Supplementary Figures 1 to 6

Intranasal vaccination with probiotic *Escherichia coli* membrane vesicles displaying pneumococcal capsular polysaccharides elicits mucosal immunity

Ryoma Nakao[1]*, Soichiro Kimura[2,3], Kimihiro Abe[1,4], Takehiro Yamaguchi[1], Eisuke Kuroda[5,6,7], Shigeto Hamaguchi[5,7], Kazuhiro Tateda[3], Yukino Ohki[8], Naoki Narisawa[8], Makoto Ohnishi[1], Yukihiro Akeda[1]

[1] Department of Bacteriology I, National Institute of Infectious Diseases, Japan Institute for Health Security (JIHS)；1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

[2] Division of Infection Prevention and Control, Faculty of Pharmaceutical Sciences, Shonan University of Medical Sciences；16–10 Kamishinano, Totsuka-ku, Yokohama, Kanagawa 244-0806, Japan

[3] Department of Microbiology and Infectious Diseases, Faculty of Medicine, Toho University School of Medicine；6-11-1 Omorinishi, Ota-ku, Tokyo 143-8540, Japan

[4] Research Center for Vaccine Development, National Institute of Infectious Diseases, Japan Institute for Health Security (JIHS)；1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

[5] Department of Transformative Analysis for Human Specimen, Osaka University Graduate School of Medicine；2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

[6] Division of Fostering Required Medical Human Resources, Center for Infectious Disease Education and Research (CiDER), Osaka University；2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

[7] Division of Infection Control and Prevention, Osaka University Hospital；2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

[8] Department of Food Bioscience and Biotechnology, College of Bioresource Science, Nihon University; 1866, Kameino, Fujisawa, Kanagawa 252-0880, Japan

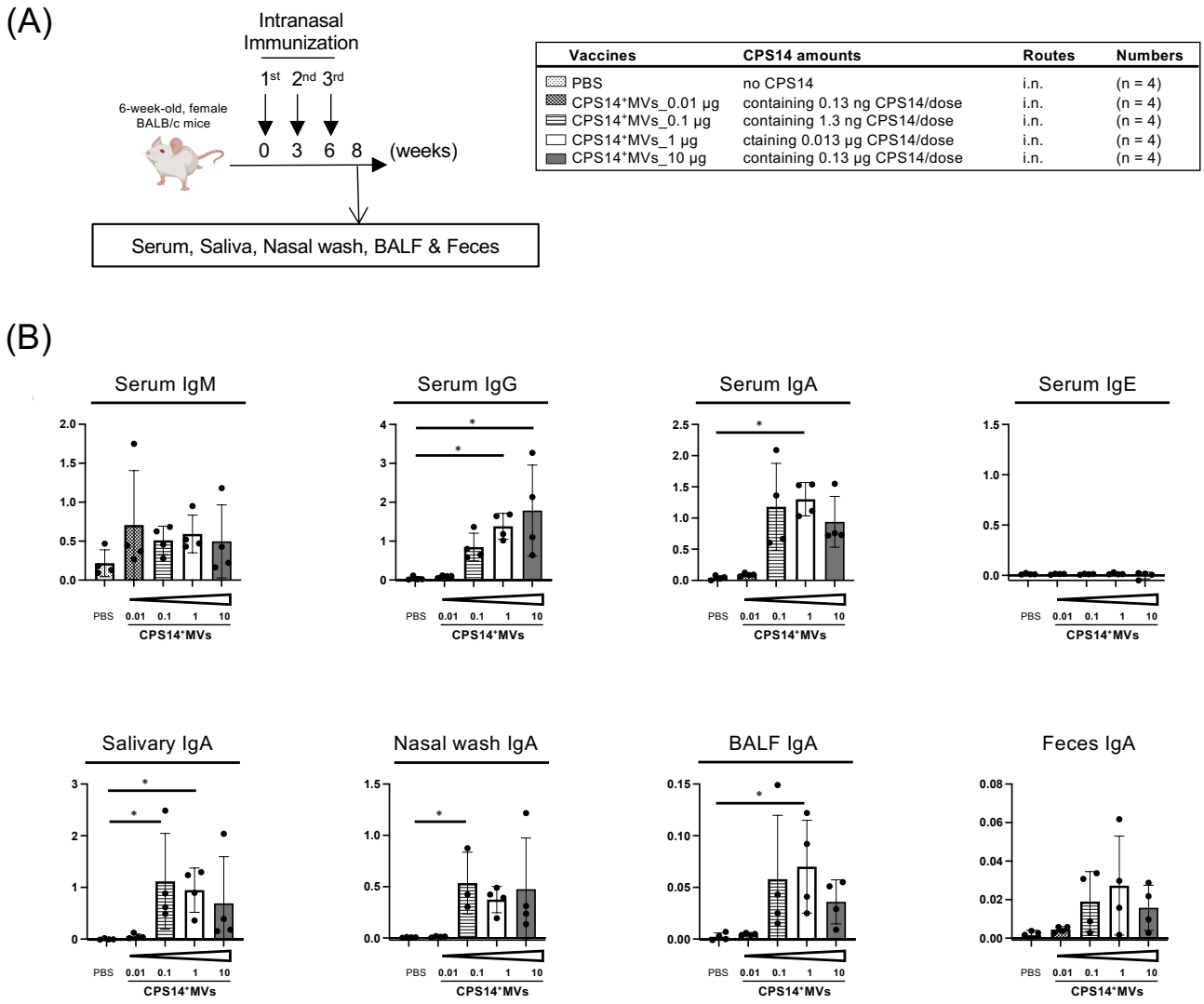
*** Corresponding author:** Ryoma Nakao

E-mail: nakao.ry@jihs.go.jp

ORCID number: 0000-0002-4802-8518

Affiliation: Department of Bacteriology I, National Institute of Infectious Diseases, Japan Institute for Health Security (JIHS)

Address: 1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan



Supplementary Figure 1. Nakao et al.

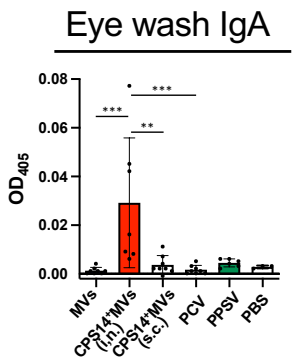
Supplementary Figure 1. CPS14-specific humoral immune responses after intranasal administration with CPS14$^+$MVs at different doses

(A) Timeline of intranasal immunization with different CPS14$^+$MV amounts.

Six-week-old female BALB/c mice (n = 20) were equally distributed into 5 groups and intranasally immunized with PBS or four different doses of CPS14$^+$MVs (0.01, 0.1, 1, and 10 μg) three times at weeks 0, 3, and 6. The CPS14 amounts per dose in the CPS14$^+$MVs at different doses (0.01, 0.1, 1, and 10 μg) were 0.13 ng, 1.3 ng, 0.013 μg, and 0.13 μg, respectively. Serum, saliva, nasal wash, BALF and feces samples were collected at week 8. Vaccination groupings of mice are described in the right table. Created in BioRender. Konig, S. (2025) https://BioRender.com/z8u1cwz.

(B) Humoral immune responses to intranasal immunization with different CPS14$^+$MV amounts.

CPS14-specific antibodies in serum, saliva nasal wash, and feces samples were quantified by ELISA. In all ELISA except for serum IgG, samples were used at 1:100 dilution. In ELISA for serum IgG, the samples were used at 1:1000 dilution. The results of IgM and IgG ELISA are expressed as OD_{405} values (mean) after a 60-min incubation with AP substrate. The results of serum IgE and all IgA ELISAs are expressed as OD_{405} values (mean ± SD) after a 120-min incubation with AP substrate. Representative data were obtained from 24 mice in one independent animal experiment. Shown are the results of the statistical analysis using Kruskal-Wallis test. *$p < 0.05$.



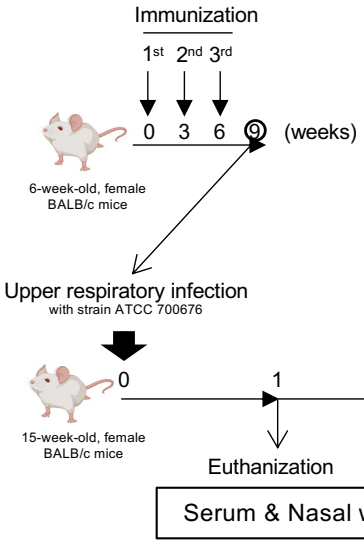
	Groups	Vaccines	CPS14/dose	Routes	Numbers
	Grp. A	MVs (vector control)	None	i.n.	n = 8
	Grp. B	CPS14+MVs	0.013 μg	i.n.	n = 7
	Grp. C	CPS14+MVs	0.013 μg	s.c.	n = 8
	Grp. D	PCV	0.039 μg	s.c.	n = 8
	Grp. E	PPSV	4.5 μg	s.c.	n = 6
	Grp. F	PBS (mock immunization)	None	s.c.	n = 3

Supplementary Figure 2. Nakao et al.

Supplementary Figure 2. CPS14-sepecific IgA antibodies in eye wash

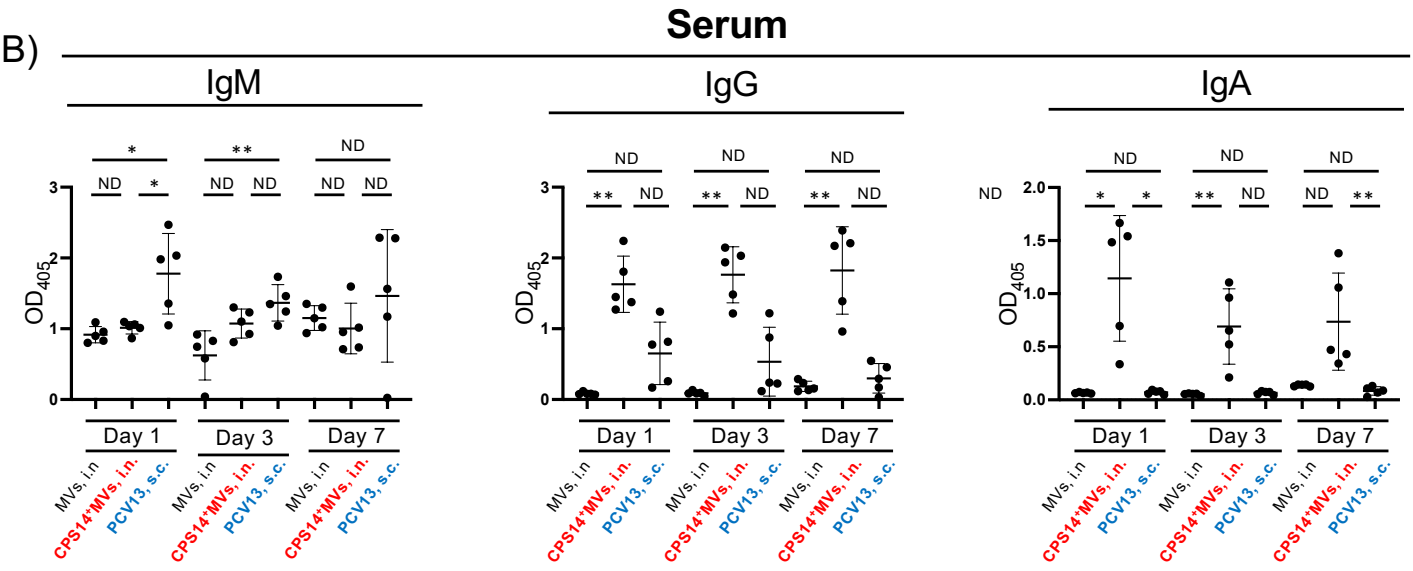
Eye wash samples were collected at week 53(Fig. 2B). CPS14-sepecific IgA antibodies in eye wash obtained from mice (n = 40) at week 53 on the timeline of the long-term study (Fig. 2B) were examined by ELISA. Eye wash samples were used at 1:100 dilution. The results are expressed as OD_{405} values (mean ± SD) after a 120-min incubation with AP substrate. Vaccination groupings of mice are described in the right table, which shows the vaccine used, the amount of CPS14 per dose, the route, and the number of mice in each group. Shown are the data from the 40 mice in one independent animal experiment. Also shown are the results of the statistical analysis using Kruskal-Wallis test. $^{**}p < 0.01$. $^{***}p < 0.001$.

(A)

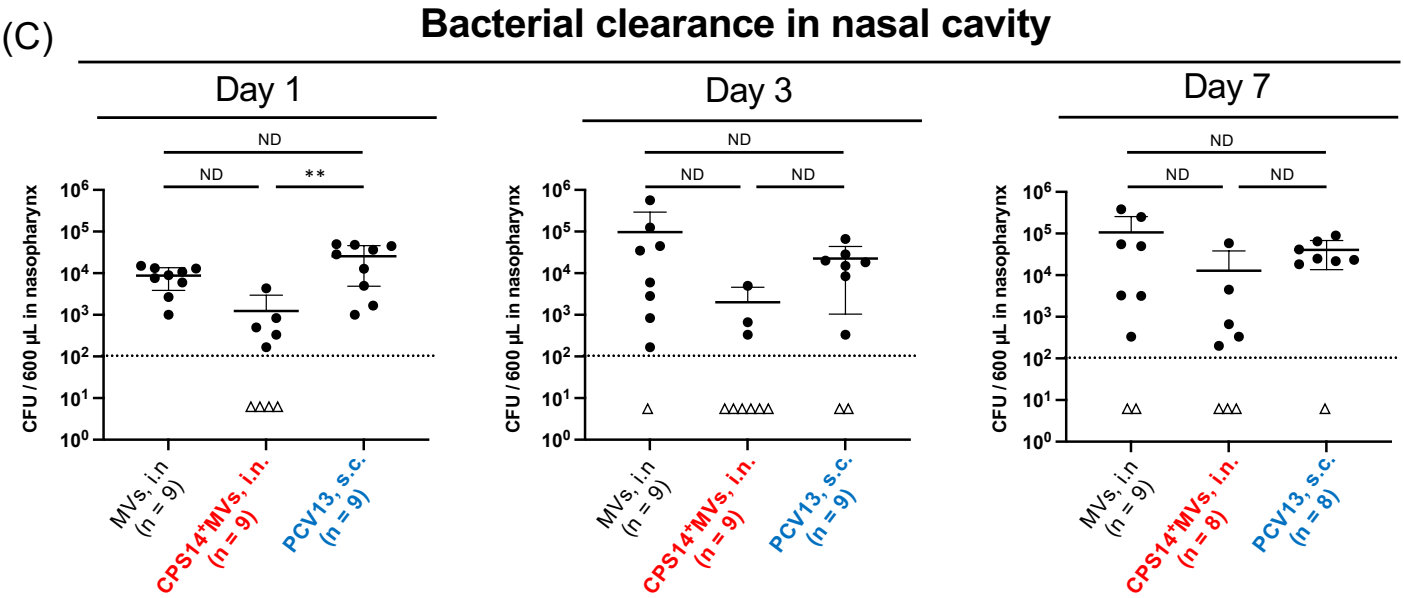


Vaccines	$CPS14_{/dose}$	Routes	Number (for Fig. S4A & B)	Number (Fig. S4B)
MVs (vector control)	None	i.n.	n = 15 (3 time points)	n = 12 (3 time points)
CPS14⁺MVs	0.013 μg	i.n.	n = 15 (3 time points)	n = 12 (3 time points)
PCV	0.039 μg	s.c.	n = 15 (3 time points)	n = 12 (3 time points)

(B)



(C)



Supplementary Figure 3. Nakao et al.

Supplementary Figure 3. Bacterial clearance at different time points after challenge

(A) Timeline of immunization, challenge with pneumococci, and sample collection.

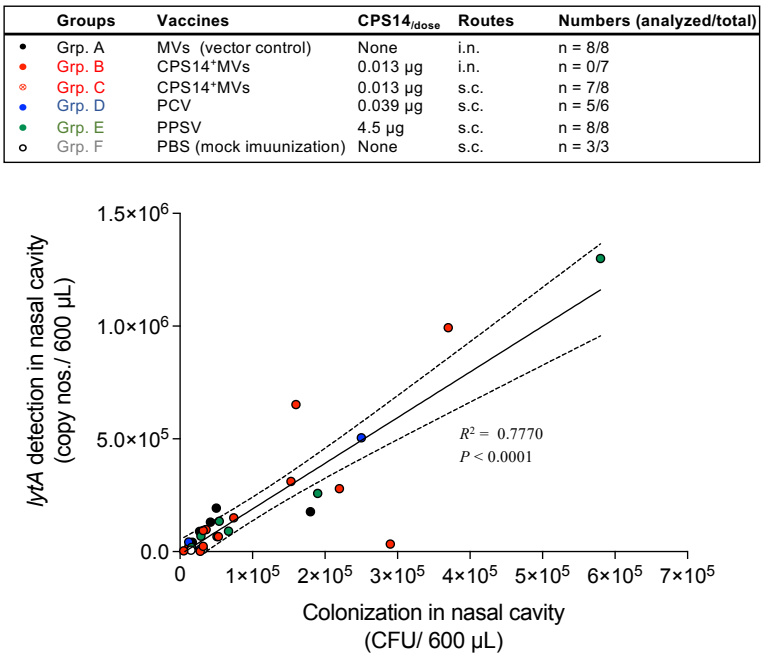
Six-week-old female BALB/c mice were intranasally immunized with MVs (vector control) or CPS14^{+}MVs, and subcutaneously immunized with PCV three times at weeks 0, 3, and 6. At week 9, mice were intranasally inoculated with pneumococcal strain ATCC 700676 at the CFU of 2 x 10^5. At 1, 3, or 7 days after the inoculation, mice were euthanized by cardiac puncture for blood sampling, and the serum and nasal wash samples were collected. BALB/c mice vaccinated with MVs (i.n., n = 15) or CPS14^{+}MVs (i.n., n = 15), and PCV (s.c., n = 15) were used for the 1st bacterial clearance assay. The CPS14-specific antibodies in the serum samples from the mice used for the 1st bacterial clearance assay (n =45) were also tested (See Fig. S4B). One third of mice in each group (n = 5 out of 15) were euthanized at each time point at day 1, 3, or 7. The whole blood and nasal wash samples collected from the mice were used for CPS14 ELISA. The 2nd bacterial clearance assay was performed using another set of mice (n = 36) equally distributed and vaccinated with the 3 groups: MVs (i.n., n = 12) or CPS14^{+}MVs (i.n., n = 12), and PCV (s.c., n = 12). In total, vaccination groupings of all these mice (n = 81) are described in the upper right table, which shows the vaccine used, the amount of CPS14 per dose, the route, and the number of mice in each group. Created in BioRender. Konig, S. (2025) https://BioRender.com/z8u1cwz.

(B) CPS14-specific IgM, IgG, and IgA in serum samples at different time points.

The serum and nasal wash samples collected from the mice at different time points (days 1, 3, and 7) were used for CPS14 ELISA. In ELISA for IgM and IgA, samples were used at 1:100 dilution. In ELISA for IgG, samples were used at 1:1000 dilution. The results are expressed as OD_{405} values (mean $\pm$ SD) after a 30-min incubation with AP substrate. Shown are results of statistical analysis between three groups (Grps A, B, and D) at each time point, using Kruskal-Wallis test. *$p < 0.05$. **$p < 0.01$. ND: no statistically significant difference. Shown are the combined data from the samples of the 45 mice in one independent animal experiment.

(C) Bacterial clearance at different time points.

The number of BALB/c mice used for the 1st and 2nd bacterial clearance assays were 45 and 36, respectively. One third of the mice in each group (n = 9) were euthanized at each time point at day 1, 3, or 7. The nasal wash samples were used for CFU assay. One mouse allocated in the CPS14^{+}MVs and PCV groups by day 7 in the 2nd bacterial clearance assay accidentally died. Shown are the means with SD of CFU/mL in nasopharynx. The detection limit of live pneumococci was 10^2 CFU, which was indicated by a horizontal dotted line in each table. Open triangles indicate the subject number under the detection limit. Statistical analysis was performed using Kruskal-Wallis test. **$p < 0.01$. ND: no statistically significant difference. Shown are the combined data of 59 mice in two independent animal experiments.

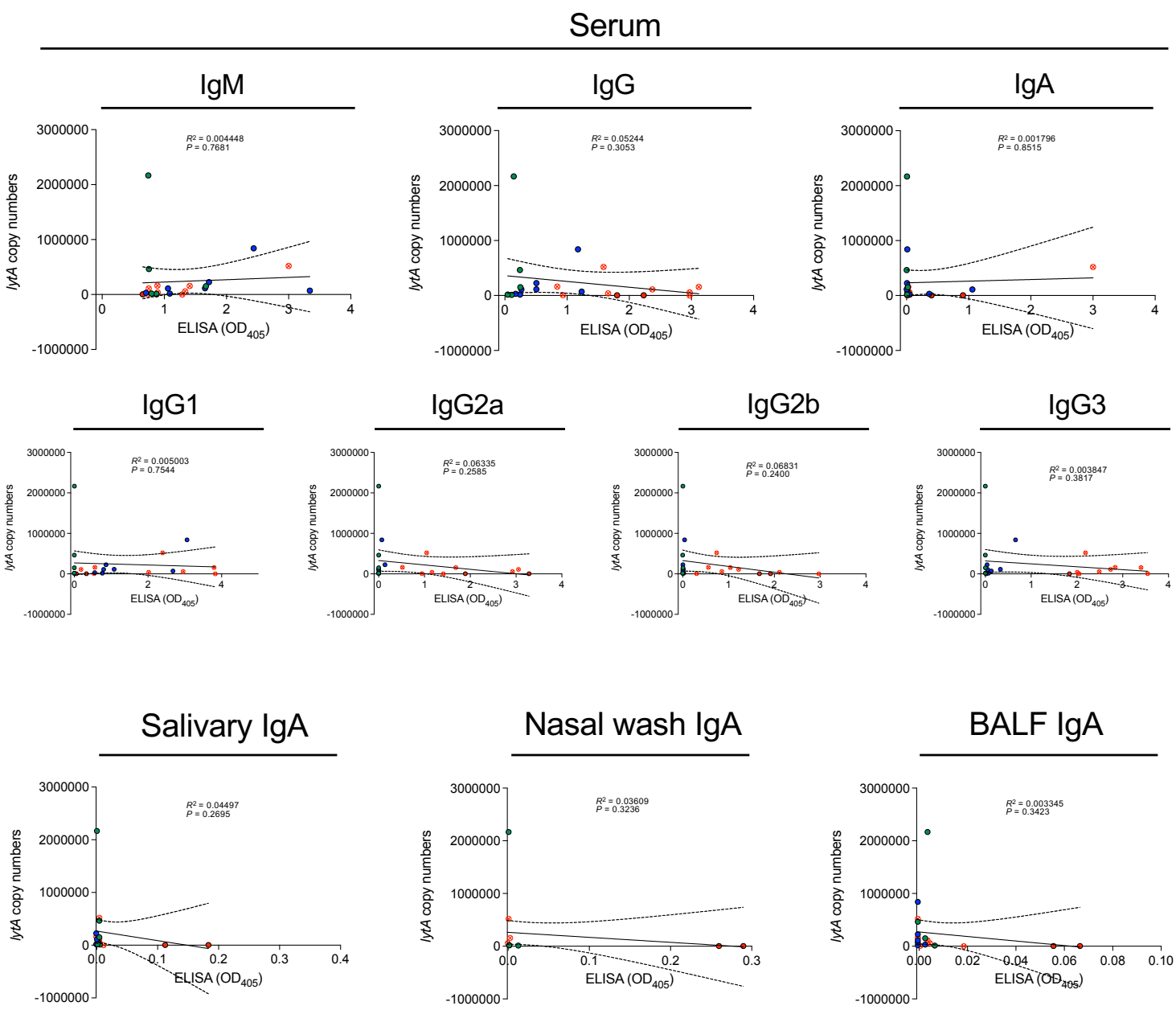


Supplementary Figure 4. Nakao et al.

Supplementary Figure 4. Correlation between CFU assay and real-time PCR-based assay for detection of pneumococci

Vaccination groupings of mice are described in the top table, which shows the vaccine used, the amount of CPS14 per dose, the route, and the number of mice in each group. Correlations between the CFU and *lytA* copy numbers in the nasal wash samples (600 μL) were assessed. Representative data were generated from the results of 31 mice (out of 40 mice) that were positive in both the CFU and real-time PCR-based assays. Of the 31 mice, 8 mice (out of 8 mice) were in Grp. A; no mice (out of 7 mice) were in Grp. B; 7 mice (out of 8 mice) were in Grp. C; 5 mice (out of 6 mice) in Grp. D; 8 mice (out of 8 mice) were in Grp. E; and 3 mice (out of 3 mice) were in Grp. F. Shown are the plotted values with the linear regression line and the 95% confidence bands (dotted lines). Also shown are *R*-squared and *P* value that were calculated using GraphPad Prism version 10.2.1.

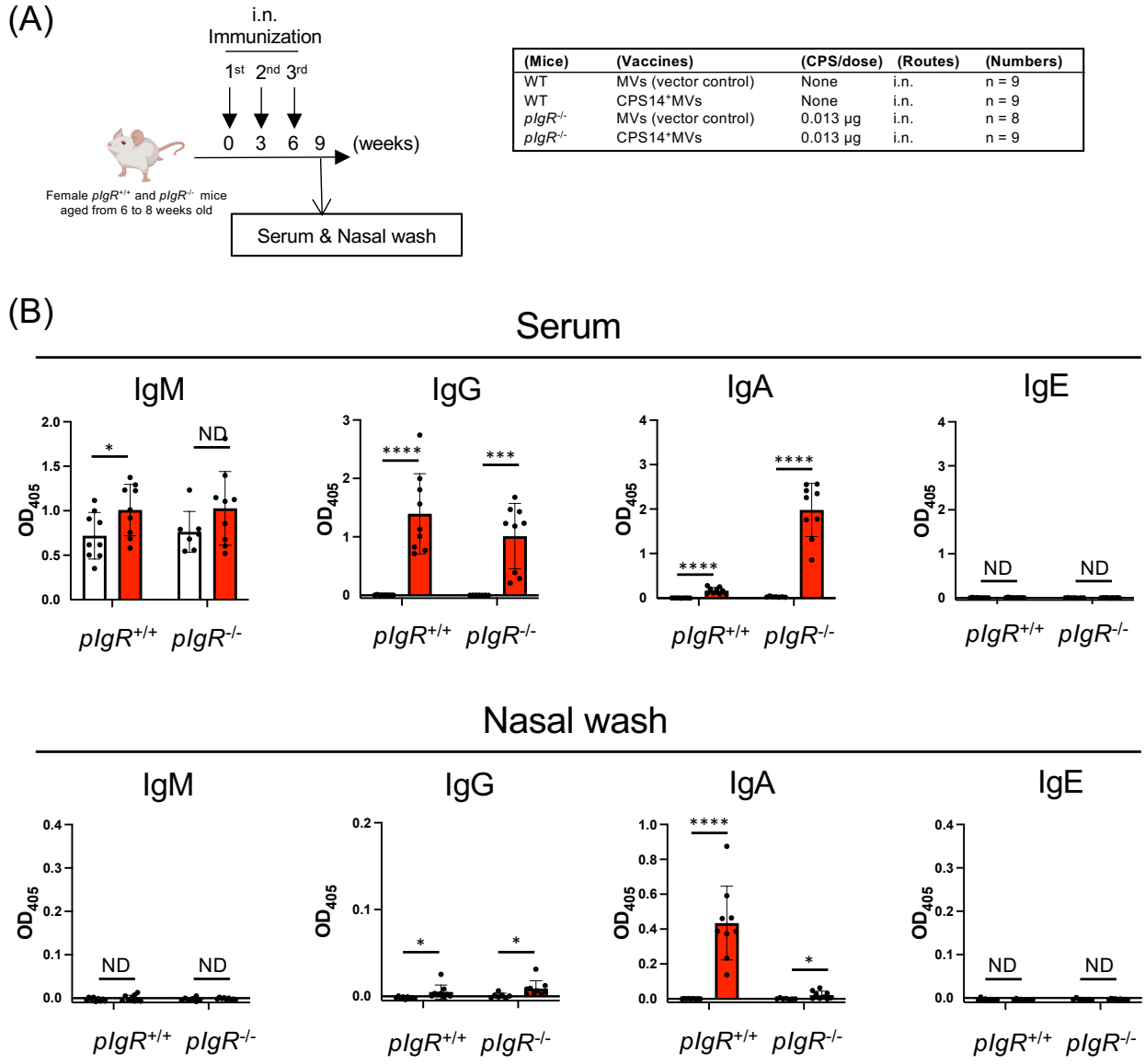
Groups	Vaccines	CPS14$_{/dose}$	Routes	Numbers (analyzed/total)
Grp. B	CPS14^{+}MVs	0.013 μg	i.n.	n = 2/7
Grp. C	CPS14^{+}MVs	0.013 μg	s.c.	n = 8/8
Grp. D	PCV	0.039 μg	s.c.	n = 7/6
Grp. E	PPSV	4.5 μg	s.c.	n = 5/8



Supplementary Figure 5. Nakao et al.

Supplementary Figure 5. Correlations of antibodies in vaccinated mice with detection of *lytA* gene in the nasal cavity.

We compared the OD_{405} values of different immunoglobulin classes and different IgG subclasses in serum samples and IgA in different mucosal sites (saliva, nasal wash and BALF samples) with the *lytA* copy numbers/mL. Vaccination groupings of mice are described in the top table, which shows the vaccine used, the amount of CPS14 per dose, the route, and the number of mice in each group. The representative data were generated from the results of ELISA (Fig. 4) and real-time PCR-based bacterial clearance assay (Fig. 7B, right) using only mice showing *lytA*-positive in nasal cavities (n = 22, out of 29 mice). Of the 22 mice, 2 mice (out of 7 mice) were in Grp. B; 8 mice (out of 8 mice) were in Grp. C; 5 mice (out of 6 mice) were in Grp. D; and 7 mice (out of 8 mice) were in Grp. E. Shown are the plotted values with the linear regression line and the 95% confidence bands in each graph. Shown are also *R*-squared and *P* values.



(Mice)	(Vaccines)	(CPS/dose)	(Routes)	(Numbers)
WT	MVs (vector control)	None	i.n.	n = 9
WT	CPS14^{+}MVs	None	i.n.	n = 9
pIgR$^{-/-}$	MVs (vector control)	0.013 μg	i.n.	n = 8
pIgR$^{-/-}$	CPS14^{+}MVs	0.013 μg	i.n.	n = 9

Supplementary Figure 6. Nakao et al.

Supplementary Figure 6. Humoral immune responses in *pIgR*$^{+/+}$ and *pIgR*$^{-/-}$ mice after nasal vaccination

(A) Timeline of intranasal immunization and sample collection.

We compared the CPS14-specific IgM, IgG, IgA, and IgE antibodies in serum and nasal wash samples of both *pIgR*$^{+/+}$ and *pIgR*$^{-/-}$ mice that were used in bacterial clearance assay (Fig. 8). Vaccination groupings of mice are described in the top table, which shows the vaccine used, the amount of CPS14 per dose, the route, and the number of mice in each group. Representative data are the combined results from 3 independent animal experiments. In total, the *pIgR*$^{+/+}$ mice (n = 18) and *pIgR* $^{-/-}$ mice (n=17) were used for the humoral immune response analysis. Created in BioRender. Konig, S. (2025) https://BioRender.com/z8u1cwz.

(B) ELISA

In all ELISAs except for serum IgG, samples were used at 1:100 dilution. In ELISA for serum IgG, the samples were used at 1:1000 dilution. The results of serum IgA ELISA are expressed as OD_{405} values (mean ± SD) after a 15-min incubation with AP substrate. The results of serum IgM and IgG ELISAs are expressed as OD_{405} values (mean ± SD) after a 30-min incubation with AP substrate. The results of nasal wash IgM, IgG, IgA, IgE, and serum IgE ELISAs are expressed as OD_{405} values (mean ± SD) after a 120-min incubation with AP substrate. Shown are the results of statistical analysis of ELISA data between mice vaccinated with MVs (vector control) and those vaccinated with CPS14^{+}MVs, using Mann-Whitney's U-test. Asterisks denote statistically significant difference. *$p < 0.05$. ***$p < 0.001$. ****$p < 0.0001$. ND: no statistically significant difference.