(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 January 2022 (20.01.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/013160 A1

(51) International Patent Classification:
A61K 39/09 (2006.01) *A61P 31/04* (2006.01)

(21) International Application Number:
PCT/EP2021/069352

(22) International Filing Date:
12 July 2021 (12.07.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2050901-4 16 July 2020 (16.07.2020) SE

(72) Inventor; and
(71) Applicant: **HENRIQUES NORMARK, Birgitta** [SE/SE]; Sturegatan 29, 2 tr, 114 36 Stockholm (SE).

(72) **Inventors: NORMARK, Staffan**; Sturegatan 29, 2 tr, 114 36 Stockholm (SE). **IOVINO, Federico**; Rudbecks väg 13, 19251 Sollentuna (SE). **NARCISO, Ana Rita**; Brogatan 10, Lgh 1002, 17441 Sundbyberg (SE). **MELLROTH, Peter**; Nybohovsbacken 67, 11764 Stockholm (SE).

(74) **Agent: NOVITAS PATENT AB**; Sveavägen 166, 113 46 Stockholm (SE).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

(54) **Title:** STREPTOCOCCAL VACCINES

(57) **Abstract:** An immunogenic composition comprising bacterial membrane vesicles (MVs) comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen, characterized in that the MVs do not comprise an immunogenic amount of a streptococcal PspA antigen. The membrane vesicles may be artificial membrane particles. Medical uses of the composition in particular for immunization against pneumococcal disease, and methods of manufacturing the composition..

WO 2022/013160 A1

STREPTOCOCCAL VACCINES

TECHNICAL FIELD

The present invention relates to the field of vaccines against *Streptococcus pneumoniae* and other streptococci, in particular serotype-independent vaccines against *Streptococcus pneumoniae*.

BACKGROUND TO THE INVENTION

Streptococcus pneumoniae (the pneumococcus) is a major cause of morbidity and mortality globally. It is the most common cause of common respiratory tract infections such as otitis and sinusitis, but also a major contributor to more severe diseases including pneumonia, as well as sepsis and meningitis, i.e., invasive pneumococcal disease (IPD). Risk groups include young children and the elderly as well as immunocompromised individuals. Also, it has been shown that influenza virus infections also predispose for pneumococcal infections. Even though pneumococci cause infections with sometimes lethal outcomes, pneumococci normally colonize healthy children in the nasopharynx from where they may spread to susceptible individuals. Several pneumococcal virulence factors have been described and a major one is the polysaccharide capsule. So far 100 different so-called capsular serotypes have been identified depending on structural differences in the capsular polysaccharide. Current pneumococcal vaccines target a limited number of these capsules either as a polysaccharide-based vaccine (PPV23) targeting 23 different capsular serotypes, or as so called pneumococcal conjugated vaccines (PCVs), where a protein has been coupled to the sugar to get a better immune response especially in the risk groups. First, a 7-valent vaccine (PCV7) was launched that was changed to either a 10-valent (PCV10) or 13-valent vaccine (PCV13) around year 2010. PCVs have been introduced in childhood vaccination programs in many countries world-wide and have resulted in significant decreases in the number of IPD cases among vaccinated children. Vaccination has also resulted in an almost complete eradication of vaccine type (VT) strains from the nasopharynx of healthy children. However, this has not decreased pneumococcal carriage rates in children, due to replacement of VT with non-vaccine type strains (NVT). This profound serotype replacement in the carrier population has led to an expansion of NVTs among IPD cases, also in non-vaccinated age groups, affecting the efficacy of the PCVs. In Sweden in 2016, the IPD incidence among the

elderly did not decrease post-vaccination, and more than 70% of the IPD cases were caused by NVTs. PCV13 includes additionally 3 serotypes not included in PCV10, i.e. serotypes 3, 6A and 19A. So far, the efficacy against IPD caused by serotype 3 is being debated, and in Sweden, serotype 3 now constitutes a major serotype among IPD cases in the elderly.

Extracellular vesicles (EVs), also termed membrane vesicles (MVs) are membrane-derived vesicles released by bacteria. Pneumococcal EVs have been isolated from liquid cultures previously (Olaya-Abril et al. J. Proteomics. 2014;106:46-60). EVs have been shown to carry many pneumococcal virulence factors and proteins known to be membrane associated, but also to contain a cytosolic cargo enriched for the LytA autolysin, and the cytotoxin pneumolysin (Codemo et al. MBio. 2018;9(2)). EVs can be internalized by host cells such as dendritic cells (DCs), and lead to DC activation and release of pro-inflammatory cytokines. *In vivo* experiments have shown that intramuscular immunization of mice with pneumococcal EVs protect mice after intranasal challenge with the same pneumococcal serotype. It has been demonstrated that pneumococci growing on solid support, mimicking the biofilm mode of growth occurring during natural infection, exhibited a different expression pattern of surface antigens as compared to planktonic growth in liquid culture (WO2018/124959).

Opsonophagocytosis is the primary mechanism for clearance of pneumococci from the host, and the measurement of opsonophagocytic antibodies appears to correlate with vaccine-induced protection. However, it has proven difficult to design vaccines that elicit an efficient response in form of opsonophagocytic antibodies.

Taken together, the above issues stress the need for novel vaccine approaches especially for adults and the elderly, where NVTs now cause the major part of severe pneumococcal infections. Thus, an objective of the present invention is the provision of alternative or improved immunogenic compositions for conferring serotype-independent immunological responses and/or alternative or improved responses in terms of opsonophagocitic antibodies and/or protection in experimental models. Further objects include provision of improved and/or alternative immunogenic compositions relating to streptococcal pathogens.

DEFINITIONS

The term membrane vesicles (MVs) refers to membrane-derived vesicles released by bacteria, in particular streptococci, such as *Streptococcus pneumoniae*. The MVs can carry many virulence factors and proteins, both soluble and membrane-associated.

***Streptococcus pneumoniae* membrane vesicle microparticles (MP)** refers to membrane vesicle microparticles of a particular type, derived from culture on solid phase medium, as described in WO2018/124959. The MPs have distinct profile of various antigens compared to membrane vesicles of the type generated by bacteria in liquid culture. The term particularly excludes membrane vesicles of the type generated by bacteria in liquid culture.

Antigen refers to structures to which antibodies generated by the adaptive immune system of vertebrates specifically bind. Antigens are often proteins, peptides or polysaccharides (sometimes containing a lipid moiety). In the context of vaccines, an antigen is an ingredient in a vaccine against which a specific antibody response is intended to be elicited in the host to which the vaccine is administered. Some vaccines contain several antigens. In the vaccine context, a vaccine antigen generally bears a structural resemblance to a pathogen antigen present in a pathogen against which the vaccine is intended to be used. A vaccine antigen may be a fragment or a modified version of the pathogen antigen, or a complete pathogen antigen presented in a non-pathogenic form e.g. as an inactivated pathogen or a recombinant protein produced in a non-pathogenic host.

PspA refers to Pneumococcal surface protein A, which is a surface exposed protein that associates with the pneumococcal cell wall via non-covalent affinity binding to phospho-choline residues through its choline binding domain. PspA has been reported to have dual properties. It is a lactoferrin-binding protein and in addition a protein that may prevent complement activation. The sequence of PspA is highly variable in both amino acid sequence and length. PspA has been suggested as a vaccine candidate but the variability of the protein among pneumococcal strains are considered as a limiting factor for cross-protection. In a number of studies PspA has been reported to be an immune-dominant protein that provides immunological protection upon immunization. However, due to the high variability of the protein it is considered to not provide good cross-protection for a diverse set of pneumococcal serotypes or isolates. See Khan N. et al. (Front. Microbiol. 2017 2;8:742) for a review on PspA. A reference sequence from strain TIGR4 is presented in SEQ ID NO: 27.

MalX is a maltose/maltodextrin-binding protein required for the uptake of maltose (a disaccharide of glucose) and maltodextrin (glucose oligosaccharides) from the host environment. Streptococcal MalX homologues are present in most streptococcal species including *Streptococcus pneumoniae, Streptococcus pyogenes*, *Streptococcus suis, Streptococcus equi, Streptococcus mitis,* and *Streptococcus pseudopneumoniae.* MalX is a lipoprotein and as such attached to a lipid (diacylglycerol) in the outer leaflet of the plasma membrane through covalent thioester bond linkage via its *N*-terminal cysteine residue of the mature protein. Pneumococcal *malX* deletion strains have been found attenuated in virulence in mouse model system and were recently identified to induce a T_H17 based immune response and immunization provided protection against pneumococcal colonization (Moffitt K.L. et al. Cell Host Microbe 2011 17;9(2):158-65). *malX* is a highly conserved gene that is present in more than 99.6 % of the sequenced *S. pneumoniae* strains (n=8351 strains) obtained from PubMLST and has a very high sequence conservation (98.5 % of these sequences with >98 % sequence identity to the TIGR4 allele on the nucleotide level). A reference sequence from strain *S. pneumoniae* TIGR4 is presented in SEQ ID NO: 25, from *Streptococcus pyogenes* in SEQ ID NO: 43, from *Streptococcus suis* in SEQ ID NO: 44 from *Streptococcus equi* in SEQ ID NO: 45, from *Streptococcus mitis* in SEQ ID NO: 46 and from *Streptococcus pseudopneumoniae* in SEQ ID NO: 47.

PrsA, also termed PpmA in the literature, is also a lipoprotein like MalX (described in the previous section). Streptococcal PrsA homologues are present in most streptococcal species including *Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus suis, Streptococcus equi, Streptococcus mitis, Streptococcus mutans* and *Streptococcus pseudopneumoniae*. It functions as a Parvulin-like peptidyl-prolyl *cis-trans* isomerase (PPI), thereby acting as a chaperon that assists secreted proteins to fold properly by catalyzing isomerization of peptidyl bonds preceding proline residues. In addition to the Parvulin-like PPI domain the protein also contains a large N-terminal domain and a short C-terminal domain of unknown function but suggested to be involved in the chaperone function as revealed by the crystal structure of the *Bacillus subtilis* PrsA orthologue (Jakob R.P. et al. J. Biol. Chem. 2015 6;290(6):3278-92). PrsA-deficient pneumococci have decreased virulent properties and have been shown to contribute to nasal colonization and to early stages of infection. Immunization of mice with the avirulent pneumococcal strain T4Δ*pab* induced

antibody responses against several antigens including PrsA (Chimalapati S. et al. Infect. Immun. 2011 79(12):4965-76). However, this type of immunization provided poor cross-protection. Immunization with an orthologue of PrsA from *Streptococcus suis* was reported to provide good cross-protection against different *S. suis* serotypes in a murine model (Ref: PMID 30629173). *prsA* is a highly conserved gene that is present in more than 99.6 % of the sequenced *S. pneumoniae* strains (n=8351 strains) obtained from PubMLST and has a very high a sequence conservation (99.5 % of these sequences with >98 % sequence identity to the TIGR4 allele on the nucleotide level). A reference sequence from strain TIGR4 is provided in SEQ ID NO: 26, from *Streptococcus pyogenes* in SEQ ID NO: 48, from *Streptococcus suis* in SEQ ID NO: 49, from *Streptococcus equi* in SEQ ID NO: 50, from *Streptococcus mitis* in SEQ ID NO: 51, from *Streptococcus mutans* in SEQ ID NO: 52 and from *Streptococcus pseudopneumoniae* in SEQ ID NO: 53.

Pneumolysin (termed Ply herein) is a 53 kDa cholesterol dependent cytolysin released by *Streptococcus pneumoniae* upon lysis. It is one of the major virulence factors of this bacterium. It forms pores in all eukaryotic cells that have cholesterol in their membranes. The formation of pores by Ply frequently results in host cell death as membrane integrity is destroyed. Ply plays a central role in protecting the pneumococcus from complement attack and aiding its spread to other tissues/organs. Ply is able to activate the classical complement pathway, even in the absence of Ply specific antibody (Mitchell T. J. et al. Subcell. Biochem. 2014;80:145-60). A reference sequence from strain TIGR4 is presented in SEQ ID NO: 28.

LytA is the major autolysin of *Streptococcus pneumoniae*. Lysis is caused by cleaving the lactyl-amide bond between the stem peptides and the glycan strands of peptidoglycan, resulting in hydrolysis of the cell wall. LytA is considered as a virulence factor since *lytA* deficient pneumococci are much less virulent in a murine infection model (Canvi J.R. et al J. Infect. Dis. 1995 172(1):119-23). However, the explicit contribution of LytA to pneumococcal virulence is still unclear. It is possible that LytA-mediated lysis releases other virulence factors such as pneumolysin. LytA could also be released to lyse neighbouring non-competent pneumococcal cells in a fratricidal manner. This would potentially facilitate genetic exchange between naturally competent pneumococcal populations that easily take up and incorporate DNA by homologous recombination. A third possibility is that LytA mediates lysis to release proteins involved in immune evasion or cell wall components that

may interfere with the host immune response (Mellroth et al. J Biol Chem. 2012;287(14):11018-29.). A reference sequence from strain TIGR4 is presented in SEQ ID NO: 29.

PspC / CbpA (choline binding protein A) is a protein that binds the phosphocholine present in the teichoic acid and the lipoteichoic acid of the cell membrane and the cell wall. It is a major pneumococcal adhesin. It promotes pneumococcal adherence via a human-specific interaction with the ectodomain of the polymeric Ig receptor. It also prevents activation of C3b and complement-mediated opsonophagocytosis of pneumococci (Bergmann& Hammerschmidt Microbiology. 2006;152(Pt 2):295-303.). The *pspC* locus is highly polymorphic and 11 major groups of this protein have been identified. Single PspC proteins are identified by sequential numbers separated from the group number by a dot (Iannelli et al. Gene. 2002;284(1-2):63-71) as follows: PspC1.1, PspC2.1, PspC2.2, PspC3.1, PspC3.4, PspC4.2/PspC10.1, PspC5.1, PspC6.1/PspC9.1, PspC7.1, PspC8.1, PspC11.1 and PspC11.4 (SEQ ID NOs: 30-41, respectively).

RrgB is the major subunit and stalk protein of the pneumococcal pilus. *S. pneumoniae* pilus 1 is encoded by a genetic islet (PI-1) present in about 30% of the pneumococcal strains and is implicated in adhesion to epithelial cells, lung infection, and virulence. Pilus 1 is composed of the backbone subunit RrgB, the minor pilin subunits RrgA, and RrgC (Barocchi, et al.Proc Natl Acad Sci U S A. 2006;103(8):2857-62). A reference sequence from strain TIGR4 is presented in SEQ ID NO: 42.

The term ***protective immunity*** in the present context refers to immunization measures resulting in any degree of reduction in the likelihood of developing the condition for which the protective immunity is relevant, including a minor, substantial or major reduction in likelihood of developing the condition as well as total prevention. Preferably, the degree of likelihood reduction is at least a minor reduction.

The term ***sequence identity*** expressed in percentage is defined as the value determined by comparing two optimally aligned sequences over a comparison window, wherein a portion of the sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences

to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Unless indicated otherwise, the comparison window is the entire length of the sequence being referred to. In this context, optimal alignment is the alignment produced by the BLASTP algorithm as implemented online by the US National Center for Biotechnology Information (see The NCBI Handbook, 2nd edition [https://www.ncbi.nlm.nih.gov/books/NBK143764/]), with the following input parameters: Word length=3, Matrix=BLOSUM62, Gap cost=11, Gap extension cost=1.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Membrane particles (MP) produced by serotype 3 pneumococci are larger and contain more cytosolic proteins than MPs from serotype 4. (A) Size distribution of MPs from the serotype 3 strain BHN428 (n=523) and the serotype 4 strain T4 (n=378), as measured from electron micrographs. **(B)** Mass spectrometry identification of proteins present in MP isolated from the serotype 3 and 4 strains respectively. The number indicates proteins found based on their predicted subcellular localization, defined as cytosolic, transmembrane, membrane-associated (lipoproteins), cell wall associated and secreted.

Figure 2. Protein content of extracellular vesicles (EVs) and membrane particles (MPs). Immunoblot detection of pneumococcal proteins and virulence factors present in EVs and MPs isolated from wild-type strain *S. pneumoniae* T4 (T4 WT), and its isogenic deficient mutants in pneumolysin (T4Δ*ply*) and LytA (T4Δ*lytA*).

Figure 3. Intranasal immunization with MP confers protection against intranasal pneumococcal infection. (A, B) T4 membrane particles (MP) confer cross-protection against infection with a serotype 1 strain. **(A)** Intranasal immunization of C57BL/6 mice MP from strain T4 of serotype 4, and then infection with strain BHN733 of serotype 1, resulted in 80% survival. 20 mice per group. **(B)** Bacteria (in colony forming units, CFU) in the lungs of immunized and infected mice in (A) after sacrifice. Each dot represents one mouse. **(C,D)** Immunization with MP of serotype 3 confers antibody-dependent protection against intranasal infection with the same strain that is dependent on proteins, but not on capsule. **(C)** Percentage of mice that survived the intranasal infection with serotype 3 bacteria of wild type C57BL/6 mice or of B-cell deficient mice (muMt knockout mice) immunized with MPs

from the same serotype 3 strain BHN428, or of wild type mice immunized with MPs from the serotype 4 strain T4. 20 mice per group. **(D)** Number of bacteria (CFU) in the lungs of mice from (C) at sacrifice. Each dot represents one mouse. **(E,F)** Protection conferred by MPs against intranasal pneumococcal challenge is not dependent on the cholesterol-binding cytotoxin pneumolysin. **(E)** Percentage survival after intranasal infection with T4 of wild type C57BL/6 mice immunized with MPs from T4 or its pneumolysin deficient strainT4Δ*ply*. 10 mice per group, 5 mice immunized with adjuvant as control. **(F)** Number of bacteria (CFU) in the lungs of mice at sacrifice. Each dot represents one mouse, * = $p<0.05$; ** = $p<0.01$; *** =$p<0.001$; **** = $p<0.0001$.

Figure 4. Intranasal immunization of mice with MP increases survival after intranasal pneumococcal challenge in a 7-day survival model. (A-D) Intranasal immunization of C57BL/6 mice with MPs from strain T4 increases survival after intranasal challenge with strain BHN733 of serotype 1 (corresponding to the experiments in Figure 3). Bacterial counts (CFU) in the blood of mice immunized with **(A)** PBS, **(B)** Adjuvant only, **(C)** MPs from T4, or **(D)** MPs+Adjuvant, and then infected with strain BHN733 of serotype 1. Each dot represents one mouse. **(E-H)** Immunization of mice with MPs from serotype 3 protects against intranasal infection with the same strain. Bacterial counts (CFU) in the blood of mice immunized with **(E)** Adjuvant only, **(F)** MPs from serotype 3+Adjuvant or **(G)** MPs from serotype 4+Adjuvant, and then infected with strain BHN428 of serotype 3. **(H)** CFU in the blood of mice deficient in B cells immunized with MPs from the serotype 3 strain BHN428, and then infected with the same strain (Figure 1C). In all graphs, each dot represents one mouse. **(I-K)** The cytotoxin pneumolysin does not affect the protective effect by MPs. CFU in the blood of mice immunized with **(I)** Adjuvant only, **(J)** MPs from T4+Adjuvant or **(K)** MPs from T4Δ*ply*+Adjuvant, and then infected with strain T4. Each dot represents one mouse.

Figure 5. Intranasal immunization with MPs elicits production of pneumococcal-specific IgG. Mice were immunized intranasally with MP from strain T4. Using ELISA, pneumococcal specific IgG were detected in wells coated with **(A)** wild type T4, or **(B)** T4R (isogenic mutant in the capsule of T4) bacteria. Each dot represents one mouse serum. The low responder mice in the MPs+Adjuvant and MP groups are those that did not survive after serotype 1 challenge. These mice are highlighted in the graphs within boxes with red borders. **(C)** Immunofluorescence microscopy of pneumococcal strains belonging to serotypes 1, 3 and

6B stained (green) using pooled sera from mice immunized with only Adjuvant or T4 MP + Adjuvant as primary antibodies. Scale bar: 5 μm. **(D)** RAW 264.7 macrophages were incubated with *S. pneumoniae* serotype 1 pre-treated with pooled sera from mice immunized with Adjuvant or MPs+Adjuvant, and bacterial adhesion to the cells was measured. **(E)** Effect on phagocytic killing by adding the sera in (E) was estimated by calculating bacterial survival inside the cells. Data are represented as means +/- SEM of three independent experiments. * = $p<0.05$.

Figure 6. Immunization with EVs, as compared to MPs (see above Figure 3-5), from T4 confers lower cross-protection against intranasal infection with serotype 1. Intranasal immunization of C57BL/6 mice with extracellular vesicles (EVs) from strain T4 of serotype 4 led to a lower survival and lower production of pneumococcal specific IgG as compared to immunization with MPs. **(A-B)** Using ELISA analysis, specific IgG was detected two weeks after the first immunization with EV (prior to the second immunization) and four weeks after the first immunization (2 weeks after the booster, prior to infection), in wells coated with **(A)** wild type T4, and **(B)** T4R (isogenic mutant in the capsule of T4) bacteria. Each dot represents one mouse serum. **(C)** Intranasal immunization of C57BL/6 mice with EVs from strain T4 of serotype 4 and infection with the strain BHN733 of serotype 1 led to lower survival (40%) compared to mice immunized with MPs from the same strain (see Figure 1). 20 mice per group. * = $p<0.05$, ** = $p<0.01$. **(D)** Bacterial counts (CFU) detected in the lungs of immunized and infected mice from (C) after sacrifice. **(E-F)** CFU detected in the blood of mice immunized with **(E)** EVs only or **(F)** EVs+Adjuvant. Each dot represents one mouse.

Figure 7. Immunization with MP reduces pneumococcal colonization of the upper respiratory tract *in vivo*. IVIS imaging of the fluorescent signal generated by pneumococci present in the upper respiratory tract of mice immunized with **(A)** adjuvant only or with **(B)** MPs from strain T4+Adjuvant, and then challenged with a strain of serotype 1 (BHN733). ROIs (Regions of Interest) have been defined per each mouse (ROIs within orange ovals) focusing on the nasopharyngeal tract. Mice that did not survive are marked with red borders and mice that survived are marked with green borders. **(C)** Per each group ("Adjuvant" and "MPs+Adjuvant") average and standard deviation of the bacterial fluorescent intensity have been calculated based on the fluorescent intensity value of each mouse per group (ten mice per group). * = $p<0.05$.

Figure 8. Cross-protection against IPD is not dependent on PspA. (A, B) Western blot analysis of bacterial lysates, supernatant samples from choline washed (cells treated with 5% choline chloride to release choline binding proteins) and from lysates of corresponding choline washed cells (Pellet CW). Binding of sera from mice immunized with T4 MPs+Adjuvant (used as primary antibody) to **(A)** T4 and T4Δ*pspA,* **(B)** serotype 1 and serotype 3 cells were analyzed. Sera came from mice included in experiments represented in Figure 1A-B. **(C-E)** Percentage of mice immunized with either T4 MP or T4Δ*pspA* MP that survived an intranasal infection with **(C)** T4, **(D)** serotype 3 (BHN428) or **(E)** serotype 1 (BHN733) bacteria. 10 mice per group (the group immunized with T4Δ*pspA* MP and infected with serotype 1 had 9 mice), 5 mice/pneumococcal strain immunized with adjuvant as control. **(F-H)** Number of bacteria (CFU) in the lungs of mice infected with **(F)** T4, **(G)** serotype 1 (BHN733) or **(H)** serotype 3 (BHN428) bacteria at sacrifice, each dot represents one mouse, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, n.s. – not significant.

Figure 9. Intranasal immunization with MP from T4Δ*pspA* elicits the same or even higher cross-protection against intranasal challenge with strains of serotype 1 and serotype 3. Intranasal immunization with MP of T4 or its isogenic mutant in PspA, T4Δ*pspA,* subsequently followed by bacterial challenge with strains T4, serotype 1 (BHN733) or serotype 3 (BHN428), in a 7-day survival experiment. **(A)** Number of bacteria (CFU) in the blood of mice, each dot represents one mouse. **(B)** Using ELISA assays, pneumococcal specific IgG was detected in wells coated with T4, serotype 1 or serotype 3 pneumococci. Each dot represents one mouse serum collected from mice used in the survival *in vivo* experiment showed in Figure 3C-E. * = $p<0.05$; ** = $p<0.01$; n.s. – not significant.

Figure 10. Cross-protection against IPD is mediated by the lipoproteins PrsA and MalX. (A, B) Validation of results from the IP experiment and MS analysis confirming the identity of the major immunoreactive proteins. Lysates from T4 wildtype (WT), single mutants T4Δ*pspA*, T4Δ*prsA*, T4Δ*malX*, double mutants T4Δ*pspA*Δ*prsA*, T4Δ*pspA*Δ*malX*, and triple mutant T4Δ*pspA*Δ*malX*Δ*prsA* as indicated, were subjected to Western blot analysis. PVDF-membranes were incubated with immune sera from mice immunized with **(A)** T4 MPs+Adjuvant or **(B)** T4Δ*pspA* MPs+Adjuvant. Sera came from mice included in experiments represented in Figure 3C-E. A second incubation with sheep anti-mouse IgG-HRP conjugated facilitated chemiluminescent detection. **(C-E)** Percentage of mice immunized with either T4

MPs or T4Δ*pspA*Δ*malX*Δ*prsA* MP that survived an intranasal infection with **(C)** T4, **(D)** serotype 1 (BHN733) or **(E)** serotype 3 (BHN428) bacteria. 10 mice per group (the group immunized with T4Δ*pspA*Δ*malX*Δ*prsA* MP and infected with T4 included 9 mice). 5 mice/pneumococcal strain were immunized with adjuvant as control. **(F-H)** CFU in the lungs of mice infected with **(F)** T4, **(G)** serotype 1 (BHN733) or **(H)** serotype 3 (BHN428) bacteria at sacrifice, each dot represents one mouse, * = p<0.05, ** = *p*<0.01, n.s. – not significant.

Figure 11. Mice immunized with MP from T4Δ*pspA*Δ*malX*Δ*prsA* are not protected against intranasal challenge with the pneumococcal strains T4, serotype 1 or serotype 3.

Intranasal immunization with MPs from T4, T4Δ*pspA* or T4Δ*pspA*Δ*malX*Δ*prsA,* subsequently followed by bacterial challenge with strains T4, serotype 1 (BHN733) or 3 (BHN428), in a 7-day survival experiment, as shown in Figure 4C-E. **(A)** Number of bacteria (CFU) in the blood of mice, each dot represents one mouse. **(B)** Using ELISA analysis, pneumococcal specific IgG was detected in wells coated with T4, serotype 1 and serotype 3 pneumococci respectively. Each dot represents one mouse serum collected from mice used in the survival *in vivo* experiment showed in Figure 4C-E. * = *p*<0.05; ** = *p*<0.01; *** = *p*<0.001; n.s. – not significant.

Figure 12. Lipoproteins PrsA and MalX in MPs from pneumococci are both required for cross protection against serotype 1 infection

(A, B) Mice were immunized with MPs purified from different serotype 4 mutants, lacking the antigens PspA, MalX and PrsA in different combinations and challenged with serotype 1 pneumococci. **(A)** Percentage of mice immunized with either T4 MPs+Adjuvant, T4Δ*pspA*Δ*malX* MPs+adjuvant, T4Δ*pspA*Δ*prsA* MPs+adjuvant, T4Δ*malX*Δ*prsA* MPs+adjuvant or T4Δ*pspA*Δ*malX*Δ*prsA* MPs + Adjuvant or Adjuvant only that survived an intranasal infection with serotype 1 (BHN733) bacteria. **(B)** CFU in the lungs of mice upon sacrifice. Each dot represents one mouse. Red-colored dots symbolize mice sacrificed before the end of the experiment. 10 mice per group (the group immunized with T4 MP included 9 mice). 5 mice/pneumococcal strain were immunized with adjuvant as control. * = p<0.05, ** = *p*<0.01.

Figure 13. MalX and PrsA are both required for protection against invasive pneumococcal disease by serotype 1

(A-F) Intranasal immunization with MPs from different T4 mutants, subsequently followed by bacterial challenge with a serotype 1 (BHN733) strain, in a 7-day survival experiment, as shown in Figure 5A,B. CFU in the blood the blood of mice is shown for the groups immunized with (A) T4 MP + Adjuvant, (B) T4Δ*pspA*Δ*malX* MP+ Adjuvant, (C) T4Δ*pspAX*Δ*prsA* MP + adjuvant, (D) T4Δ*malX*Δ*prsA* MP + Adjuvant, (E) T4Δ*pspA*Δ*malX*Δ*prsA* MP + Adjuvant and (F) Adjuvant only. Each dot represents one mouse. Each dot represents one mouse. Red-colored dots represent mice that were sacrificed at that time point.

Figure 14. Detection of MalX and PrsA in membrane particles as compared to purified recombinant proteins. Western blot using pooled sera of mice that were immunized with membrane particles from T4Δ*pspA* for antigen detection. MalX and PrsA were identified as main antigens. Secondary antibody anti-mouse IgG was used for detection.

SUMMARY OF THE INVENTION

The present invention relates to the following items. The subject matter disclosed in the items below should be regarded disclosed in the same manner as if the subject matter were disclosed in patent claims.

1. An immunogenic composition comprising bacterial (preferably streptococcal) membrane vesicles (MVs), said MVs comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen, **wherein** the MVs preferably do **not** comprise an immunogenic amount of a streptococcal PspA antigen, wherein the MVs are preferably *Streptococcus pneumoniae* MVs.

2. An immunogenic composition comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen, **characterized in that** the composition does **not** comprise an immunogenic amount of a streptococcal PspA antigen, wherein when present, the sequence(s) of the MalX antigen and/or the PrsA antigen is/are preferably derived from *Streptococcus pneumoniae*.

3. An immunogenic composition comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen, **characterized in that** the MalX antigen and/or the PrsA antigen are **not** associated with a membrane vesicle derived from the same strain as the antigen(s), wherein when present, the sequence(s) of the MalX antigen and/or the PrsA antigen is/are preferably derived from *Streptococcus pneumoniae*.

4. The composition according to any of the preceding items, wherein composition comprises the MalX antigen.

5. The composition according to any of the preceding items, wherein composition comprises the PrsA antigen.

6. The composition according to any of the preceding items, wherein composition comprises both the MalX antigen and the PrsA antigen.

7. The composition according to any of the preceding items, wherein the MalX antigen is present at a concentration of at least 0.001 µg/ml, preferably at least 0.05 µg/ml, more preferably at least 0.1 µg/ml, most preferably at least 1 µg/ml.

8. The composition according to any of the preceding items, wherein the PrsA antigen is present at a concentration of at least 0.001 µg/ml, preferably at least 0.05 µg/ml, more preferably at least 0.1 µg/ml, most preferably at least 1 µg/ml.

9. The composition according to any of the preceding items, wherein both the PrsA antigen and the MalX antigen are present at relative amounts of 10:1 to 1:10 by weight, preferably 5:1 to 1:5, more preferably 2:1 to 1:2, most preferably about 1:1.

10. The composition according to any of the preceding items, wherein the PspA antigen is absent or present at a concentration of less than 0.01 µg/ml, preferably less than 0.005 µg/ml, more preferably less than 0.001 µg/ml, most preferably absent.

11. The composition according to any of the preceding items, wherein the MalX antigen comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to any one of SEQ ID NOs: 25 or 43-47 over a stretch of at least 50 amino-acids, preferably at least 100 amino acids, more preferably at least 200 amino acids, most preferably over the entire sequence presented in said SEQ ID NO.

12. The composition according to any of the preceding items, wherein the MalX antigen comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 25.

13. The composition according to any of the preceding items, wherein the PrsA antigen comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to any one of SEQ ID NOs: 26 or 48-53 over a stretch of at least 50 amino-acids, preferably at least 100 amino acids, more preferably at least 200 amino acids, most preferably over the entire sequence presented in said SEQ ID NO.

14. The composition according to any of the preceding items, wherein the PrsA antigen comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 26.

15. The composition according to any of the preceding items, wherein the PspA antigen comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 27.

16. The composition according to item 1 or item dependent thereon, wherein the membrane vesicles are of the type *Streptococcus pneumoniae* membrane vesicle microparticles.

17. The composition according to any of items 2-3, or any items dependent thereon, wherein the antigens are associated with MVs, which may be native or artificial MVs.

18. The composition according to item 1 or any item dependent thereon or item, 17, wherein the MVs comprise MalX at the level of ≥0.001 µg/µg, preferably ≥0.01 µg/µg, more preferably ≥0.02 µg/µg, yet more preferably ≥0.05 µg/µg, most preferably ≥0.1 µg/µg of the total protein in the MVs.

19. The composition according to item 1 or any item dependent thereon or item 17 or any item dependent thereon, wherein the MVs comprise PrsA at the level of ≥0.001 µg/µg, preferably ≥0.01 µg/µg, more preferably ≥0.02 µg/µg, yet more preferably ≥0.05 µg/µg, most preferably ≥0.1 µg/µg of the total protein in the MVs.

20. The composition according to item 1 or any item dependent thereon or item 17 or any item dependent thereon, wherein the MVs lack PspA, or PspA is present at a level which is less than 0.01 µg/µg, preferably less than 0.05 µg/µg, yet more

preferably less than 0.001 µg/µg of the total protein in the MVs, most preferably the MVs lack PspA.

21. The composition according to item 1 or any item dependent thereon or item 17 or any item dependent thereon, wherein MVs are 5-1000 nm in diameter, preferably 5-300 nm, more preferably 15-175 nm, most preferably 10-125 nm.

22. The composition according to item 1 or any item dependent thereon or item 17 or any item dependent thereon, comprising MVs in an amount of 1 µg/ml, preferably 5 µg/ml, more preferably 10 µg/ml, most preferably 100 µg/ml.

23. The composition according to item 1 or any item dependent thereon or item 17 or any item dependent thereon, wherein the MVs are derived from any *Streptococcus pneumoniae* strain, preferably any strain/s of serotype 1, 3, 4, 5, 6A, 6B, 6C, 6D, 7F, 8, 9N, 9V, 10A, 11A, 11B, 12F, 13, 14, 15A, 15B, 15C, 16F, 17F, 18C, 19A, 19F, 20, 21, 22F, 23A, 23B, 23F, 24F, 31, 33F, 34, 35B, 35F, 37, 38, including strains TIGR4, P1031, BHN418, and A66.

24. The composition according to any of the preceding items, wherein the composition comprises the MalX antigen and/or the PrsA antigen associated with membrane vesicles from a host cell being a firmicute.

25. The composition according to any of the preceding items, wherein the antigen(s) is/are associated with membrane vesicles from a host cell, where the antigen(s) is/are heterologous in relation to the host cell.

26. The composition according to item 25, wherein the host cell is of the genus *Lactobacillus* or *Lactococcus*.

27. The composition according to item 26, wherein the host cell is a *Lactococcus lactis*-cell.

28. The composition according to item 1 or any item dependent thereon or item 17 or any item dependent thereon, wherein the membrane vesicles are artificial membrane particles.

29. The composition according to item 1 or any item dependent thereon or item 17 or any item dependent thereon, wherein the membrane vesicles comprise disrupted bacterial cells, preferably mechanically disrupted cells.

30. The composition according to item 1 or any item dependent thereon or item 17 or any item dependent thereon, wherein the membrane vesicles comprise membrane vesicles derived from mechanically disrupted bacterial cells.

31. The composition according to item 2 or any item dependent thereon, wherein the composition comprises the MalX antigen and/or the PrsA antigen **not** associated with streptococcal (preferably *Streptococcus pneumoniae*) membrane vesicles.

32. The composition according to any of the preceding items, wherein the composition comprises the MalX antigen and/or the PrsA antigen as isolated proteins, preferably recombinant proteins, or as peptides such as synthetic peptides or truncated recombinant proteins.

33. The composition according to any of the preceding items, wherein the composition further comprises a nanoparticle carrier.

34. The composition according to item 33, wherein the nanoparticle carrier comprises an ISCOM, an ISCOM matrix, a liposome, a polymeric nanoparticle, an inorganic nanoparticle, a non-biodegradable nanoparticle, a calcium phosphate particle, an emulsion or a virus-like particle.

35. The composition according to any of the preceding items, wherein the composition is devoid of whole *Streptococcus* cells, preferably *Streptococcus pneumoniae* cells.

36. The composition according to any of the preceding items, further comprising capsular polysaccharides from a *Streptococcus sp.,* preferably *Streptococcus pneumoniae.*

37. The composition according to any of the preceding items, for use as a medicament.

38. The composition according to any of the preceding items, wherein the composition is a vaccine.

39. The composition according to any of the preceding items, wherein the composition further comprises an adjuvant, preferably aluminium hydroxide, a lipid-based

substance such as squalene, an immune-stimulating component or a protein from a microbe such as diphtheria (preferably CRM), cholera (preferably choleratoxoid CTB or mmCT) or *Escherichia coli* (preferably non-toxic heat-labile toxin such as LTK63 or dmLT).

40. The composition according to any of the preceding items, wherein the composition is capable of eliciting serotype independent antibodies against a *Streptococcus sp.*, preferably *Streptococcus pneumoniae,* when administered to a mammalian host.

41. The composition according to any of the preceding items, wherein the composition is capable of eliciting opsonophagocitic antibodies against a *Streptococcus sp.*, preferably *Streptococcus pneumoniae,* when administered to a mammalian host.

42. The composition according to any of the preceding items, wherein the composition is capable of eliciting serotype-independent antibodies against a *Streptococcus sp.*, preferably *Streptococcus pneumoniae,* when administered to a mammalian host.

43. The composition according to any of the preceding items, for use in a method for inducing protective immunity against a *Streptococcus sp.* in a subject.

44. The composition according to any of the preceding items, for use in a method for inducing protective immunity against *Streptococcus pneumoniae* in a subject.

45. The composition according to any of the preceding items, for use in a method for inducing protective immunity against a *Streptococcus sp.* in a subject, wherein the immunity is protective against a condition selected from pneumococcal sinusitis, pneumococcal otitis, pneumococcal pneumonia and invasive pneumococcal disease including but not limited to pneumococcal sepsis and pneumococcal meningitis, preferably invasive pneumococcal disease.

46. The composition according to any of the preceding items, for use according to any of items 43-45, wherein the protective immunity is serotype-independent.

47. A method for manufacturing a composition according to item 28 or any item dependent thereon, comprising:

    a. expressing a streptococcal MalX antigen and/or a streptococcal PrsA antigen in a suitable host cell, preferably heterologously;

b. generating membrane vesicles by disrupting the host cell expressing said antigens; and

c. recovering the generated membrane vesicles to form an immunogenic composition comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen associated with membrane vesicles.

48. The method according to item 47, wherein the host cells are mechanically disrupted, preferably by shear forces, most preferably with a French press.

49. The method according to any of items 47-48, wherein the recovering step comprises precipitation by centrifugation followed by resuspension.

DETAILED DESCRIPTION

The present invention is based on the inventor's studies on membrane vesicles secreted by *Streptococcus pneumoniae*. These vesicles are known to differ in properties between cells grown in liquid culture and on solid media. Intranasal immunization with the membrane vesicles confers serotype-independent cross-protection against invasive pneumococcal disease (Example 1), reduces pneumococcal load in the airways (Example 1) and leads to production of pneumococcal-specific antibodies with opsonophagocytic activity (Example 2). The membrane vesicles can confer antibody-dependent, and mainly serotype–independent, protection against challenge with serotype 3 pneumococci (Examples 3 and 4).

The inventors conducted further studies to elucidate the specific antigens responsible for the immunogenic effects of the membrane vesicles. The cytotoxin pneumolysin Ply (while a known vaccine candidate) was found not to be required (Example 5).

In addition, a major membrane vesicle protein PspA, while a dominant immunogen in the MPs and a known vaccine antigen candidate, was unexpectedly found to be not required for the cross-protection. In fact, the inventors surprisingly discovered that better protection could in fact be achieved with membrane vesicle preparation devoid of PspA (Example 6). The inventors further unexpectedly discovered that cross-protection mediated by membrane vesicles against invasive pneumococcal disease is dependent on the lipoproteins PrsA and MalX (Example 7). Thus, the inventors have discovered the use a cocktail of MP

from different serotypes lacking PspA, but presenting MalX and PrsA, as a novel vaccine approach that can target most non-vaccine pneumococci, especially in the elderly.

It was also demonstrated that antibodies raised against MPs containing MalX and/or PrsA recognized their native antigens much more efficiently than plain protein recombinant versions, indicating that the antibody binding is to a significant degree dependent on features missing from the plain recombinant proteins (Example 9). Such features include the presence of the signal peptide and the lipid modification of the terminal cysteine residue, e.g. N-palmitoyl cysteine or S-diacylglycerol cysteine. Therefore, the antigens as presented in their native context incorporated in MPs are superior as vaccine antigens compared to plain recombinant proteins.

Finally, the inventors demonstrated that MalX and/or PrsA-containing membrane vesicles may be generated artificially through mechanical cell disruption, for large-scale production (Examples 10 and 11).

The above studies resulted in the invention of novel immunogenic compositions and methods disclosed below.

Immunogenic compositions

In a first aspect, the present invention provides an immunogenic composition comprising bacterial membrane vesicles (preferably streptococcal, most preferably *Streptococcus pneumoniae* membrane vesicles) (MVs), preferably said MVs comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen. Preferably, the MVs do **not** comprise an immunogenic amount of a streptococcal PspA antigen. In a preferred combination, both MalX and PrsA are present, but PspA absent. It should be understood that the MVs of the first aspect may be of the type produced by bacteria through biological processes, or be artificial MVs produced through disruption of bacteria as described herein.

In a second aspect, the present invention provides an immunogenic composition comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen, **characterized in that** the composition does **not** comprise an immunogenic amount of a streptococcal PspA antigen. In a preferred variation of the second aspect, there is provided an immunogenic composition comprising a streptococcal MalX antigen and a streptococcal PrsA antigen associated with membrane vesicles from a host cell, characterized in that the MalX antigen and the PrsA

antigen are not associated with a membrane vesicle derived from the same strain as the antigens, and wherein the sequence(s) of the MalX antigen and the PrsA antigen are derived from *Streptococcus pneumoniae*. It should be understood that the antigens in the composition of the second aspect may be associated with MVs produced by bacteria through biological processes, or with artificial MVs produced through disruption of bacteria as described herein.

In a third aspect, the present invention provides an immunogenic composition comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen, **characterized in that** the MalX antigen and/or the PrsA antigen are **not** associated with a membrane vesicle derived from the same strain as the antigen(s).

The antigens may be associated with membrane vesicles from host organisms to which they have been introduced by genetic engineering. In this case, the antigen(s) would be associated with a membrane vesicle but in a non-native context. Preferably, the antigens are heterologous in relation to the membrane vesicles. It is also contemplated that the MalX antigen and/or the PrsA antigen may not be associated with any membrane vesicle. The antigens in the composition of the third aspect may be associated with MVs either produced by bacteria through biological processes, or with artificial MVs produced through disruption of bacteria as described herein.

Antigens

Preferably, the composition of the first, second or third aspect comprises the MalX antigen. Also preferably, the composition comprises the PrsA antigen. More preferably, the composition comprises both the MalX antigen and the PrsA antigen. When both the PrsA antigen and the MalX antigen are present, they may be present at relative amounts of 10:1 to 1:10 by weight, preferably 5:1 to 1:5, more preferably 2:1 to 1:2, most preferably about 1:1. Alternatively, the PrsA antigen and the MalX antigen may be present at relative amounts of 10:1 to 1:10 on a molar basis, preferably 5:1 to 1:5, more preferably 2:1 to 1:2, most preferably about 1:1.

On weight basis, the MalX antigen may be present at a concentration of at least 0.001 µg/ml, preferably at least 0.05 µg/ml, more preferably at least 0.1 µg/ml, most preferably at least 1 µg/ml. The PrsA antigen may present at a concentration of at least 0.001 µg/ml, preferably at least 0.05 µg/ml, more preferably at least 0.1 µg/ml, most preferably at least 1

µg/ml. The PspA antigen may be absent or present at a concentration of less than 0.01 µg/ml, preferably less than 0.005 µg/ml, more preferably less than 0.001 µg/ml, most preferably absent.

On molar basis, the MalX antigen may be present at a concentration of at least 0.025 nM, preferably at least 1.25 nM, more preferably at least 2.5 nM, most preferably at least 25 nM. The PrsA antigen may present at a concentration of at least 0.025 nM, preferably at least 1.25 nM, more preferably at least 2.5 nM, most preferably at least 25 nM. The PspA antigen may be absent or present at a concentration of less than 0.25 nM, preferably at less than 0.05 nM, more preferably less than 0.01 nM, most preferably absent.

The MalX and/or PrsA antigens may be proteins, peptides or peptidomimetics sharing a sequence similarity with the naturally occurring antigens. The antigens may be a fragment or a modified version of the naturally occurring antigen, or a complete antigen.

The sequence of the MalX antigen is preferably derived from *Streptococcus pneumoniae.* The MalX antigen may comprise a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to any one of SEQ ID NOs: 25 or 43-47. The MalX antigen may comprise a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 25.

The sequence of the PrsA antigen is preferably derived from *Streptococcus pneumoniae.* The PrsA antigen may comprise a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to any one of SEQ ID NOs: 26 or 48-53.

The PrsA antigen may comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 26.

The sequence of the PspA antigen is preferably derived from *Streptococcus pneumoniae.* The PspA antigen may comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 27.

The sequence identity referred to above may be determined over a stretch of at least 50 amino acids, preferably at least 100 amino acids, more preferably at least 200 amino acids, most preferably over the entire sequence presented in the SEQ ID NO serving as the reference.

Membrane vesicle composition

Preferably, the composition of the first aspect comprises the MalX antigen and/or the PrsA antigen, most preferably both. The antigens are regarded present when they are present in at least immunogenic amounts, i.e., such amount that the composition is capable of inducing an immune response against the antigen after the composition has been administered to a vertebrate host, such as a human or a mouse.

The membrane vesicles (MVs) of the composition of the first aspect may be of the type *Streptococcus pneumoniae* membrane vesicle microparticles. Such *Streptococcus pneumoniae* membrane vesicle microparticle may be characterized by that they comprise:

i. the protein **Ply** at the level of ≥0.070 μg/μg total protein in the MP;
ii. the protein **LytA** at the level of ≥0.070 μg/μg total protein in the MP;
iii. the protein **PspC** at the level of ≥0.130 μg/μg total protein in the MP; and/or
iv. the protein **RrgB** at the level of ≥0.020 μg/μg total protein in the MP.

The microparticle may comprise the protein Ply at the level of ≥0.070 μg/μg total protein in the MP. The microparticle may comprise the protein Ply at the level of ≥0.15, preferably ≥0.2, more preferably ≥0.3, most preferably ≥0.35 μg/μg total protein in the MP.

The microparticle may comprise the protein LytA at the level of ≥0.070 μg/μg total protein in the MP. The microparticle may comprise the protein LytA at the level of ≥0.08, preferably ≥0.09, yet more preferably ≥0.1 μg/μg, most preferably ≥0.2 μg/μg total protein in the MP.

The microparticle, MP, may comprise the protein PspC at the level of ≥0.130 μg/μg total protein in the MP. The microparticle may comprise comprising the protein PspC at the level of ≥0.15, preferably ≥0.18, more preferably ≥0.2, most preferably ≥0.3 μg/μg total protein in the MP.

The microparticle may comprise the protein RrgB at the level of ≥0.02 μg/μg total protein in the MP. The microparticle may comprise the protein RgrB at the level of ≥0.022, preferably ≥0.025, most preferably ≥0.028 μg/μg total protein in the MP.

The protein Ply may comprise a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 28.

The protein LytA may comprise a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 29.

The protein PspC may comprise a sequence having at least 40% or 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NOs: 30.

The protein PspC may comprise a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to any one of SEQ ID NOs: 30-41.

The protein RgrB may comprise a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 42.

The membrane vesicle microparticles may be as described in WO 2018/123959.

Preferably, the antigens in the compositions of the first, second and/or third aspects are associated with MVs.

The MVs may comprise MalX at the level of ≥0.001 μg/μg, preferably ≥0.01 μg/μg, more preferably ≥0.02 μg/μg, yet more preferably ≥0.05 μg/μg, most preferably ≥0.1 μg/μg total protein in the MVs.

The MVs may comprise PrsA at the level of ≥0.001 μg/μg, preferably ≥0.01 μg/μg, more preferably ≥0.02 μg/μg, yet more preferably ≥0.05 μg/μg, most preferably ≥0.1 μg/μg total protein in the MVs.

The MVs may lack PspA, or PspA may be present at a level which is less than 0.01 μg/μg, preferably less than 0.05 μg/μg, yet more preferably less than 0.001 μg/μg total protein in the MV. Most preferably, the MVs lack PspA.

The MVs may be 5-1000 nm in diameter, preferably 5-300 nm, more preferably 15-175 nm, most preferably 10-125 nm.

The composition of the first, second or third aspects may comprise MVs in an amount of 1 μg/ml, preferably 5 μg/ml, more preferably 10 μg/ml, most preferably 100 μg/ml.

The MVs may be derived from any *Streptococcus pneumoniae* strains, preferably any strain/s of serotype 1, 3, 4, 5, 6A, 6B, 6C, 6D, 7F, 8, 9N, 9V, 10A, 11A, 11B, 12F, 13, 14, 15A, 15B, 15C, 16F, 17F, 18C, 19A, 19F, 20, 21, 22F, 23A, 23B, 23F, 24F, 31, 33F, 34, 35B, 35F, 37, 38, including strains TIGR4, P1031, BHN418 and A66.

Composition of membrane vesicles from other bacteria than streptococci

The composition of the first, second or third aspects may comprise the MalX antigen and/or the PrsA antigen **not** associated with streptococcal membrane vesicles (in particular *Streptococcus pneumoniae* membrane vesicles). Instead, the antigens may be associated with other bacterial membrane vesicles from gram-positive and/or gram-negative bacteria such as firmicutes including Lactobacillales order, Bacillales order, proteobacteria such as *E. coli* and a *Klebsiella* species, a *Neisseria* species, and a *Haemophilus* species, or associated with artificially made lipid droplets (e.g. liposomes).

Streptococcus pneumoniae growing on solid medium produce highly immunogenic MP, but the yield is low. After identifying pneumococcal MalX and PrsA as the two major protective MP antigens the inventors heterologously over-expressed these two proteins in *Lactococcus lactis.* This non-pathogenic Gram-positive organism is widely used in dairy industry and a natural member of the vaginal microbiota, making is suitable as a platform for large scale production of native membrane bound antigens for vaccine purposes.

Thus, in some cases, the membrane vesicles and the antigen(s) may be derived from different species. The antigen(s) may be recombinantly expressed in the host producing the membrane vesicle, the antigen(s) preferably being heterologous to the host.

The host cell is preferably of the genus *Lactococcus*. More preferably, the host cell is a *Lactococcus lactis*-cell.

The membrane vesicles are preferably artificial membrane particles (aMP) i.e., particles that are generated by treatment of the host cells, e.g., by mechanical disruption. The aMPs are preferably enriched, isolated or purified from other cell debris (i.e. cell components not part of the aMPs) derived from the disrupted host cells, e.g., by filtration, selective precipitation, gradient centrifugation, ultracentrifugation, combination thereof or similar techniques. By isolated in this context is meant that the relative amount of cell debris to aMPs is reduced by at least 50% in the final composition compared to the composition immediately after the aMPs were generated by treatment of the host cells. Preferably, the amount of cell debris is reduced by at least 70%, more preferably by at least 80%, even more preferably by at least 90%, still more preferably by at least 95%, most preferably by at least 99%.

The artificial membrane particles are to be understood to be distinct from membrane vesicles or membrane particles produced by cells through biological processes. Thus, the membrane vesicles preferably comprise disrupted bacterial cells, more preferably mechanically disrupted. Preferably, the membrane vesicles comprise membrane vesicles derived from mechanically disrupted bacterial cells. The membrane vesicles of the artificial type may be derived from *S. pneumoniae* or another suitable host such as *Lactococcus lactis* heterologously expressing the necessary pneumococcal antigens.

Non-membrane vesicle compositions

The composition of the second or third aspects may comprise the MalX antigen and/or the PrsA antigen **not** associated with streptococcal (in particular *Streptococcus pneumoniae*) membrane vesicles.

The composition of the second or third aspects may comprise the MalX antigen and/or the PrsA antigen as isolated proteins, preferably recombinant proteins, or as peptides such synthetic peptides or truncated recombinant proteins, or as peptidomimetics.

Preferably, the MalX and/or the PrsA antigen of the second or third aspects are recombinant proteins with a post-translational modification of the terminal cysteine being bound to a lipid. More preferably, the terminal cysteine residue is N-palmitoyl cysteine or S-diacylglycerol cysteine. Preferably, the MalX and/or the PrsA antigen are recombinant proteins comprising the native signal peptide sequence.

Preferred features of the compositions

The compositions according to the first, second or third aspects may further comprise a nanoparticle carrier. Such nanoparticle carrier may comprise an ISCOM, an ISCOM matrix, a liposome, a polymeric nanoparticle, an inorganic nanoparticle, a non-degradable nanoparticle, an emulsion or a virus-like particle.

The compositions according to the first, second or third aspects may be devoid of whole streptococcal (in particular *Streptococcus pneumoniae*) cells.

The compositions according to the first, second or third aspects may further comprise capsular polysaccharides from a *Streptococcus sp.,* preferably *Streptococcus pneumoniae.*

The compositions according to the first, second or third aspects may be formulated as a vaccine, preferably for intranasal, intramuscular or subcutaneous administration.

The compositions according to the first, second or third aspects may further comprise an adjuvant, preferably aluminium hydroxide, a lipid-based substance such as squalene, an immune-stimulating component or a proteins from a microbe such as diphtheria (preferably CRM), cholera (preferably choleratoxoid CTB or mmCT) or *Escherichia coli* (preferably non-toxic heat-labile toxin such as LTK63 or dmLT).

The compositions according to the first, second or third aspects may be capable of eliciting serotype independent antibodies against a *Streptococcus sp*, preferably *Streptococcus pneumoniae,* when administered to a mammalian host.

The compositions according to the first, second or third aspects may be capable of eliciting opsonophagocitic antibodies against a *Streptococcus sp*, preferably *Streptococcus pneumoniae,* when administered to a mammalian host.

The compositions according to the first, second or third aspects may be capable of eliciting antibodies against *Streptococcus pneumoniae* serotype 3, when administered to a mammalian host.

Prevention of pneumococcal disease

In a fourth aspect of the present invention, the compositions according to the first, second or third aspects may be for use as a medicament, preferably in a method for inducing

protective immunity against a *Streptococcus sp*, preferably *Streptococcus pneumoniae,* in a subject.

The fourth aspect also encompasses a method for inducing protective immunity against a *Streptococcus sp*, preferably *Streptococcus pneumoniae,* in a subject in need thereof, comprising administering an effective amount of the composition according to the first, second or third aspects to the subject. The fourth aspect also encompasses the use of a composition according to the first, second or third aspects in the manufacture of a vaccine for immunization against a *Streptococcus sp*, preferably *Streptococcus pneumoniae.*

The protective immunity may be an immunity reducing the likelihood of a condition selected from pneumococcal sinusitis, pneumococcal otitis, pneumococcal pneumonia and invasive pneumococcal disease including but not limited to pneumococcal sepsis and pneumococcal meningitis, preferably invasive pneumococcal disease. Preferably, the subject to be immunized is a young child (e.g. less than 7 years of age) or an elderly person (e.g. over 65 years of age), but also other age groups could be targeted. Preferably, the protective immunity is at least partially serotype-independent, more preferably substantially serotype-independent, yet more preferably essentially serotype-independent, most preferably fully serotype-independent.

Preferably, the protective immunity encompasses protective immunity against serotype 3 pneumococci in addition to protective immunity against at least one other serotype, preferably serotype 1 and/or serotype 4.

For immunization, the composition may be administered to the subject in various manners known in the art, including but not limited to by way of injection (e.g. intramuscular, intracutaneous, subcutaneous, intravenous), buccal, oral and intranasal administration as well as inhalation.

Manufacturing a composition with artificial membrane particles

Streptococcus pneumoniae growing on solid medium produce highly immunogenic MP, but the yield is lower than desirable for many practical purposes such as large-scale vaccine production. Thus, it may be desirable to manufacture MVs as artificial membrane particles.

In a fifth aspect, the present invention provides method for manufacturing a composition according to first, second or third aspects, comprising:

a. expressing a streptococcal MalX antigen and/or a streptococcal PrsA antigen in a suitable host cell, preferably heterologously;

b. generating membrane vesicles by disrupting the host cell expressing said antigens; and

c. recovering the generated membrane vesicles to form an immunogenic composition comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen associated with membrane vesicles.

The host cells are preferably mechanically disrupted to generate the membrane vesicles, more preferably by shear forces, most preferably with a French press or a similar type of mechanical homogenization device.

The method may comprise isolating/purifying the artificial membrane particles by removing some or all of the cell debris derived from the disrupted host cells e.g., by filtration, gradient centrifugation, selective precipitation, ultracentrifugation, combination thereof or similar techniques.

By isolated in this context is meant that the relative amount of cell debris to aMPs is reduced by at least 50% in the final composition compared to the composition immediately after the aMPs were generated by treatment of the host cells. Preferably, the amount of cell debris is reduced by at least 70%, more preferably by at least 80%, even more preferably by at least 90%, still more preferably by at least 95%, most preferably by at least 99%.

The recovering step may comprise precipitation by ultracentrifugation followed by resuspension.

The host cells may be a *S. pneumoniae* strain expressing the appropriate antigens. The host cells may also be another suitable host such as a *Lactococcus* such as *Lactococcus lactis* heterologously expressing the necessary pneumococcal antigens by way of genetic engineering.

Discussion relating to present disclosure

At any time point up to 30 to 60% of healthy preschool children are colonized by *Streptococcus pneumoniae* in the nasopharynx. It is believed that pneumococcal colonization is an immunization event, explaining why nasopharyngeal colonization

decreases with age. Serum taken from healthy adults frequently contain antibodies directed against pneumococcal membrane proteins, many of which are highly conserved between pneumococcal strains. *Streptococcus pneumoniae* produces spontaneously, during growth on solid and liquid media, membrane protrusions across the cell wall that pinch off from the bacterial cell without observed lysis resulting in membrane vesicle of different sizes confined by the plasma membrane and enclosing a cytosolic cargo (Fig 1)(Codemo M. et al. mBio. 2018 Apr 10;9(2) e00559-18). We suggest that such membrane protrusions are spontaneously produced during pneumococcal colonization. As membrane vesicle contain only low levels of capsular polysaccharide and cell wall material, their plasma membranes are readily targeted by complement C3 deposition facilitating uptake into antigen presenting cells (Codemo et al., supra). Membrane vesicles contain the entire set of known cytoplasmic membrane proteins including all known lipoproteins, hydrophilic proteins anchored to the outer leaflet of the plasma-membrane through their N-terminal lipid moiety attached to a conserved cysteine (Fig 1) (Codemo et al., supra). In addition, many choline binding proteins are also found on membrane vesicle, probably associated to the choline residues decorating the membrane associated lipoteichoic acids. Many membrane bound proteins have specialized localizations, such as those involved in septum formation and cell division. We hypothesize that the superior immuno-protective effects found here of membrane particles isolated from solid agar plates (MPs) over extracellular vesicles (EVs), isolated from liquid growing cells (Fig 6), could be that the former are enriched for membrane proteins localized to the septum region, whereas the latter are also the result of vesicle formation following bacterial autolysis.

There is an urgent need for a new pneumococcal vaccine approach especially for the elderly against IPD. It has been demonstrated that high age is the main risk factor for attracting IPD. The underlying mechanisms are not known but could be due to a waning immune memory from nasopharyngeal exposure events during childhood. In the post-PCV vaccination era the elderly are protected from IPD caused by pneumococcal strains expressing vaccine type capsules (VT), since VT strains have been eliminated from the nasopharynx of vaccinated children. However, PCV vaccination in children has resulted in replacement of VT with non-vaccine types (NVT) strains also in non-vaccinated age groups such as the elderly. An elderly vaccine should ideally be protective irrespective of serotype thereby including NVT-strains

and strains producing a serotype 3 capsule. Even though serotype 3 is included in PCV13, it is still a major serotype causing IPD particularly among the elderly. Here, we show that intranasal immunization of mice with MP from serotype 4 protect against heterologous challenge with strains of serotype 1 and 3. Also, we find that this serotype-independent protection is antibody-dependent, but not dependent on the cytotoxin pneumolysin, since deletion of pneumolysin in the MP does not affect the protective effect. Moreover, we find that although the highly diverse choline binding protein PspA is a dominant immunogen in the MP, it only marginally contributes to the homologous serotype protection observed, and it is not required for cross-protection. In fact, the MP antigens from hosts with deleted PspA unexpectedly conferred *better* cross-protection than native MP antigens (Fig 8 C-E).

Importantly, we identify two highly conserved pneumococcal membrane-anchored liproproteins, MalX and PrsA, as the major protective antigens in the MP, and they are both needed for protection. MalX is part of an ABC transporter complex and binds maltooligosaccharides of various sizes. MalX was in a signature mutagenesis screen identified as a protein required for lung-infection in an intranasal challenge model (Hava and Camilli, 2002, Mol Microbiol *45*, 1389-1406), and serum antibodies directed against MalX have been detected in healthy adults (Giefing et al., 2008 J Exp Med *205*, 117-131). PrsA is a conserved cis-trans prolyl isomerase that facilitate protein secretion by promoting extracellular folding of several secreted proteins. PrsA has been shown to contribute to nasopharyngeal colonization in a murine model, and also enhances bacterial resistance to phagocytosis. It was suggested that these effects by PrsA may by indirect by promoting proper folding of adhesins and other virulence associated surface proteins (Cron et al., 2009 Microbiology *155*, 2401-2410). We suggest that intranasal immunization with membrane particles elicits neutralizing antibodies to the two natively folded MalX and PrsA that prevent bacterial proliferation in the respiratory tract. The antigenic epitopes generating protective antibodies to MalX and PrsA are not known, and remain to be studied. However, human antibodies have been demonstrated to recognize an epitope present in the signal peptide of MalX (Giefing et al., 2008, supra), an epitope only present in the nascently secreted polypeptide prior to its excision by the leader peptidase.

General statements relating to the present disclosure

The term "comprising" is to be interpreted as including, but not being limited to. All references are hereby incorporated by reference. The arrangement of the present disclosure into sections with headings and subheadings is merely to improve legibility and is not to be interpreted limiting in any way, in particular, the division does not in any way preclude or limit combining features under different headings and subheadings with each other.

EXAMPLES

The following examples are not to be regarded as limiting. For further information on the experimental details, the skilled reader is directed to a separate section titled Materials and Methods.

Example 1: Intranasal immunization with MPs confers serotype-independent cross-protection against IPD.

The inventors isolated vesicles from plate-grown pneumococci as a proxy for biofilm growing pneumococci during nasopharyngeal carriage, and called them membrane particles (MP) (see Figs 1A,B and Fig 2 for characteristics). Wild-type mice (C57BL/6) were immunized twice intranasally with MP isolated from the strain T4 (TIGR4), alone or combined with the adjuvant aluminium hydroxide, or with phosphate-buffered saline (PBS), or adjuvant alone for the control groups. After four weeks, mice were infected intranasally with 5×10^6 colony forming units (CFU) of the serotype 1 strain BHN733. Mice were followed for seven days and survival was recorded. Heterologous serotype challenge with immunization using MP+adjuvant from T4, and infection with serotype 1, conferred 80% survival. Also, in the absence of adjuvant the inventors observed a significant protection (65% survival) (Fig.3A). Bacterial loads in the lungs were consistent with the survival data, with lower bacterial numbers in immunized mice as compared to control mice treated with adjuvant only (Fig.3B). Bacterial CFU in the blood was monitored daily until sacrifice (Fig.4A-D). For the PBS and adjuvant groups, only mice with no bacteraemia survived. Seven of the mice vaccinated with MP+adjuvant cleared the bacteria from the blood, suggesting that even though the immunization was intranasal, immune clearance occured systemically. The following experiments were done using MP (and not EV isolated from liquid cultures) since intranasal immunization of mice with MPs from strain T4 gave higher protection, lower

pneumococcal numbers in the upper respiratory tract, and higher antibody levels after immunization than what was observed using EV from the same strain (Fig.3A-B, Fig.4A-D, and Fig.6). The effect of MP-immunization on colonization was studied using the IVIS imaging system to monitor mice after sacrifice. The inventors found that the bacterial density in the nasopharyngeal tract of mice immunized intranasally with MP+adjuvant was significantly lower than what was observed in mice immunized with adjuvant alone. Indeed, the eight surviving mice immunized with MP+adjuvant showed no fluorescent signal (Fig. 7). In summary, intranasal immunization with MP gives a serotype independent cross-protection against IPD, and also reduces the pneumococcal load in the upper airways.

Example 2: Immunization with MPs leads to production of pneumococcal-specific antibodies with opsonophagocytic activity

Next, the inventors analyzed the presence of pneumococcal-specific antibodies in sera of mice immunized intranasally with MP from T4. Using enzyme-linked immunosorbent assay (ELISA), they detected IgG specific for whole heat-inactivated T4 in sera from mice two and four weeks after immunization, and the IgG levels were higher in immunized mice than in the adjuvant and PBS control groups already two weeks after immunization, and significantly higher after four weeks (Fig.5A). Furthermore, the inventors detected IgG with affinity for the non-encapsulated mutant strain T4R, thus indicating reactivity to non-capsular antigens (Fig.5B). In order to further assess the presence of pneumococcal-specific antibodies, the inventors performed immunofluorescence staining of three pneumococcal strains, BHN733, BHN428, and BHN191, of serotypes 1, 3, and 6B respectively, using sera from mice immunized with T4 MP+adjuvant as the source of primary antibodies. For all the three pneumococcal serotypes, the inventors observed fluorescence signals on the bacterial surface (Fig.5C), while no fluorescence signals were detected when sera from mice treated with adjuvant only was used (Fig. 5C).. To investigate the opsonophagocytic activity of the cross-reactive antibodies, serotype 1 pneumococci were incubated with sera from mice immunized with T4 MP+adjuvant, and subsequently incubated with RAW mouse macrophages. The ratio of adhered bacteria was significantly higher when pneumococci were incubated with sera from mice immunized with MP+adjuvant, compared to adjuvant alone (Fig.5D). Also, these pneumococci with better adherence were significantly less able to survive inside the cells compared to pneumococci incubated with control serum (Fig.5E).

Example 3: MPs from serotype 4 confer antibody-dependent protection against challenge with serotype 3 pneumococci

Since there is an urgent need for a vaccine against serotype 3, the inventors purified and characterized MP from the serotype 3 strain BHN428. These MP were larger than those from T4 with diameters up to 850 nm, possessed more cytosolic proteins and less predicted cell wall associated, and secreted proteins (Fig. 1A). Mice were immunized intranasally with MP of serotype 3. In the seven-days-survival experiment, all mice immunized with MP of serotype 3+adjuvant survived, while the adjuvant treated control mice all died within four days (Fig. 3C). Immunized mice showed lower bacterial numbers in the lungs and blood as compared to adjuvant-treated mice (Fig.3D, Fig. 4E-F). Immunization using MP from T4 and challenge with serotype 3 bacteria (BHN428), provided 50% cross-protection (Fig.3C), and the bacterial load in the lungs of surviving mice tended to be lower than for the adjuvant control, and for those that succumbed to infection (Fig.3D). The ten mice that showed lower lung counts had no detectable bacteria in the blood (Fig 4G). These data show that MPs of type 3 show full protection against homologous challenge in mice. These data also indicate that T4 MP evoke cross-protection against genetically distant pneumococcal lineages, such as of serotype 3.

Example 4: Protection conferred by MPs is antibody-dependent

To investigate whether the protection is antibody-dependent, the inventors immunized B cell-deficient mice (MuMt knock-out mice) with MP from serotype 3 and challenged intranasally with the same strain. In the seven-days survival experiment, none of the B cell-deficient mice survived, demonstrating that the protection is antibody-dependent (Fig.3C). Bacterial numbers in the lungs and blood were high and similar to what was detected in wild-type mice given adjuvant only, and significantly higher than in wild-type mice immunized with the same MP (Fig.3D, Fig. 4F-H). Thus, data indicates that intranasal immunization with MP provides an antibody-mediated protection.

Example 5: The cytotoxin pneumolysin is not required for MPs to confer protection against IPD

The pneumococcal cytotoxin pneumolysin (Ply) has been discussed as a vaccine candidate since Ply-deficient strains are highly attenuated in mice infection models. The inventors investigated whether protection is Ply-dependent using MP isolated from an isogenic strain

lacking Ply (T4Δ*ply*). Importantly, mice immunized with MP from T4Δ*ply* and challenged with T4 showed 100% protection (Fig.3E). Also, these mice showed similar CFU in the lungs as mice immunized with MP from T4, and had no bacteria in the blood (Fig.3F, Fig. 4I-K). The inventors conclude that Ply is not crucial for the protection against IPD.

Example 6: PspA is a major protein in MPs, but the cross-protection is not dependent on PspA

To identify which pneumococcal proteins in MP are responsible for the protection, T4 was grown in liquid medium, and then washed with elevated concentrations of choline chloride to release choline binding proteins that non-covalently associate with the cell surface. Western blot analyses of the cell pellets and supernatants of choline washed (cw) cells were performed using sera from immunized mice (with T4 MP) as the source of primary antibodies. A major band of approximately 80 kDa was observed mainly in the supernant fraction, that was absent in a T4Δ*pspA* mutant, thus identifying it as the highly immunogenic choline binding pneumococcal surface protein PspA (Fig.8A). PspA has been described as a putative vaccine candidate against IPD and colonization, but its sequence is highly variable among clinical strains, which likely explains why no PspA bands were observed in choline washed samples from BHN733 (serotype 1) and BHN428 (serotype 3) (Fig.8B). However, two additional protein bands of slightly less than 40 and 50 kDa respectively were observed in the lysates and pellet fractions of choline-washed cells of T4, T4Δ*pspA,* and of the serotype 1 and 3 strains (Fig.8A and B).

To study the role played by PspA for protection by MP, the inventors immunized mice with MP from the mutant strain, T4Δ*pspA*, and challenged with T4. Immunized mice showed 80% protection (Fig.8C), suggesting that antigens other than PspA provide protection. Indeed, when mice immunized with T4Δ*pspA* MP were challenged with the serotype 1 strain BHN733, a 100% cross-protection was observed, reinforcing that the protective antigens in MP were neither PspA, nor the capsular polysaccharide (Fig.8D). Similar results were obtained when mice were challenged with serotype 3 pneumococci (BHN428). Here, a 60% protection was observed with T4Δ*pspA* MP which was in the same range as for T4 MP (Fig.8E). For all three infection experiments, the bacterial loads in the lungs (Fig.8F-H) and blood (Fig. 9A) were low in surviving mice. ELISA assays, and coating with serotype 1, 3 and 4 pneumococci respectively, showed higher IgG titers in mice immunized with MP from

T4Δ*pspA* compared to T4 MP (Fig. 9B). Unexpectedly, the T4Δ*pspA* MPs were better in conferring protection against Serotype 1 infection than wild-type T4 MPs (Fig 8D, G), while being indistinguishable in efficacy against Serotype 3 or 4 infection (Fig 8CF, 8EH)

Example 7: Cross-protection mediated by membrane vesicles against invasive pneumococcal disease is dependent on the lipoproteins PrsA and MalX

Since the inventors found that PspA in MP was not important for heterologous serotype protection, the inventors next performed a series of immunoprecipitation (IP) experiments to capture additional putative antigens using sera from mice immunized with MPs from T4 and T4Δ*pspA*, or from adjuvant treated mice as a control. Antibody-enriched proteins in lysates of T4 were subjected to mass spectrometry analysis for protein identification. Importantly, the hits with the highest scores from IP using sera from T4Δ*pspA* MP-immunized mice were two lipoproteins, PrsA and MalX. These proteins were not detected in IP with sera from the adjuvant control. As expected, PspA was identified as one of the top hits in the IP with sera from T4 MP, but MalX was also enriched with this sera, albeit with a lower score (Tables A-C). Additionally, the masses of MalX and PrsA corresponded to the immunoreactive bands of ~40 and ~50 kDa that the inventors observed in the lysates and cell pellet fractions by Western blot analysis, see above (Fig. 8A and 8B). To further validate that PspA, PrsA and MalX were the major reactive proteins from the immune sera, the inventors complemented the IP experiment with a Western blot analysis where blotted lysates from wt T4, and mutants in *pspA*, *prsA* and *malX* were incubated with sera from mice immunized with MP from T4 or T4Δ*pspA* (Fig. 10A-B). Indeed, sera from T4 MP-immunized mice reacted with three major proteins, and absence of these protein bands in the corresponding mutant background confirmed these proteins to be PspA, PrsA and MalX (Fig. 10A). Thus, although PrsA was not enriched in IP with the T4 MP immune sera, the Western blot analysis clearly showed that this sera still reacted to this protein. Likewise, using sera from mice immunized with T4Δ*pspA* MPs revealed two major bands of slightly less than 40 and 50 kDa, absent in mutants having a *prsA* or a *malX* deletion, thus confirming the identity of these proteins (Fig. 10B).

To further investigate if the identified PrsA and MalX proteins were responsible for the cross-protection, the inventors first isolated MP from a mutant lacking both *prsA* and *malX* in the T4Δ*pspA* background,T4Δ*pspA*Δ*prsA*Δ*malX*. Mice were immunized with either MP

from T4 or from the triple mutant, and then infected intranasally with either T4, BHN733 (serotype 1) or BHN428 (serotype 3). Immunization with T4 MP showed again 100%, 80%, and 60% protection against these strains respectively, confirming the previous results (Fig. 8C-E). However, when MP from T4Δ*pspA*Δ*prsA*Δ*malX* were used for immunization, mice showed only 20% protection towards T4 and type 1 (BHN733), and no protection towards type 3 (BHN428) (Fig. 10C-E). Lung and blood CFU were in accordance with these findings, as were IgG titers (Fig.10F-H, Fig.9A-B).

To further study whether the crossprotection depends on both lipoproteins, three new mutant strains were created in the strain T4 where *pspA* and *malx*, or *pspA* and *prsA* or *malX* and *prsA* were deleted (Fig. 12A-B, Fig. 13A-F). MPs from the three strains were used to immunize mice and then the mice were challenged with a serotype 1 strain. The data suggest that both MalX and PrsA are needed for cross-protection since mice immunized with MPs from either of the mutant strains showed a lower survival than mice immunized with MPs from the wild type strain (Fig. 12A). Also, the bacterial numbers in the lungs and blood were consistent with the survival data (Fig. 12B, Fig. 13A-F). The inventors concluded that both MalX and PrsA are needed in MPs for cross-protection against a pneumococcal infection.

Example 8: MalX and PrsA are conserved lipoproteins in *S. pneumoniae*

To investigate the conservation of *prsA* and *malX*, the inventors blasted the nucleotide sequences of these genes against the annotated PubMLST database containing 8351 pneumococcal genomes belonging to several serotypes and sequence types. For *prsA*, encoding a membrane bound lipoprotein acting as a *cis-trans* prolyl-isomerase, 99.5% of the pneumococcal genomes (8325/8351 genomes) had a *prsA* sequence more than 98% identical to the T4 sequence. Similarly, for *malX,* encoding a lipoprotein acting as an ABC maltose transporter, 98.5% of the genomes (8318/8351) had a *malX* sequence more than 98% identical to the T4 sequence. This indicates a very high sequence conservation of these two genes across different strains and serotypes of pneumococci. However, single nucleotide polymorphisms, leading to amino-acid replacements, were found.

Table A. Proteins identified with LC-MS/MS after immunoprecipitation with immune sera from T4 MPs immunized mice.

Accession	Protein	Gene number	Annotation	Score	Coverage	# Unique Peptides	# Peptides	MW [kDa]	pI

P95829	DnaK	SP_0517	Chaperone protein	42,90	33,94	12	12	64,8	4,77
A0A0H2UMZ8	PspA	SP_0117	Pneumococcal surface protein A	23,97	16,40	7	7	82,7	4,91
A0A0H2UNM7	RrgB	SP_0463	Cell wall surface anchor family protein	9,68	10,08	4	4	71,3	5,15
Q97SV2	RplB	SP_0212	50S ribosomal protein L2	9,13	31,77	5	5	29,9	10,68
P61182	RplV	SP_0214	50S ribosomal protein L22	8,67	42,98	3	3	12,2	10,76
P06653	LytA	SP_1937	Autolysin	6,54	11,95	3	3	36,5	5,34
P59213	MalX	SP_2108	Maltose/maltodextrin-binding protein	6,33	8,51	3	3	45,3	5,22
Q97SU6	RplR	SP_0226	50S ribosomal protein L18	5,83	29,66	2	2	12,9	10,40
P66359	RpsK	SP_0235	30S ribosomal protein S11	3,74	22,05	2	2	13,4	11,43

Table B. Proteins identified with LC-MS/MS after immunoprecipitation with immune sera from T4Δ*pspA* MPs immunized mice.

Accession	Protein	Gene number	Annotation	Score	Coverage	# Unique Peptides	# Peptides	MW [kDa]	pI
Q97R51	PrsA	SP_0981	Foldase protein	81,08	53,99	21	21	34,4	5,12
P59213	MalX	SP_2108	Maltose/maltodextrin-binding protein	77,02	63,36	22	22	45,3	5,22
P95829	DnaK	SP_0517	Chaperone protein	42,96	37,40	16	16	64,8	4,77
Q97SV2	RplB	SP_0212	50S ribosomal protein L2	25,95	40,79	8	8	29,9	10,68
P61182	RplV	SP_0214	50S ribosomal protein L22	24,40	58,77	5	5	12,2	10,76
A0A0H2UMZ8	PspA	SP_0117	Pneumococcal surface protein A	22,64	20,70	8	8	82,7	4,91
A0A0H2UNM7	RrgB	SP_0463	Cell wall surface anchor family protein	17,81	15,04	6	6	71,3	5,15
P66112	RplT	SP_0961	50S ribosomal protein L20	12,36	41,18	4	4	13,7	10,83
P66524	RpsU	SP_1414	30S ribosomal protein S21	10,67	34,48	4	4	7,0	11,30

Q97SQ4	RpsG	SP_0272	30S ribosomal protein S7	10,51	23,72	4	4	17,7	10,51
P06653	LytA	SP_1937	Autolysin	9,28	27,36	7	7	36,5	5,34
A0A0H2URD1		SP_1683	Sugar ABC transporter	7,06	12,67	4	4	48,3	5,44
Q2MGH6		SP_0368	Endo-alpha-N-acetylgalactosaminidase	6,97	4,47	7	7	195,9	6,07
P18791	AmiA	SP_1891	Oligopeptide-binding protein	6,62	6,37	3	3	72,4	5,06
P66581	RpsE	SP_0227	30S ribosomal protein S5	6,58	24,39	3	3	17,0	9,52
P65144	InfC	SP_0959	Translation initiation factor IF-3	5,40	16,22	2	2	21,2	9,85
Q97SU6	RplR	SP_0226	50S ribosomal protein L18	4,91	29,66	2	2	12,9	10,40
Q97QS2	Eno	SP_1128	Enolase	4,87	11,29	3	3	47,1	4,81
A0A0H2UQS8	MltG	SP_1518	Endolytic murein transglycosylase	4,70	10,89	5	5	60,8	5,16
Q97SN4	RpsI	SP_0295	30S ribosomal protein S9	4,68	16,92	2	2	14,2	10,86
P64030	EF-Tu	SP_1489	Elongation factor Tu	4,49	13,07	4	4	43,9	4,97
Q9L7Q2	ZmpB	SP_0664	Zinc metalloprotease	4,41	4,25	5	5	213,4	5,25
A0A0H2UPZ3	Hup	SP_1113	DNA-binding protein HU	4,40	35,16	2	2	9,6	9,48
P0A4C3	RpsC	SP_0215	30S ribosomal protein S3	4,06	12,44	2	2	24,0	9,70
P66359	RpsK	SP_0235	30S ribosomal protein S11	3,85	21,26	2	2	13,4	11,43
A0A0H2UNF0	RplM	SP_0294	50S ribosomal protein L13	3,72	14,19	2	2	16,1	9,95
A0A0H2XFA2	LytC	SP_1573	Lysozyme	3,16	4,69	2	2	57,3	6,93
A0A0H2US50	CbpA	SP_2190	Choline binding protein A	2,90	9,96	3	3	77,7	5,81
P66565	RpsD	SP_0085	30S ribosomal protein S4	2,59	11,33	2	2	23,0	10,15

P0A4A7	RpsL	SP_0271	30S ribosomal protein S12	2,21	15,33	2	2	15,1	11,53
Q97T80	ZmpC	SP_0071	Zinc metalloprotease	1,95	6,52	8	8	206,6	5,57
Q04707	PBP-1A	SP_0369	Penicillin-binding protein 1A	1,93	5,29	3	3	79,7	5,60

Table C. Proteins identified with LC-MS/MS after immunoprecipitation with sera from adjuvant challenged mice

Accession	Protein	Gene number	Annotation	Score	Coverage	# Unique Peptides	# Peptides	MW [kDa]	pI
P95829	DnaK	SP_0517	Chaperone protein	64,30	51,24	23	23	64,8	4,77
A0A0H2UNM7	RrgB	SP_0463	Cell wall surface anchor family protein	18,23	28,42	13	13	71,3	5,15
Q97SV2	RplB	SP_0212	50S ribosomal protein L2	16,80	32,13	6	6	29,9	10,68
P61182	RplV	SP_0214	50S ribosomal protein L22	14,97	52,63	4	4	12,2	10,76
A0A0H2UMZ8	PspA	SP_0117	Pneumococcal surface protein A	14,78	14,11	5	5	82,7	4,91
Q97SQ4	RpsG	SP_0272	30S ribosomal protein S7	8,81	19,23	2	2	17,7	10,51
P64030	EF-Tu	SP_1489	Elongation factor Tu	6,97	10,80	3	3	43,9	4,97
P06653	LytA	SP_1937	Autolysin	6,82	26,10	7	7	36,5	5,34
Q97SU6	RplR	SP_0226	50S ribosomal protein L18	6,42	37,29	3	3	12,9	10,40
Q97PI9	RpsO	SP_1626	30S ribosomal protein S15	5,70	21,35	3	3	10,5	10,18
P66359	RpsK	SP_0235	30S ribosomal protein S11	5,43	33,07	3	3	13,4	11,43
P65144	InfC	SP_0959	Translation initiation factor IF-3	5,40	16,22	2	2	21,2	9,85
P0A4C3	RpsC	SP_0215	30S ribosomal protein S3	5,19	19,35	3	3	24,0	9,70
Q2MGH6		SP_0368	Endo-alpha-N-acetylgalactosaminidase	3,91	1,70	3	3	195,9	6,07

A0A0H2XFA2	LytC	SP_1573	Lysozyme	2,85	4,69	2	2	57,3	6,93
P66565	RpsD	SP_0085	30S ribosomal protein S4	2,69	10,34	2	2	23,0	10,15
P18791	AmiA	SP_1891	Oligopeptide-binding protein	2,52	5,16	3	3	72,4	5,06
P66524	RpsU	SP_1414	30S ribosomal protein S21	2,49	34,48	4	4	7,0	11,30
A0A0H2UNF0	RplM	SP_0294	50S ribosomal protein L13	2,44	14,19	2	2	16,1	9,95
P66112	RplT	SP_0961	50S ribosomal protein L20	2,29	29,41	2	2	13,7	10,83
P66581	RpsE	SP_0227	30S ribosomal protein S5	2,16	18,90	2	2	17,0	9,52
A0A0H2US50	CbpA	SP_2190	Choline binding protein A	2,12	4,91	2	2	77,7	5,81
Q9L7Q2	ZmpB	SP_0664	Zinc metalloprotease	2,01	2,47	3	3	213,4	5,25
P0A4A7	RpsL	SP_0271	30S ribosomal protein S12	2,00	15,33	2	2	15,1	11,53
Q97QP7	ZmpA	SP_1154	Immunoglobulin A1 protease	1,69	1,25	2	2	223,8	5,38

Example 9: Antibodies raised against MPs containing MalX and/or PrsA recognize the respective native lipoproteins in MPs considerably better than to recombinant PrsA , and MalX

Sera from mice immunized with MPs isolated from T4 Δ*pspA* were used in Western blots on after SDS-PAGE separation of MP-proteins and recombinant PrsA and MalX respectively (Figure 14). More PrsA and MalX specific antibodies bound the native proteins present in MPs as compared to the respective recombinant protein, despite the much lower content of the antigen present in MPs as compared to the recombinant form. The recombinant form of the protein lacks the amino-terminal sequence corresponding to the signal peptide, and also lacks lipid modification of the terminal cysteine residue required for the formation of membrane bound lipoproteins. These observations indicate that both MalX and PrsA need to be post-translationally modified into lipoproteins (which natively occurs in the N-terminal cysteine of the mature polypeptide) in order to elicit a protective immune response and/or that the

dominant antigenic epitopes are not localized in the mature part of the protein but rather on the signal peptide.

Example 10: Artificial membrane particles (aMP), prepared from high pressure lysates of *Lactococcus lactis,* containing pneumococcal MalX and PrsA antigens

To find a potential platform for vaccine production, the inventors next constructed a *Lactococcus lactis* strain carrying both pneumococcal *malX* and *prsA* using an expression vector. *L. lactis* also expresses homologues to pneumococcal MalX and PrsA, but their amino-acid sequences are sufficiently different to not cross-react antigenically. *L. lactis* is not known to spontaneously produce membrane particles during growth like pneumococci, so the inventors purified aMP from high pressure ruptured *L. lactis* cells.

Antisera from mice immunized with T4pspA MPs reacted strongly with the aMPs (Fig 15), indicating that immune reactive MalX and PrsA antigens were present in the aMPs. Notably, the aMPs did not contain pneumococcal PspA antigen. In conclusion, the aMPs may be used in place of native streptococcal MPs as vaccine antigens, immunogenic compositions and other purposes.

Example 11: Artificial membrane particles (aMP), prepared from high pressure lysates of *S. pneumoniae,* containing pneumococcal MalX and PrsA antigens

As an additional alternative source of MalX and PrsA antigens, similar aMPs as described in Example 10 and using similar protocol will be made using cultured *S. pneumoniae* cells expressing MalX and PrsA, but deficient in PspA (see Table D strain BHN2258). Similar to Example 10, the isolated aMPs will contain antigenic MalX and PrsA antigens and can be used in lieu of native streptococcal MPs as vaccine antigens, immunogenic compositions and other purposes.

MATERIALS AND METHODS

Pneumococcal strains

Strains of *S. pneumoniae* that were used: T4 (TIGR4 of serotype 4)(Tettelin et al., 2001 Science *293*, 498-506), its isogenic mutant expressing bioluminescence for IVIS tracking (TIGR4lux), isogenic mutants lacking pneumolysin (T4*Δply*) (Littmann et al., 2009 EMBO Mol Med *1*, 211-222), or the capsule (T4R) (Fernebro et al., 2004 J Infect Dis *189*, 328-338), or *pspA*, *prsA*, and *malX* (Table D), BHN733 of serotype 1, BHN428 of serotype 3, and BHN191 of serotype 6B

(Iovino et al., 2016a J Clin Invest *126*, 2821-2826; Pathak et al., 2018 Nat Commun *9*, 3398). T4, BHN733, BHN428 and BHN191 were grown in C+Y-medium, pH 7.9-8.0, at 37ºC. Growth was followed by measuring optical density (OD) at 600 nm with a spectrophotometer (Genesys 20, Thermo Spectronic).

Table D. *S. pneumoniae* strains used in the study.

Strain	**Identifier**	**Comment**
T4 (TIGR4)	BHN38	Wild-type TIGR4 serotype 4
Serotype 1	BHN733	Wild-type serotype 1
Serotype 3	BHN428	Wild-type serotype 3
Serotype 6B	BHN191	Invasive meningitis clinical isolate serotype 6B
T4Δ*pspA*	BHN2258	*pspA* (*SP0117*) replaced with an *ermB* cassette (promoter plus Orf)
T4Δ*malX*::*kanR*	BHN1696	*malX* (*SP2108*) Orf replaced with a *kanR* Orf
T4Δ*prsA*::*tetM*	BHN1697	*prsA* (*SP0981*) Orf replaced with a tetM Orf
T4Δ*pspA*::*ermB*Δ*malX*::*kanR*	BHN1698	*pspA* (*SP0117*) replaced with an *ermB* cassette (promoter plus Orf); *malX* (*SP2108*) Orf replaced with a *kanR* Orf
T4Δ*pspA*::*ermB*Δ*prsA*::*tetM*	BHN1699	*pspA* (*SP0117*) replaced with an *ermB* cassette (promoter plus Orf); *prsA* (*SP0981*) Orf replaced with a *tetM* Orf
T4Δ*pspA*::*ermB*Δ*prsA*::*tetM*Δ*malX*::*kanR*	BHN1700	*pspA* (*SP0117*) replaced with an *ermB* cassette (promoter plus Orf); *prsA* (*SP0981*) Orf replaced with a *tetM* Orf; *malX* (*SP2108*) Orf replaced with a *kanR* Orf

Preparation of EVs and MPs

EV were isolated from liquid cultures as described before (Codemo et al, 2018 MBio *9*). Briefly, bacteria were grown in C+Y medium until $OD_{600\ nm}$=0.9, and removed by centrifugation, the

supernatant filtered and centrifuged. Vesicles were washed twice and resuspended in PBS. MPs were isolated from plate-grown bacteria. Briefly, bacteria were grown overnight on blood agar plates at 37ºC with 5% CO_2, scraped off from the plates, resuspended in PBS and removed by centrifugation (17000 rcf, 30 min). The supernatant was passed through a 0.2 µm filtering device (Filtropur S 0.2, Sarstedt) (except for type 3), and ultracentrifuged (170000 rcf, 12 hours). Pelleted vesicles were resuspended in PBS. Both EV and MP were further purified using Optiprep™ Density Gradient Medium (Sigma). After being resuspended in PBS, EV and MP protein concentrations were assessed by Pierce BCA Protein Assay Kit (Thermo Scientific) and preparations were stored at -80°C.

Animal experiments

All animal experiments were approved by the local ethical committee (Stockholms Norra djurförsöksetiska nämnd). For each immunization experiment, male C57BL/6 wild-type mice 5 weeks old were used. Before immunization, mice were anesthetized by isofluorane (Abbott) inhalation and then challenged intranasally with 50 µl/mouse (10 µg of total protein content/mouse in 50µl) of EV or MP preparation, either alone or in combination with the adjuvant aluminium hydroxide (Sigma Aldrich, 10 mg/ml in PBS) or PBS or the adjuvant alone for the control groups. Immunization was repeated after two weeks from the first immunization following the same procedure as described above. After four weeks of immunization, mice were infected intranasally (50 µl/mouse) with 5×10^6 colony forming units (CFUs) for T4 and type 1, or 1×10^6 CFUs for type 3. Mice were anesthetized by isofluorane inhalation prior to bacterial challenge. After the infection, clinical symptoms of the mice were monitored multiple times daily (in accordance with the ethical permit). Blood samples (5 µl/mouse) were taken every infection day and bacteremia levels were assessed by plating serial dilutions of blood samples onto blood-agar plates. Mice that reached humane end-points were sacrificed according to ethical regulations. For IVIS imaging, mice were anesthetized by isofluorane inhalation and treated intranasally (50 µl/mouse) with Bacterisense 645 (Perkin Elmer) approximately 30 minutes prior to imaging, which was performed after sacrifice. Lungs and spleens were collected for further analyses. Bacterial CFUs were calculated after plating serial dilutions of lung homogenates onto blood-agar plates. To define the ROIs for each mouse with the IVIS system, the fluorescent signal intensity was quantified using the imaging software Living Image 4.5 (Perkin Elmer). MuMt knock-out

mice in the C57BL/6 background (Jackson Laboratories) were used as the B cell deficient model.

SDS-PAGE and western blotting

Detection of pneumococcal proteins in EV and MP was performed as previously described (Codemo M. et al. mBio. 2018 Apr 10;9(2) e00559-18; Mellroth et al. J Biol Chem. 2012;287(14):11018-29). Briefly, Pierce™ BCA Protein Assay Kit (Life Technologies) was used to determine the total protein quantity in EV or MP. Samples were resolved by SDS-PAGE using NuPAGE 4-12% Bis-Tris protein gels (Invitrogen) and electroblotted onto PVDF membranes. Membranes were blocked with 5% skim milk in PBS containing 0.1% Tween-20 and incubated with sera from immunized mice (as primary antibodies, 1:5000) and horseradish peroxidase (HRP)-conjugated secondary antibodies. Membranes were developed with Amersham™ ECL Plus Western blotting detection system (GE Healthcare Life Sciences), using a ChemiDoc™ XRS+ (Bio-Rad Laboratories). For Western blot detection using bacterial lysates, T4, T4Δ*pspA*, T4Δ*prsA*, T4Δ*malX*, T4Δ*pspA*Δ*prsA*, T4Δ*pspA*Δ*malX*, and T4Δ*pspA*Δ*prsA*Δ*malX* were grown overnight on blood agar plates and inoculated in C+Y medium at 37 ºC. These pre-cultures were grown to mid-log phase and used to start new cultures in C+Y media at OD_{620}=0.05. When the cultures reached OD_{620}=0.5 (0.5 mL), each strain was centrifugated (10000 rpm, 5 min) and the cell pellet washed with 1 ml PBS, centrifugated (10000 rpm, 5 min), dissolved in 250 µL of 1x SDS-PAGE loading buffer including 25 mM 1,4-dithiothreitol (DTT) and then boiled for 5 min. For assessment of sera binding to pneumococcal choline binding proteins (CBPs), cells were after growth incubated with 5% choline chloride for 10 minutes at 37 ºC, centrifuged and the supernatant collected. The pellet was washed with 5% choline chloride to remove CBPs. Pellets and supernatant fractions were mixed with loading buffer and Western Blot analysis was performed as described above.

Immunoprecipitation

A column-based immunoprecipitation protocol was set up using the Protein G HP SpinTrap/Ab Spin Trap kit (GE Healthcare). The resin from the Protein G sepharose kit columns were washed three times with PBS and then removed from the columns and transferred to Eppendorf tubes in a 300 µL slurry in PBS. Samples (100 µL) of ten-fold diluted immune sera from mice immunized with MPs from T4 and T4Δ*pspA* and of sera from adjuvant challenged mice were added to the resin in separate tubes and incubated for 30 min in room

temperature. Following incubation, the resins were washed twice with 400 µL PBS using centrifugation (100 rfc, 1 min). The pelleted resin fractions, containing IgG molecules from the immune sera, were resuspended in 400 µL of a pneumococcal cell lysate from T4 generated from mid-log phase cultures grown in C+Y media at 37ºC, and transferred to Eppendorf tubes (500 µL per tube) and treated with 5 µL 10% Triton X100 (0.1% end concentration) for 30 min to induce prominent autolysin-mediated lysis of the cultures. These lysates were centrifugated (14000 rpm, 5 min) to remove cell wall debris and 400 µL of the supernatant was used per sample. The lysate/antisera-Protein G sepharose mixtures were incubated for 30 min in room temperature after which the samples were transferred back to the spin columns, fitted into collection tubes and centrifugated (100 rfc, 1 min) to remove unbound proteins. The columns were successively washed five times with 400 µL PBS (with centrifugation (100 rfc, 1 min)) and elution was done with 400 µL 100 mM sodium citrate buffer, pH 2.5, with centrifugation (100 rfc, 1 min) and the samples were collected into tubes containing 40 µL 1 M Tris buffer pH 8.0 to neutralize the pH. Samples were concentrated using SpedVac (Thermo Fisher) to 100 µL, frozen, and sent for mass spectrometry analysis.

Mass spectrometry

Analysis of EVs and MP was performed as described previously (Codemo M. et al. mBio. 2018 Apr 10;9(2) e00559-18). Briefly, a urea-containing buffer was used to lyse EV and MP. Proteins were reduced, alkylated, and digested in-solution by trypsin. A Pierce C18 Spin Column (Thermo Scientific) was used to purify the sample that was dried and resolved in 0.1% formic acid. Peptides were separated in reversed-phase on a C18-column and electrosprayed on-line to a Q Exactive Plus mass spectrometer (Thermo Finnigan). Tandem mass spectrometry was performed applying HCD. The Sequest algorithm in Proteome Discoverer 1.4 (Thermo Scientific™) was used to search databases towards a FASTA database of TIGR4 (for serotype 4) or SP3-BS71 (for serotype 3) proteins from UniProtKB. Criteria for protein identification were at least two matching peptides of 95% confidence level. In order to avoid false positives, only proteins with a score of 20 or above were included in the analysis. Subcellular localizations of proteins were predicted as before (Codemo M. et al. mBio. 2018 Apr 10;9(2) e00559-18).

For analysis of the immunoprecipitation samples, the following mass spectrometry setup was used. *Chemicals and reagents.* Acetonitrile (ACN), formic acid (FA), and ammonium

bicarbonate (NH_4HCO_3) were obtained from Merck. Protease inhibitor cocktail and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich. For tryptic digestion, iodoacetamide (IAA), urea, and dithiothreitol (DTT) were obtained from Sigma-Aldrich and grade modified trypsin (V5073) was from Promega. Ultrapure water was prepared by Milli-Q water purification system (Millipore). *In-solution tryptic digestion of proteins.* The whole volume of the samples (100 µL) was used for in-solution digestion. A volume of 10 µL of 45mM DTT was added and the mixtures were incubated at 50°C for 15 min and then cooled to ambient temperature. Then 10 µl 100mM IAA was added, and the mixtures were incubated for 15 minutes at room temperature in darkness. Finally, trypsin solution was added to yield a final trypsin/protein concentration of 5% (w/w). The tryptic digestion was performed at 37°C overnight in darkness. Thereafter the samples were desalted using the SPE Pierce C18 Spin Columns (Thermo Scientific). These columns were activated by 2×200 µL 50% ACN and equilibrated with 2×200 µL 0.5% TFA. The tryptic peptides were adsorbed to the media using two repeated cycles of 40 µL sample loading and the column was washed using 3×200 µL 0.5% TFA. Finally, the peptides were eluted in 3×50 µL 70% ACN and dried. Dried peptides were resolved in 30 µL 0.1% FA prior to nano-LC-MS/MS. *LC-MS/MS analysis.* The nanoLC-MS/MS experiments were performed using Q Exactive Orbitrap mass spectrometer (ThermoFisher Scientific, Bremen, Germany) equipped with a nano electrospray ion source. The peptides were separated by C18 reversed phase liquid chromatography using an EASY-nLC 1000 system (Thermo Fisher Scientific). A set-up of pre-column and analytical column was used. The precolumn was 2 cm EASYcolumn (ID 100 µm, 5 µm particles) (Thermo Fisher Scientific) while the analytical column was 10 cm EASY-column (ID 75 µm, 3 µm particles, Thermo Fisher Scientific). Peptides were eluted with a 90 min linear gradient from 4% to 100% acetonitrile at 250 nL min-1. The mass spectrometer was operated in positive ion mode acquiring a survey mass spectrum with resolving power 70,000 (full width half maximum), m/z 400-1750 using an automatic gain control (AGC) target of 3×10^6. The 10 most intense ions were selected for higher-energy collisional dissociation (HCD) fragmentation (25% normalized collision energy) and MS/MS spectra were generated with an AGC target of 5×105 at a resolution of 17,500. The mass spectrometer worked in data-dependent mode. *Data analysis.* The acquired data (.RAW-files) were processed by Proteome Discoverer software (Thermo Scientific, version [nr 1.4.1.14]) using the Sequest algorithm towards a combined database containing protein sequences from the strain TIGR4 proteome (2178 entries) downloaded from Uniprot 2019-

10. The following parameters were used for data processing: maximum 10 ppm and 0.02 13 Da error tolerances for the survey scan and MS/MS analysis, respectively, trypsin as digesting enzyme, carbamidomethylation of cysteins as fixed modification, oxidation of methionine as variable modification, maximum of two miss cleavages sites. The target decoy PSM validator was used to calculate false discovery rate (FDR). An FDR of maximum 5% for peptide identification was accepted and the search criteria for protein identification were set to at least two matching peptides per protein.

Quantification of fluorescence signal *in vivo*

Mice treated with Bacterisense 645 were imaged at the IVIS Spectrum Imaging System focusing on the upper respiratory tract. To define the ROIs for each mouse, the fluorescent signal intensity was quantified using the imaging software Living Image 4.5 (Perkin Elmer).

Mouse IgG ELISA assay

To detect EV- or MP-specific mouse IgG in sera of immunized mice, 96 well optical plates (Sarstedt) were coated with 1 µg/ml of EV or MP in 0.1 M Sodium Carbonate buffer pH 9.5, overnight at 4°C. Wells were washed three times with PBS containing 0.05% Tween-20 and incubated with 200 µl PBS with 10% FBS for 1 hour at room temperature. After three washes, wells were incubated with 100 µl mice sera diluted 1:500 in PBS with 10% FBS for 2 hours, and then washed three times and incubated with 100 µl anti-mouse IgG-HRP (GE Healthcare) diluted 1:500 in PBS 10% FBS for 2 hours, at room temperature. After three washes, wells were incubated with TMB substrate (BD Bioscience) for 10 minutes and the reaction was stopped with 1 M H_3PO_4. Absorbance at 450 nm was measured with a plate reader.

To detect T4 and T4R-specific mouse IgG in sera of T4 EV, T4 MP and T4R MP immunized mice, bacteria were grown on blood agar plates overnight at 37°C, resuspended in PBS and heat-inactivated for 2 hours at 60°C. After diluting the bacteria to OD_{600} 0.6, optical plates were coated with 100 µl bacteria in 0.1 M Sodium Carbonate buffer pH 9.5 overnight at 4°C. Wells were washed three times and incubated with 200 µl PBS with 2.5% skim milk for 2 hours, and after three washes incubated with 100 µl mice sera diluted 1:500 in PBS for one hour, at room temperature. Wells were washed three times and incubated with 100 µl anti-mouse IgG-HRP diluted 1:500 in PBS for one hour at room temperature, and the procedure was continued as described above.

To detect T4, serotype 1 (BHN733) and serotype 3 (BHN428)-specific mouse IgG in sera of mice immunized with T4 MP and T4Δ*pspA* MP, bacteria were grown on blood agar plates overnight at 37°C, resuspended in PBS and heat-inactivated for 2 hours at 60°C. After diluting the bacteria to OD_{600} 0.6, optical plates were coated with 100 µl bacteria in PBS with complete Protease Inhibitor Cocktail (Roche) overnight at 4°C. Wells were washed three times and incubated with 200 µl PBS with 2.5% skim milk for 2 hours, and after three washes incubated with 100 µl mice sera diluted 1:1000 in PBS with 1% BSA, for one hour in room temperature. Wells were washed three times and incubated with 100 µl anti-mouse IgG-HRP diluted 1:1000 in PBS with 1% BSA for one hour at room temperature, and and the procedure was continued as described above.

Construction of pneumococcal mutant strains

For generation of pneumococcal mutants using transformation, a general procedure was employed as previously described (Balaban et al., 2014 Proc Natl Acad Sci U S A *111*, E758-765). To generate double and triple mutants, the same procedure was iterated using sequenced mutants that had acquired the corresponding mutations. Confirmation of mutants were done with Sanger sequencing of PCR amplicons covering the cognate loci (Tables D, E).

Pneumococcal mutants were created in which the open reading frames (Orfs) of *pspA (SP0117)*, *prsA (SP0981)* and *malX (SP2108)*, (gene numbers in parenthesis), were deleted and replaced with antibiotic resistance Orfs (*prsA* and *malX*) or a cassette consisting of a promoter and Orf (*pspA*) as indicated in Extended Data Table 4. Transformation constructs were made with sequential extension overlap PCR were the ~700 bp upstream and ~700 bp downstream regions flanking the target genes were PCR amplified from genomic DNA isolated from T4 with primers given in Table E. These primers contained non-annealing overhang sequences complementary to the 5' and 3' ends of the antibiotic Orfs or cassette to be fused. In a second PCR ~700 bp up- and down-stream regions were fused to the corresponding antibiotic cassette (or Orf) through thermal annealing followed by overlap extension PCR. The final PCR products that contained the joined (700 bp upstream region plus the antibiotic resistance Orf or cassette plus the 700 bp downstream region) were purified (PCR clean up kit (Qiagen)) and used to transform the T4 strain.

Table E. Primers used in the study

SED ID NO	Primer	Sequence[1]	Comment
1	pspA-UpFr-800	ggagtttgtcgttgaaattac	Anneals ~800bp upstream of *pspA* Start codon
2	pspA-UpFr-700	gtatttagagattttcaaagtg	Anneals ~700bp upstream of *pspA* Start codon
3	pspA-UpRe-ErmCass-OH	TGCAAGTCACACGAACACGAActaaatttacctcttttctgatag	Anneals directly upstream of *pspA* start codon with an overhang seq. complementary to the *ermB* cassette
4	pspA-DoFr-ErmCass-OH	CTATTATTTAACGGGAGGAAATAAgccgattaaattaaatcatg	Anneals directly downstream of *pspA* stop codon with an overhang seq. complementary to the *ermB* cassette
5	pspA-DoRe-700bp	acgtccggatttggcgtgc	Anneals ~700bp downstream of *pspA* Stop codon
6	pspA-DoRe-800bp	ccactcgagcataatgcc	Anneals ~800bp downstream of *pspA* Stop codon
7	Erm-cass-frw	ttcgtgttcgtgtgacttgca	
8	Erm-cass-rev	ttatttcctcccgttaaataatag	
9	prsA-UpFr-800	aggattggcataaaatggttg	Anneals ~800bp upstream of *prsA* Start codon
10	prsA-UpFr-700	ggtttcttgaaagaattgga	Anneals ~700bp upstream of *prsA* Start codon
11	prsA-UpRe-TetOH	TAATTTTCATGTGATTTTCCTCCATgtctactcctttgagataagtg	Anneals directly upstream of *prsA* start codon with an overhang seq. complementary to the beginning of the *tetM* Orf
12	prsA-DoFr-TetOH	ATATATGTTCAATAAAATAACTTAGtccaaatcaatgagtcaggga	Anneals directly upstream of *prsA* stop codon with an overhang seq. complementary to the end of the *tetR* Orf
13	prsA-DoRe-800	aacgcatcatatcaggtgtac	Anneals ~800bp downstream of *prsA* Stop codon
14	prsA-DoRe-700	aacgcatcatatcaggtgtac	Anneals ~700bp downstream of *prsA* Stop codon
15	TetOrf-frw	atggaggaaaatcacatgaaaatta	

16	TetOrf-rev	ctaagttattttattgaacatatat	
17	malX-UpFr-800	gcttccaacaaaccttgctc	Anneals ~800bp upstream of *malX* Start codon
18	malX-UpFr-700	gctcctaatggaaggatttg	Anneals ~700bp upstream of *malX* Start codon
19	malX-UpRe-KanOH	ctattctttaggaggaatacactATGGCTAAAATGAGAATATCA	Anneals directly upstream of *malX* start codon with an overhang seq. complementary to the beginning of the kanR Orf
20	malX-DoFr-KanOH	acccccccttgaacaaatttttCTAAAACAATTCATCCAGTAAA	Anneals directly upstream of *malX* stop codon with an overhang seq. complementary to the end of the *kanR* Orf
21	malX-DoRe-800	gaaaataacaccaaagatacgt	Anneals ~800bp downstream of *malX* Stop codon
22	malX-DoRe-700	agcccaaatgatagtccaag	Anneals ~700bp downstream of *malX* Stop codon
23	KanOrf-frw	atggctaaaatgagaatatca	
24	KanOrf-rev	ctaaaacaattcatccagtaaa	

Notes: [1] annealing sequences in lower case and non-annealing overhang sequences in upper case letters

Pneumococcal transformation

For pneumococcal transformation, a general procedure was employed as previously described (Mellroth P. et al. J. Biol. Chem. 2012 30;287(14):11018-29). To generate double and triple mutants the same procedure was iterated using sequenced mutant isolates that had acquired the corresponding mutations. Confirmation of mutants were done with Sanger sequencing of PCR amplicons from genomic DNA of isolated mutants using primers that annealed ~800bp of the cognate loci (thus outside the constructs used for transformation) (primer sequences in Table E). Strains produced are listed in Table D.

Immunofluorescence microscopy

After growth, pneumococci (serotypes 1, 3 and 6B) were stained using sera from immunized mice as primary antibody (dilution 1:100 in PBS 1% BSA) followed by incubation with the secondary antibody Alexa Fluor 488 goat anti mouse (dilution 1:500 in PBS 1% BSA). Imaging was performed using a DV Elite microscope (Applied Precision) and a scientific

complementary metal-oxide-semiconductor (sCMOS) camera. Images were acquired with FITC Laser intensity 50% and exposure time 200 ms using Softworx (Applied Precision).

Quantification of the signal detected on the bacteria after immunofluorescence staining

Using the functions Image>Adjust>Threshold and Analyze>Measure of ImageJ, the area covered by the bacteria and the area covered by the signal detected on the bacteria after staining with sera were selected, defined and measured. The final signal ratio was calculated by dividing the area of the signal (detected using the sera) by the total area of the bacteria.

Opsonophagocytosis assay with RAW cells

RAW 264.7 murine macrophages were grown and maintained at 37ºC, 5% CO_2 in RPMI medium (Gibco) supplemented with 10% (v/v) Fetal bovine serum (FBS) (HyClone). To assess the opsonophagocytic activity of antibodies in immunized mice sera, 2×10^5 RAW 264.7 cells were seeded in 24-well plates and incubated overnight at 37ºC. Serotype 1 bacteria were incubated for 30 minutes at 37ºC with 5% CO_2 with 20% serum from mice. RAW cells were then washed with PBS and incubated for 1.5 hours with 2.5×10^7/well of pre-treated bacteria. Cells were washed three times with PBS to remove unattached bacteria. To measure total uptake of bacteria, cells were incubated with a 50/50 solution of 2% saponin (Sigma) and trypsin-EDTA (Gibco) for 15 minutes at 37°C, to lyse eukaryotic cells, and total bacteria were plated for enumeration. To evaluate phagocytosis, 300 µg/ml of Gentamicin (Sigma) and 0,12 mg/ml of Penicillin G (Sigma) were added to separate wells and incubated 15 minutes at 37°C to kill extracellular bacteria. Cells were washed three times with PBS and incubated with a 50/50 solution of 2% saponin and trypsin-EDTA for 15 minutes at 37°C to lyse eukaryotic cells. To evaluate bacterial killing inside macrophages, separate wells were treated with antibiotics (as for phagocytosis), washed three times with PBS and incubated for one hour at 37°C with medium. Cells were washed three times with PBS and incubated with a 50/50 solution of 2% saponin and trypsin-EDTA for 15 minutes at 37°C to lyse eukaryotic cells.

Preparation of *L. lactis* artificial membrane particles (aMP) containing pneumococcal MalX and PrsA

DNA Techniques and Primer Sequences

Primers were designed based on the genomes of *S. pneumoniae* TIGR4 and *L. lactis* subsp. *cremoris* MG1363. All PCRs were conducted with Phusion Master Mix HF (Thermo

Scientific). Obtained plasmids were confirmed by PCR and sequencing. All primers used are listed in Table E.

Cloning of P23, prsA and malX into pORI

The strong *L. lactis* promotor P23 was cloned in plasmid pORI (provided by Ulrich von Pawel-Rammingen, Umeå University, Sweden; Que et al. 2020 Infection and Immunity Vol. 68, No. 6) by FastCloning (Li et al., 2011 BMC Biotechnol *11*, 92) resulting in plasmid pORI-P23 (SEQ ID NO: 64). The plasmid backbone was amplified with primers CS-pORI-new-r (GACTAGCAAATACTAACAACAAG) and CS-pORI-new-f (GGATCCGTCGACCTGCAG), and the insert was amplified from genomic DNA from *L. lactis* subsp. *cremoris* MG1363 (provided by Harold Marcotte, Karolinska Institutet, Sweden) with primers CS-P23-f2 (CTTGTTGTTAGTATTTGCTAGTCGAATTCGAAAAGCCCTGACAACGC) and CS-P23-r (CTGCAGGTCGACGGATCCAACATCATTGTCATTCATATTTTTC).

prsA and *malX* were amplified *S. pneumoniae* TIGR4 with primers CS-prsA-f (GGGAGGCCAAATATAATGAAGAAAAAATTATTGGCAG), CS-prsA+4nt-r (GACATAGTGTATTCCTCCTAAATGGACTATTCGTTTGATGTAC), CS-malX-17nt-f (GTACATCAAACGAATAGTCCATTTAGGAGGAATACACTATGTC) and CS-malX+56nt-r (AAGCGGAAGAGCGTCTATCTCTATTGATAAATTCAAAGG). The two fragments were joined by Gibson Assembly system (New England Biolabs). The assembled fragment encoding an artificial *prsA-malX* operon was amplified by PCR with the flanking primers. This fragment was then cloned into pORI-P23 by FastCloning using primers CS-pORI23-oriCol1-f (TAGACGCTCTTCCGCTTC) and CS-pORI23-P23-r (TCATTATATTTGGCCTCCC) for plasmid backbone amplification and directly transformed through electroporation into *L. lactis* subsp. *cremoris* MG1363 as previously described (King et al., 2015 In Methods in Enzymology, A.K. Shukla, ed. (Academic Press), pp. 77-97). In addition, also empty pORI23 was transformed into the same background. The resulting *L. lactis* strains carrying the plasmid pORI-P23-prsA-malX and pORI-P23 were used for subsequent preparation of artificial membrane particles (aMP).

TABLE F: Sequences for Lactobacillus experiments

SED ID NO	Designation	Sequence[1]
54	CS-pORI-new-r	gactagcaaatactaacaacaa g
55	CS-pORI-new-f	ggatccgtcgacctgcag
56	CS-P23-f2	cttgttgttagtatttgctagtcga attcgaaaagccctgacaacgc
57	CS-P23-r	ctgcaggtcgacggatccaaca tcattgtcattcatatttttc
58	CS-prsA-f	gggaggccaaatataatgaaga aaaaattattggcag
59	CS-prsA+4nt-r	gacatagtgtattcctcctaaatg gactattcgtttgatgtac
60	CS-malX-17nt-f	gtacatcaaacgaatagtccattt aggaggaatacactatgtc
61	CS-malX+56nt-r	aagcggaagagcgtctatctcta ttgataaattcaaagg
62	CS-pORI23-oriCol1-f	tagacgctcttccgcttc
63	CS-pORI23-P23-r	tcattatatttggcctccc
64	pORI-P23	

Preparation of L. lactis artificial membrane particles (aMP)

L. lactis strains carrying the plasmids pORI-P23-*prsA-malX* and pORI-P23 were grown overnight at 30 °C without aeration or agitation in M17 (Sigma-Aldrich) supplemented with 1% Glucose and 5 µg/ml erythromycin. The following day the cultures were diluted to OD 0.1 and grown in the same condition to OD 0.85. Cells were then collected by centrifugation for 20 min at 11'000 g and washed with PBS. Cells were disrupted by applying one time 30 kpsi with a ´Pressure cell´ Homogeniser EP FPG12805 (Homogenising Systems Ltd). The cell lysate was clarified by centrifugation at 11'000 g for 20 min prior to sterile filtration of the supernatant through a 0.45 µm filter. MVs were then collected by ultra-centrifugation at 31'000 g for 16 h. The resulting pellet was washed in PBS by another round of ultra-centrifugation for 3 h. Pellets were thereafter suspended in PBS and protein concentrations

of the MV preparations were assessed by Pierce BCA Protein Assay Kit (Thermo Scientific) prior to storage at -80 °C. Similar procedure is also applicable to e.g. *S. pneumoniae* cells.

Statistical analysis

Statistical analysis for multiple comparisons was done using a nonparametric ANOVA test, and Dunn´s test was applied to assess differences between pairs. Two groups comparisons were analyzed using a nonparametric two-tailed Mann-Whitney test. Analysis of survival curves was done using a log-rank Mantel-Cox test. Statistically significant data was defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** = $p < 0.0001$.

CLAIMS

1. An immunogenic composition comprising bacterial membrane vesicles (MVs) comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen, **characterized in that** the MVs do **not** comprise an immunogenic amount of a streptococcal PspA antigen.

2. An immunogenic composition comprising a streptococcal MalX antigen and a streptococcal PrsA antigen associated with membrane vesicles from a host cell, **characterized in that** the MalX antigen and the PrsA antigen are **not** associated with a membrane vesicle derived from the same strain as the antigens, and wherein the sequence(s) of the MalX antigen and the PrsA antigen are derived from *Streptococcus pneumoniae*.

3. The composition according to claim 1, wherein the MVs comprise streptococcal MVs.

4. The composition according to any of the preceding claims, wherein the antigen(s) is/are associated with membrane vesicles from a host cell, where the antigen(s) is/are heterologous in relation to the host cell.

5. The composition according to claim 4, wherein the host cell is of the genus *Lactococcus*.

6. The composition according to claim 5, wherein the host cell is a *Lactococcus lactis*-cell.

7. The composition according to any of the preceding claims, wherein the membrane vesicles are artificial membrane particles.

8. The composition according to any of the preceding claims, wherein the membrane vesicles comprise disrupted bacterial cells.

9. The composition according to any of the preceding claims, wherein the membrane vesicles comprise membrane vesicles derived from mechanically disrupted bacterial cells.

10. The composition according to claim 1 or any claim dependent thereon, wherein composition comprises the MalX antigen.

11. The composition according to claim 1 or any claim dependent thereon, wherein composition comprises the PrsA antigen.

12. The composition according to claim 1 or any claim dependent thereon, wherein composition comprises both the MalX antigen and the PrsA antigen.

13. The composition according to any of the preceding claims, wherein the MalX antigen is present at a concentration of at least 0.001 μg/ml, preferably at least 0.05 μg/ml, more preferably at least 0.1 μg/ml, most preferably at least 1 μg/ml.

14. The composition according to any of the preceding claims, wherein the PrsA antigen is present at a concentration of at least 0.001 μg/ml, preferably at least 0.05 μg/ml, more preferably at least 0.1 μg/ml, most preferably at least 1 μg/ml.

15. The composition according to any of the preceding claims, wherein both the PrsA antigen and the MalX antigen are present at relative amounts of 10:1 to 1:10 by weight, preferably 5:1 to 1:5, more preferably 2:1 to 1:2, most preferably about 1:1.

16. The composition according to any of the preceding claims, wherein the PspA antigen is absent or present at a concentration of less than 0.01 μg/ml, preferably less than 0.005 μg/ml, more preferably less than 0.001 μg/ml, most preferably absent.

17. The composition according to any of the preceding claims, wherein the MalX antigen comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to any one of SEQ ID NOs: 25 or 43-47 over a stretch of at least 50 amino-acids, preferably at least 100 amino acids, more preferably at least 200 amino acids, most preferably over the entire sequence presented in said SEQ ID NO.

18. The composition according to any of the preceding claims, wherein the MalX antigen comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 25.

19. The composition according to any of the preceding claims, wherein the PrsA antigen comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence

identity to any one of SEQ ID NOs: 26 or 48-53 over a stretch of at least 50 amino-acids, preferably at least 100 amino acids, more preferably at least 200 amino acids, most preferably over the entire sequence presented in said SEQ ID NO.

20. The composition according to any of the preceding claims, wherein the PrsA antigen comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 26.

21. The composition according to any of the preceding claims, wherein the PspA antigen comprises a sequence having at least 70%, preferably 80%, more preferably 85%, yet more preferably 90%, still more preferably 95%, most preferably 100% sequence identity to SEQ ID NO: 27.

22. The composition according to any of the preceding claims, wherein the membrane vesicles are of the type *Streptococcus pneumoniae* membrane vesicle microparticles.

23. The composition according to any of the preceding claims, wherein the MVs comprise MalX at the level of ≥0.001 μg/μg, preferably ≥0.01 μg/μg, more preferably ≥0.02 μg/μg, yet more preferably ≥0.05 μg/μg, most preferably ≥0.1 μg/μg of the total protein in the MVs.

24. The composition according to any of the preceding claims, wherein the MVs comprise PrsA at the level of ≥0.001 μg/μg, preferably ≥0.01 μg/μg, more preferably ≥0.02 μg/μg, yet more preferably ≥0.05 μg/μg, most preferably ≥0.1 μg/μg of the total protein in the MVs.

25. The composition according to any of the preceding claims, wherein the MVs lack PspA, or PspA is present at a level which is less than 0.01 μg/μg, preferably less than 0.05 μg/μg, yet more preferably less than 0.001 μg/μg of the total protein in the MVs, most preferably the MVs lack PspA.

26. The composition according any of the preceding claims, wherein MVs are 5-1000 nm in diameter, preferably 5-300 nm, more preferably 15-175 nm, most preferably 10-125 nm.

27. The composition according any of the preceding claims, comprising MVs in an amount of 1 µg/ml, preferably 5 µg/ml, more preferably 10 µg/ml, most preferably 100 µg/ml.

28. The composition according any of the preceding claims, wherein the MVs are derived from any *Streptococcus pneumoniae* strain, preferably any strain/s of serotype 1, 3, 4, 5, 6A, 6B, 6C, 6D, 7F, 8, 9N, 9V, 10A, 11A, 11B, 12F, 13, 14, 15A, 15B, 15C, 16F, 17F, 18C, 19A, 19F, 20, 21, 22F, 23A, 23B, 23F, 24F, 31, 33F, 34, 35B, 35F, 37, 38, including strains TIGR4, P1031, BHN418, and A66.

29. The composition according to any of the preceding claims, wherein the composition comprises the MalX antigen and/or the PrsA antigen associated with membrane vesicles from a host cell being a firmicute.

30. The composition according to any of the preceding claims, wherein the composition further comprises a nanoparticle carrier.

31. The composition according to claim 30, wherein the nanoparticle carrier comprises an ISCOM, an ISCOM matrix, a liposome, a polymeric nanoparticle, an inorganic nanoparticle, a non-biodegradable nanoparticle, a calcium phosphate particle, an emulsion or a virus-like particle.

32. The composition according to any of the preceding claims, wherein the composition is devoid of whole *Streptococcus* cells, preferably *Streptococcus pneumoniae* cells.

33. The composition according to any of the preceding claims, further comprising capsular polysaccharides from a *Streptococcus sp.,* preferably *Streptococcus pneumoniae.*

34. The composition according to any of the preceding claims, for use as a medicament.

35. The composition according to any of the preceding claims, wherein the composition is a vaccine.

36. The composition according to any of the preceding claims, wherein the composition further comprises an adjuvant, preferably aluminium hydroxide, a lipid-based substance such as squalene, an immune-stimulating component or a protein from a microbe such as diphtheria (preferably CRM), cholera (preferably choleratoxoid CTB

or mmCT) or *Escherichia coli* (preferably non-toxic heat-labile toxin such as LTK63 or dmLT).

37. The composition according to any of the preceding claims, wherein the composition is capable of eliciting serotype independent antibodies against a *Streptococcus sp.*, preferably *Streptococcus pneumoniae,* when administered to a mammalian host.

38. The composition according to any of the preceding claims, wherein the composition is capable of eliciting opsonophagocitic antibodies against a *Streptococcus sp.*, preferably *Streptococcus pneumoniae,* when administered to a mammalian host.

39. The composition according to any of the preceding claims, wherein the composition is capable of eliciting serotype-independent antibodies against a *Streptococcus sp.*, preferably *Streptococcus pneumoniae,* when administered to a mammalian host.

40. The composition according to any of the preceding claims, for use in a method for inducing protective immunity against a *Streptococcus sp.* in a subject.

41. The composition according to any of the preceding claims, for use in a method for inducing protective immunity against *Streptococcus pneumoniae* in a subject.

42. The composition according to any of the preceding claims, for use in a method for inducing protective immunity against a *Streptococcus sp.* in a subject, wherein the immunity is protective against a condition selected from pneumococcal sinusitis, pneumococcal otitis, pneumococcal pneumonia and invasive pneumococcal disease including but not limited to pneumococcal sepsis and pneumococcal meningitis, preferably invasive pneumococcal disease.

43. The composition according to any of the preceding claims, for use according to any of claims 40-42, wherein the protective immunity is serotype-independent.

44. A method for manufacturing a composition according to any of the preceding claims, comprising:

    a. heterologously expressing a streptococcal MalX antigen and/or a streptococcal PrsA antigen in a suitable host cell;

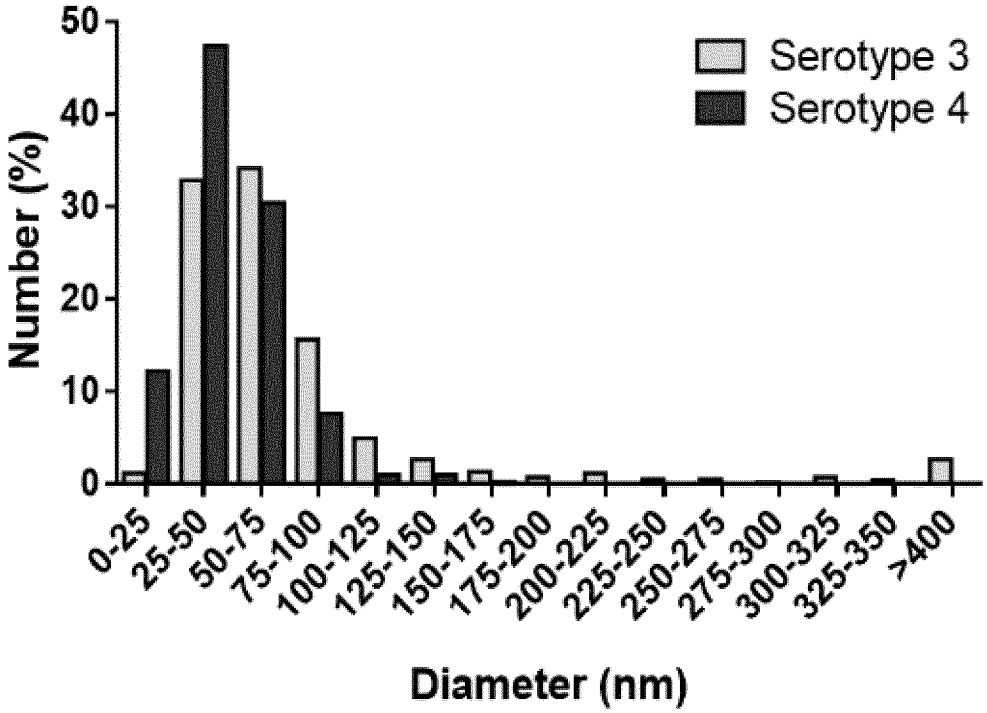
    b. generating membrane vesicles by disrupting the host cell expressing said antigens; and

c. recovering the generated membrane vesicles to form an immunogenic composition comprising a streptococcal MalX antigen and/or a streptococcal PrsA antigen associated with membrane vesicles.

45. The method according to any of claims 44, wherein the host cells are mechanically disrupted, preferably by shear forces, most preferably with a French press.

46. The method according to any of claims 44-45, wherein the recovering step comprises precipitation by centrifugation followed by resuspension.

A



B

Prediction of subcellular localization	Total no. of proteins	
	Serotype 3	Serotype 4
Cytosolic	320	115
Transmembrane	53	53
Lipoproteins	23	26
Cell wall	2	12
Secreted proteins	4	10

Figure 1.

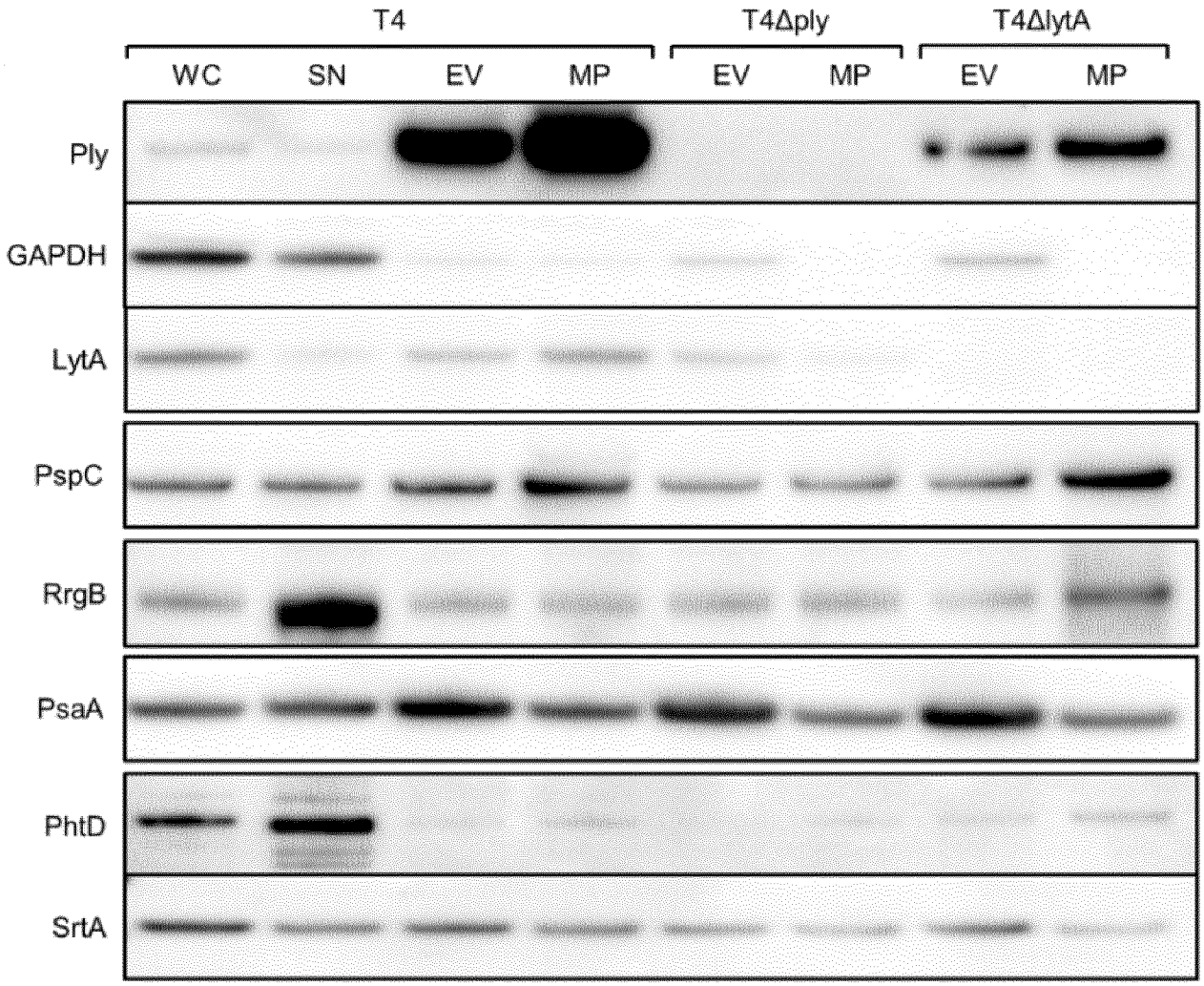

T4
T4Δply
T4ΔlytA
WC
SN
EV
MP
EV
MP
EV
MP
Ply
GAPDH
LytA
PspC
RrgB
PsaA
PhtD
SrtA

Figure 2.

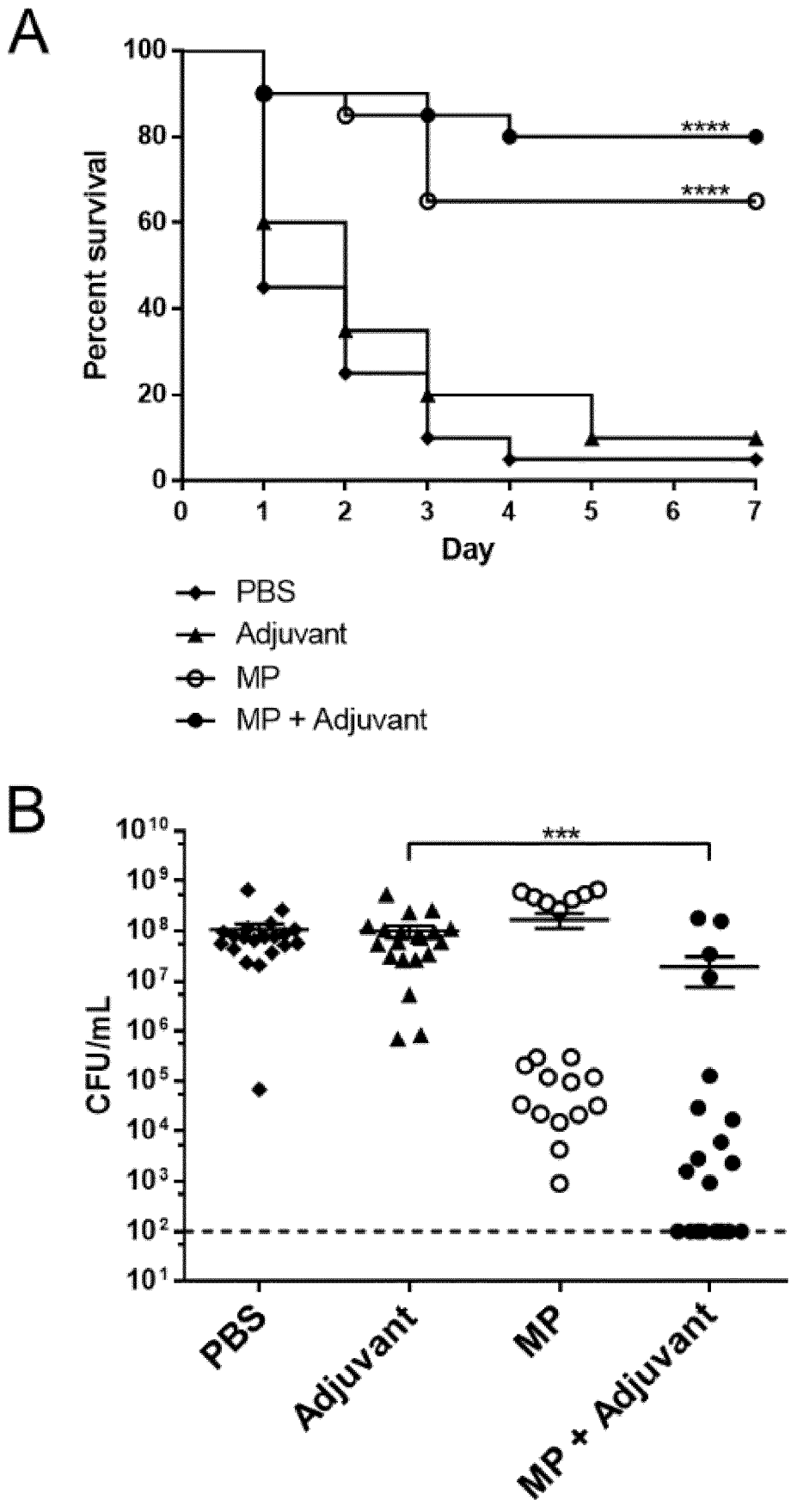
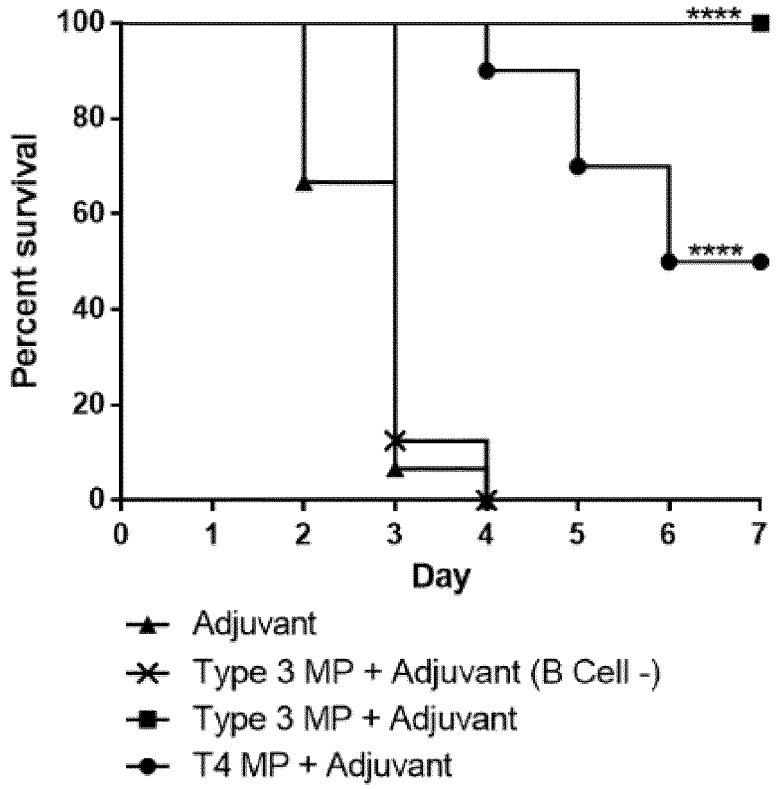

A
Percent survival
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Day
****
****
PBS
Adjuvant
MP
MP + Adjuvant
B
CFU/mL
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
***
PBS
Adjuvant
MP
MP + Adjuvant

Figure 3AB.

C



D

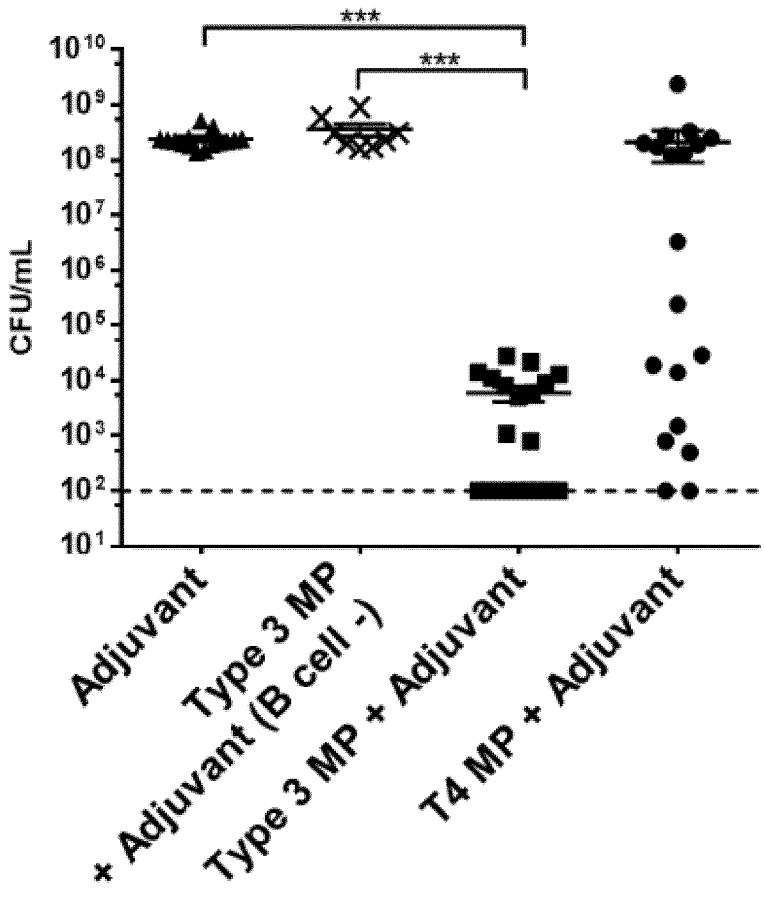
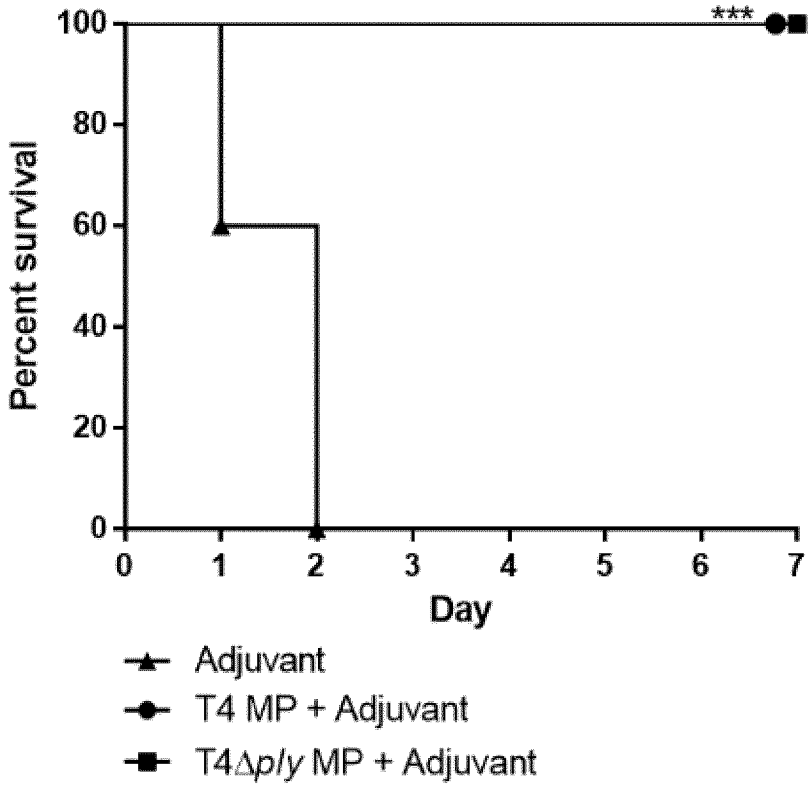


Figure 3CD.

E



F

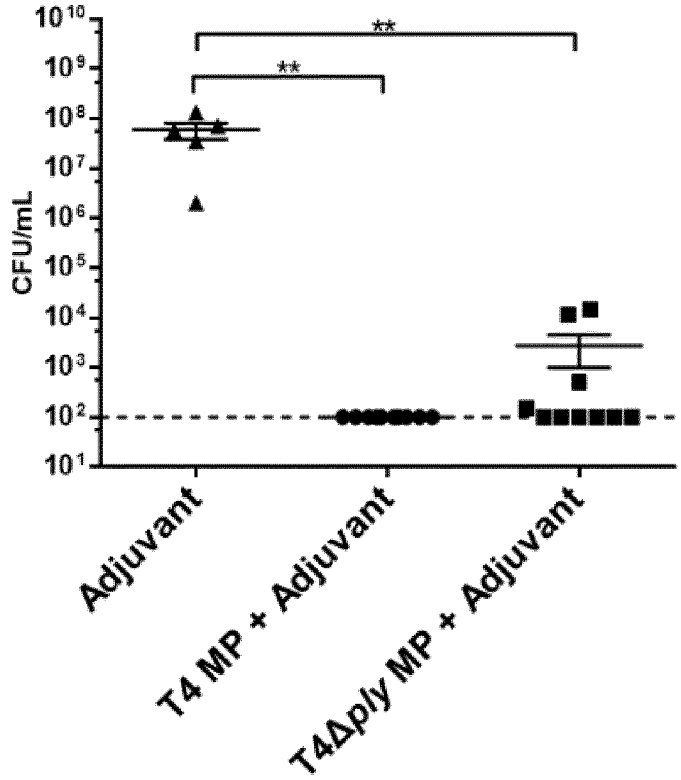


Figure 3EF.

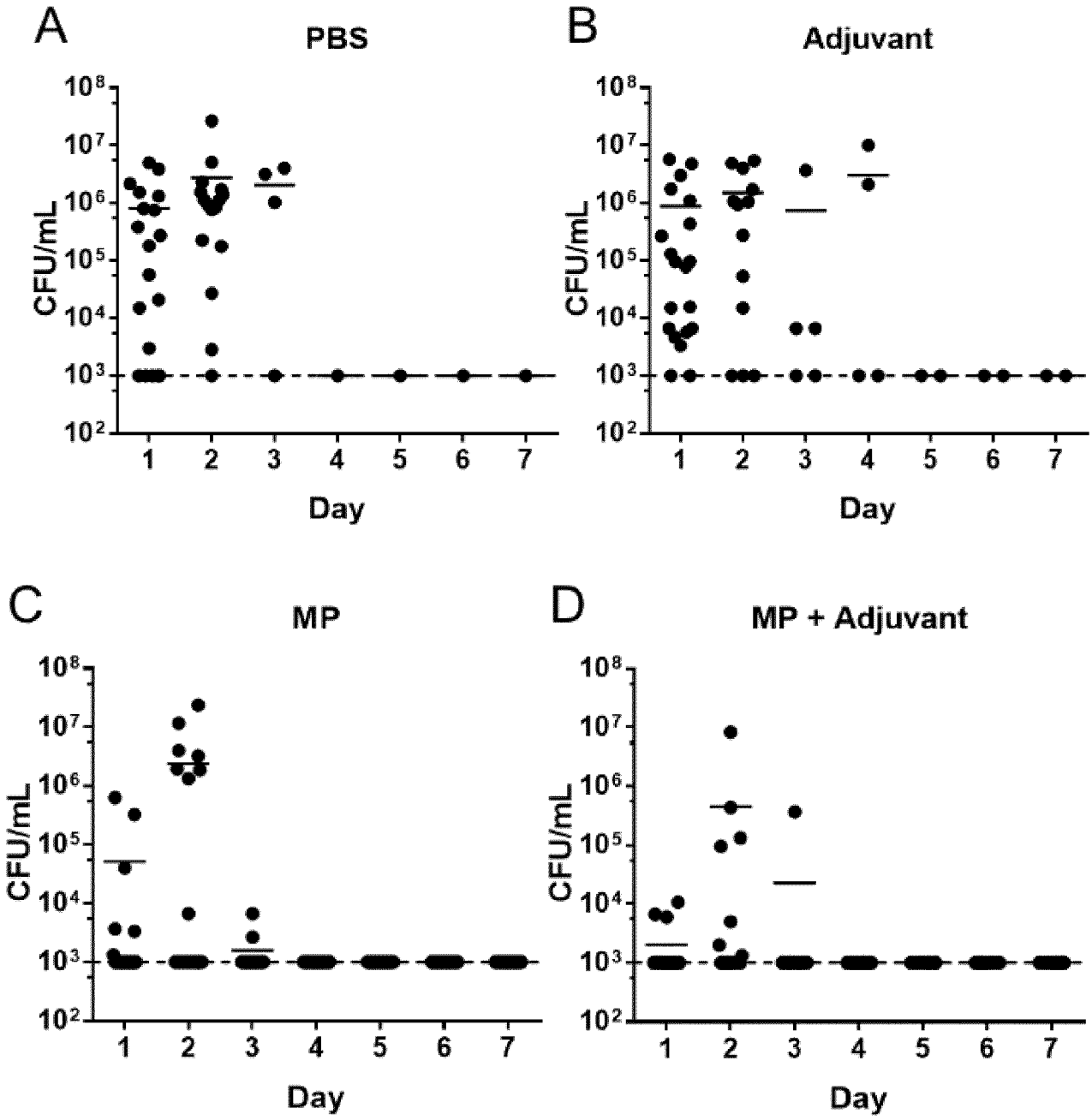

A
PBS
B
Adjuvant
C
MP
D
MP + Adjuvant
CFU/mL
Day
10^8
10^7
10^6
10^5
10^4
10^3
10^2
1 2 3 4 5 6 7

Figure 4A-D.

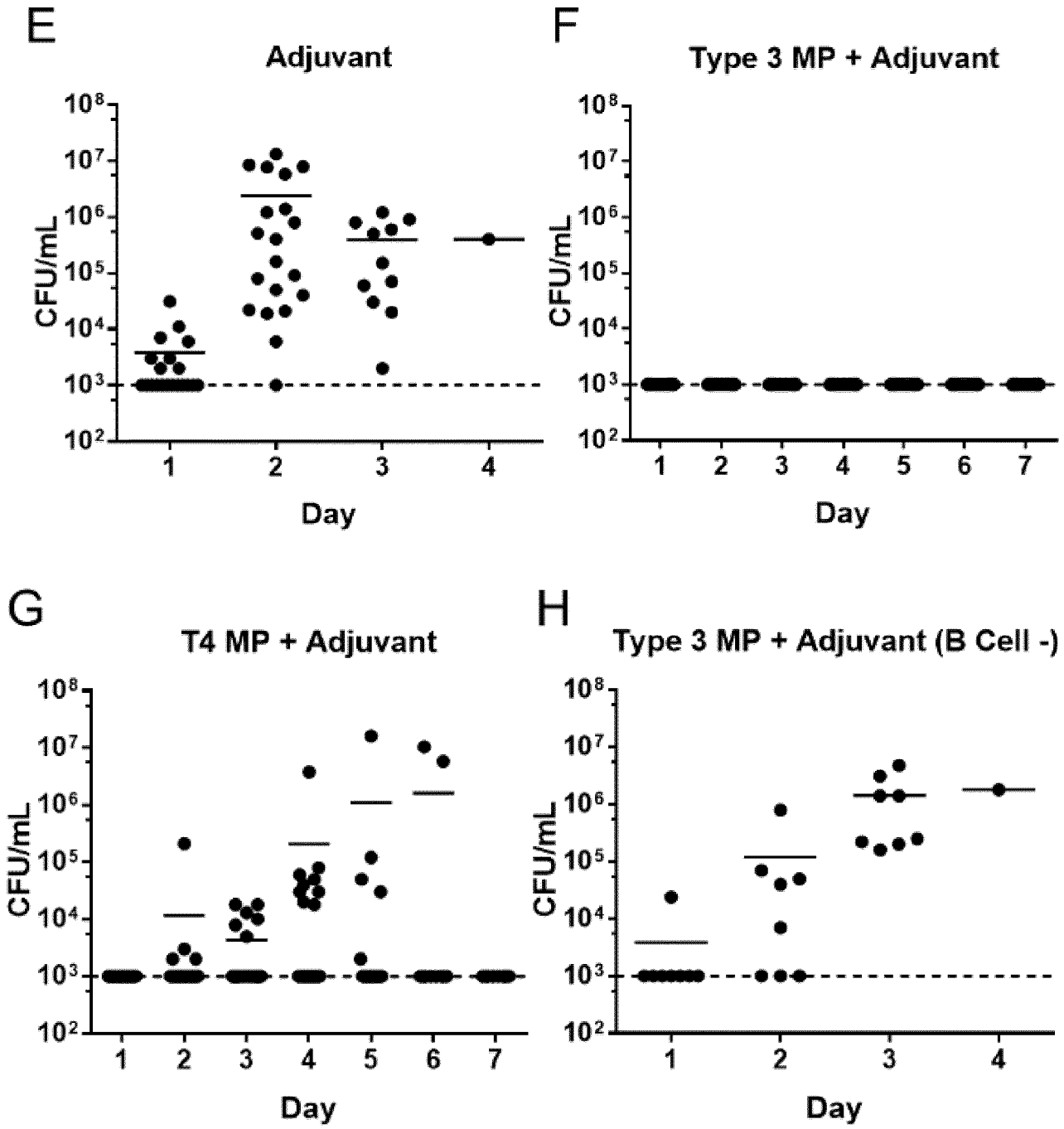

E
Adjuvant
F
Type 3 MP + Adjuvant
G
T4 MP + Adjuvant
H
Type 3 MP + Adjuvant (B Cell -)
CFU/mL
Day

Figure 4E-H

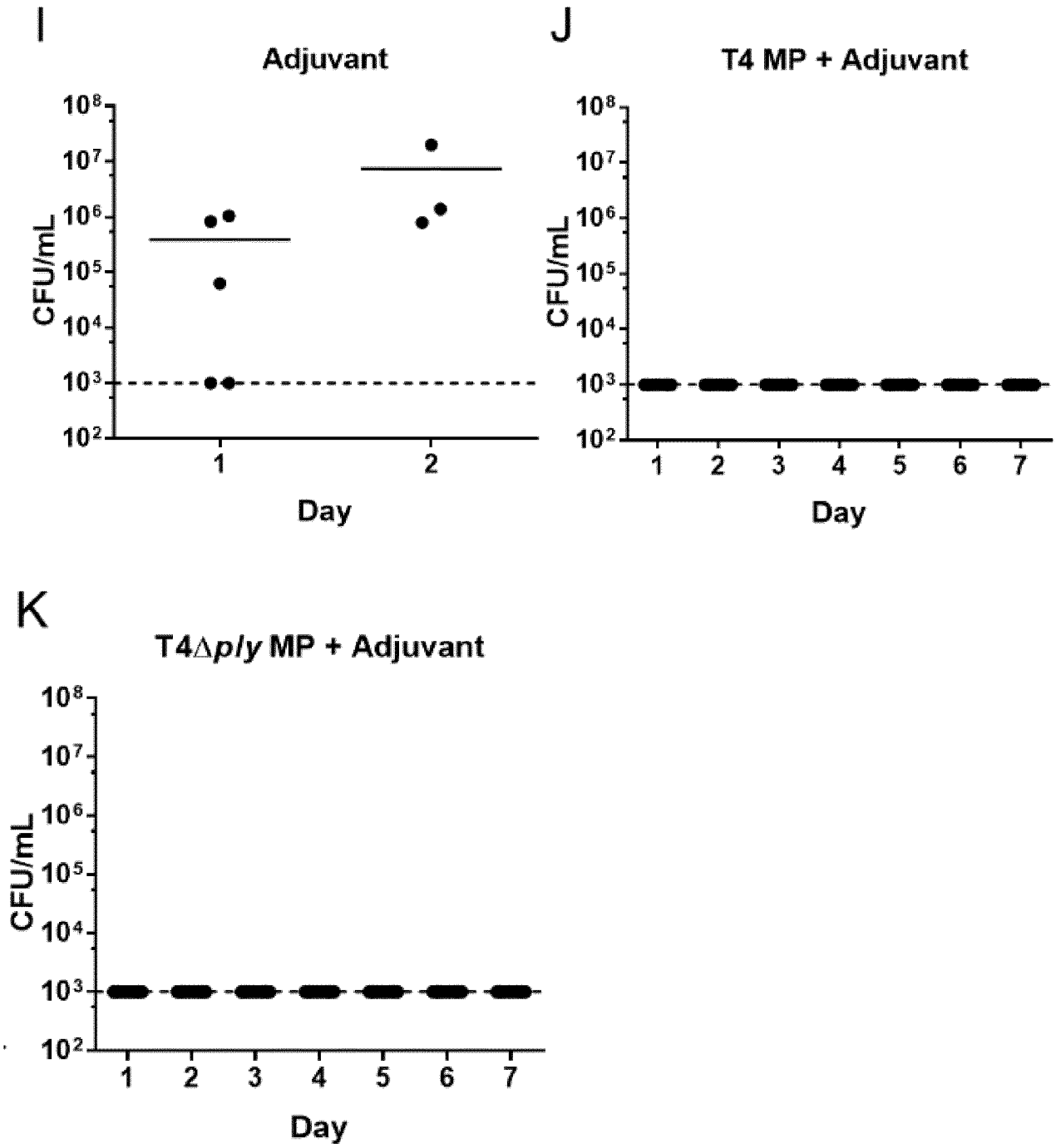

I
Adjuvant
J
T4 MP + Adjuvant
K
T4Δply MP + Adjuvant
CFU/mL
Day
10^8
10^7
10^6
10^5
10^4
10^3
10^2
1
2
3
4
5
6
7

Figure 4I-K.

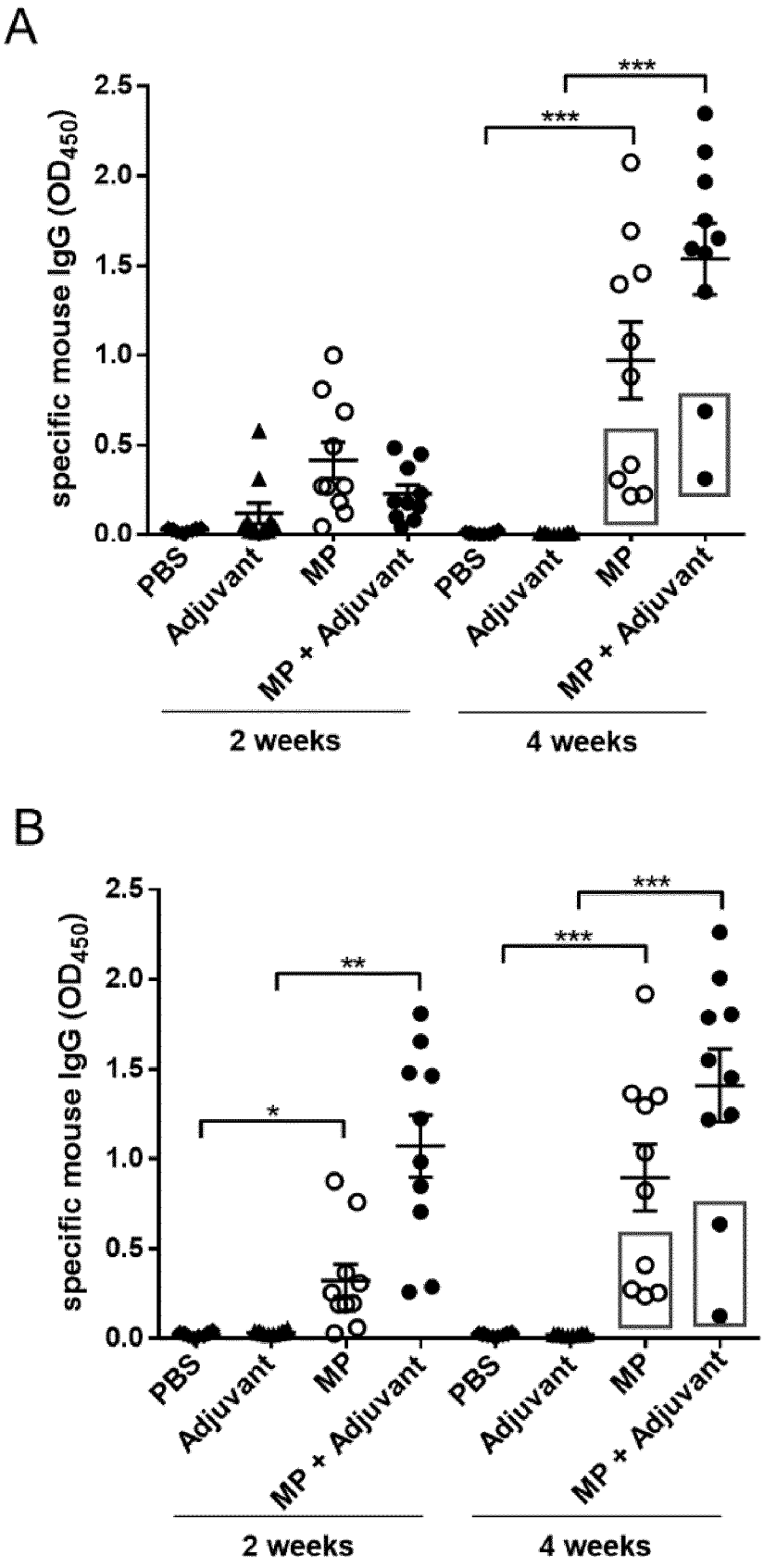
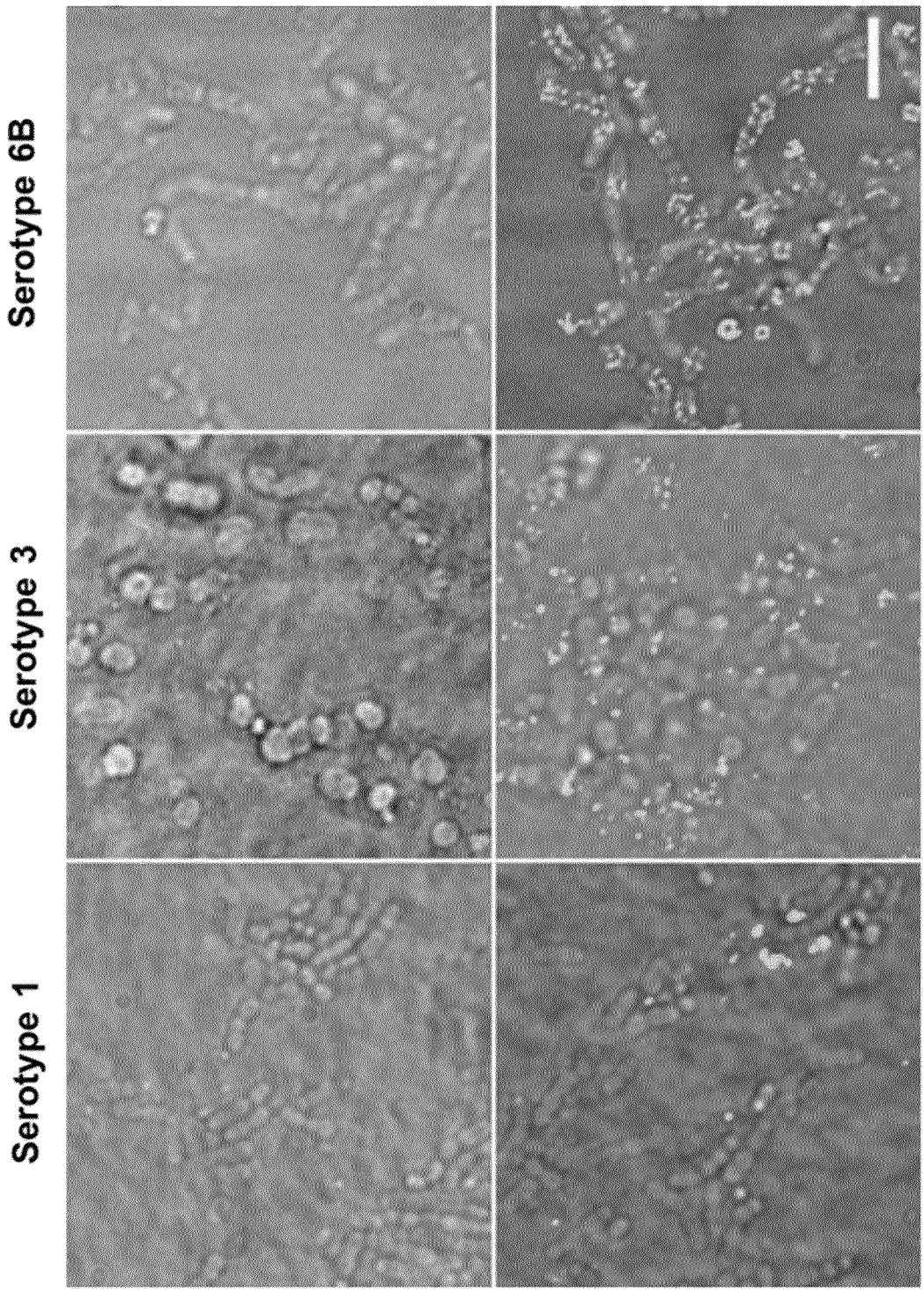

A
specific mouse IgG (OD450)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
PBS
Adjuvant
MP
MP + Adjuvant
PBS
Adjuvant
MP
MP + Adjuvant
2 weeks
4 weeks
B
specific mouse IgG (OD450)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
***
***
PBS
Adjuvant
MP
MP + Adjuvant
PBS
Adjuvant
MP
MP + Adjuvant
2 weeks
4 weeks

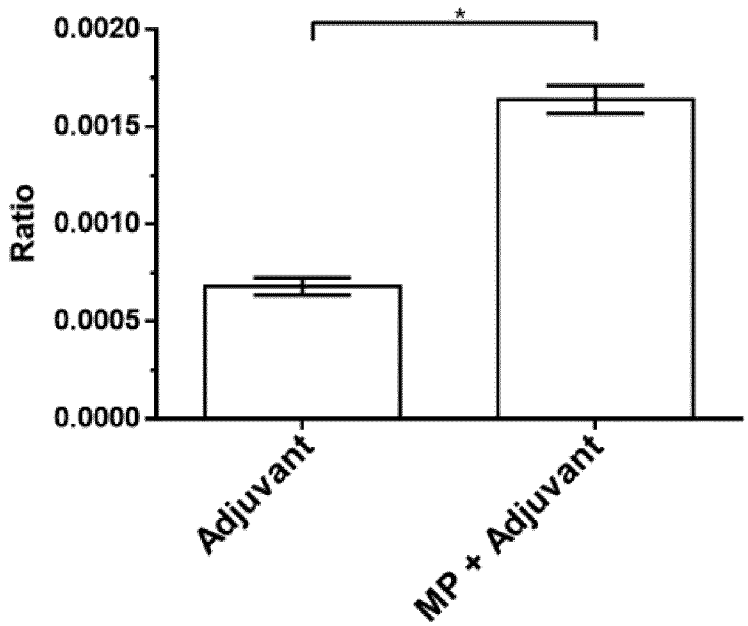
Figure 5 AB



C

Figure 5C

D



E

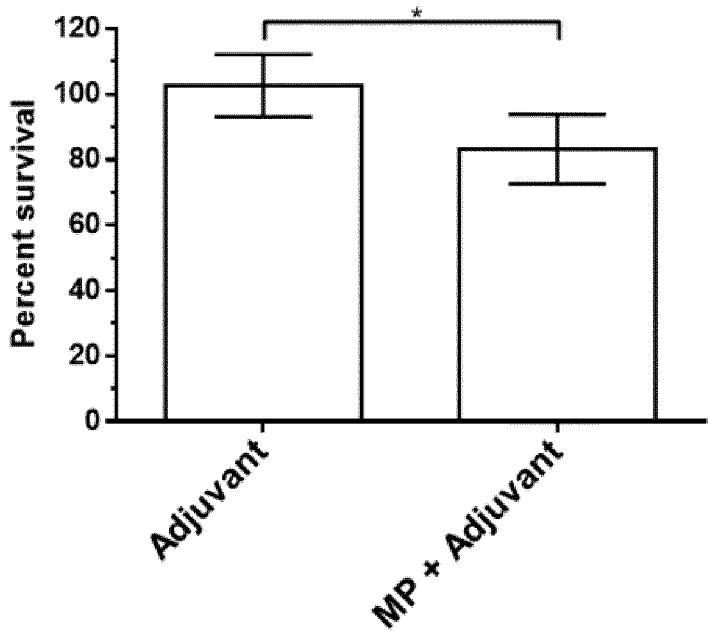


Figure 5DE

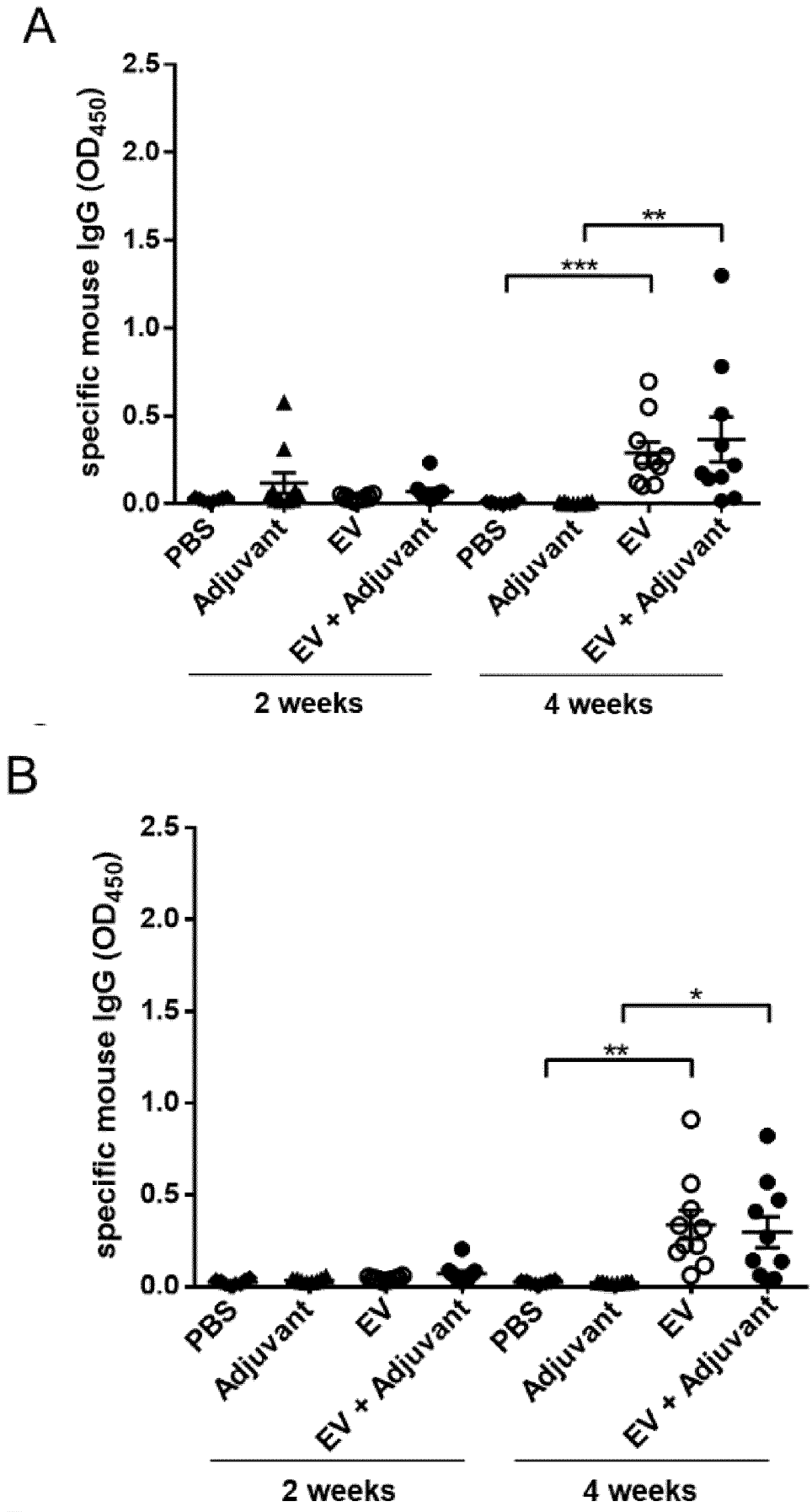

A
specific mouse IgG (OD450)
2.5
2.0
1.5
1.0
0.5
0.0
**
***
PBS
Adjuvant
EV
EV + Adjuvant
PBS
Adjuvant
EV
EV + Adjuvant
2 weeks
4 weeks
B
specific mouse IgG (OD450)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
PBS
Adjuvant
EV
EV + Adjuvant
PBS
Adjuvant
EV
EV + Adjuvant
2 weeks
4 weeks

Figure 6AB

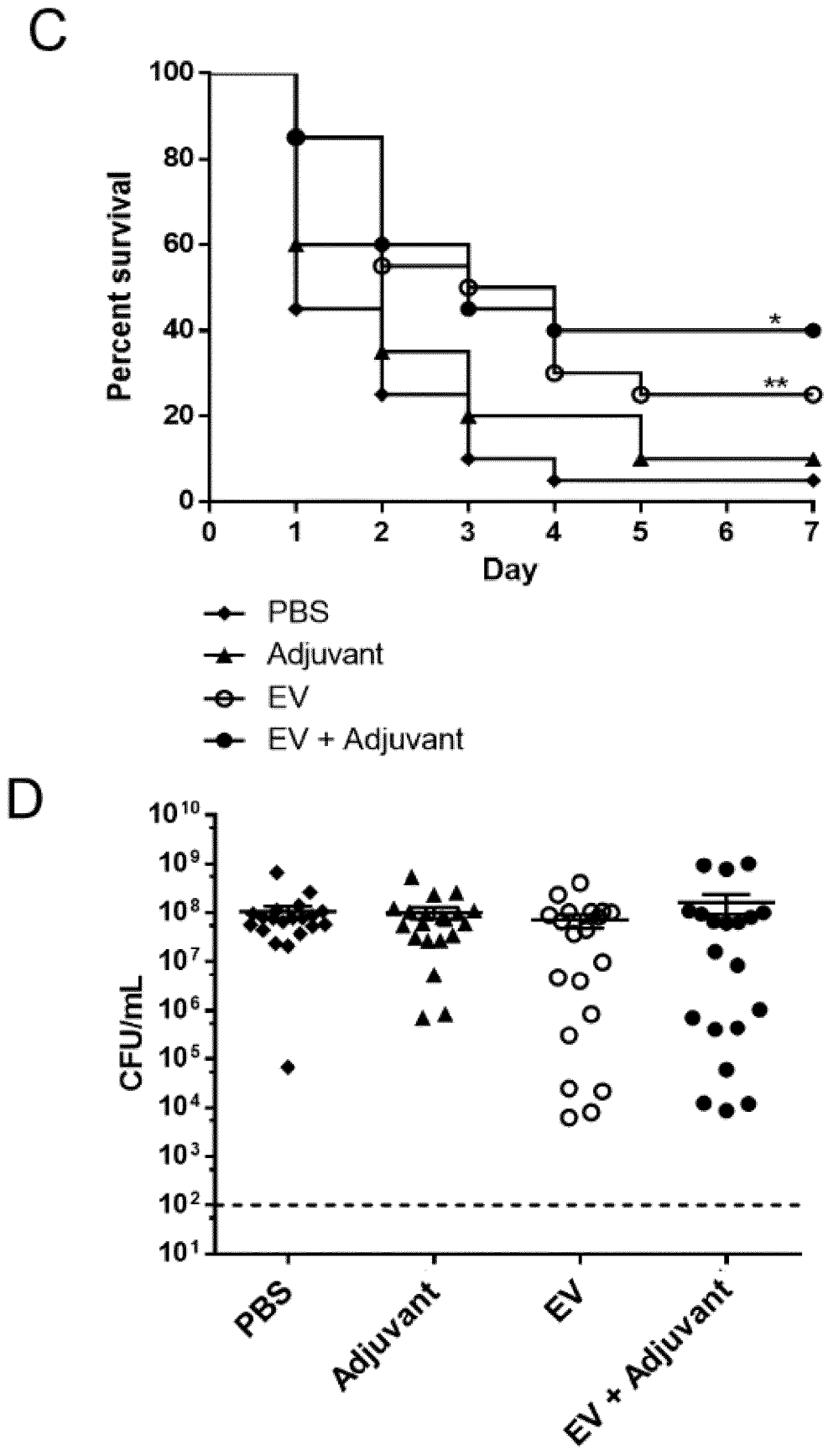

C
Percent survival
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Day
*
**
PBS
Adjuvant
EV
EV + Adjuvant
D
CFU/mL
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
PBS
Adjuvant
EV
EV + Adjuvant

Figure 6CD

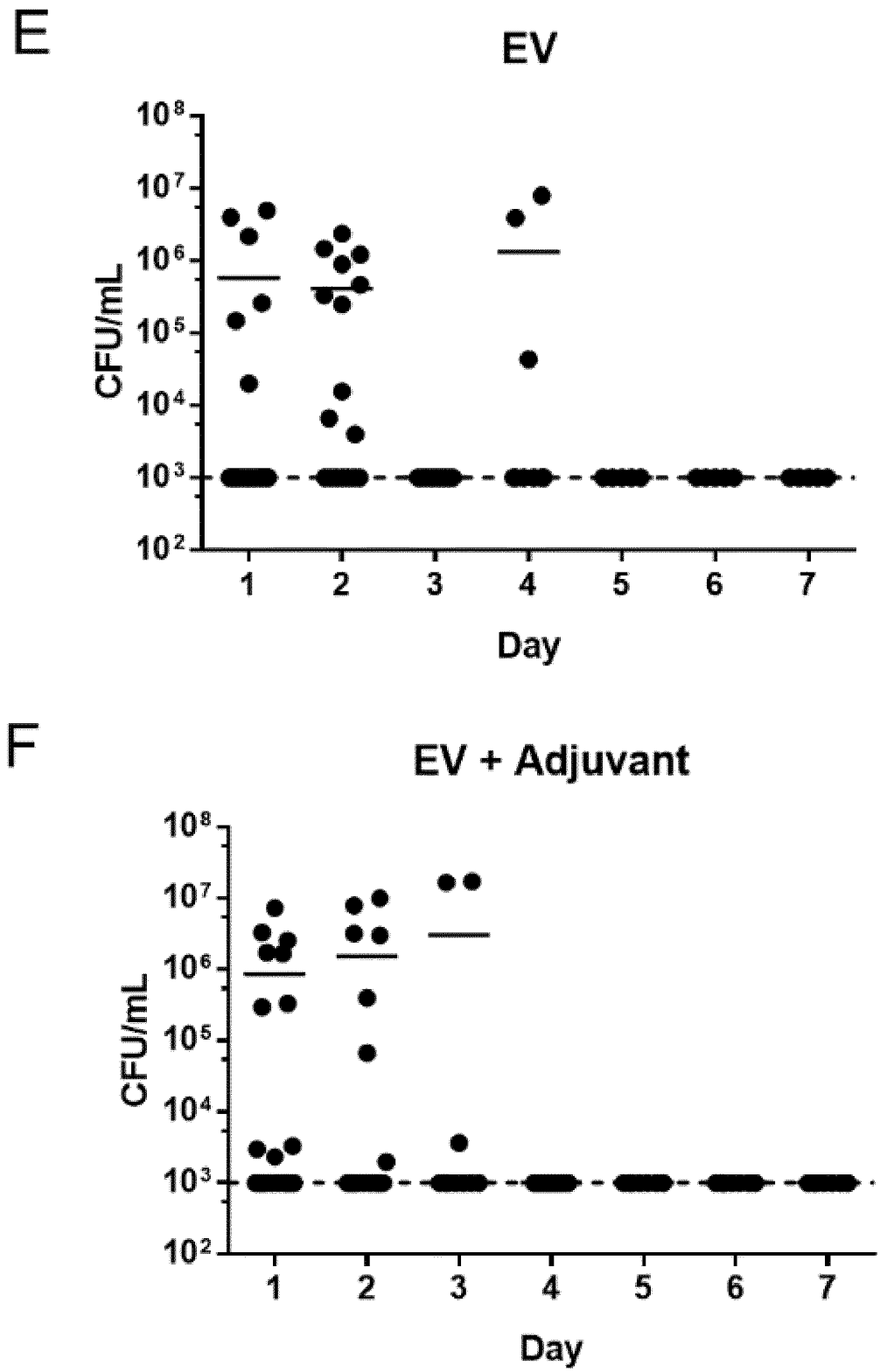

E
EV
CFU/mL
10^8
10^7
10^6
10^5
10^4
10^3
10^2
1
2
3
4
5
6
7
Day
F
EV + Adjuvant
CFU/mL
10^8
10^7
10^6
10^5
10^4
10^3
10^2
1
2
3
4
5
6
7
Day

Figure 6EF

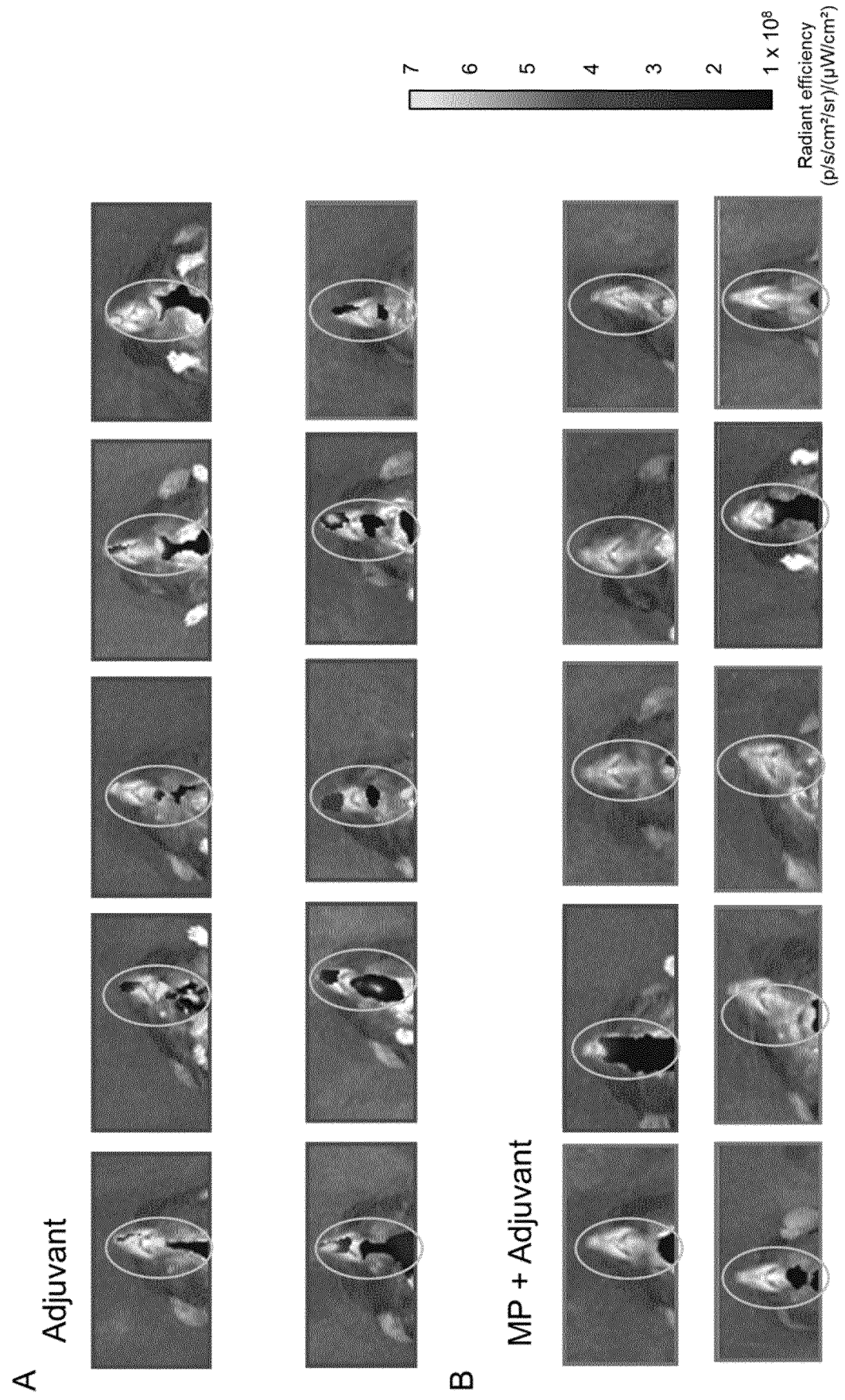

A
Adjuvant
B
MP + Adjuvant
7
6
5
4
3
2
1 x 10⁸
Radiant efficiency
(p/s/cm²/sr)/(µW/cm²)

Figure 7 AB

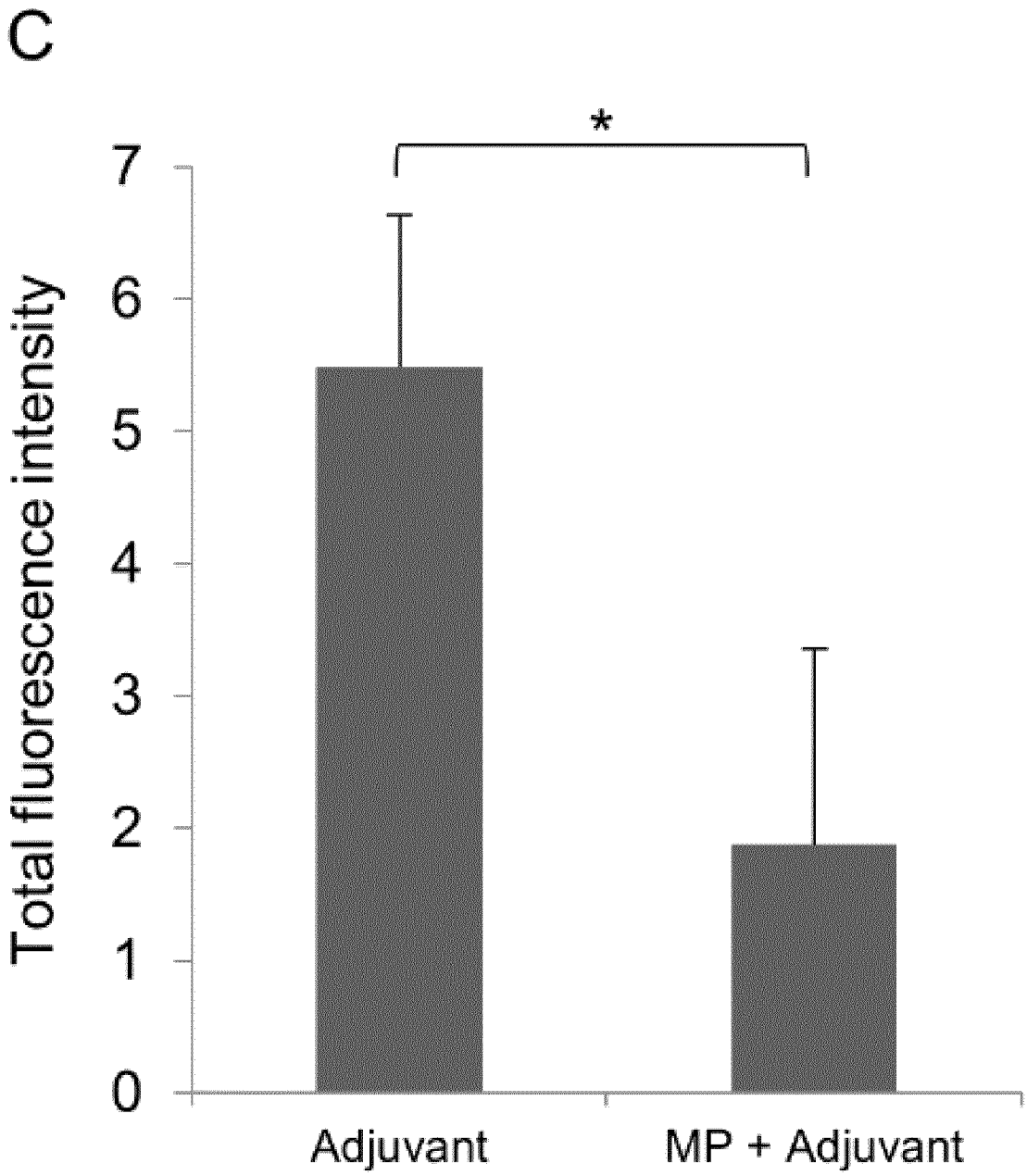

C
*
Total fluorescence intensity
0
1
2
3
4
5
6
7
Adjuvant
MP + Adjuvant

Figure 7C

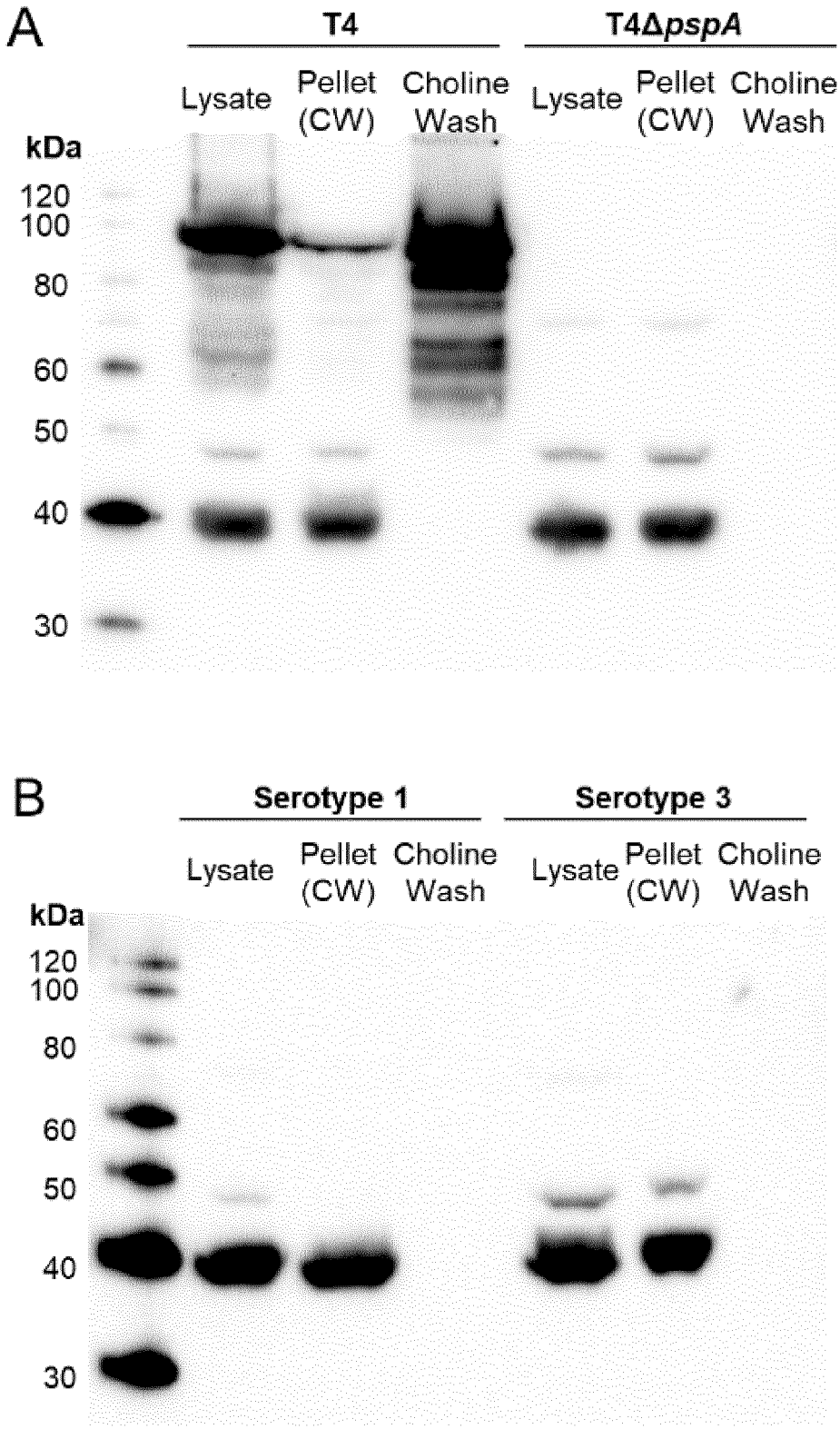

A
T4
T4ΔpspA
Lysate
Pellet (CW)
Choline Wash
Lysate
Pellet (CW)
Choline Wash
kDa
120
100
80
60
50
40
30
B
Serotype 1
Serotype 3
Lysate
Pellet (CW)
Choline Wash
Lysate
Pellet (CW)
Choline Wash
kDa
120
100
80
60
50
40
30

Figure 8AB.

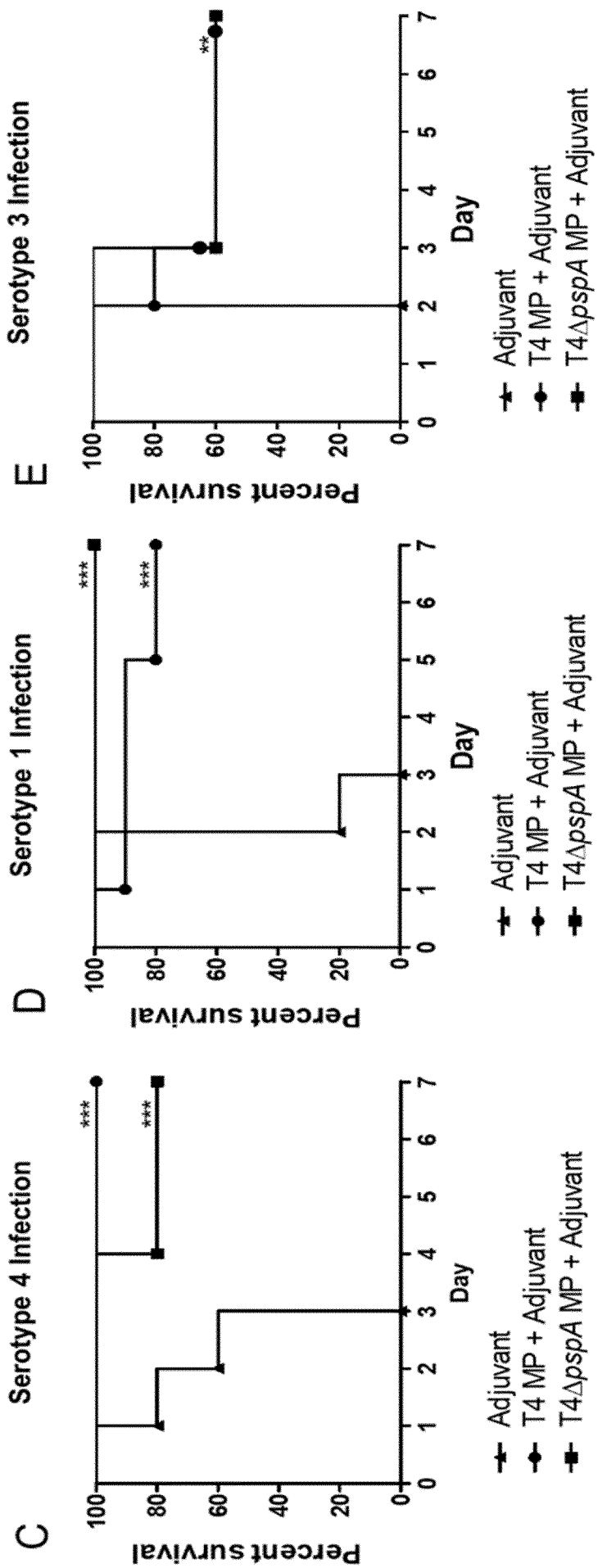

C
Serotype 4 Infection
D
Serotype 1 Infection
E
Serotype 3 Infection
Percent survival
Day
Adjuvant
T4 MP + Adjuvant
T4ΔpspA MP + Adjuvant
***
**

Figure 8C-E.

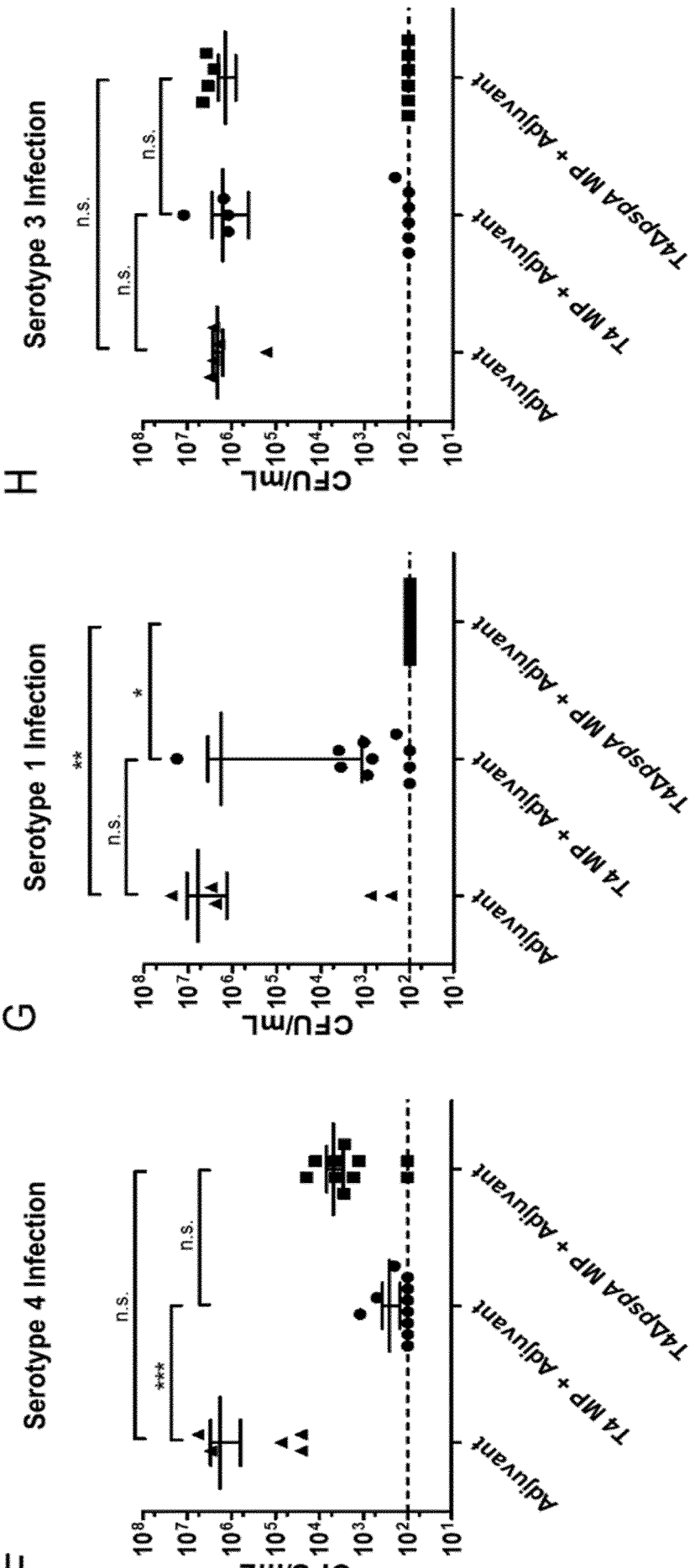

F
Serotype 4 Infection
G
Serotype 1 Infection
H
Serotype 3 Infection
CFU/mL
n.s.
***
**
*
Adjuvant
T4 MP + Adjuvant
T4ΔPspA MP + Adjuvant

Figure 8F-H

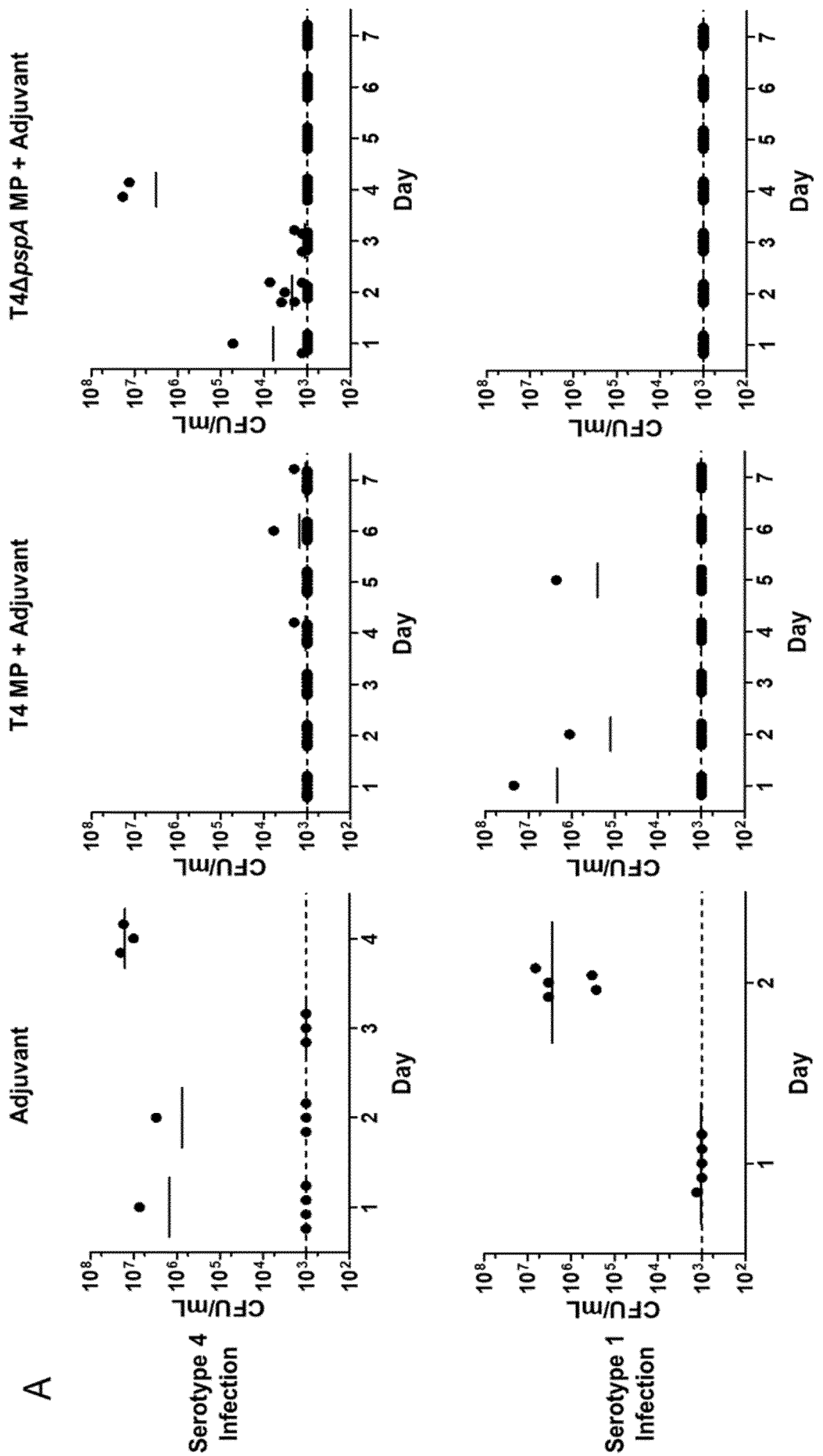

A
Serotype 4 Infection
Serotype 1 Infection
Adjuvant
T4 MP + Adjuvant
T4ΔpspA MP + Adjuvant
CFU/mL
Day

Figure 9A

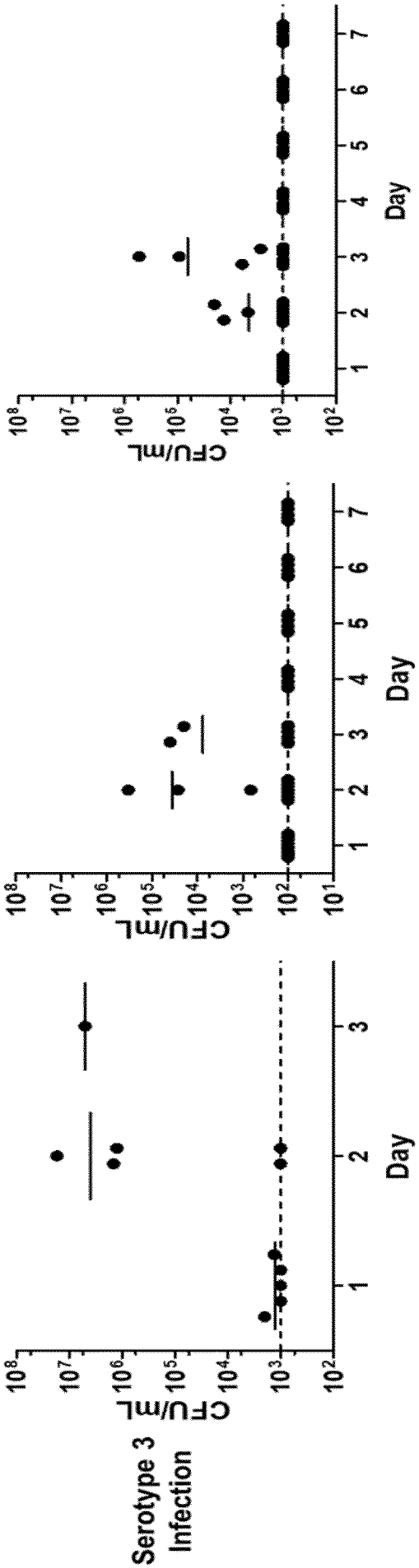


Figure 9A (cont.)

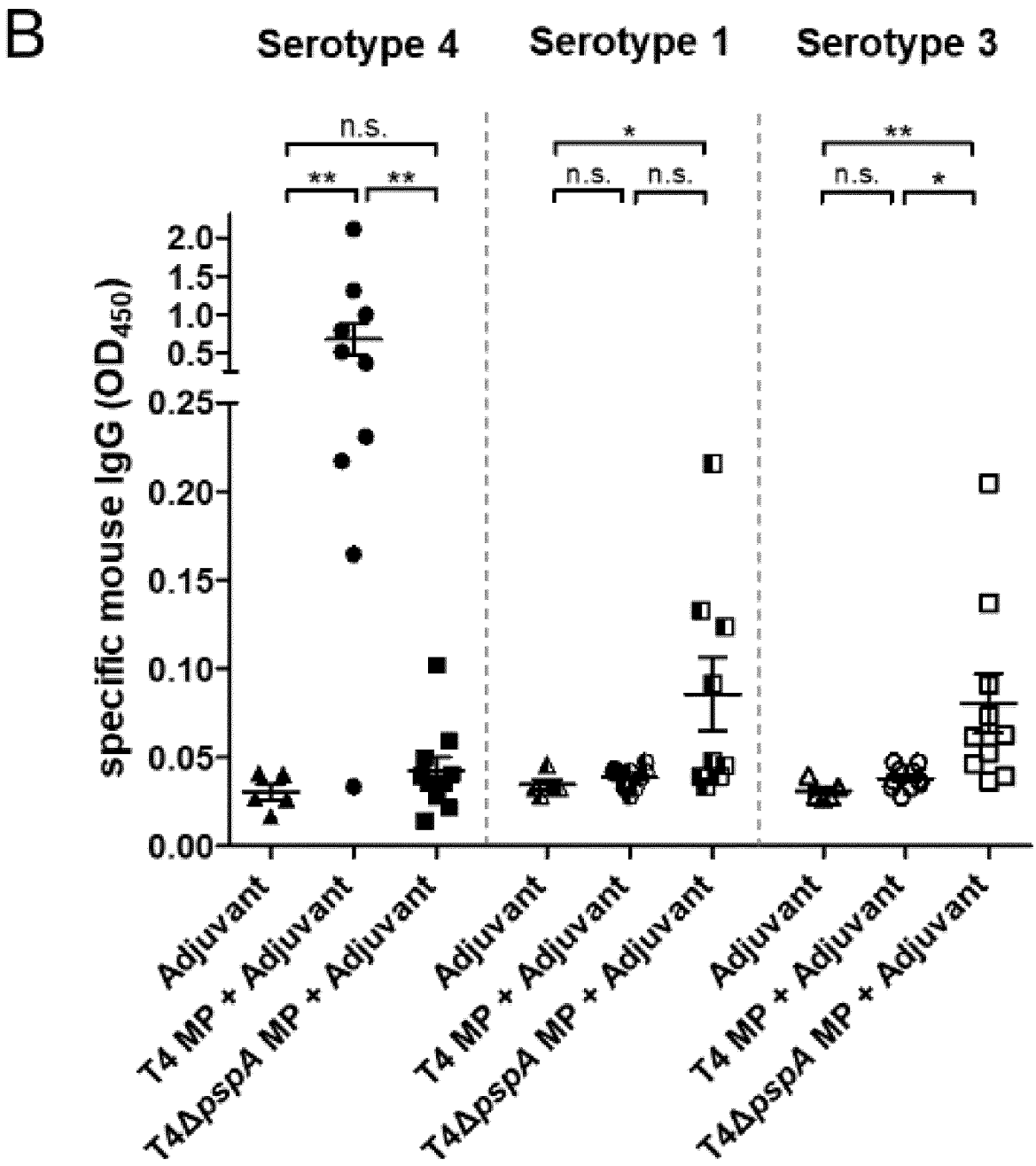

B
Serotype 4
Serotype 1
Serotype 3
n.s.
**
**
*
n.s.
n.s.
**
n.s.
*
specific mouse IgG (OD450)
2.0
1.5
1.0
0.5
0.25
0.20
0.15
0.10
0.05
0.00
Adjuvant
T4 MP + Adjuvant
T4ΔpspA MP + Adjuvant
Adjuvant
T4 MP + Adjuvant
T4ΔpspA MP + Adjuvant
Adjuvant
T4 MP + Adjuvant
T4ΔpspA MP + Adjuvant

Figure 9B

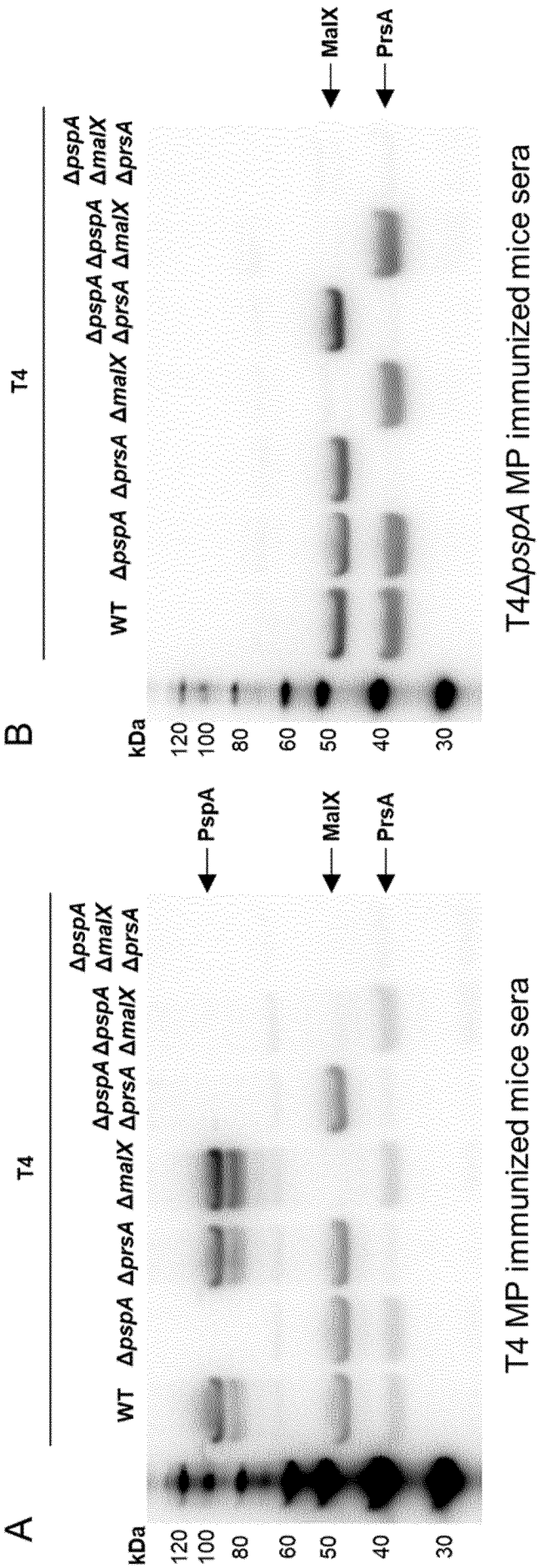

A
T4
WT
ΔpspA
ΔprsA
ΔmalX
ΔpspA ΔprsA
ΔpspA ΔmalX
ΔprsA ΔmalX
ΔpspA ΔmalX ΔprsA
kDa
120
100
80
60
50
40
30
PspA
MalX
PrsA
T4 MP immunized mice sera
B
T4
WT
ΔpspA
ΔprsA
ΔmalX
ΔpspA ΔprsA
ΔpspA ΔmalX
ΔprsA ΔmalX
ΔpspA ΔmalX ΔprsA
kDa
120
100
80
60
50
40
30
MalX
PrsA
T4ΔpspA MP immunized mice sera

Figure 10AB

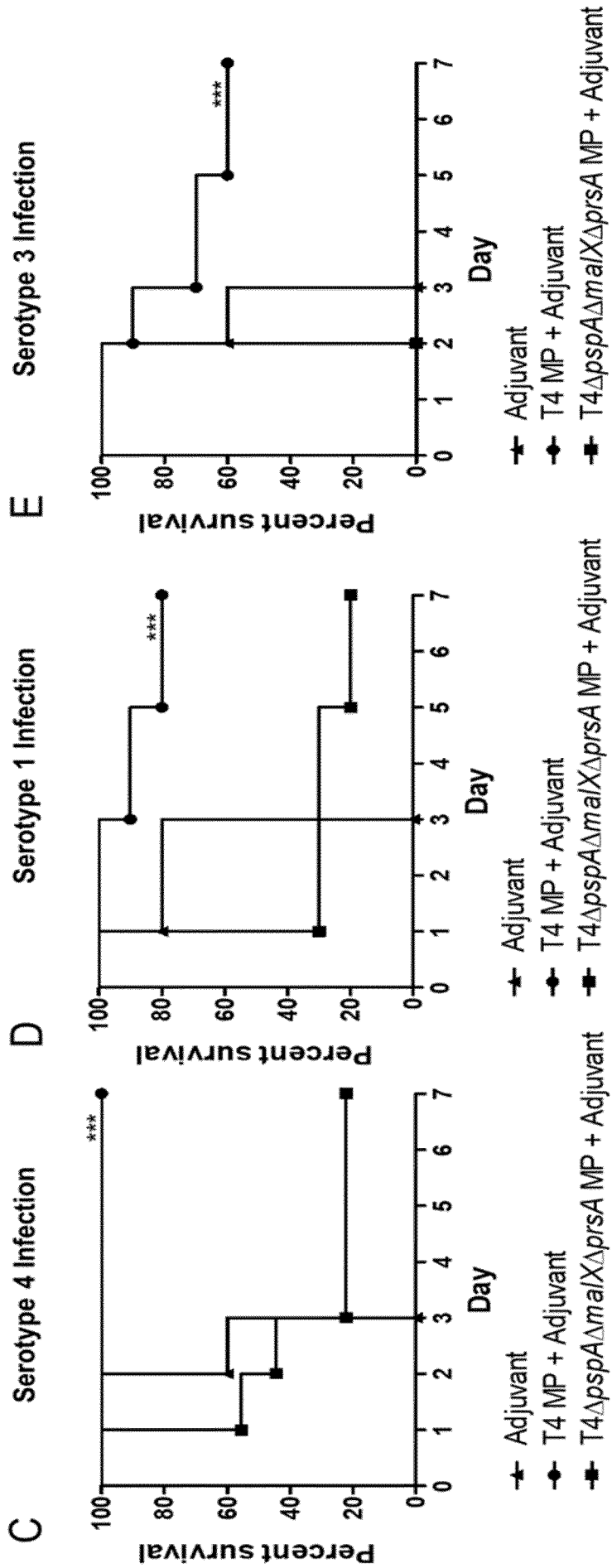

C
Serotype 4 Infection
D
Serotype 1 Infection
E
Serotype 3 Infection
Percent survival
Day
***
Adjuvant
T4 MP + Adjuvant
T4ΔpspAΔmalXΔprsA MP + Adjuvant

Fig 10 C-E

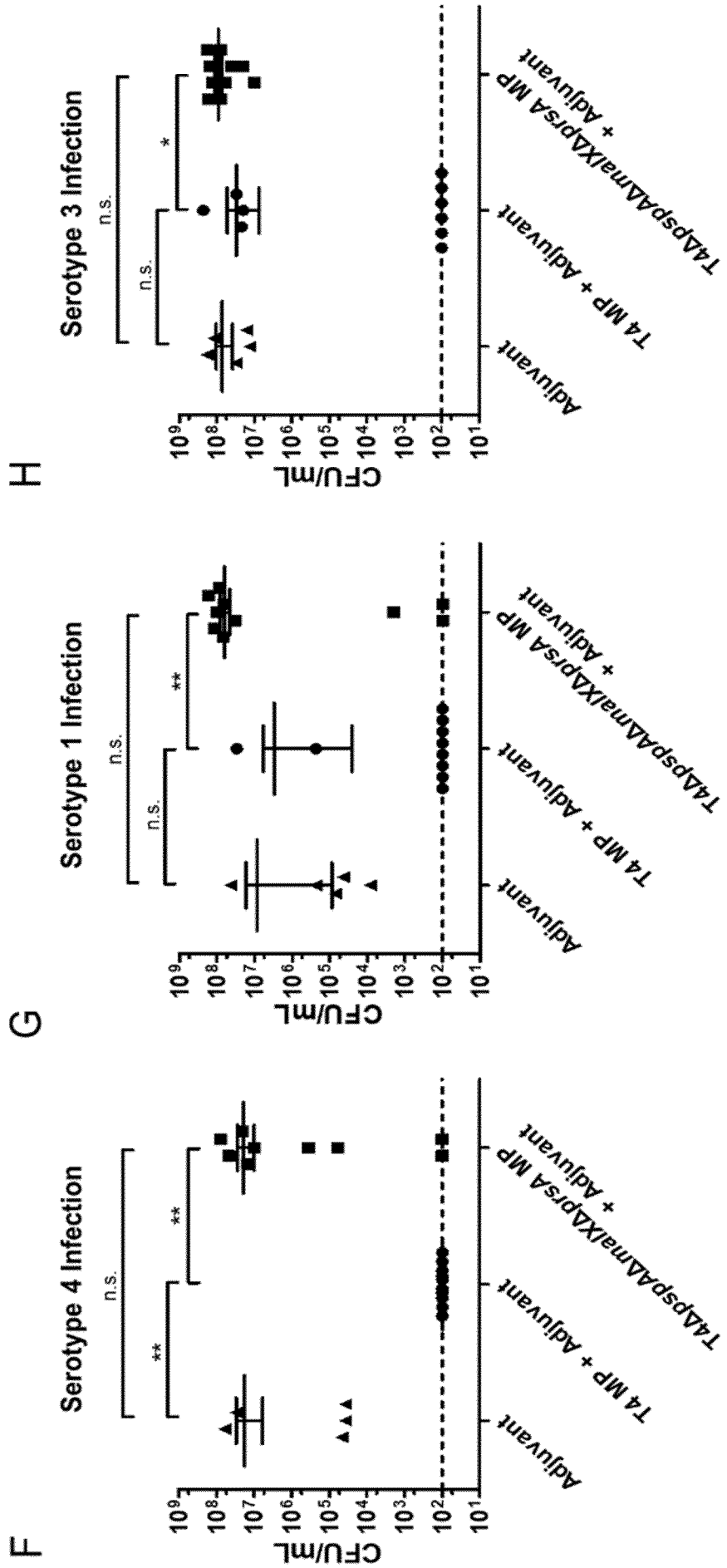

F
Serotype 4 Infection
G
Serotype 1 Infection
H
Serotype 3 Infection
CFU/mL
n.s.
**
*
Adjuvant
T4 MP + Adjuvant
T4ΔPspAΔmalXΔPrsA MP + Adjuvant

Figure 10F-H

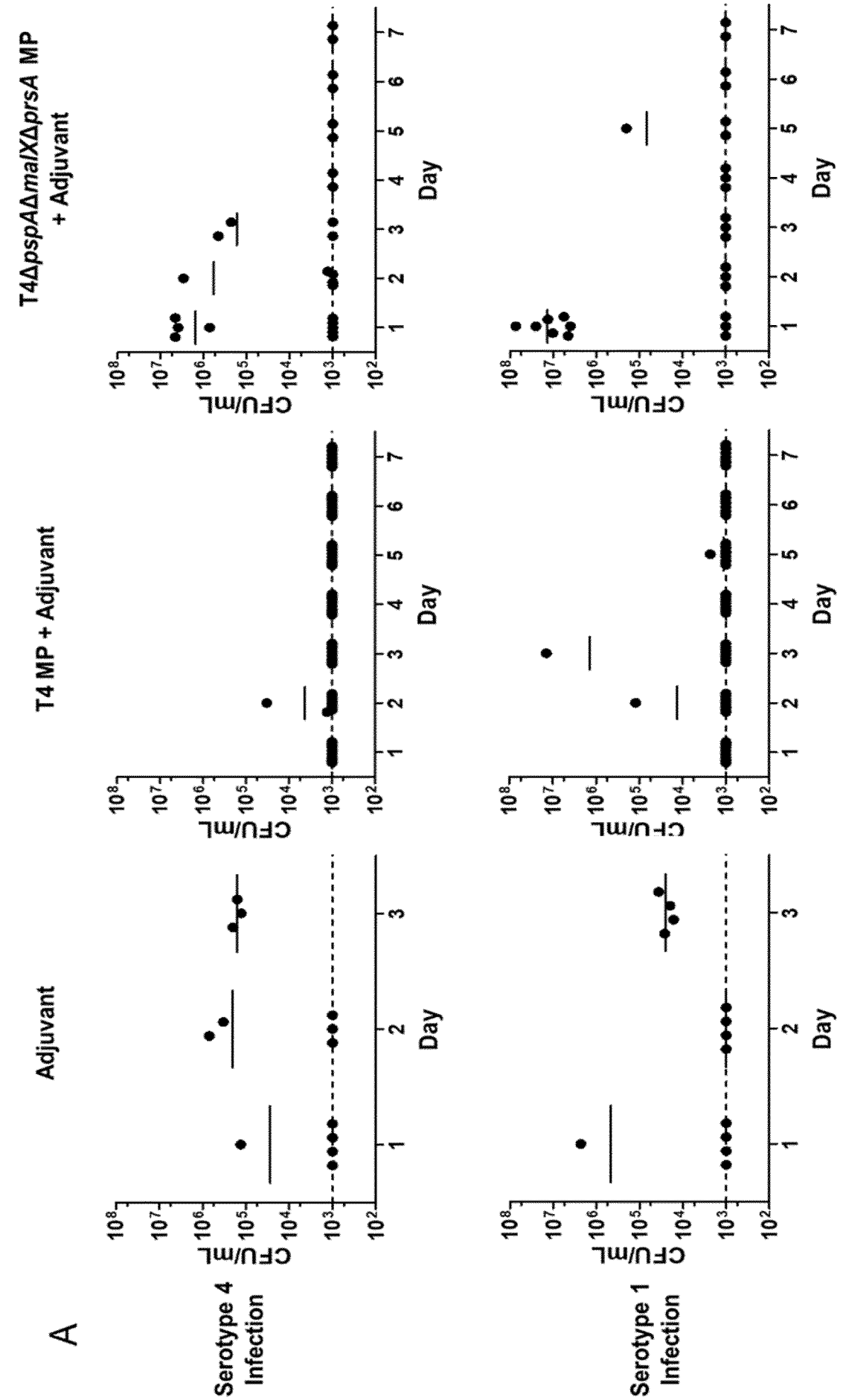

A
Serotype 4 Infection
Serotype 1 Infection
Adjuvant
T4 MP + Adjuvant
T4ΔpspAΔmalXΔprsA MP + Adjuvant
CFU/mL
Day

Fig 11A

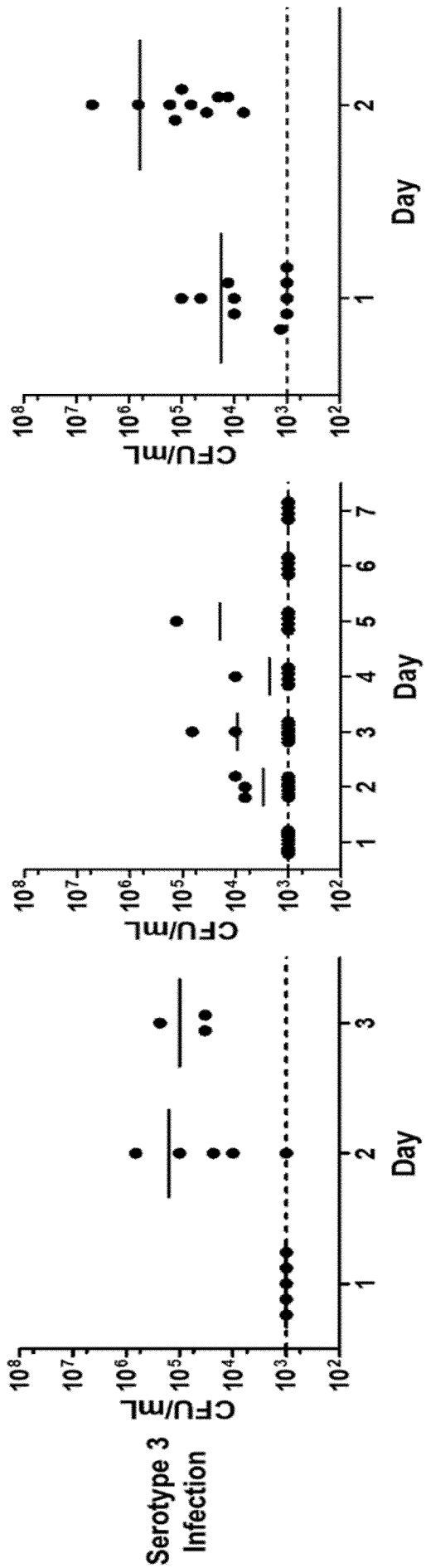


Figure 11A (cont.).

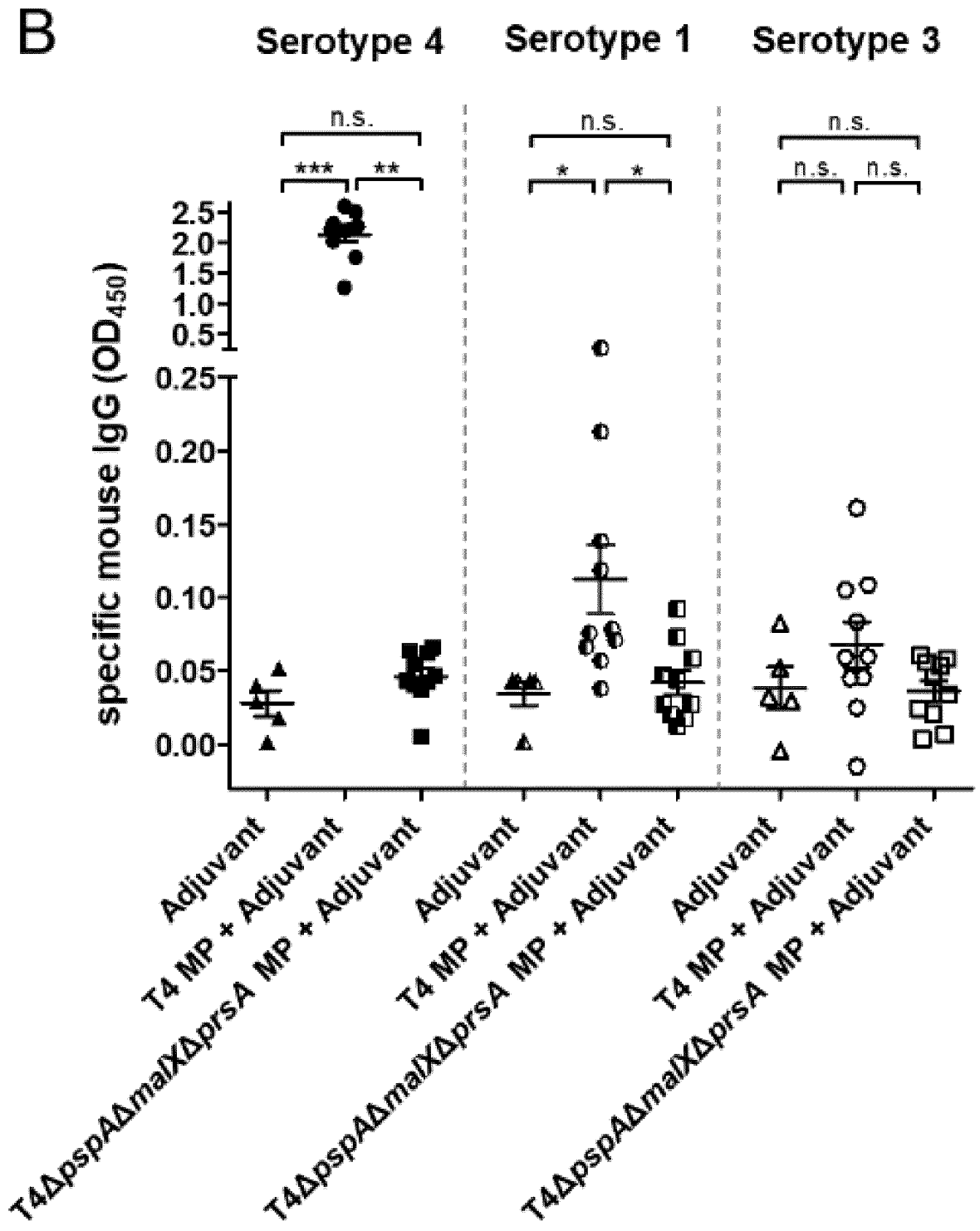

B
Serotype 4
Serotype 1
Serotype 3
n.s.
***
**
*
*
n.s.
n.s.
n.s.
specific mouse IgG (OD450)
2.5
2.0
1.5
1.0
0.5
0.25
0.20
0.15
0.10
0.05
0.00
Adjuvant
T4 MP + Adjuvant
T4ΔpspAΔmalXΔprsA MP + Adjuvant

Figure 11B

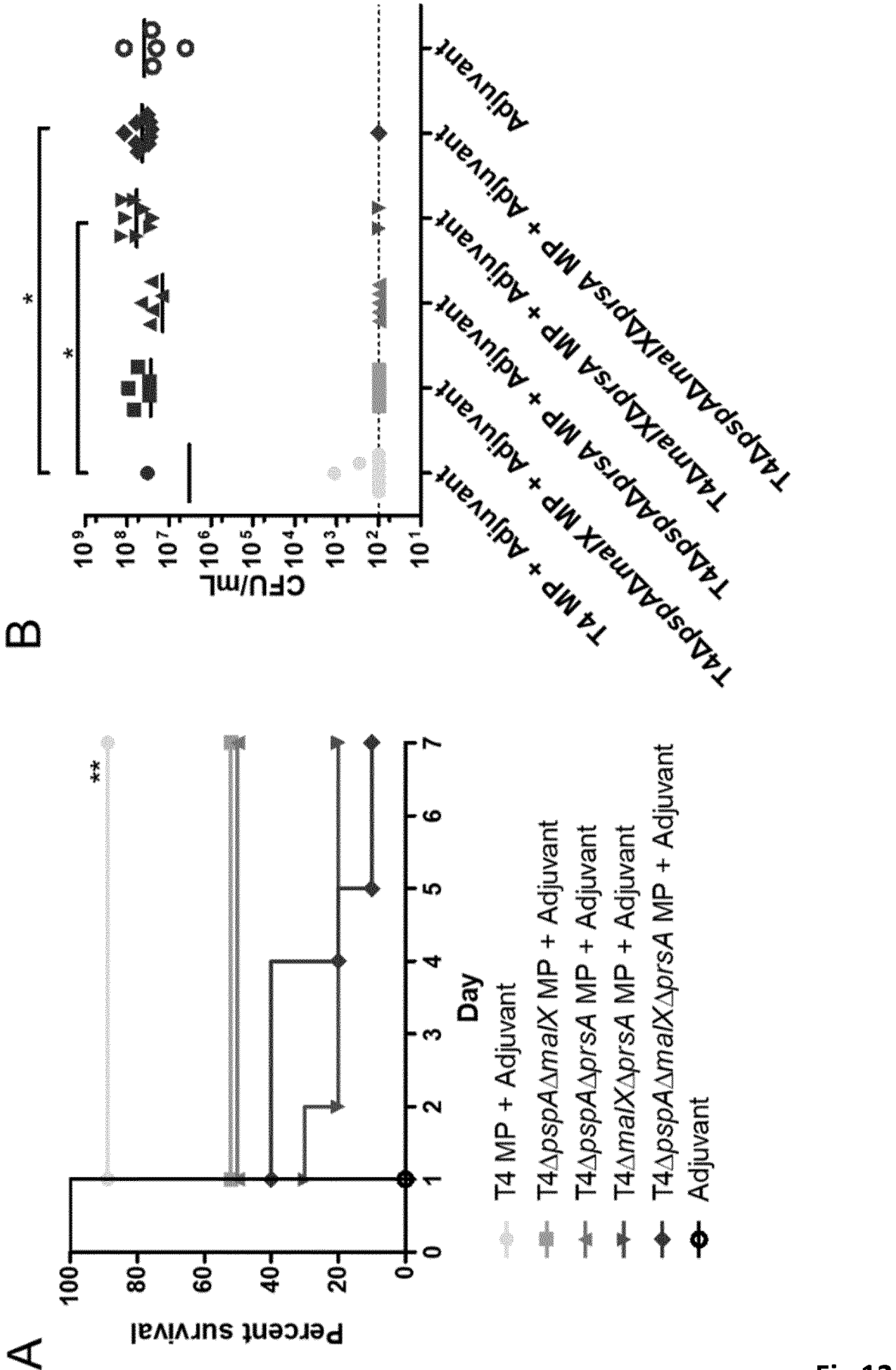

A
Percent survival
Day
T4 MP + Adjuvant
T4ΔpspAΔmalX MP + Adjuvant
T4ΔpspAΔprsA MP + Adjuvant
T4ΔmalXΔprsA MP + Adjuvant
T4ΔpspAΔmalXΔprsA MP + Adjuvant
Adjuvant
**
B
CFU/mL
*
*
T4 MP + Adjuvant
T4ΔpspAΔmalX MP + Adjuvant
T4ΔpspAΔprsA MP + Adjuvant
T4ΔmalXΔprsA MP + Adjuvant
T4ΔpspAΔmalXΔprsA MP + Adjuvant
Adjuvant

Fig 12

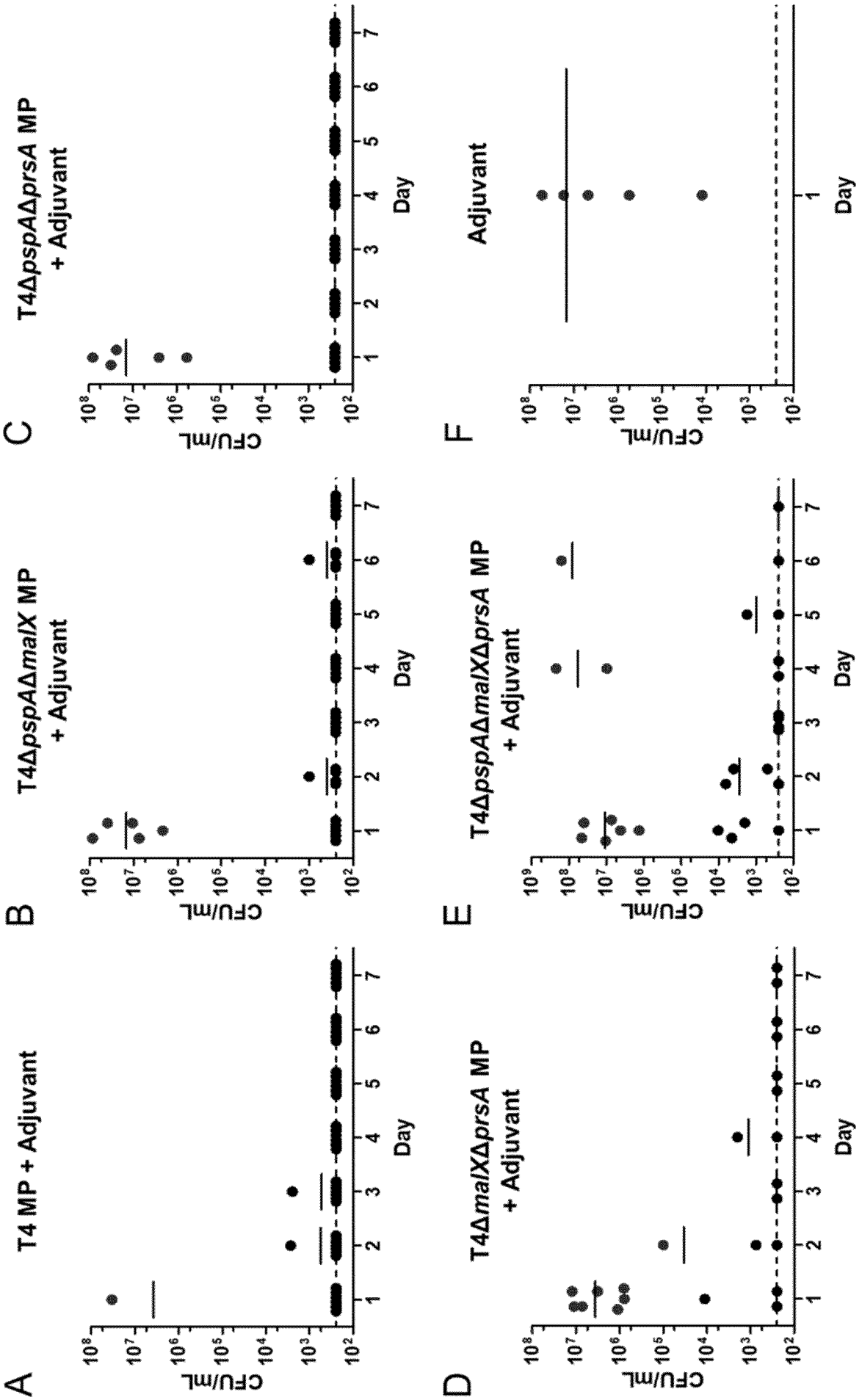

A
T4 MP + Adjuvant
B
T4ΔpspAΔmalX MP
+ Adjuvant
C
T4ΔpspAΔprsA MP
+ Adjuvant
D
T4ΔmalXΔprsA MP
+ Adjuvant
E
T4ΔpspAΔmalXΔprsA MP
+ Adjuvant
F
Adjuvant
CFU/mL
Day

Fig 13

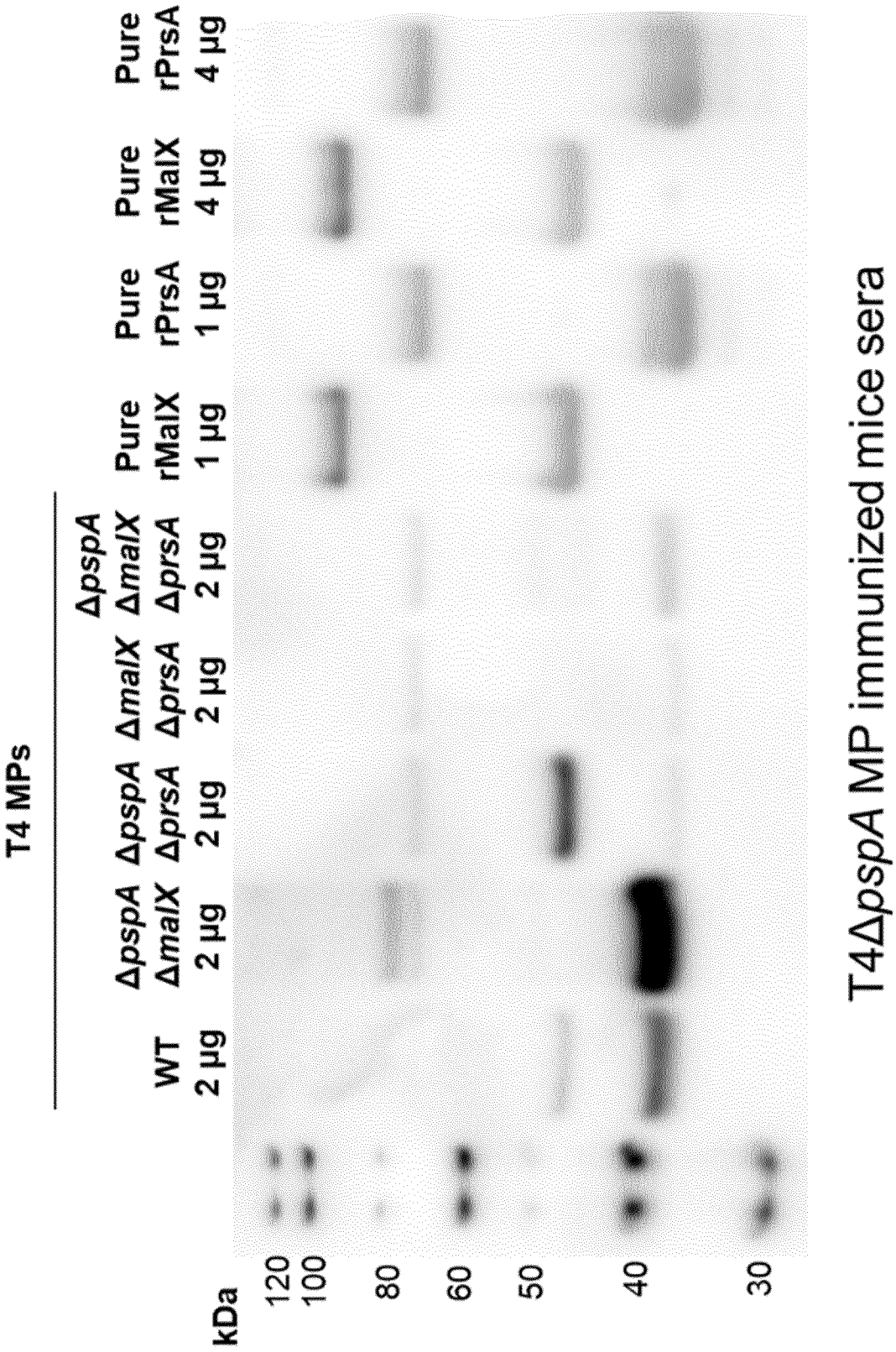

T4 MPs
WT 2 µg
ΔpspA ΔmalX 2 µg
ΔpspA ΔprsA 2 µg
ΔmalX ΔprsA 2 µg
ΔpspA ΔmalX ΔprsA 2 µg
Pure rMalX 1 µg
Pure rPrsA 1 µg
Pure rMalX 4 µg
Pure rPrsA 4 µg
kDa
120
100
80
60
50
40
30
T4ΔpspA MP immunized mice sera

Figure 14

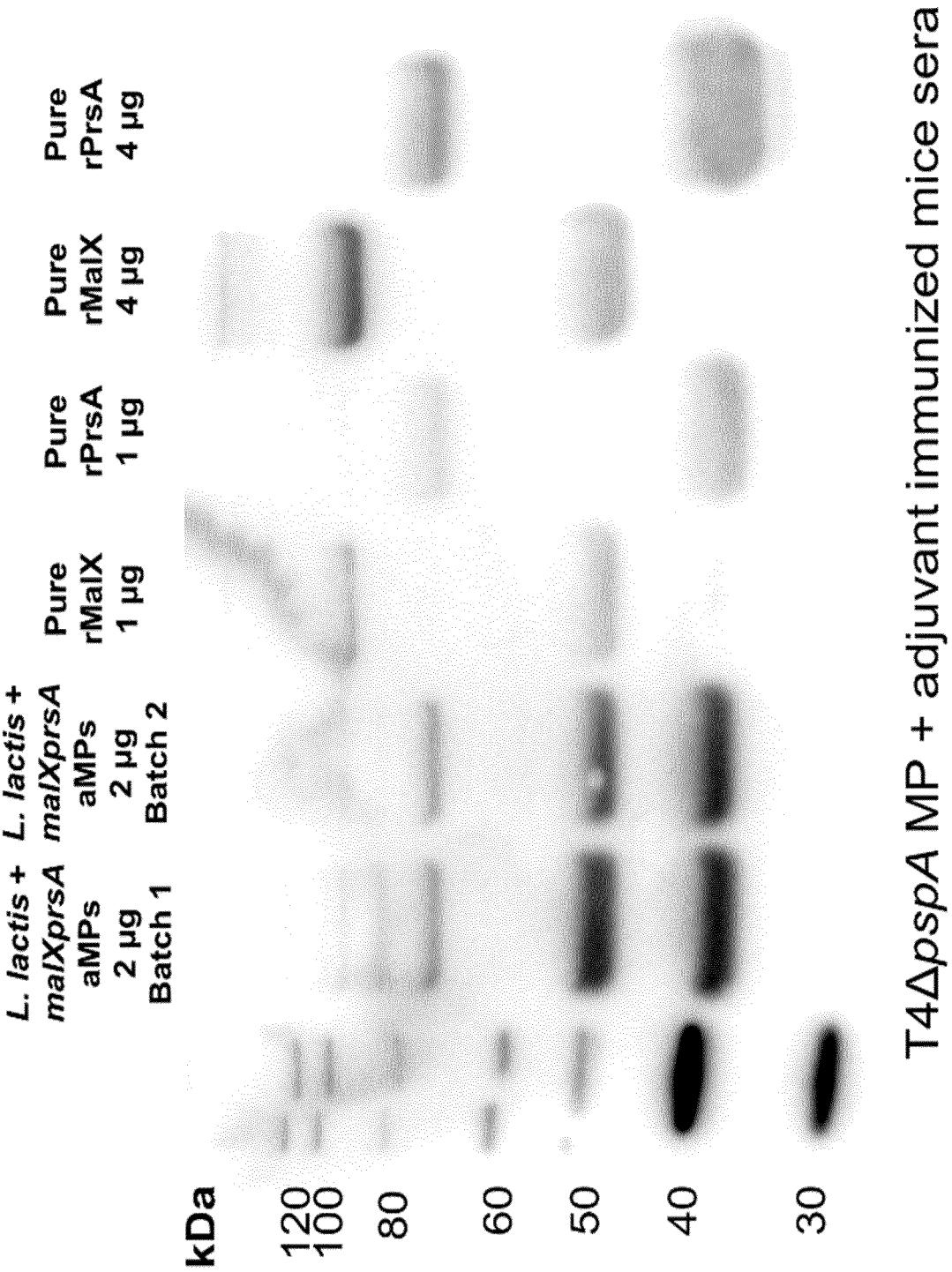

T4ΔpspA MP + adjuvant immunized mice sera
kDa
120
100
80
60
50
40
30
L. lactis + malXprsA aMPs 2 µg Batch 1
L. lactis + malXprsA aMPs 2 µg Batch 2
Pure rMalX 1 µg
Pure rPrsA 1 µg
Pure rMalX 4 µg
Pure rPrsA 4 µg

Figure 15

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/069352

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/09 A61P31/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHOI CHI-WON ET AL: "Potential Usefulness of Streptococcus pneumoniae Extracellular Membrane Vesicles as Antibacterial Vaccines", JOURNAL OF IMMUNOLOGY RESEARCH, vol. 2017, 22 January 2017 (2017-01-22), pages 1-8, XP055856180, US ISSN: 2314-8861, DOI: 10.1155/2017/7931982 Retrieved from the Internet: URL:http://downloads.hindawi.com/journals/jir/2017/7931982.xml> 3.3; page 5, right-hand column; figure 3 ----- -/--	1,3,7-43

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 2021	11/11/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Renggli-Zulliger, N

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANNIE FRELET-BARRAND ET AL: "Lactococcus lactis, an Alternative System for Functional Expression of Peripheral and Intrinsic Arabidopsis Membrane Proteins", PLOS ONE, vol. 5, no. 1, 1 January 2010 (2010-01-01) , pages e8746-e8746, XP055083269, ISSN: 1932-6203, DOI: 10.1371/journal.pone.0008746 page 12, left-hand column ----- MURALINATH M ET AL: "Immunization with Salmonella enterica serovar Typhimurium-derived outer membrane vesicles delivering the pneumococcal protein PspA confers protection against challenge with Streptococcus pneumoniae", INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 79, no. 2, 1 February 2011 (2011-02-01), pages 887-894, XP002715780, ISSN: 0019-9567, DOI: 10.1128/IAI.00950-10 [retrieved on 2010-11-29] cited in the application abstract ----- WO 2018/124959 A2 (HENRIQUES NORMARK BIRGITTA [SE]) 5 July 2018 (2018-07-05) cited in the application the whole document ----- CODEMO MARIO ET AL: "Immunomodulatory Effects of Pneumococcal Extracellular Vesicles on Cellular and Humoral Host Defenses", MBIO, vol. 9, no. 2, 10 April 2018 (2018-04-10), pages 1-15, XP055856186, DOI: 10.1128/mBio.00559 Retrieved from the Internet: URL:https://journals.asm.org/doi/pdf/10.1128/mBio.00559-18> cited in the application abstract page 10, paragraph 1 -----	1,2,4-46 1,2,4, 7-46 1,3, 10-43 1-46

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2021/069352

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2018124959 A2	05-07-2018	EP 3562838 A2	06-11-2019
		US 2019328861 A1	31-10-2019
		WO 2018124959 A2	05-07-2018