Supplementary Information

A vaccine platform targeting lung-resident memory CD4$^+$ T-cells provides protection against heterosubtypic influenza infections in mice and ferrets

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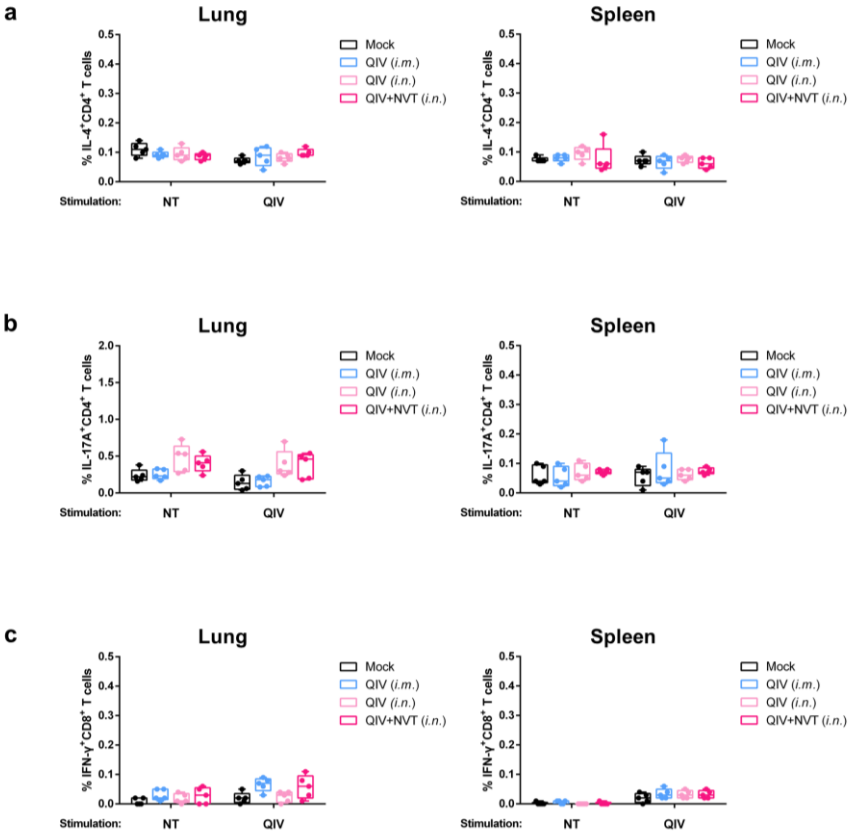
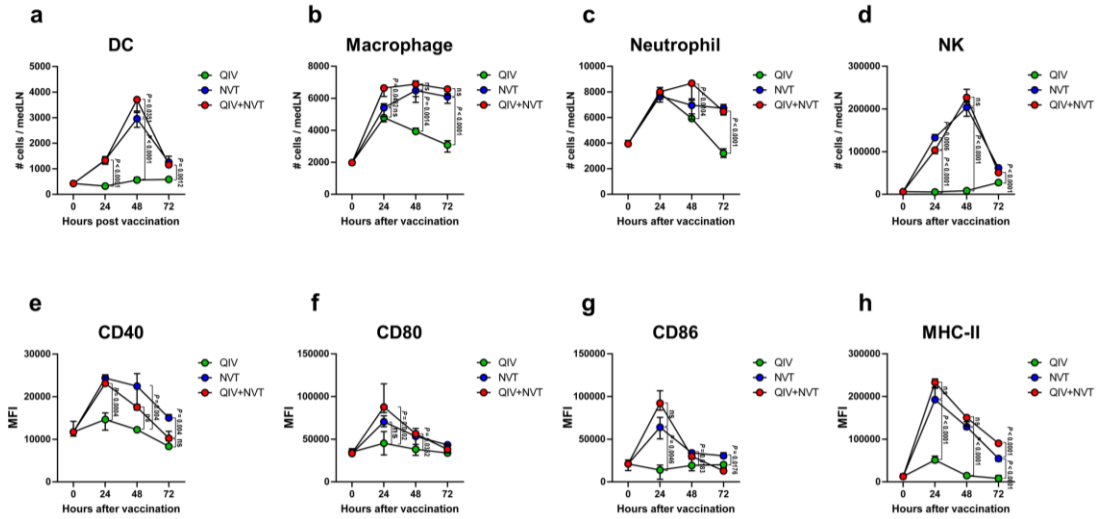
1. Supplementary figures and figure legends

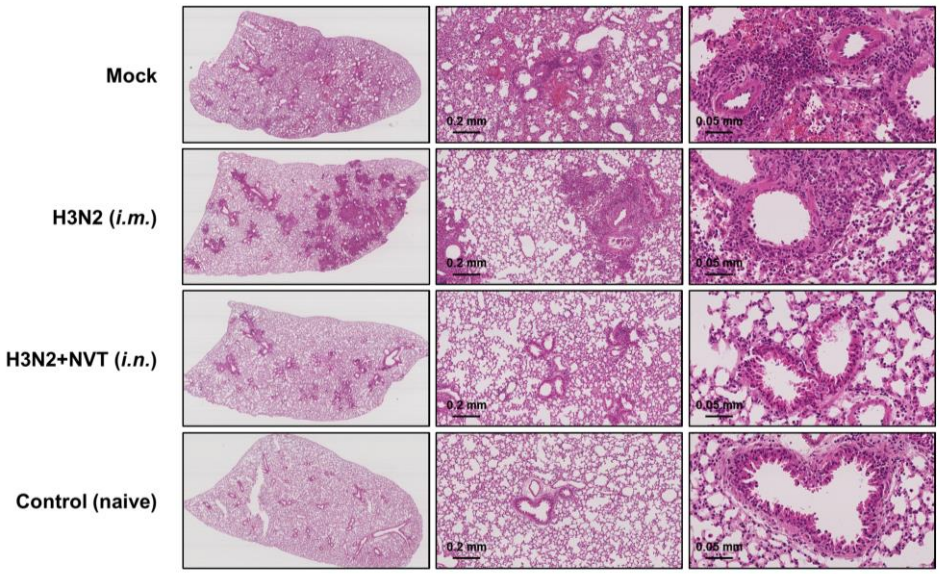
2. Supplementary Tables



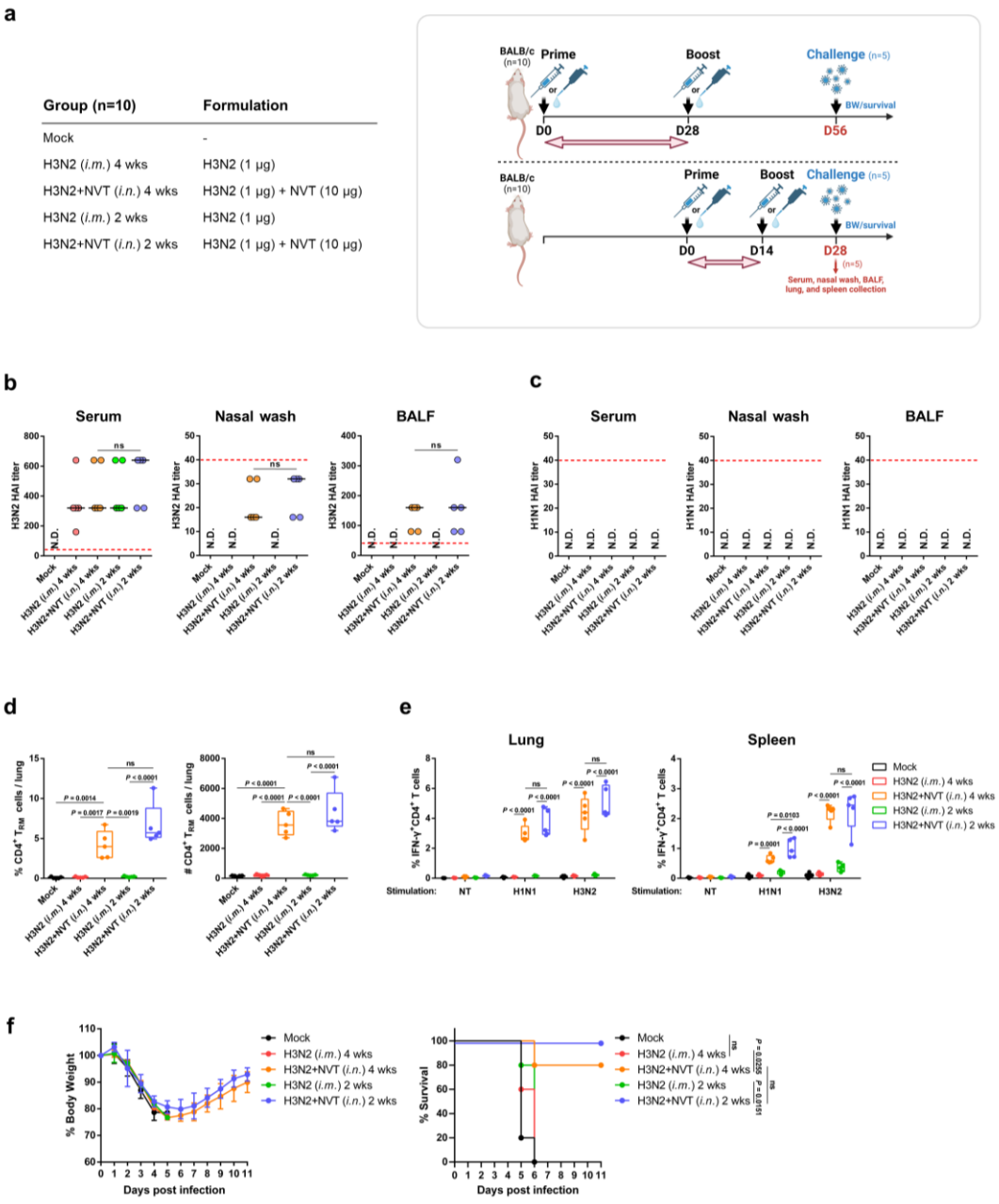
Supplementary Fig. 1. Intranasal administration of QIV+NVT did not induce Th2, Th17, or CTL responses. BALB/c mice were vaccinated as shown in Fig. 1A. After stimulating lung and spleen cells with QIV, (**a**) Th2 (IL-4$^+$CD4$^+$ T-cells), (**b**) Th17 (IL-17A$^+$CD4$^+$ T-cells), and (**c**) CTL (IFN-γ$^+$CD8$^+$ T-cells) responses were determined via flow cytometry. Box and whisker plots show the median (center), 25th and 75th percentiles (box), and lowest and highest values (whiskers). This study was performed independently at least three times, and one representative set is shown. NT, non-treated. Source data are provided as a Source Data file.



Supplementary Fig. 2. Intranasal immunization with NVT prompts innate immune responses. BALB/c mice (n=12 per group) were *i.n.* administered with QIV, NVT, or QIV+NVT. At 0, 24, 48, and 72 h, the mediastinal lymph nodes (n=3 per group at each time point) were collected and dissociated into single cells, after which whole cells were counted. **a-d** (**a**) DCs (MHC-II$^+$CD11c$^+$), (**b**) macrophages (CD11b$^+$F4/80$^+$), (**c**) neutrophils (CD11b$^+$Ly6G$^+$), and (**d**) NK cells (F4/80$^-$NKp46$^+$) were evaluated via flow cytometry, and the absolute numbers were calculated by multiplying the total number of cells by their percentage. **e-h** The mean fluorescence intensities (MFIs) of (**e**) CD40, (**f**) CD80, (**g**) CD86, and (**h**) MHC-II expression on DCs were determined by flow cytometry. All the data are expressed as median and range. Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test. ns, not significant. Source data are provided as a Source Data file.

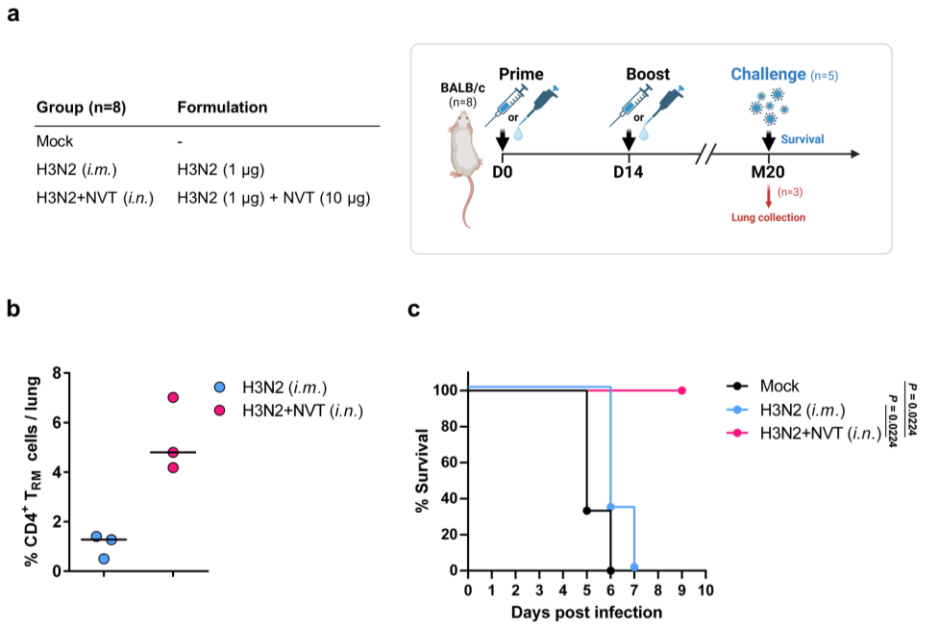


Supplementary Fig. 3. Lung histopathology of mice challenged with H1N1 virus. BALB/c mice were immunized *i.m.* with H3N2 or *i.n.* with H3N2+NVT twice at two-week intervals. Two weeks after the last immunization, the mock-treated and vaccinated mice were *i.n.* challenged with H1N1 virus (A/Korea/2785/2009). On day 6 after challenge, lungs from challenged and control (naïve) mice were harvested, and histological sections were stained with hematoxylin and eosin.

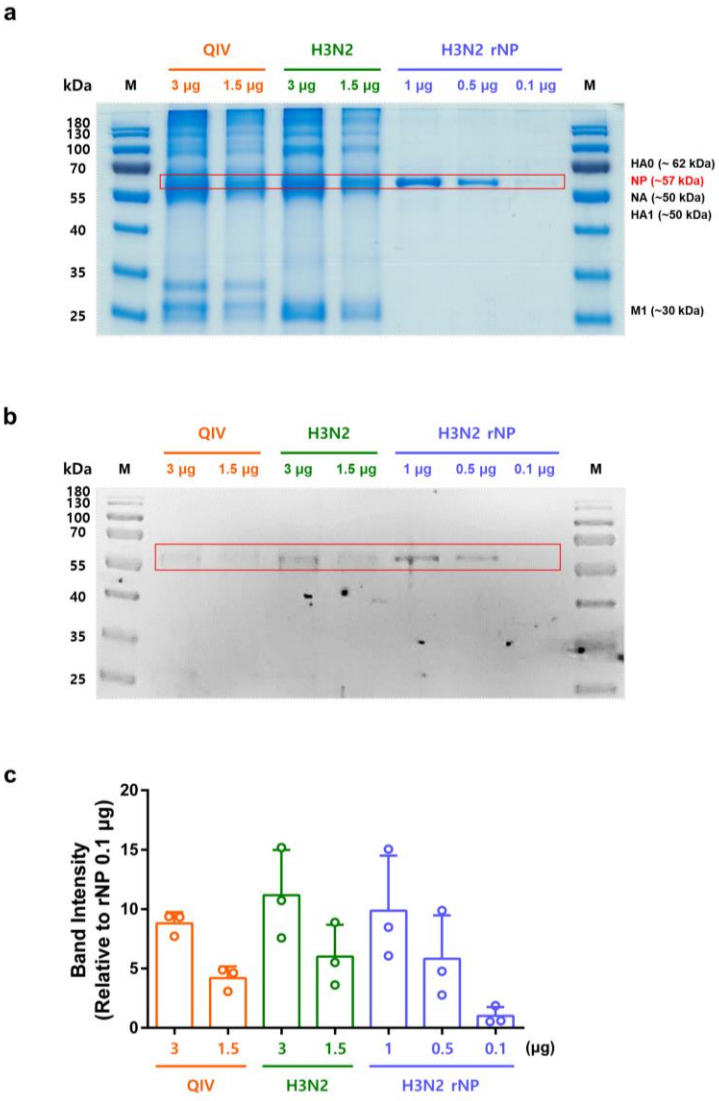


Supplementary Fig 4. Comparison of protective immunity between vaccination at 2-week intervals and vaccination at 4-week intervals. a Experimental design. BALB/c mice (n=10 per group) were immunized *i.m.* with H3N2 or *i.n.* with H3N2+NVT twice, 2 or 4 weeks apart. For the 2-week interval group, 2 weeks after the last vaccination, immune response analysis (n=5 per group) (**b-e**) and H1N1 virus (A/KOREA/2785/2009) challenge (n=5 per group) (**f**) were carried out; and for the 4-week interval group, 4 weeks after the last vaccination, the same analyses were performed. Schematic image was created in BioRender. Han, S. (2024) BioRender.com/g49q384. **b, c** HAI titers against H3N2 or H1N1 in serum, nasal wash, and BALF were measured via HAI assay. An HAI antibody titer of 1:40 (red dotted line) was considered protective against influenza infection. The data are expressed as dot plots, with

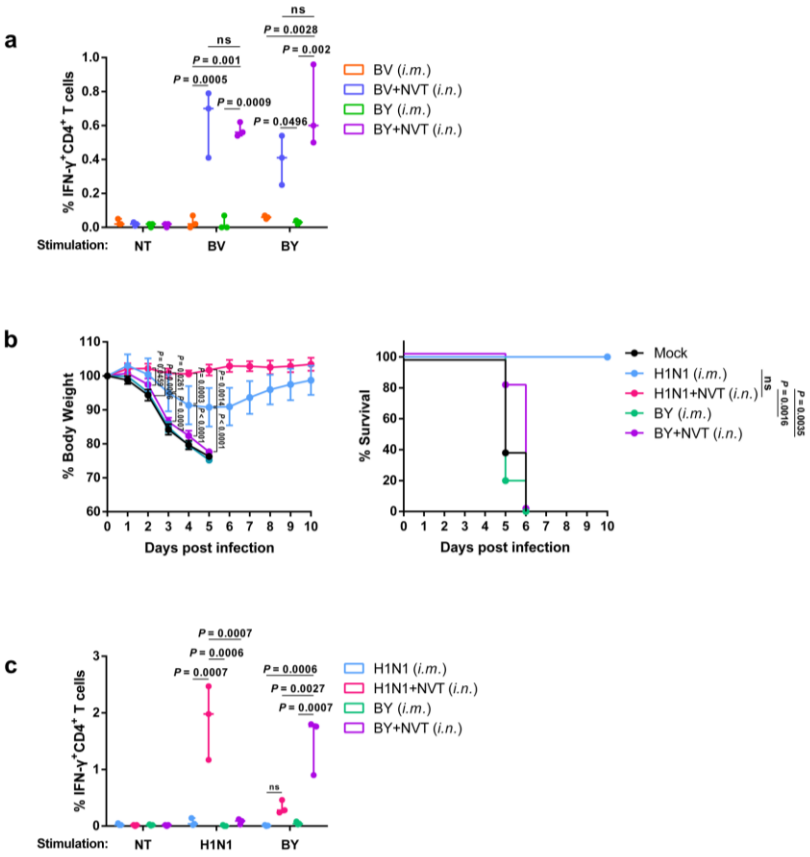
horizontal lines representing the medians. **d, e** (**d**) The proportion and numbers of lung $CD4^+$ T_{RM} cells and (**e**) the IFN-γ^+CD4^+ T-cell response to H1N1 or H3N2 in lung and spleen were determined by flow cytometry. Box and whisker plots show the median (center), 25th and 75th percentiles (box), and lowest and highest values (whiskers). **f** The vaccinated mice were infected *i.n.* with 4 LD_{50} doses of H1N1 virus. Body weight and survival (n=5 per group) were monitored daily. Weight-loss data are shown as mean value ± SD. Survival data are represented as Kaplan–Meier survival curves, and the significant differences in survival rates were calculated by the log-rank test. **b, d, e** Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test or two-sided Mann–Whitney U test. This study was performed once. BW, body weight; N.D., not detected; ns, not significant; NT, non-treated. Source data are provided as a Source Data file.



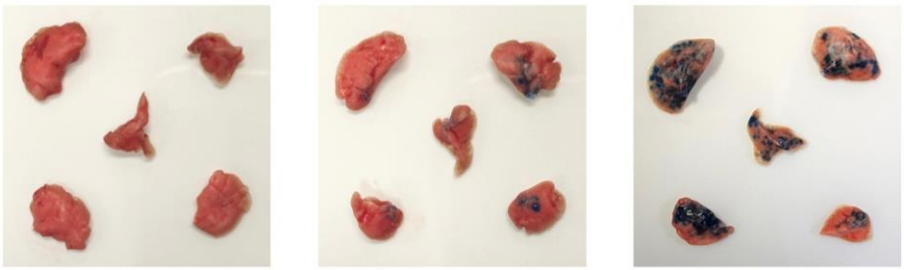
Supplementary Fig. 5. Lung CD4$^+$ T_{RM} cells provide long-term protection against heterosubtypic virus challenge responses. a Experimental design. BALB/c mice (n=8 per group) were immunized twice *i.m.* with H3N2 or *i.n.* with H3N2+NVT at two-week intervals. Twenty months after the last vaccination, lung CD4$^+$ T_{RM} cell analysis (n=3 per group) (**b**) and H1N1 virus (A/Korea/2785/2009) challenge (n=5 per group) (**c**) were performed. Schematic image was created in BioRender. Han, S. (2024) BioRender.com/s14e934. **b** The lung CD4$^+$ T_{RM} cell population (CD4$^+$CD44$^+$CD62L$^-$CD69$^+$) was determined using flow cytometry. The data are expressed as dot plots, with horizontal lines representing the medians. **c** The vaccinated mice were challenged *i.n.* with 4 LD_{50} doses of H1N1 virus, and survival was monitored daily. Survival data are represented as Kaplan–Meier survival curves, and the significant differences in survival rates were calculated by the log-rank test. This study was performed once. Source data are provided as a Source Data file.



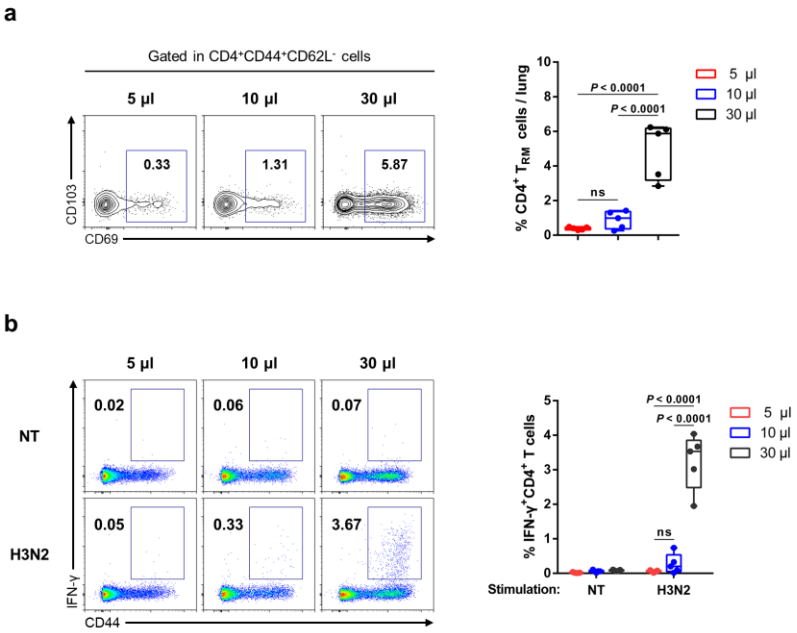
Supplementary Fig. 6. Commercial vaccine preparations contain NPs. Two sets of QIV (3 or 1.5 μg of HA content), H3N2 (3 or 1.5 μg of HA content), and H3N2 rNPs (1, 0.5, or 0.1 μg) were loaded on a 12% SDS-PAGE gel. After electrophoresis, **a** one set of gels was stained with Coomassie Protein Stain. **b** The other set of unstained gels was subjected to Western blotting using serum from mice vaccinated with H3N2 rNPs. Lanes M were loaded with marker proteins, with the corresponding molecular weight presented to the left. **c** Band intensities were measured three times using ImageJ software, with the H3N2 rNP 0.1 μg band as a relative reference. The data are shown as mean value ± SD. Source data are provided as a Source Data file.



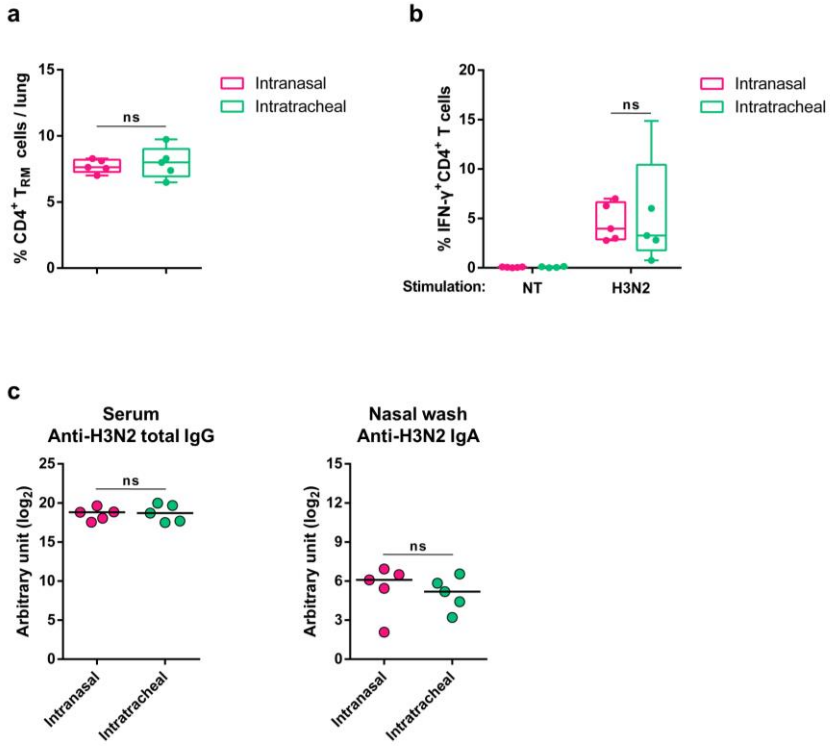
Supplementary Fig. 7. A correlation between cross-reactivity and cross-protection was observed for influenza types A and B. a BALB/c mice (n=3 per group) were immunized twice *i.m.* with BV or BY, or *i.n.* with BV+NVT or BY+NVT twice at two-week intervals. After stimulating the lung cells with BV or BY, the IFN-γ^{+}CD4^{+} T-cell response was determined via flow cytometry. The data are expressed as dot plots, with horizontal lines representing the medians. **b, c** BALB/c mice (n=8 per group) were immunized twice *i.m.* with H1N1 or BY, or *i.n.* with H1N1+NVT or BY+NVT twice at two-week intervals. Two weeks after the last immunization, lung CD4^{+} T-cell response analysis (n=3 per group) and H1N1 virus (A/Korea/2785/2009) challenge (n=5 per group) were performed. **b** Mice were infected *i.n.* with 4 LD_{50} doses of H1N1 virus, and body weight and survival were monitored daily. Weight-loss data are shown as mean value ± SD. Survival data are represented as Kaplan–Meier survival curves, and the significant differences in survival rates were calculated by the log-rank test. **c** After stimulating lung cells with H1N1 or BY, the IFN-γ^{+}CD4^{+} T-cell response was determined by flow cytometry. The data are expressed as dot plots, with horizontal lines representing the medians. **a-c** Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test. This study was performed once. ns, not significant; NT, non-treated. Source data are provided as a Source Data file.



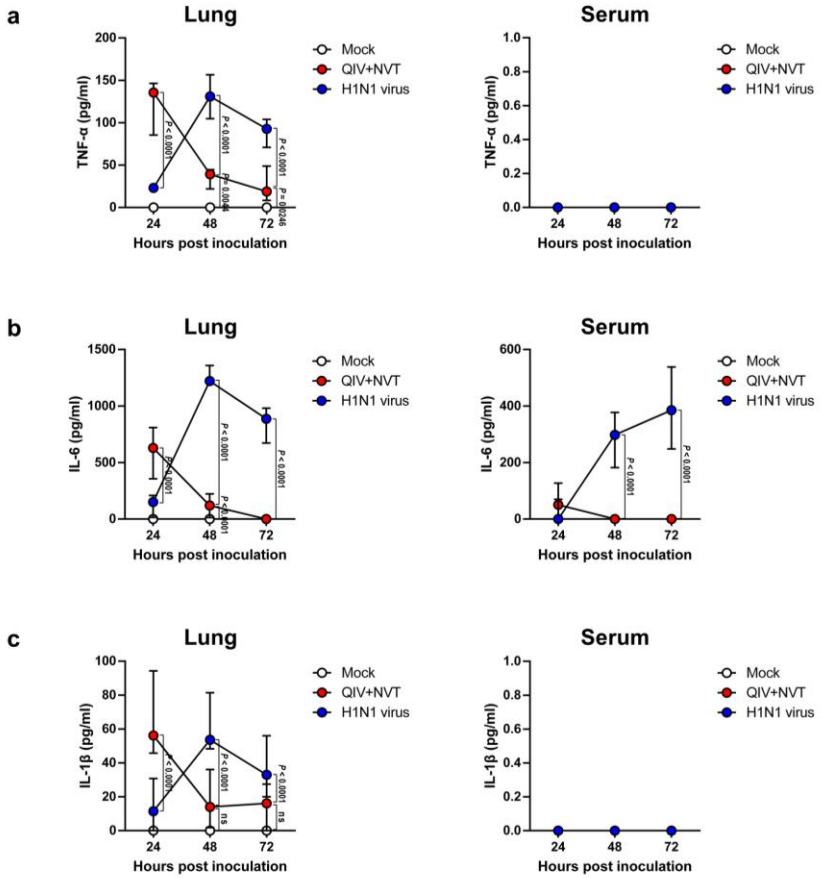
Supplementary Fig. 8. The amount delivered to the lungs varies depending on the intranasal instillation volume. BALB/c mice were administered 5, 10, or 30 µl of 2% Evans blue dye *i.n.*, after which the lungs were collected. The lung lobes stained with the dye are shown.



Supplementary Fig. 9. The generation of lung CD4$^+$ T$_{RM}$ cells varies depending on the intranasal instillation volume. a, b BALB/c mice (n=5 per group) were immunized twice *i.n.* with 5, 10, or 30 µl of H3N2+NVT twice at two-week intervals. Two weeks after the last immunization, (**a**) the proportion of lung CD4$^+$ T$_{RM}$ cells and (**b**) the IFN-γ$^+$CD4$^+$ T-cell response to H3N2 were determined by flow cytometry. Box and whisker plots show the median (center), 25th and 75th percentiles (box), and lowest and highest values (whiskers). Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test. This study was independently performed at least three times, and one representative set is shown. ns, not significant; NT, non-treated. Source data are provided as a Source Data file.



Supplementary Fig. 10. Administration methods using nasal instillation (intranasal) and an endotracheal intubation device (intratracheal) induce similar levels of immune response. a-c BALB/c mice (n=5 per group) were immunized *i.n.* or intratracheally with H3N2+NVT via a microsprayer (Model IA-1C, PennCentury, Wyndmoor, PA) twice at two-week intervals. Two weeks after the last immunization, (**a**) the proportion of lung $CD4^+$ T_{RM} cells was determined using flow cytometry. (**b**) After stimulating lung cells with H3N2, the IFN-γ^+CD4^+ T-cell response was determined via flow cytometry. **a, b** Box and whisker plots showing the median (center), 25th and 75th percentiles (box), and lowest and highest values (whiskers). **c** H3N2-specific total IgG in the serum and H3N2-specific IgA in the nasal wash were assessed via ELISA. The data are expressed as dot plots, with horizontal lines representing the medians. **a-c** The two-sided Mann–Whitney U test was used for statistical analysis. This study was performed independently twice, and one representative set is shown. ns, not significant; NT, non-treated. Source data are provided as a Source Data file.



Supplementary Fig. 11. The NVT-adjuvanted influenza vaccine does not induce excessive levels of inflammatory cytokines. BALB/c mice (n=15 per group) were immunized *i.n.* with QIV+NVT. At 24, 48, and 72 h, (**a**) TNF-α, (**b**) IL-6, and (**c**) IL-1β levels in lung lysates and serum samples (n=5 per group at each time point) were measured via ELISA. All the data are expressed as median and range. Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test. ns, not significant. Source data are provided as a Source Data file.

Supplementary Table 1. List of mouse antibodies used in this study

Antibody	Fluorochrome	Clone	Purpose	Vendor	Cat no.
Viability dye	eFluor780	-	Flow cytometry	eBioscience	65-0865-14
mCD11b	PE/Cyanine7	M1/70	Flow cytometry (For innate cells)	BioLegend	101216
mCD11c	APC	N418		BioLegend	117310
mLy6G	PerCP/Cyanine5.5	1A8		BioLegend	127616
mF4/80	PE	BM8		BioLegend	123110
mCD40	FITC	3/23	Flow cytometry (For DC activation)	BioLegend	124607
mCD80	PE	16-10A1		BioLegend	104708
mCD86	PE/Cyanine5	GL-1		BioLegend	105016
mMHC-II	PE/Cyanine7	AF6-120.1		BioLegend	116420
mCD4	PE/Cyanine7	RM4-5	Flow cytometry (For T-cell analysis)	BioLegend	100528
mCD8a	FITC	53-6.7		BioLegend	100706
mCD44	APC	IM7		BioLegend	103011
mCD62L	PE/Cyanine5	MEL-14		BioLegend	104410
mCD69	PE	H1.2F3		BioLegend	104507
mCD103	PerCP/Cyanine5.5	2E7		BioLegend	121415
mIFN-γ	PE	XMG1.2		BioLegend	505808
mIL-4	PerCP/Cyanine5.5	11B11		BioLegend	504123
mIL-17A	eFlour 660	eBio17B7		Invitrogen	50-7177-82
mIgG-HRP	-	-	ELISA	SouthernBiotech	1030-05
mIgG1-HRP	-	-		SouthernBiotech	1070-05
mIgG2a-HRP	-	-		SouthernBiotech	1080-05
mIgA-HRP	-	-		SouthernBiotech	1040-05

Supplementary Table 2. Seasonal influenza vaccines used in this study

Influenza season (northern hemisphere)	Vaccine strain
2018-2019	A/Michigan/45/2015, pdm09-like (H1N1)
	A/Singapore/INFIMH-16-0019/2016-like strain (H3N2)
	B/Colorado/06/2017-like strain (B/Victoria/2/87 lineage, BV)
	B/Phuket/3073/2013-like strain (B/Yamagata/16/88 lineage, BY)
2019-2020	A/Brisbane/02/2018, pdm09-like (H1N1)
	A/Kansas/14/2017 (H3N2)
	B/Colorado/06/2017-like strain (BV)
	B/Phuket/3073/2013-like strain (BY)
2020-2021	A/Victoria/2570/2019 (IVR-215) (H1N1)
	A/Cambodia/E0826360/2020 (IVR-224) (H3N2)
	B/Victoria/705/2018 (BVR-11) (BV)
	B/California/12/2015 (NYMC BX-59B) (BY)
2022-2023	A/Victoria/2570/2019 (IVR-215) (H1N1)
	A/Darwin/9/2021 (SAN-010) (H3N2)