Supplementary material for

Membrane particles evoke a serotype-independent cross-protection against pneumococcal infection that is dependent on the conserved lipoproteins MalX and PrsA

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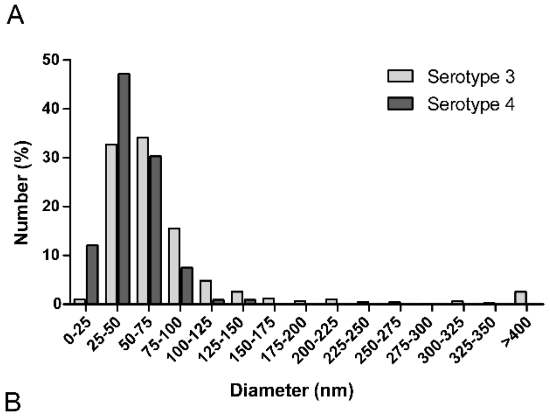
Supplementary Figures

Supplementary Figure S1. Membrane particles (MPs) 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 MPs 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 (see also Supplementary Table S1).

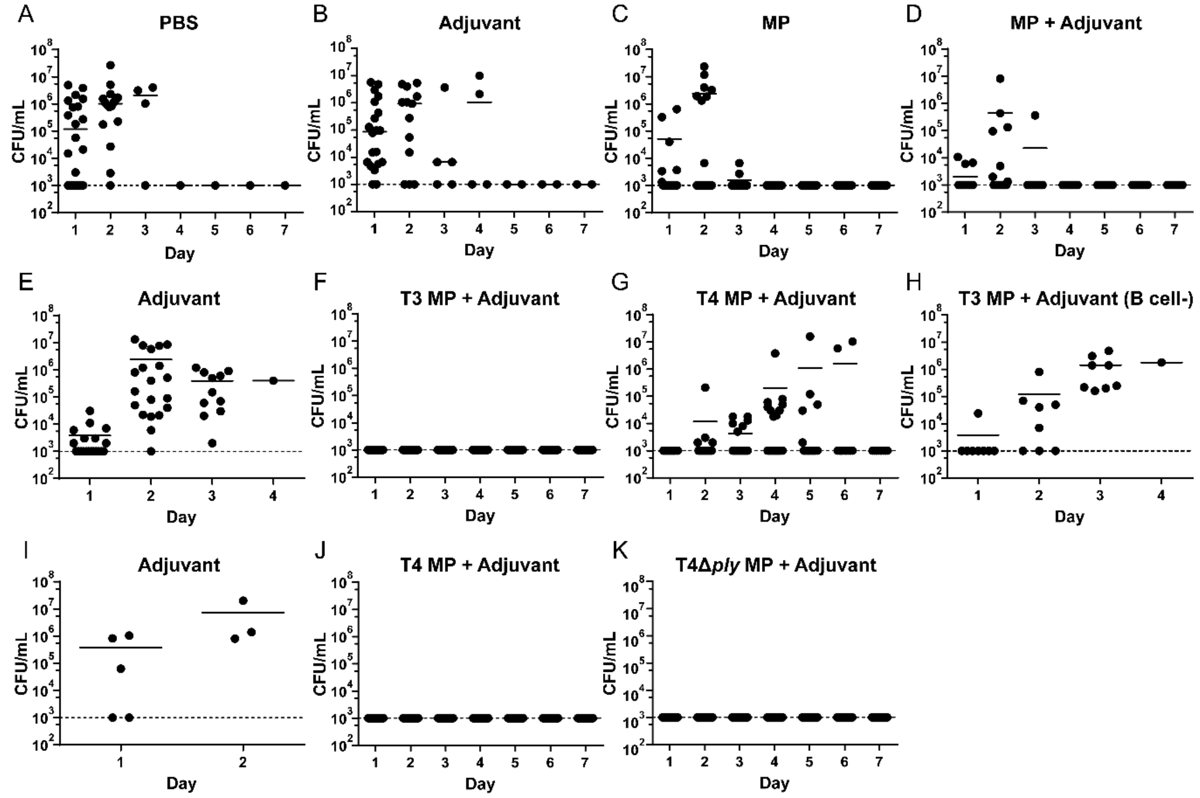


B

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

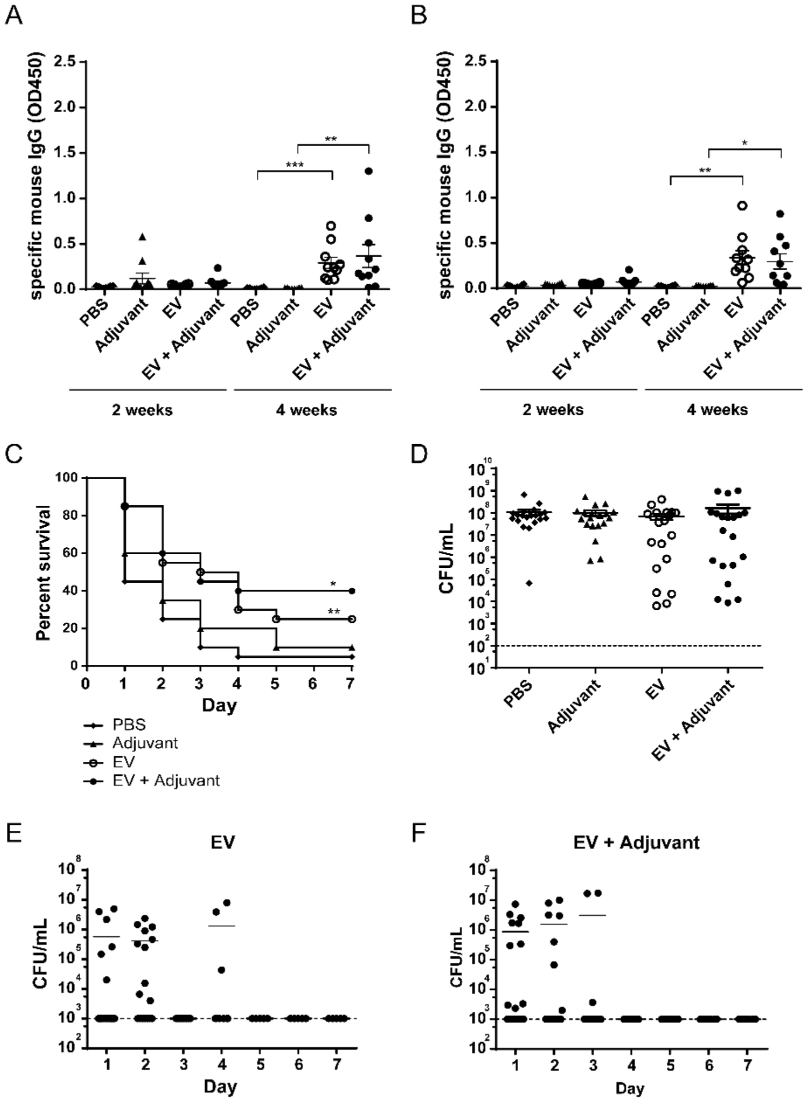
Supplementary Figure S2. Blood counts after intranasal immunization of mice with MPs and intranasal pneumococcal challenge in a 7-day survival model

(A-D) Intranasal immunization of C57BL/6 mice with MPs from strain T4 increases survival after intranasal challenge with strain BHN733 of serotype 1. Bacterial counts (CFU) in the blood of mice immunized with **(A)** PBS, **(B)** Adjuvant only, **(C)** MPs from T4, or **(D)** T4 MPs + adjuvant, and then infected with strain BHN733 of serotype 1, corresponding to the survival experiment in Figure 1A. 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, corresponding to the survival experiment in Figure 1C. **(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, corresponding to survival experiment in Figure 1E. Each dot represents one mouse.



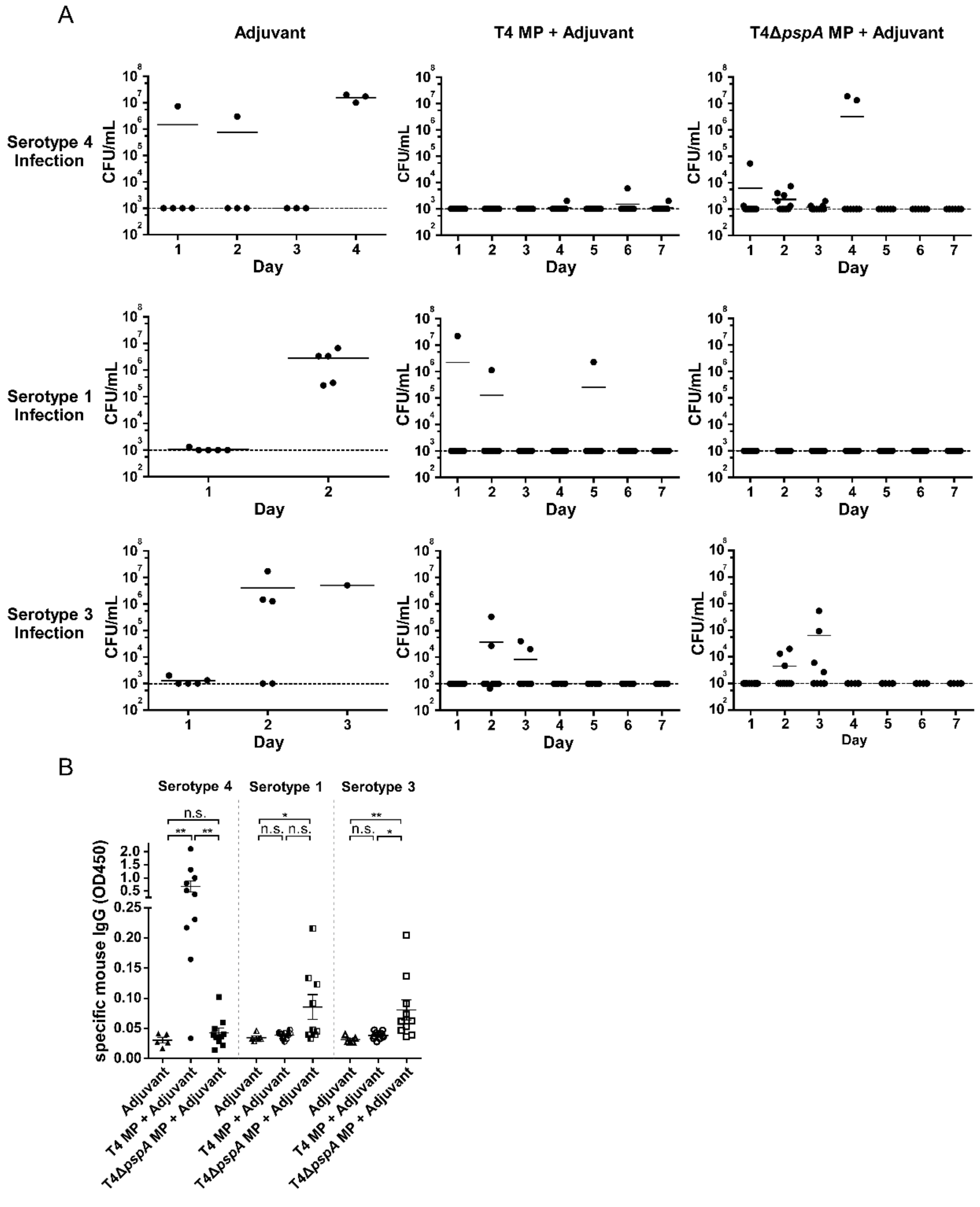
Supplementary Figure S3. Immunization with EVs, as compared to MPs, from T4 confers lower cross-protection against intranasal infection with serotype 1

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. This experiment was conducted in parallel with experiments presented in Fig. 1.


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 weeks
4 weeks
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
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
1 2 3 4 5 6 7
Day

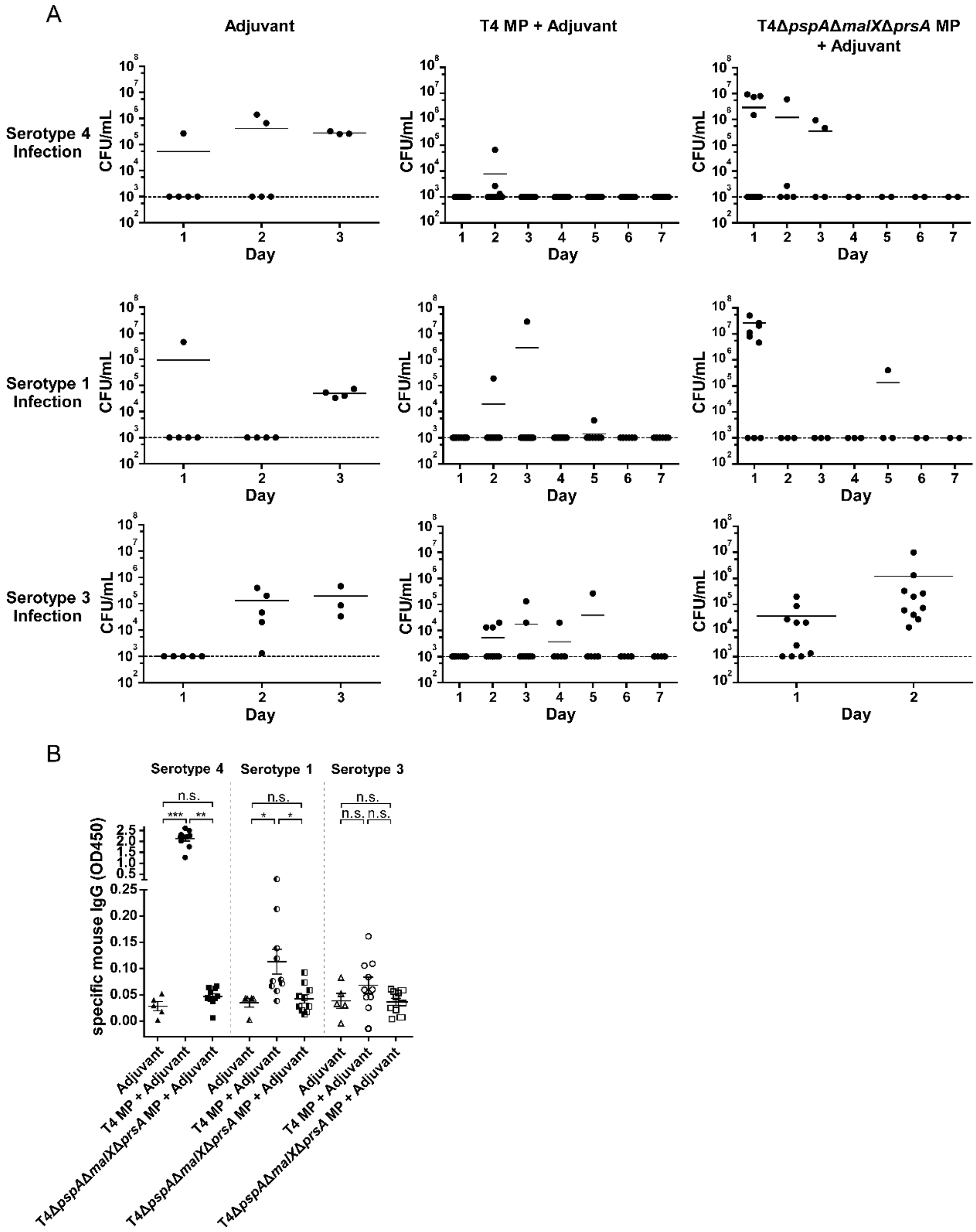
Supplementary Figure S4. Blood counts and IgG levels after intranasal immunization with MPs from T4Δ*pspA* and intranasal challenge with strains of serotype 1 and serotype 3

Intranasal immunization with MPs 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, as shown in Figure 3C-E. **(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.


A
Adjuvant
T4 MP + Adjuvant
T4Δ*pspA* MP + Adjuvant
Serotype 4 Infection
Serotype 1 Infection
Serotype 3 Infection
CFU/mL
10^2
10^3
10^4
10^5
10^6
10^7
10^8
Day
1
2
3
4
5
6
7
B
Serotype 4
Serotype 1
Serotype 3
n.s.
**
*
specific mouse IgG (OD450)
0.00
0.05
0.10
0.15
0.20
0.25
0.5
1.0
1.5
2.0
Adjuvant
T4 MP + Adjuvant
T4Δ*pspA* MP + Adjuvant

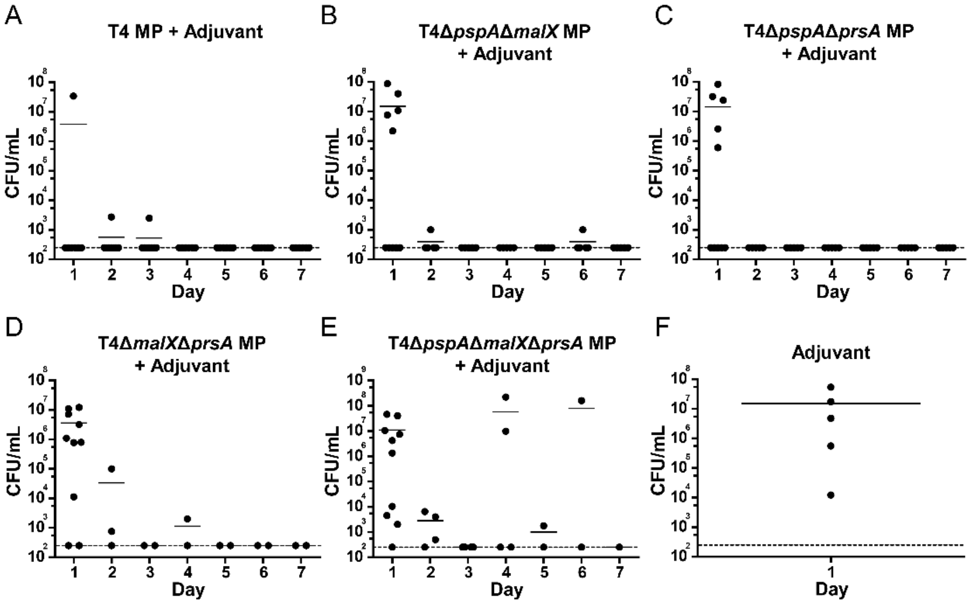
Supplementary Figure S5. Blood counts and IgG levels after intranasal immunization with MPs from T4Δ*pspA*Δ*malX*Δ*prsA* and intranasal challenge with the pneumococcal strains T4, serotype 1 or serotype 3

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.


A
Adjuvant
T4 MP + Adjuvant
T4ΔpspAΔmalXΔprsA MP + Adjuvant
Serotype 4 Infection
Serotype 1 Infection
Serotype 3 Infection
CFU/mL
10^2
10^3
10^4
10^5
10^6
10^7
10^8
Day
1
2
3
4
5
6
7
B
Serotype 4
Serotype 1
Serotype 3
n.s.
***
**
*
specific mouse IgG (OD450)
0.00
0.05
0.10
0.15
0.20
0.25
0.5
1.0
1.5
2.0
2.5
Adjuvant
T4 MP + Adjuvant
T4ΔpspAΔmalXΔprsA MP + Adjuvant

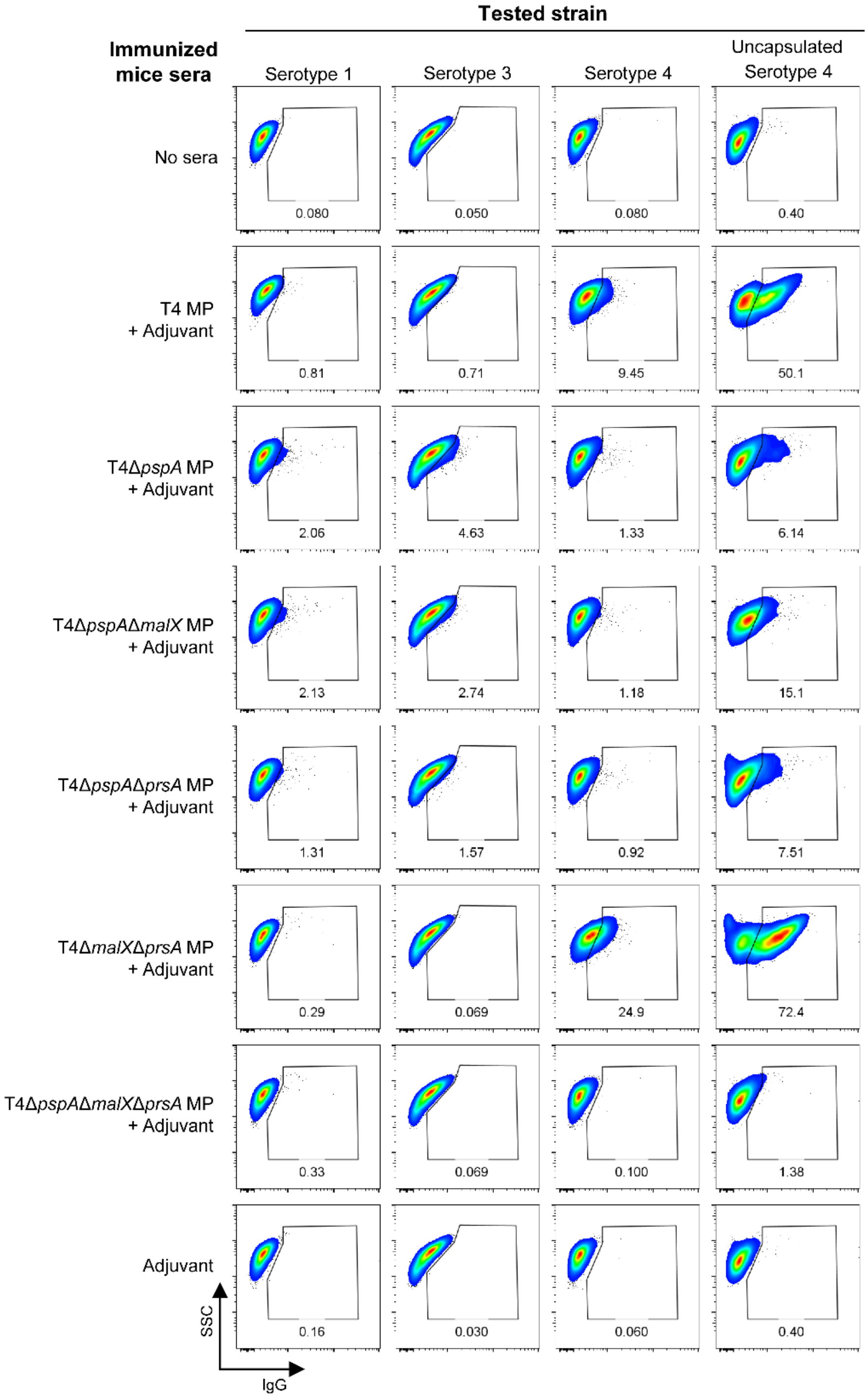
Supplementary Figure S6. Blood counts showing that presence of both MalX and PrsA in MPs are required for protection against invasive pneumococcal disease by serotype 1

(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.

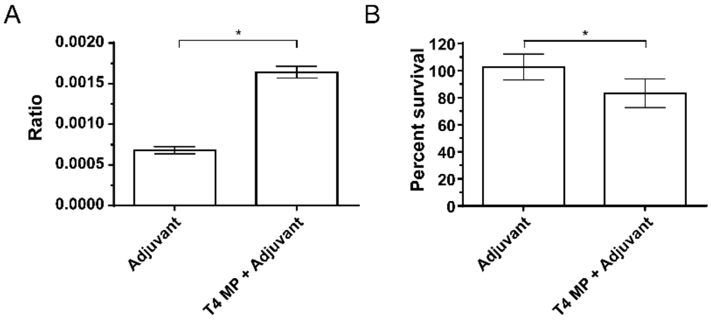


Supplementary Figure S7. Density-colored dot plots of the flow cytometry data displayed in Figure 5C.

Bacterial cells of *S. pneumoniae* serotypes 1, 3, 4 and uncapsulated serotype 4 (T4R), were incubated with sera from mice immunized with MPs harvested from the indicated pneumococcal strains. Subsequently, the bacteria were stained with antibody Alexa Fluor® 488 goat anti-mouse IgG (H+L) and analyzed by flow cytometry to determine the percentage of bacteria bound to mice IgGs, indicated below the gate windows and plotted in Figure 5C. Side scattering (SSC) versus fluorescence (Bound IgG) plots are displayed and the gating was determined based on the cells distribution in the control samples where the bacteria were not incubated with sera.


Tested strain
Immunized mice sera
Serotype 1
Serotype 3
Serotype 4
Uncapsulated Serotype 4
No sera
0.080
0.050
0.080
0.40
T4 MP + Adjuvant
0.81
0.71
9.45
50.1
T4Δ*pspA* MP + Adjuvant
2.06
4.63
1.33
6.14
T4Δ*pspA*Δ*malX* MP + Adjuvant
2.13
2.74
1.18
15.1
T4Δ*pspA*Δ*prsA* MP + Adjuvant
1.31
1.57
0.92
7.51
T4Δ*malX*Δ*prsA* MP + Adjuvant
0.29
0.069
24.9
72.4
T4Δ*pspA*Δ*malX*Δ*prsA* MP + Adjuvant
0.33
0.069
0.100
1.38
Adjuvant
0.16
0.030
0.060
0.40
SSC
IgG

Supplementary Figure S8. Opsonophagocytic activity of cross-reactive antibodies. RAW mouse macrophages were incubated with serotype 1 pneumococci pre-treated with pooled sera from mice immunized with Adjuvant or T4 MP+adjuvant. **A)** Bacterial adhesion to the cells was measured. **(B)** The effect on phagocytic killing by adding the sera in (A) was estimated by calculating bacterial survival inside the cells. Data are represented as means +/- SEM of three independent experiments. * = p<0.05.



Supplementary Tables

Supplementary Table S1. Proteins identified with LC-MS/MS after immunoprecipitation with immune sera from mice immunized with MPs from T4

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

Supplementary Table S2. Proteins identified with LC-MS/MS after immunoprecipitation with immune sera from mice immunized with MPs from T4Δ*pspA*

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

Supplementary Table S3. Proteins identified with LC-MS/MS after immunoprecipitation with sera from mice challenged with adjuvant only

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

Supplementary Table S4. *S. pneumoniae* strains used in this 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
T4Δ*pspA*::*ermB*	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*prsA*::*tetM*Δ*malX*::*kanR*	BHN2044	*prsA* (*SP0981*) Orf replaced with a *tetM* Orf; *malX* (*SP2108*) Orf replaced with a *kanR* 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

Supplementary Table S5. Primers used in this study

Primer	**Sequence**[1]	**Comment**
pspA-UpFr-800	ggagtttgtcgttgaaattac	Anneals ~800bp upstream of *pspA* Start codon
pspA-UpFr-700	gtatttagagattttcaaagtg	Anneals ~700bp upstream of *pspA* Start codon
pspA-UpRe-ErmCass-OH	TGCAAGTCACACGAACACGAActaaatttacctcttttctgatag	Anneals directly upstream of *pspA* start codon with an overhang seq. complementary to the *ermB* cassette
pspA-DoFr-ErmCass-OH	CTATTATTTAACGGGAGGAAATAAgccgattaaattaaatcatg	Anneals directly downstream of *pspA* stop codon with an overhang seq. complementary to the *ermB* cassette
pspA-DoRe-700bp	acgtccggatttggcgtgc	Anneals ~700bp downstream of *pspA* Stop codon
pspA-DoRe-800bp	ccactcgagcataatgcc	Anneals ~800bp downstream of *pspA* Stop codon
Erm-cass-frw	ttcgtgttcgtgtgacttgca	
Erm-cass-rev	ttatttcctcccgttaaataatag	
prsA-UpFr-800	aggattggcataaaatggttg	Anneals ~800bp upstream of *prsA* Start codon
prsA-UpFr-700	ggtttcttgaaagaattgga	Anneals ~700bp upstream of *prsA* Start codon
prsA-UpRe-TetOH	TAATTTTCATGTGATTTTCCTCCATgtctactcctttgagataagtg	Anneals directly upstream of *prsA* start codon with an overhang seq. complementary to the beginning of the *tetM* Orf
prsA-DoFr-TetOH	ATATATGTTCAATAAAATAACTTAGtccaaatcaatgagtcaggga	Anneals directly upstream of *prsA* stop codon with an overhang seq. complementary to the end of the *tetR* Orf
prsA-DoRe-800	caagttttatctacaacctca	Anneals ~800bp downstream of *prsA* Stop codon
prsA-DoRe-700	aacgcatcatatcaggtgtac	Anneals ~700bp downstream of *prsA* Stop codon
TetOrf-frw	atggaggaaaatcacatgaaaatta	
TetOrf-rev	ctaagttattttattgaacatatat	
malX-UpFr-800	gcttccaacaaaccttgctc	Anneals ~800bp upstream of *malX* Start codon
malX-UpFr-700	gctcctaatggaaggatttg	Anneals ~700bp upstream of *malX* Start codon
malX-UpRe-KanOH	ctattctttaggaggaatacactATGGCTAAAATGAGAATATCA	Anneals directly upstream of *malX* start codon with an overhang seq. complementary to the beginning of the kanR Orf
malX-DoFr-KanOH	acccccccttgaacaaatttttCTAAAACAATTCATCCAGTAAA	Anneals directly upstream of *malX* stop codon with an overhang seq. complementary to the end of the *kanR* Orf
malX-DoRe-800	gaaaataacaccaaagatacgt	Anneals ~800bp downstream of *malX* Stop codon
malX-DoRe-700	agcccaaatgatagtccaag	Anneals ~700bp downstream of *malX* Stop codon
KanOrf-frw	atggctaaaatgagaatatca	
KanOrf-rev	ctaaaacaattcatccagtaaa	

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