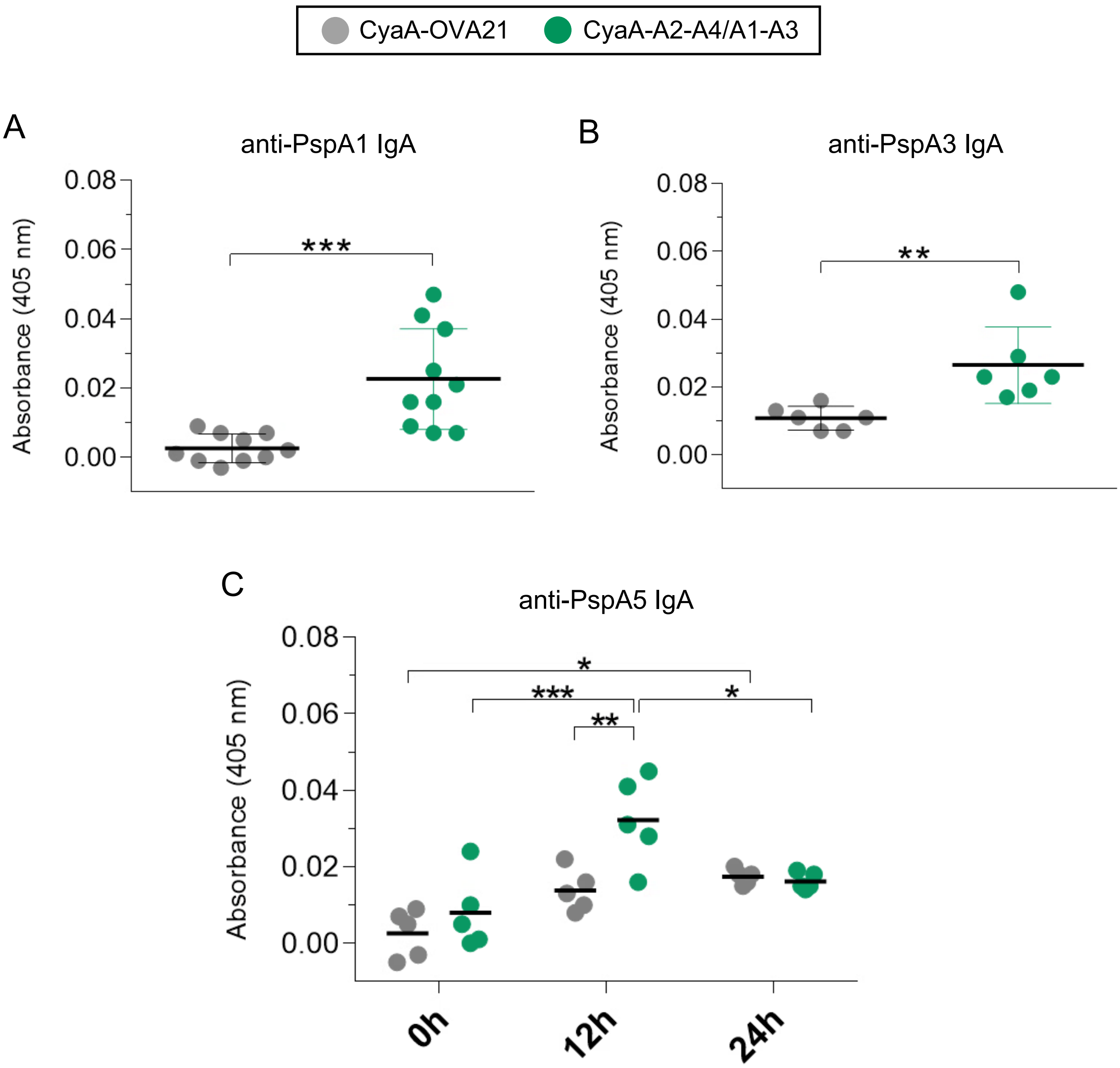
Supplementary Fig. 8. Sera from CyaA-A2-A4/A1-A3 immunized mice present high levels of reactivity with different pneumococcal strains.

Mice were immunized with three s.c. doses of CyaA-OVA21, CyaA-A2-A4 or CyaA-A2-A4/A1-A3. Sera collected after the third dose (n=4 per group) were 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. Statistical analyses were performed by One-way ANOVA with Tukey's post-test: *P<0,05; **P<0,01; ***P<0,001 and ****P<0.0001. Circles indicate the median fluoresce intensity (MFI) for each histogram of FITC-fluorescence, bars represent the mean for each group ± SEM, and the dashed lines indicate the MFI produced by negative control bacteria, incubated with anti-mouse IgG-FITC, without previous incubation with immune sera. Serotype (st) and PspA clade of each strain are indicated.



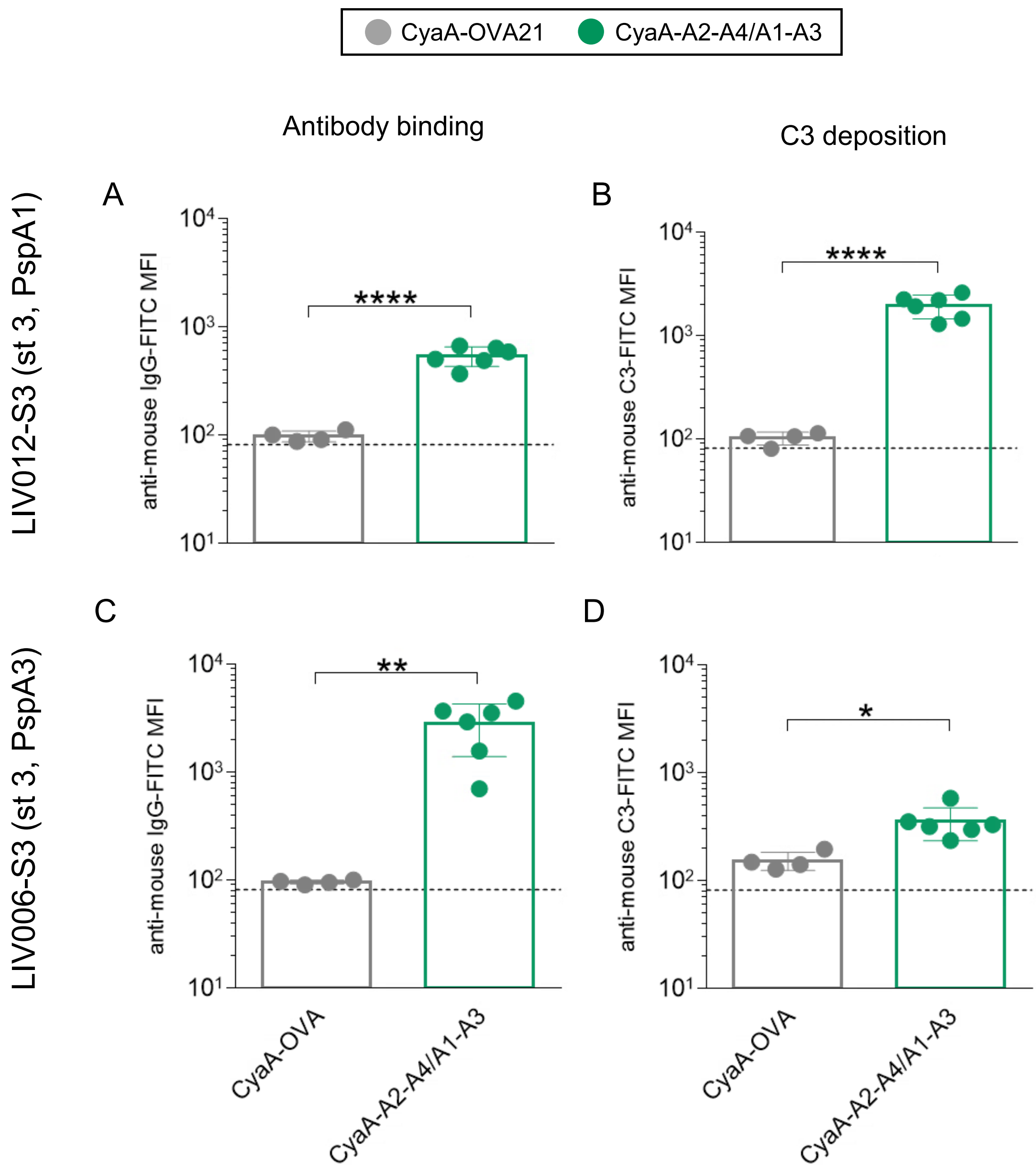
Supplementary Fig. 9. Complement deposition onto pneumococcal surface after incubation with sera from CyaA-A2-A4/A1-A3 immunized mice.

Mice were immunized with three s.c. doses of CyaA-OVA21, CyaA-A2-A4 or CyaA-A2-A4/A1-A3. Sera collected after the third dose (n=4 per group) were heat-inactivated and incubated with the indicated pneumococcal strains. Bacteria were then incubated with complement source (naive mice sera), followed by FITC-conjugated anti-mouse C3-FITC. Samples were analyzed by flow cytometry with 15,000 events recorded. Results were analyzed using the FlowJo V10 software. Statistical analyses were performed by One-way ANOVA with Tukey's post-test: *P<0,05; **P<0,01; ***P<0,001 and ****P<0.0001. Circles indicate the median fluoresce intensity (MFI) for each histogram of FITC-fluorescence, bars represent the mean for each group ± SEM, and the dashed lines indicate the MFI produced by negative control bacteria, incubated with complement source and anti-mouse C3-FITC, without previous incubation with immune sera. Serotype (st) and PspA clade of each strain are indicated.



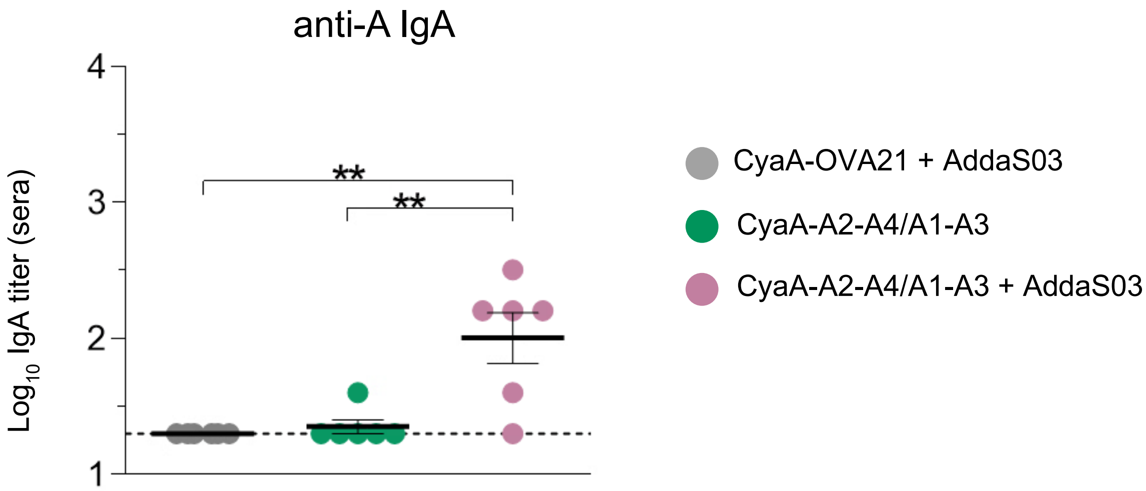
Supplementary Fig. 10. Immunization with CyaA-A2-A4/A1-A3 minimal levels of anti-PspA IgA in the LRT of mice.

Mice were immunized with three s.c. doses of CyaA-OVA21 or CyaA-A2-A4/A1-A3. Three weeks after the third dose, animals were intranasally challenged with pneumococcal strains (**A**) EF3030 (serotype 19F, PspA1), (**B**) TIGR4 (serotype 4, PspA3), or (**C**) ATCC6303 (serotype 3, PspA5). BALF samples were collected at (**A-C**) 24 h or (**c**) at 12 h post-challenge; BALF from (**C**) non-infected mice (0 h) were also collected. Samples were analyzed by ELISA for (**A**) anti-PspA1, (**B**) anti-PspA3, or (**C**) anti-PspA5 IgA, and the absorbance values at 1:2 dilution are shown. (**A, B**) Student's T test or (**C**) Two-way ANOVA with Tukey's post-test was used to analyze results: *P<0.05; **P<0.01; ***P<0.001. Circles represent individual data; lines represent the mean for each group ± SEM.



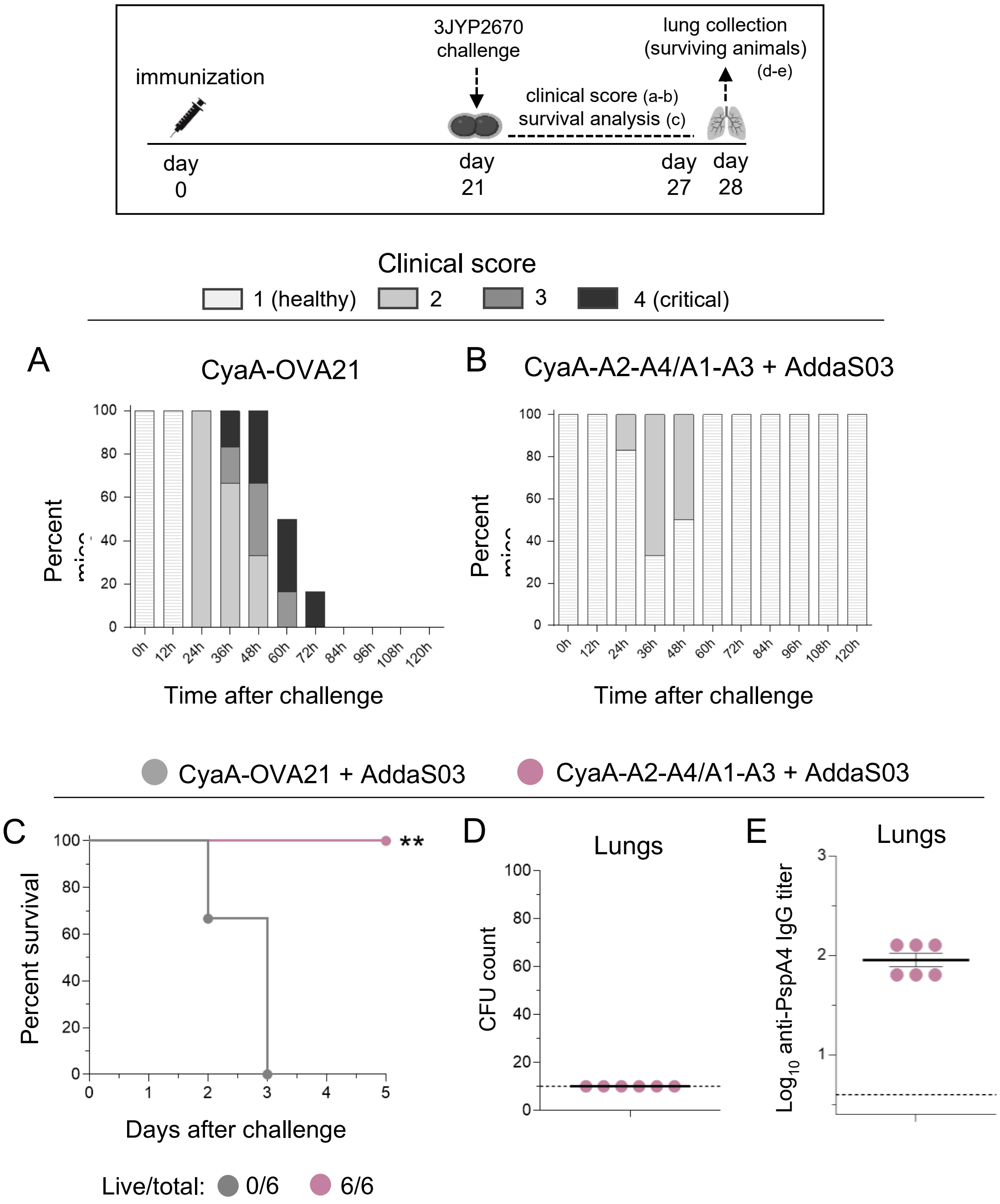
Supplementary Fig. 11. Sera from CyaA-A2-A4/A1-A3 immunized mice induce significant levels of antibody binding and complement deposition onto the surface of serotype 3 strains circulating in the post-PCV period.

Mice were immunized with three s.c. doses of CyaA-OVA21, CyaA-A2-A4 or CyaA-A2-A4/A1-A3. Sera collected after the third dose (n= 4 or 6 mice per group) were heat-inactivated and incubated with the pneumococcal strains (**A, B**) LIV012-S3 or (**C, D**) LIV006-S3. (**A, C**) Antibody binding was detected by incubation with anti-mouse IgG-FITC. (**B, D**) Bacteria pre-incubated with immune sera were then incubated with complement source (naive mice sera), followed by FITC-conjugated anti-mouse C3-FITC. All samples were analyzed by flow cytometry, with 15,000 events recorded. Results were analyzed using the FlowJo V10 software. Statistical analyses were performed by One-way ANOVA with Tukey's post-test: *P<0,05; **P<0,01; and ****P<0.0001. Circles indicate the median fluoresce intensity (MFI) for each histogram of FITC-fluorescence, bars represent the mean for each group ± SEM. The dashed lines indicate the MFI produced by negative control bacteria, (**A, C**) incubated with anti-mouse IgG-FITC or (**B, D**) complement source and anti-mouse C3-FITC, in both cases without previous incubation with immune sera. Serotype (st) and PspA clade of each strain are indicate



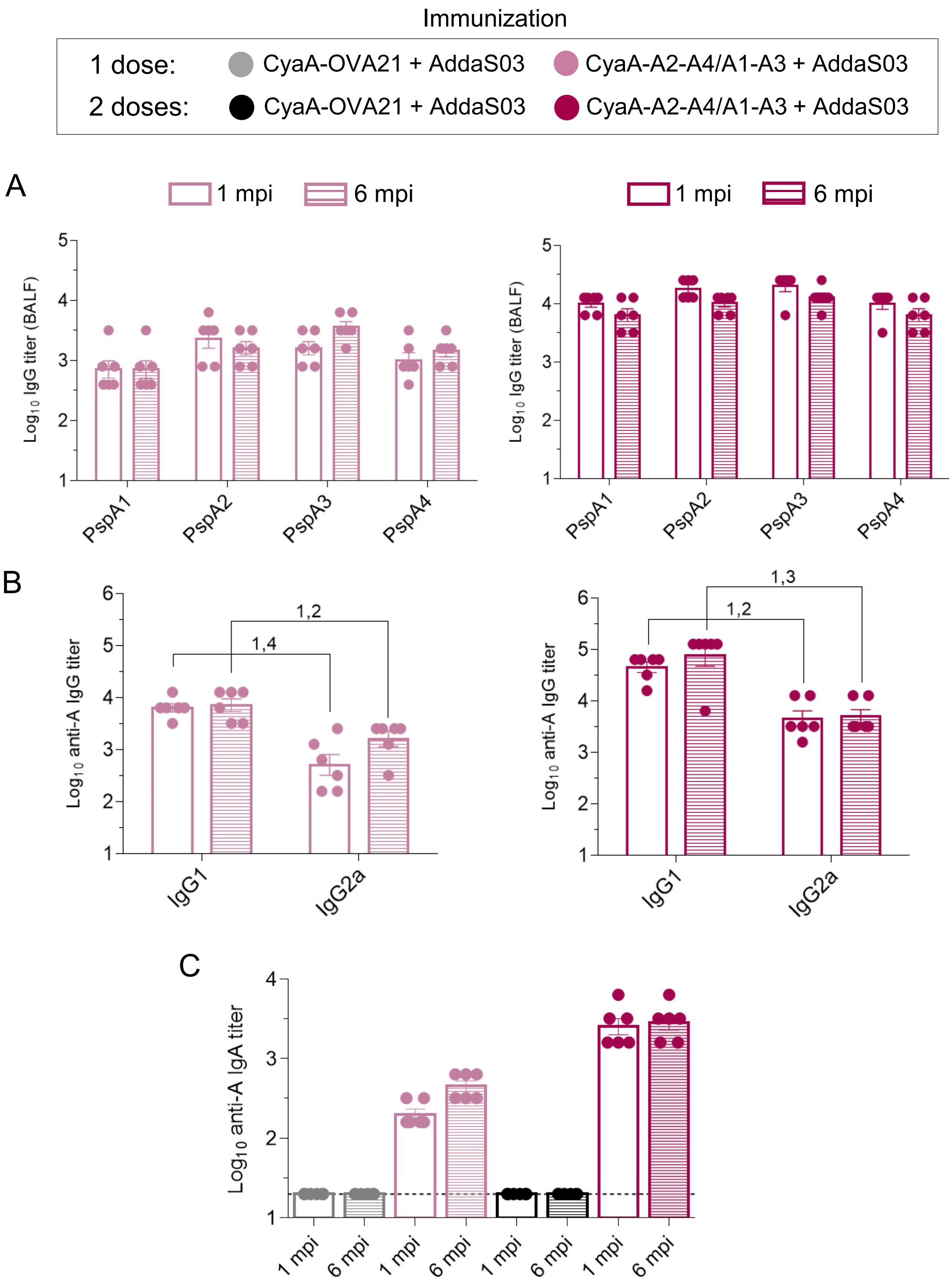
Supplementary Fig. 12. Immunization with CyaA-A2-A4/A1-A3 induces circulating anti-PspA IgA.

Mice (n = 6 per group) were immunized with one s.c. dose of CyaA-OVA21, CyaA-A2-A4/A1-A3 or CyaA-A2-A4/A1-A3 + AddaS03. Sera collected 2 weeks after immunization were analyzed by ELISA for the levels of specific IgA against a mixture of the A fragments (A1, A2, A3 and A4). Results were analyzed by One-way ANOVA with Tukey's post-test: **P<0,01. Circles represent individual data; lines represent the mean for each group ± SEM and the dashed line indicates the limit of detection.



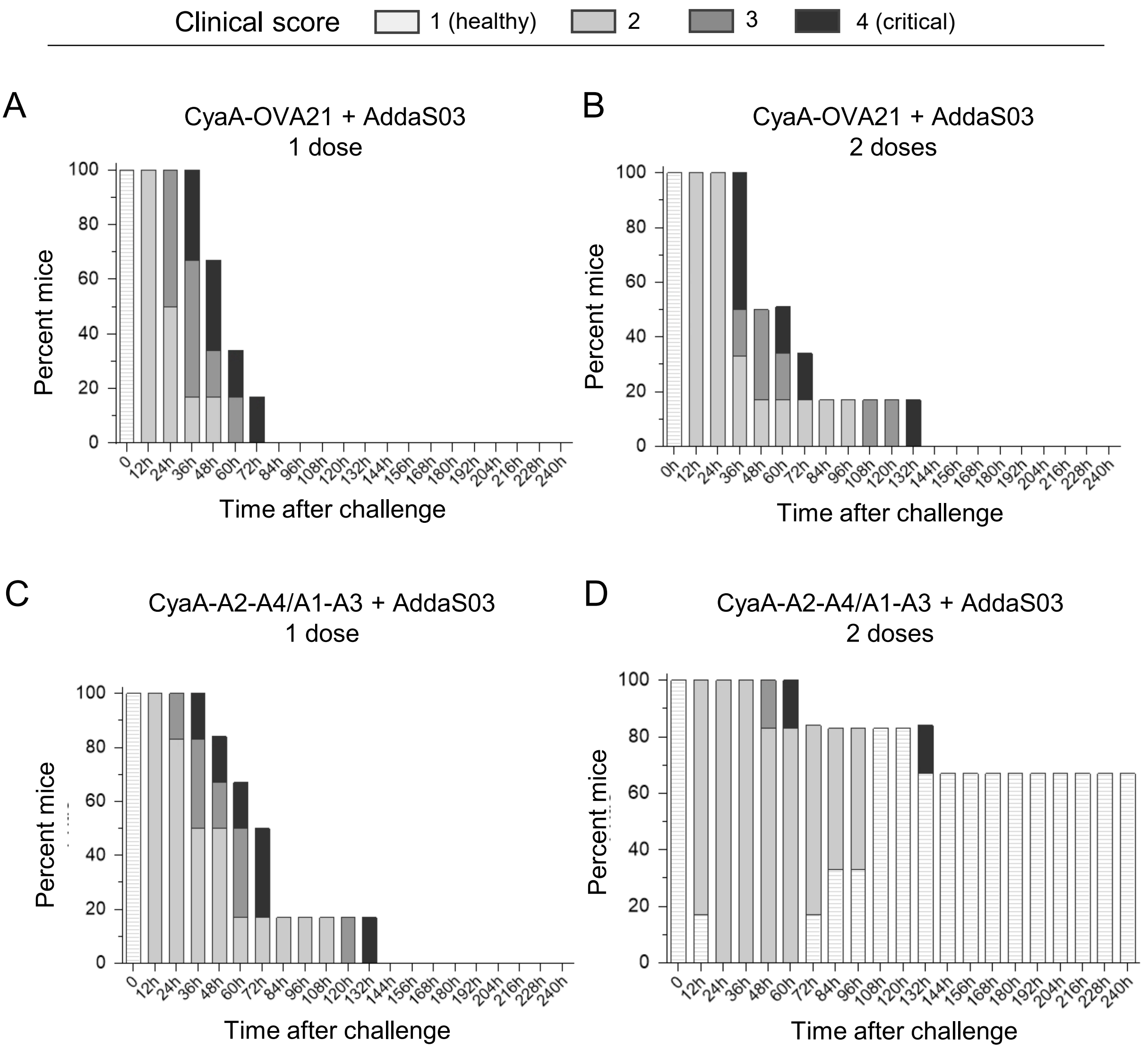
Supplementary Fig. 13. Mice immunized with CyaA-A2-A4/A1-A3 + AddaS03 presented no critical symptoms of disease and no detectable levels of bacteria in the lungs after challenge with 3JYP2670.

Mice were immunized subcutaneously with one dose of CyaA-A2-A4/A1-A3 + AddaS03 and the control group received CyaA-OVA21 (n = 6 per group); schematic representation of immunization protocol is shown at the top of the figure. Three weeks after immunization, animals were challenged with pneumococcal strain 3JYP2670 (serotype 3, PspA4). (**A, B**) Animals were analyzed for symptoms of disease twice a day for 5 days, being scored from 1 (healthy) to 4 (critical, humane endpoint). (**C**) Survival was analyzed using the Log-Rank survival curve with Mantel-Cox post-test: **P<0.001 (comparison with CyaA-OVA21). At the 5th day after challenge, lungs from surviving animals were collected and macerates were (**D**) plated on blood agar for CFU counting and (**e**) analyzed by ELISA for anti-PspA4 IgG titers. Circles represent individual data; lines represent the mean for each group ± SEM and dashed lines indicate the limit of detection.



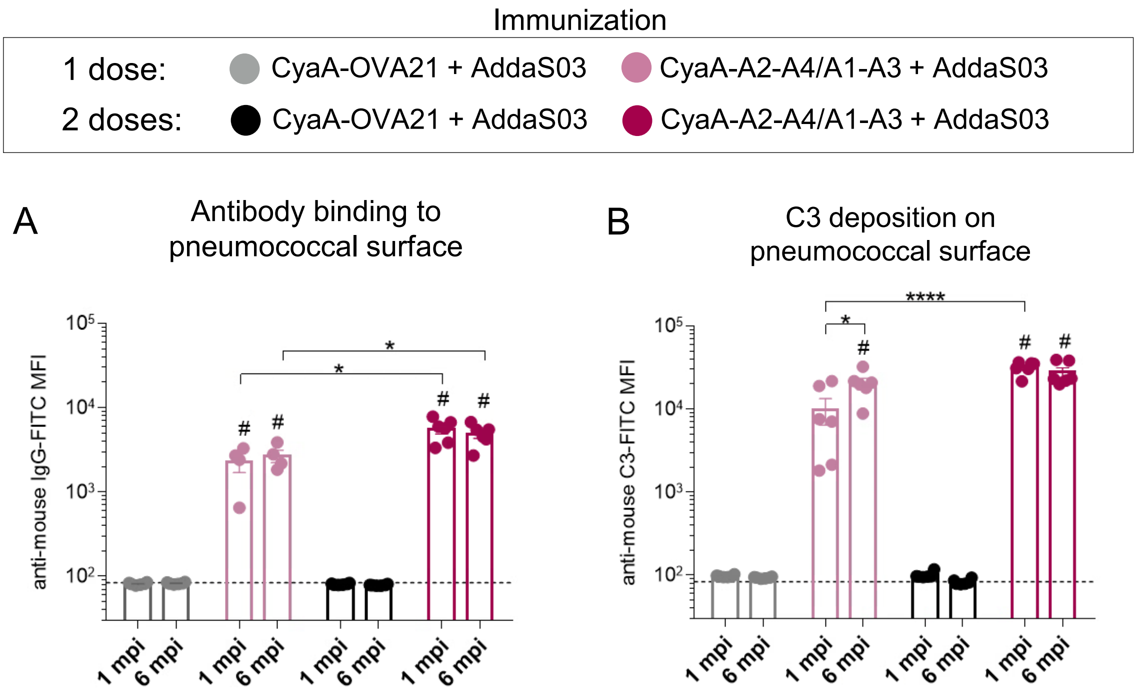
Supplementary Fig. 14. Sera collected at 1 or 6 months post immunization with CyaA-A2-A4/A1-A3 + AddaS03 present similar antibody levels.

Mice were immunized with one or two doses of CyaA-A2-A4/A1-A3 + AddaS03 (n = 6 per group). Sera collected at 1 or 6 months post immunization (mpi) were analyzed by ELISA for (**A**) the levels of specific IgG against the N-terminal region of PspAs from clades 1 to 4, (**B**) IgG1 and IgG2a or (**C**) IgA against a mixture of the A fragments (A1, A2, A3 and A4). (**B**) The IgG1/IgG2a ratios are indicated in the graph. Circles represent individual data, lines represent the mean for each group ± SEM and dashed lines indicate the limit of detection. For each group, results obtained for sera collected at 1 or 6 mpi were analyzed by Student's T test.



Supplementary Fig. 15. Clinical score of mice challenged with 3JYP2670 at 6 months post-immunization with CyaA-A2-A4/A1-A3 + AddaS03.

Mice were immunized subcutaneously with (**A**) one or (**B**) two doses of CyaA-OVA21 + AddaS03, or (**C**) one or (**D**) two doses of CyaA-A2-A4/A1-A3 + AddaS03 (n = 6 per group). Six months after immunization, animals were challenged with pneumococcal strain 3JYP2670 (serotype 3, PspA4) and analyzed for symptoms of disease twice a day for 10 days, being score from 1 (healthy) to 4 (critical, humane endpoint).



Supplementary Fig. 16. Sera collected at 1 or 6 months post immunization with CyaA-A2-A4/A1-A3 + AddaS03 present similar levels of antibody binding and complement deposition onto the surface of pneumococcal strain 3JYP2670.

Mice were immunized with one or two doses of CyaA-A2-A4/A1-A3 + AddaS03. Sera collected at 1 or 6 months post immunization (mpi) (n= 4 or 6 mice per group) were heat-inactivated and incubated with the pneumococcal strain 3JYP2670. **(A)** Antibody binding was detected by incubation with anti-mouse IgG-FITC. **(B)** Bacteria pre-incubated with immune sera were then incubated with complement source (naive mice sera), followed by FITC-conjugated anti-mouse C3-FITC. All samples were analyzed by flow cytometry, with 15,000 events recorded. Results were analyzed using the FlowJo V10 software. Statistical analyses were perfomed by Two-way ANOVA with Tukey's post-test: *P<0,05 and ***P<0.001 (comparison between the groups indicated by the bars); #P<0.0001, in comparison to CyaA-OVA21 + AddaS03 immunized groups, regardless of the number of doses and time of collection of sera. Circles indicate the median fluoresce intensity (MFI) for each histogram of FITC-fluorescence, bars represent the mean for each group ± SEM, and dashed lines indicate the MFI produced by negative control bacteria, incubated with anti-mouse IgG-FITC **(A)** or complement source and anti-mouse C3-FITC **(B)**, without previous incubation with immune sera.

Supplementary Tables

Supplementary Table 1. Primers used for amplification of A1 and A3 from pAE-*pspA1*[1] and pAE-*pspA3*[2], respectively.

Primer	5’ – 3’ sequence
A1 forward	GGATCCCTCAAAGAGATTGATGAGTCTGATTCAGAAG
A1 reverse	**AAGCTT**TCAAGGAGCCGGGCGTGGTTTTGGTTGTTCAGCTGG
A3 forward	GGATCCGATGGCACAGAAGTTATAGAAGCTAAATTAAAC
A3 reverse	**AAGCTT**TCATGGTGCAGGAGCTGGTTGCTCTGGTTTTGGTGCAGG

BamH I restriction site underlined; Hind III restriction site in bold.

Supplementary Table 2: Synthetic DNAs used for cloning of A1and A3 into pCACT plasmids.

I	CCGAAACCGGCACCGGCTCCGGGTACTAGTGACGGTACTGAAGTGATTGAGGCAAAGCTTAATAAGGGCGAGGCTGAGCTTAATGCAAAGCAAGCCGAGTTGGCTAAGAAGCAAACAGAGCTCGAGAAGTTATTGGATAGCCTCGATCCCGAGGGAAAGACGCAAGATGAGTTGGATAAGGAAGCAGAGGAAGCCGAGTTGGATAAGAAGGCCGATGAGTTACAAAATAAGGTCGCAGATCTTGAGAAGGAGATTTCAAATCTCGAGATTTTGCTCGGCGGAGCAGATAGCGAGGATGATACCGCAGCTCTGCAAAATAAGTTGGCCACAAAGAAGGCAGAGTTAGAGAAGACTCAAAAGGAGCTTGATGCAGCTTTGAATGAGTTGGGCCCAGATGGGGATGAGGAAGAGACGCCTGCTCCTGCCCCTCAACCAGAGCAACCAGCACCAGCACCAAAACCAGAACAACCTGCCCCAGCACCG**GGTACC**GCCGCCAGCGAGGCCACGGG (Bcu I restriction site underlined; Acc65 I restriction site in bold)
II	CCGAAACCGGCACCGGCTCCGACCAGTGACGGTACTGAAGTTATAGAGGCTAAGCTCAATAAGGGAGAGGCAGAGCTCAATGCCAAGCAAGCAGAGCTTGCTAAGAAGCAAACTGAGCTTGAGAAGCTCCTCGATAGCTTAGATCCTGAGGGCAAGACGCAAGATGAGCTTGATAAAGAGGCCGAAGAGGCTGAGTTAGATAAGAAGGCCGATGAGTTACAAAATAAGGTGGCCGATTTAGAGAAGGAGATTTCCAATCTTGAGATTCTCTTAGGTGGCGCCGATAGCGAGGATGATACGGCTGCCTTACAAAATAAGCTCGCTACTAAGAAGGCTGAGTTAGAGAAGACACAAAAGGAGTTAGATGCAGCCCTCAATGAGTTGGGACCCGATGGGGATGAAGAAGAGACTCCAGCACCCGCTCCTCAACCAGAGCAACCCGCACCCGCACCAAAGCCCGAGCAACCTGCTCCGGCACCG**ACTAGT**GAAAGCCAGATGCTCACGCGC (Bcu I restriction site in bold)
III	CTGGCGCGTACGCGTAGGCCTGCTAGCCTGAAAGAAATCGACGAATCTGACTCTGAAGACTACGTTAAAGAAGGTCTGCGTGCGCCGCTGCAGTTCGAGCTGGACGTTAAACAGGCGAAACTGTCTAAACTGGAAGAACTGTCTGACAAAATCGACGAACTGGACGCGGAAATCGCGAAACTGGAAAAAGACGTTGAAGACTTCAAAAACTCTGACGGTGAACAGGCGGGTCAGTACCTGGCGGCGGCGGAAGAAGACCTGGTTGCGAAAAAAGCGGAACTGGAAAAAACCGAAGCGGACCTTAAGAAAGCGGTTAACGAACCGGAAAAACCGGCGGAAGAAACCCCGGCGCCGGCGCCGAAACCGGAACAGCCGGCGGAACAGCCGAAACCGGCACCGGCTCCG**GGTACC**GCCGCCAGCGAGGCCACGGG (Nhe I restriction site underlined; Kpn I restriction site in bold)
IV	AAGATTTTCGTCGTATCGGCCACCGGTCTGAAAGAAATCGACGAATCTGACTCTGAAGACTACGTTAAAGAAGGTCTGCGTGCGCCGCTGCAGTTCGAGCTGGACGTTAAACAGGCGAAACTGTCTAAACTGGAAGAACTGTCTGACAAAATCGACGAACTGGACGCGGAAATCGCGAAACTGGAAAAAGACGTTGAAGACTTCAAAAACTCTGACGGTGAACAGGCGGGTCAGTACCTGGCGGCGGCGGAAGAAGACCTGGTTGCGAAAAAAGCGGAACTGGAAAAAACCGAAGCGGACCTTAAGAAAGCGGTTAACGAACCGGAAAAACCGGCGGAAGAAACCCCGGCGCCGGCGCCGAAACCGGAACAGCCGGCGGAACAGCCGAAACCGGCACCGGCTCCG**ACTAGT**GAAAGCCAGATGCTCACGCG (BshT I restriction site underlined; Bcu I restriction site in bold.

Supplementary References

1. Moreno, A.T. *et al.* Immunization of mice with single PspA fragments induces antibodies capable of mediating complement deposition on different pneumococcal strains and cross-protection. *Clinical and vaccine immunology : CVI* **17**, 439-446 (2010).

2. Darrieux, M. *et al.* Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. *J Med Microbiol* **57**, 273-278 (2008).