Supplementary Materials:

Preclinical evaluation of a universal inactivated influenza B vaccine based on the mosaic hemagglutinin-approach

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A

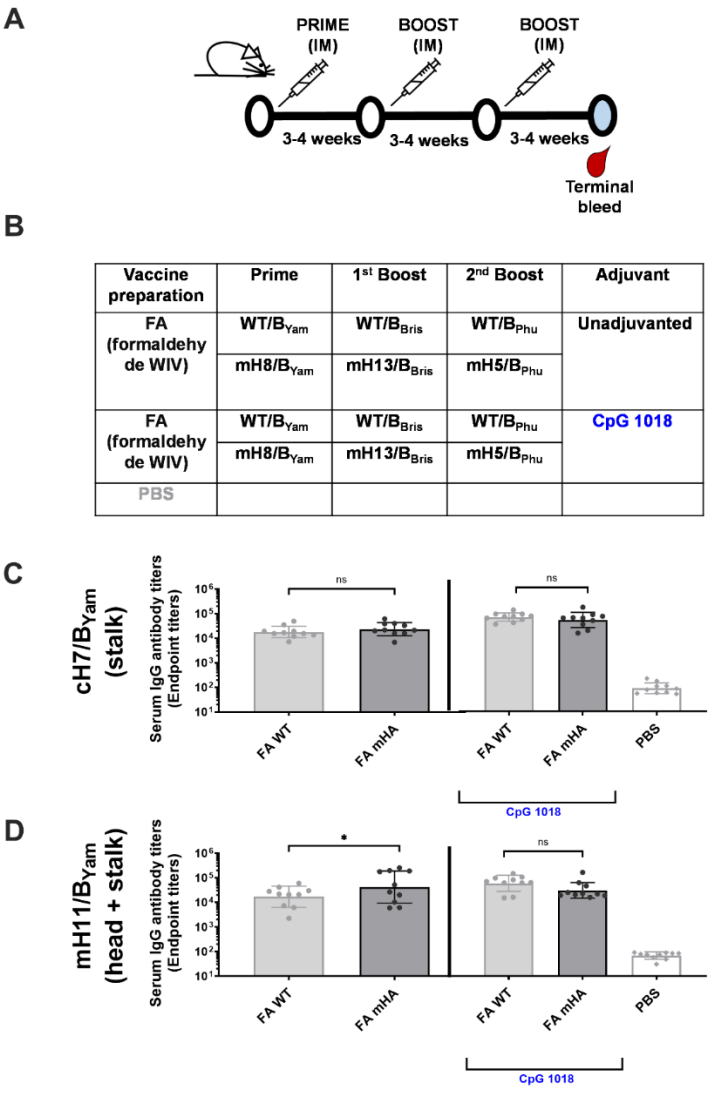
Vaccine name	Database	ID
B/Beijing/184/93-like virus	**Influenza virus database**	AF050061
B/Shangdong/7/97-like virus	**Influenza virus database**	AF486836
B/Sichuan/379/99-like virus	**Influenza virus database**	AJ784040
B/Hong Kong/330/2001-like virus	**Influenza virus database**	CY018709
B/Shanghai/361/2002-like virus	**Influenza virus database**	AJ784056
B/Malaysia/2506/2004-like virus	**Influenza virus database**	CY040449
B/Brisbane/60/2008-like virus	**Influenza virus database**	CY073893
B/Florida/4/2006-like virus	**Influenza virus database**	EU515937
B/Wisconsin/1/2010-like virus	**NA**	
B/Massachusetts/2/2012-like virus	**Influenza virus database**	MT056027
B/Phuket/3073/2013-like virus	**GISAID**	EPI_ISL_168822
B/Colorado/06/2017-like virus	**Influenza virus database**	CY232066
B/Washington/02/2019 -like virus	**Influenza virus database**	MK676294
B/Austria/1359417/2021-like virus	**GISAID**	EPI_ISL_1519459
Reference sequences	**Database**	ID
B/Lee/1940	**Influenza virus database**	J02093
B/Yamagata/16/1988	**Influenza virus database**	M58419
B/Victoria/2/1987	**Influenza virus database**	M58428

B

B/Victoria/2/1987-like lineage	Year	B/Yamagata/16/1988-like lineage	Year
B/Shangdong/7/97-like virus	**2000**	**B/Beijing/184/93-like virus**	**1999**
B/Hong Kong/330/2001-like virus	**2002-2004**	**B/Sichuan/379/99-like virus**	**2001-2002**
B/Malaysia/2506/2004-like virus	**2006-2008**	**B/Shanghai/361/2002-like virus**	**2004-2006**
B/Brisbane/60/2008-like virus	**2009-2018**	**B/Florida/4/2006-like virus**	**2008-2009**
B/Colorado/06/2017-like virus	**2018-2020**	**B/Wisconsin/1/2010-like virus**	**2012-2013**

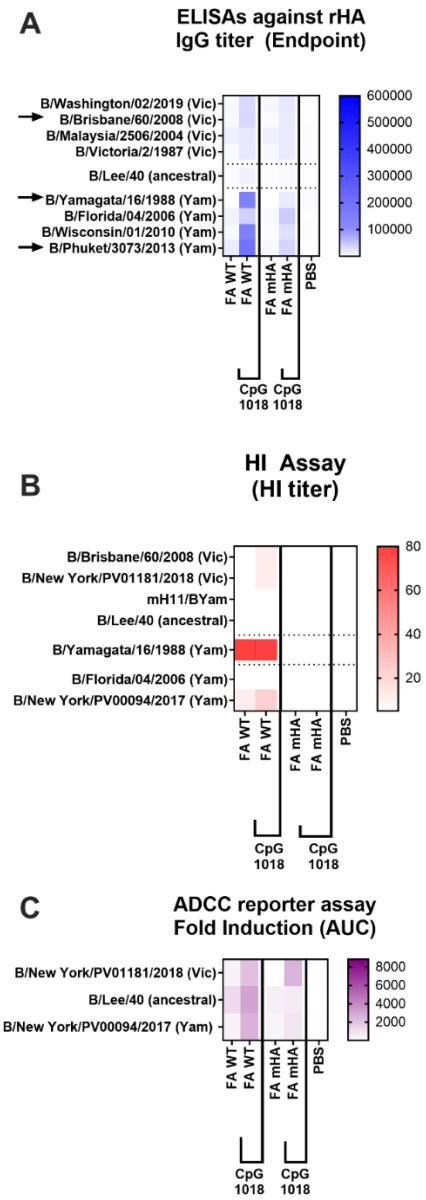
B/Washington/02/2019 -like virus	**2020-2022**	**B/Massachusetts/2/2012-like virus**	**2013-2015**
B/Austria/1359417/2021-like virus	**2022-2024**	**B/Phuket/3073/2013-like virus**	**2015-2023**

Supplementary Figure 1. Phylogenetic sequences of historical annual formulation of influenza B virus vaccines of Fig. 1C. (A) IBV HA sequences of historical annual formulation for IBV vaccine strains from 1999 to 2023 used to construct the phylogenetic tree using the maximum likelihood method and Tamura-Nei model and was visualized through Mega11 [14,15]. (B) Historical annual formulation for IBV vaccine strains from 1999 to 2023 (Global Influenza Programme (who.int) as of 1st Aug 2023).



Supplementary Figure 2. Antibody responses against the conserved HA stalk and immuno-subdominant head domains elicited by WT HA and mHA constructs prepared by inactivation with formaldehyde as whole inactivated virus vaccines. Whole inactivated virus (WIV) with formaldehyde (FA) for two different vaccination strategies with WT viruses or mHA viruses. Vaccines were tested without adjuvant or with the addition of CpG 1018 (30 µg). (A-B) Vaccination regimen and groups. BALB/c mice were vaccinated in a three-dose vaccination experiment with a dose of 1µg of HA

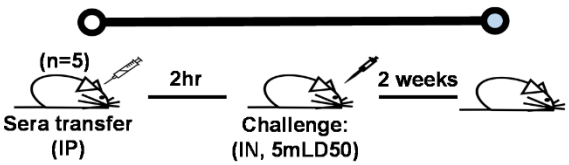
in a 3–4-week interval. An unvaccinated group (PBS) was included as control. (C-D) Binding of serum antibodies towards the immuno-subdominant epitopes. A cH7/B_{Yam} protein with a group 2 avian H7 head and the B/Yamagata/16/1988 HA stalk was used to measure stalk-specific antibodies in the unadjuvanted and adjuvanted groups (C). A mH11/B_{Yam} protein displaying the H11 sequences at the major antigenic sites within the B/Yamagata/16/1988 HA was used to measure antibody binding to conserved epitopes in the head and stalk domains in the unadjuvanted and adjuvanted groups (D). The geometric mean endpoint titer was calculated as the readout. The statistics were calculated using an unpaired one-tailed t test (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P≤0.0001).



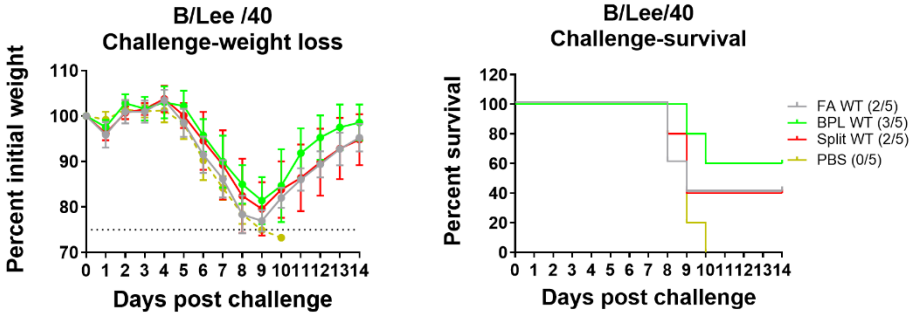
Supplementary Figure 3. Cross-reactivity, HI-activity, and Fc effector function antibody responses elicited by FA-WIV wildtype and mHA vaccines. (A) Heatmap of binding serum antibodies against a panel of recombinant influenza B HA proteins. (B) Heatmap of hemagglutination inhibition (HI) titer against a panel of influenza B viruses.

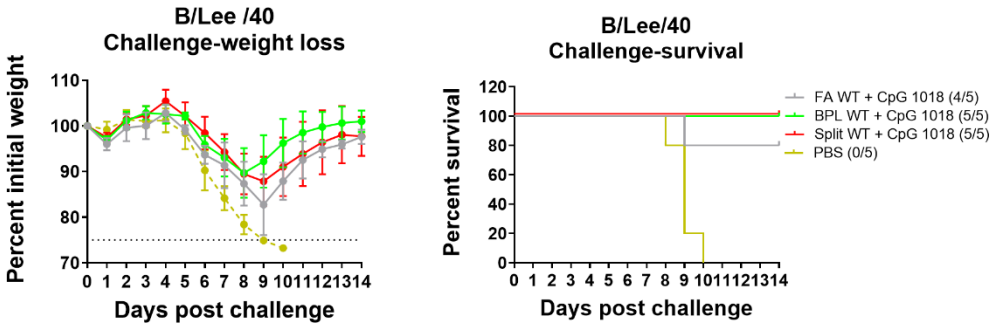
Mean HI titer of each group (pooled sera) measured in triplicate is depicted. (C) Heatmap of ADCC activity against three influenza B viruses. To perform the ADCC reporter assay, MDCK cells were infected with each virus at an MOI of 5 (single-cycle replication). One day after infection, mouse sera were added to the cells and incubated for 30 min and genetically modified Jurkat cells expressing the mouse FcγRIV with a luciferase reporter gene under transcriptional control of the nuclear factor-activated T (NFAT) cell promoter were then added and incubated for 6h. Later luciferase activity was measured. The geometric mean AUC of fold induction of each group (pooled sera) was measured in technical duplicate.

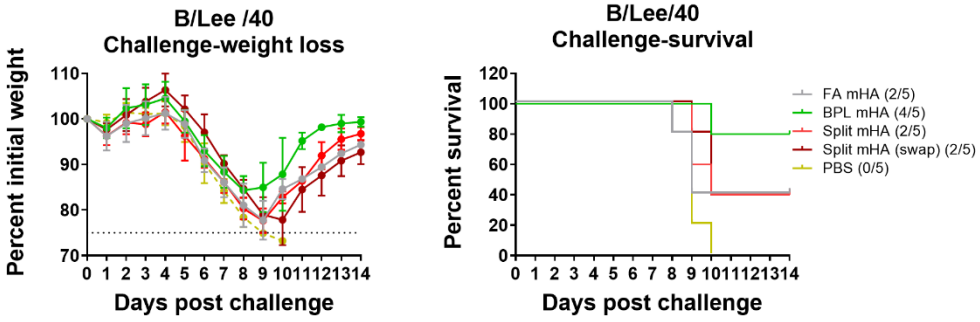
A

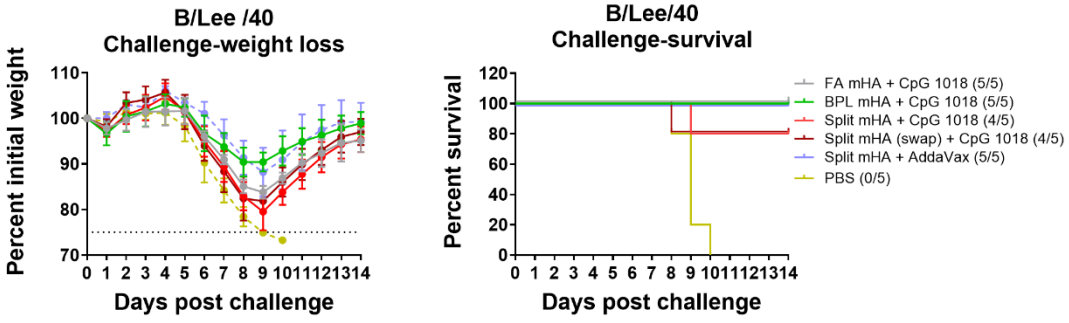

(n=5)
2hr
2 weeks
Sera transfer
(IP)
Challenge:
(IN, 5mLD50)

B

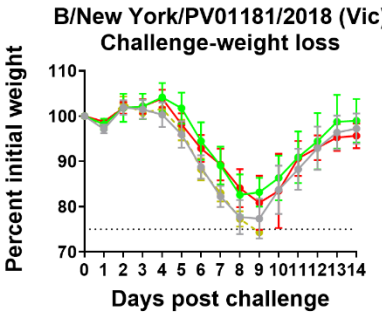

B/Lee /40
Challenge-weight loss
Percent initial weight
Days post challenge
B/Lee/40
Challenge-survival
Percent survival
Days post challenge
FA WT (2/5)
BPL WT (3/5)
Split WT (2/5)
PBS (0/5)

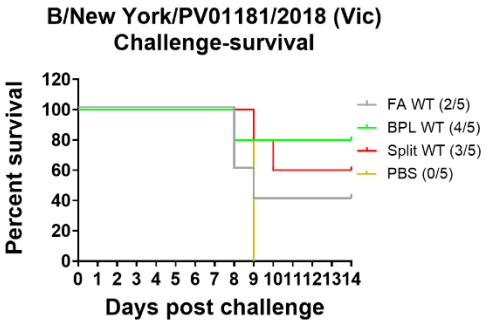

B/Lee /40
Challenge-weight loss
Percent initial weight
Days post challenge
B/Lee/40
Challenge-survival
Percent survival
Days post challenge
FA WT + CpG 1018 (4/5)
BPL WT + CpG 1018 (5/5)
Split WT + CpG 1018 (5/5)
PBS (0/5)

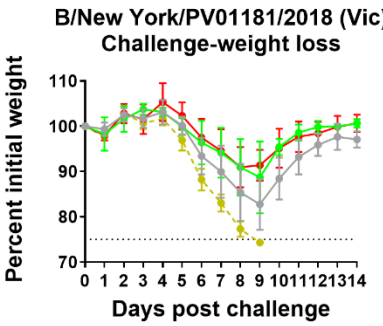

B/Lee /40
Challenge-weight loss
Percent initial weight
Days post challenge
B/Lee/40
Challenge-survival
Percent survival
Days post challenge
FA mHA (2/5)
BPL mHA (4/5)
Split mHA (2/5)
Split mHA (swap) (2/5)
PBS (0/5)

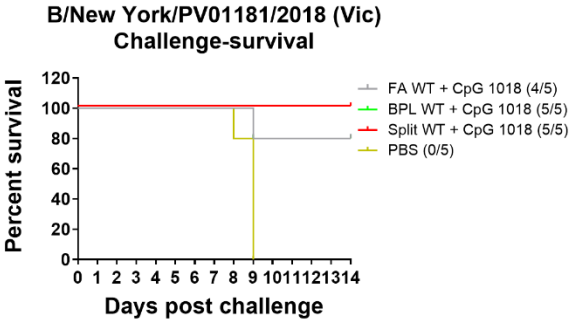

B/Lee /40
Challenge-weight loss
Percent initial weight
Days post challenge
B/Lee/40
Challenge-survival
Percent survival
Days post challenge
FA mHA + CpG 1018 (5/5)
BPL mHA + CpG 1018 (5/5)
Split mHA + CpG 1018 (4/5)
Split mHA (swap) + CpG 1018 (4/5)
Split mHA + AddaVax (5/5)
PBS (0/5)

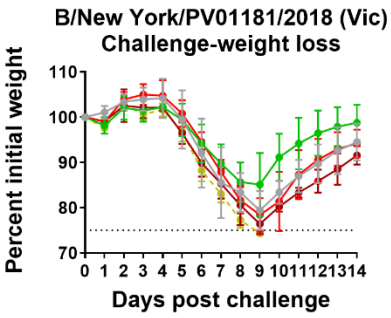
C

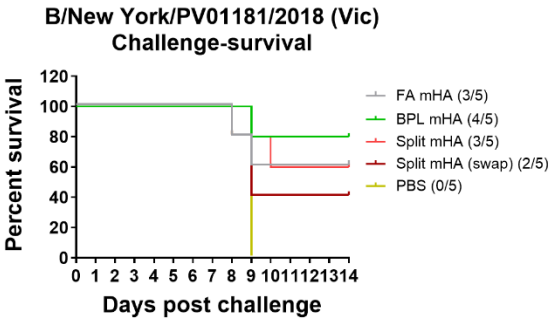

B/New York/PV01181/2018 (Vic)
Challenge-weight loss
Percent initial weight
Days post challenge

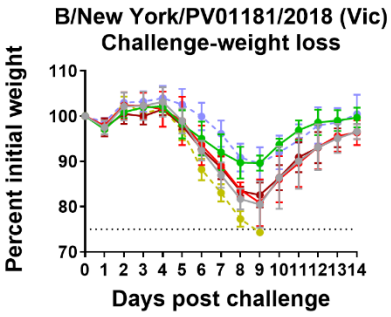

B/New York/PV01181/2018 (Vic)
Challenge-survival
Percent survival
Days post challenge
FA WT (2/5)
BPL WT (4/5)
Split WT (3/5)
PBS (0/5)

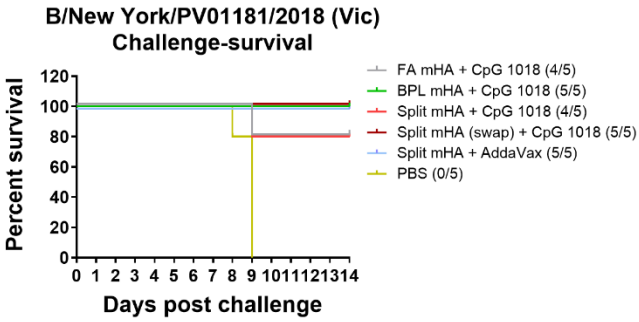

B/New York/PV01181/2018 (Vic)
Challenge-weight loss
Percent initial weight
Days post challenge


B/New York/PV01181/2018 (Vic)
Challenge-survival
Percent survival
Days post challenge
FA WT + CpG 1018 (4/5)
BPL WT + CpG 1018 (5/5)
Split WT + CpG 1018 (5/5)
PBS (0/5)

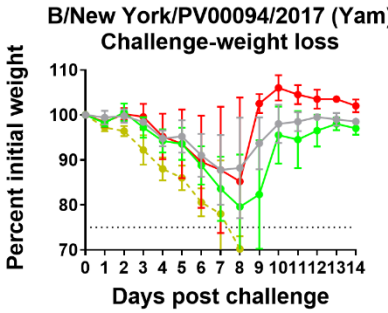

B/New York/PV01181/2018 (Vic)
Challenge-weight loss
Percent initial weight
Days post challenge

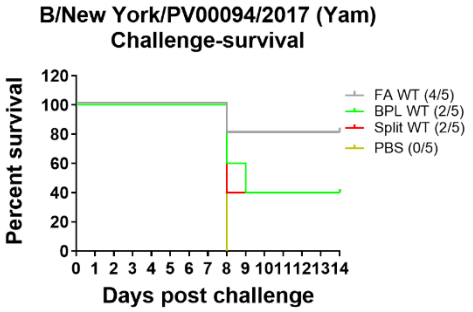

B/New York/PV01181/2018 (Vic)
Challenge-survival
Percent survival
Days post challenge
FA mHA (3/5)
BPL mHA (4/5)
Split mHA (3/5)
Split mHA (swap) (2/5)
PBS (0/5)

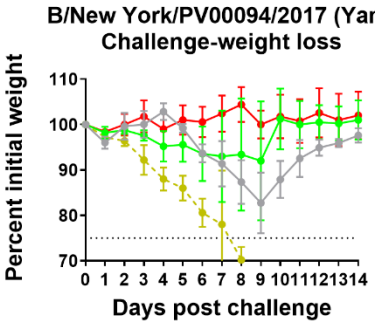

B/New York/PV01181/2018 (Vic)
Challenge-weight loss
Percent initial weight
Days post challenge

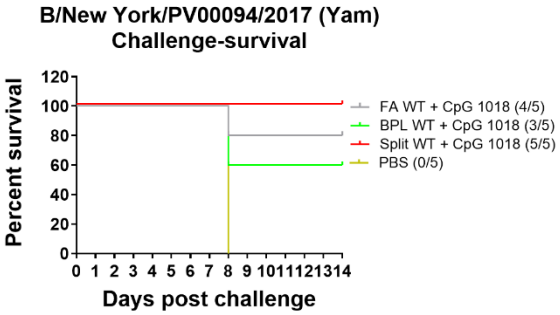

B/New York/PV01181/2018 (Vic)
Challenge-survival
Percent survival
Days post challenge
FA mHA + CpG 1018 (4/5)
BPL mHA + CpG 1018 (5/5)
Split mHA + CpG 1018 (4/5)
Split mHA (swap) + CpG 1018 (5/5)
Split mHA + AddaVax (5/5)
PBS (0/5)

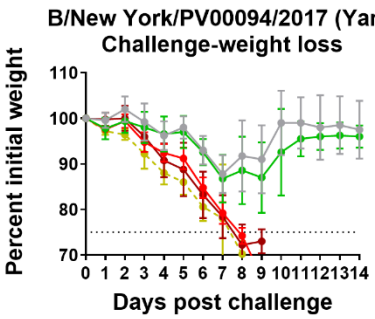
D

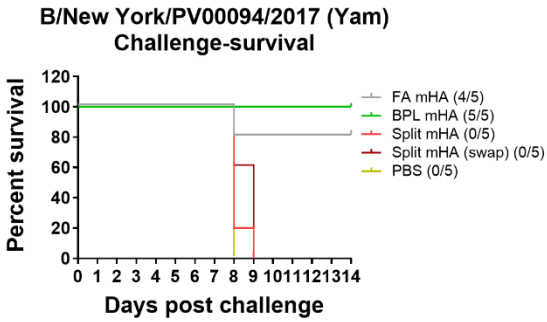

B/New York/PV00094/2017 (Yam)
Challenge-weight loss
Percent initial weight
Days post challenge

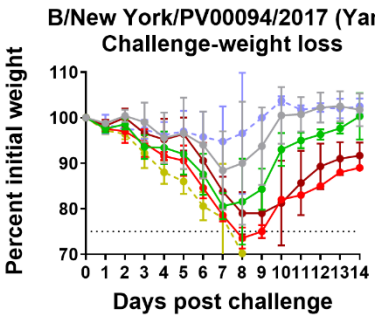

B/New York/PV00094/2017 (Yam)
Challenge-survival
Percent survival
Days post challenge
FA WT (4/5)
BPL WT (2/5)
Split WT (2/5)
PBS (0/5)

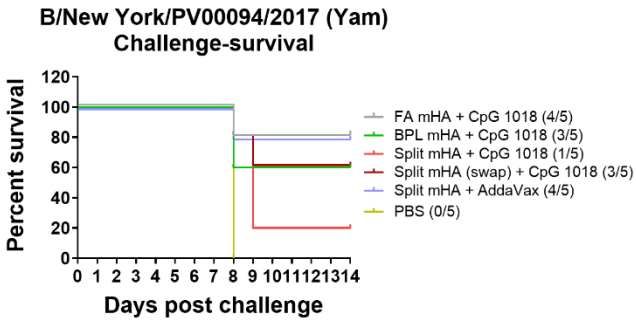

B/New York/PV00094/2017 (Yam)
Challenge-weight loss
Percent initial weight
Days post challenge

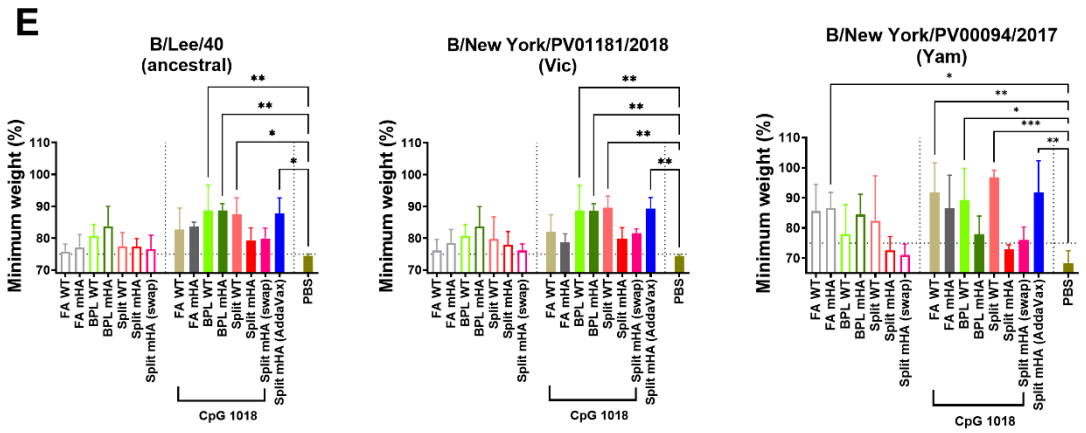

B/New York/PV00094/2017 (Yam)
Challenge-survival
Percent survival
Days post challenge
FA WT + CpG 1018 (4/5)
BPL WT + CpG 1018 (3/5)
Split WT + CpG 1018 (5/5)
PBS (0/5)


B/New York/PV00094/2017 (Yam)
Challenge-weight loss
Percent initial weight
Days post challenge

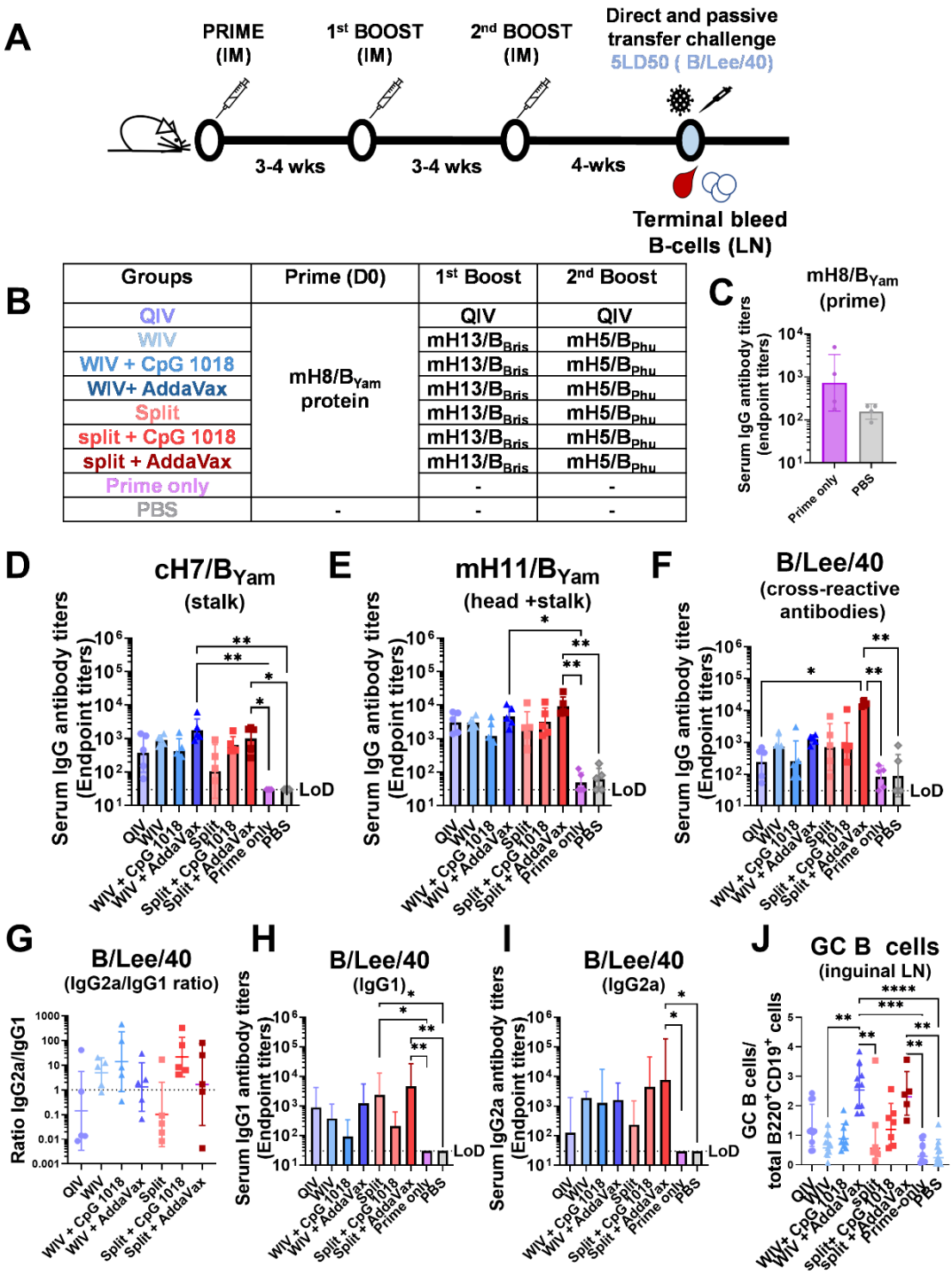

B/New York/PV00094/2017 (Yam)
Challenge-survival
Percent survival
Days post challenge
FA mHA (4/5)
BPL mHA (5/5)
Split mHA (0/5)
Split mHA (swap) (0/5)
PBS (0/5)


B/New York/PV00094/2017 (Yam)
Challenge-weight loss
Percent initial weight
Days post challenge


B/New York/PV00094/2017 (Yam)
Challenge-survival
Percent survival
Days post challenge
FA mHA + CpG 1018 (4/5)
BPL mHA + CpG 1018 (3/5)
Split mHA + CpG 1018 (1/5)
Split mHA (swap) + CpG 1018 (3/5)
Split mHA + AddaVax (4/5)
PBS (0/5)

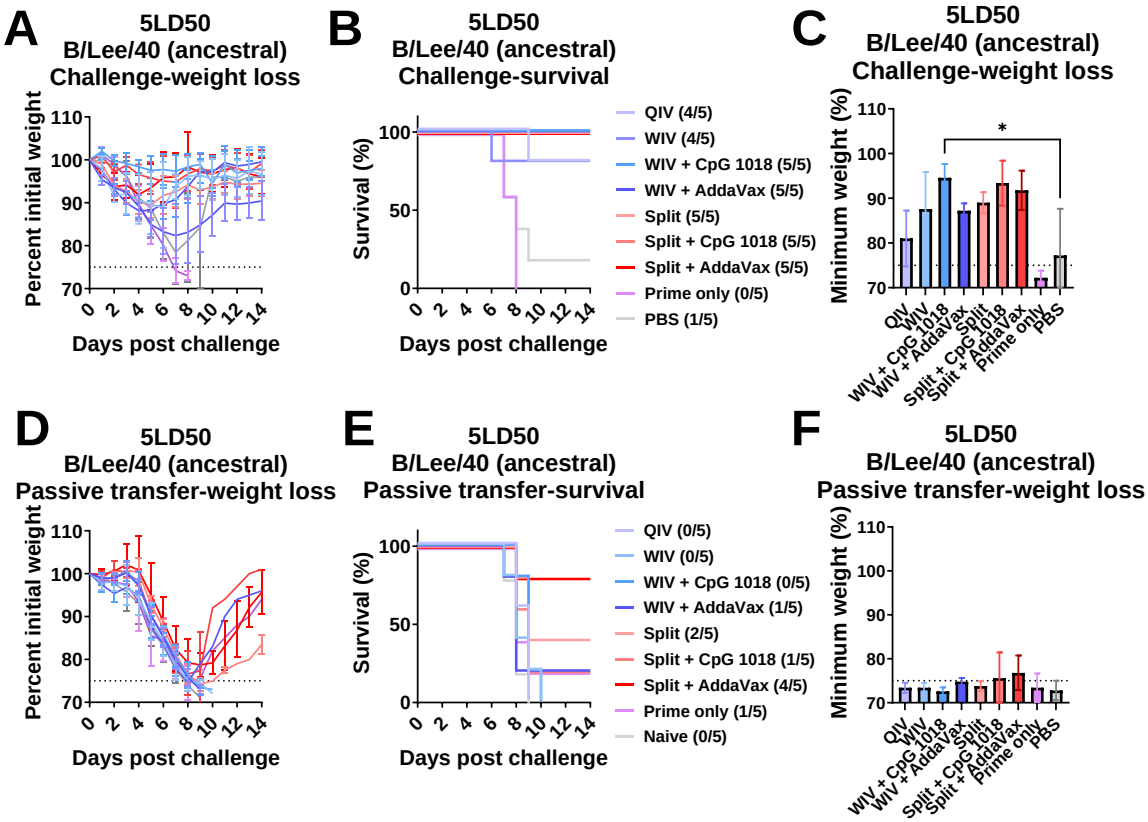


Supplementary Figure 4. Protection of WT and mHA inactivated vaccines by three different preparation methods of Fig. 2. Serum passive transfer experiment against B/Lee/40 virus (A), B/New York/PV01181/2018 virus (Victoria-lineage) (B) and B/New York/PV00094/2017 virus (Yamagata-lineage) (C). (A-D) Weight loss and survival of vaccination groups challenged with each virus. BALB/c mice received 100 µL of pooled sera intraperitoneally and 2 hours later were challenged intranasally with 5×mLD50 with each challenge virus in a total volume of 30 µL. Weight loss and survival of mice were monitored for 2 weeks with a humane endpoint of ≥25% loss of the initial weight. In the survival plots, the proportion of surviving animals in each group is shown. (E) Minimum weight reported during the passive transfer experiment. Mean ± SD for each group and statistical significance calculated using the Kruskal-Wallis test corrected using Dunn's test for multiple comparisons against PBS group is depicted (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

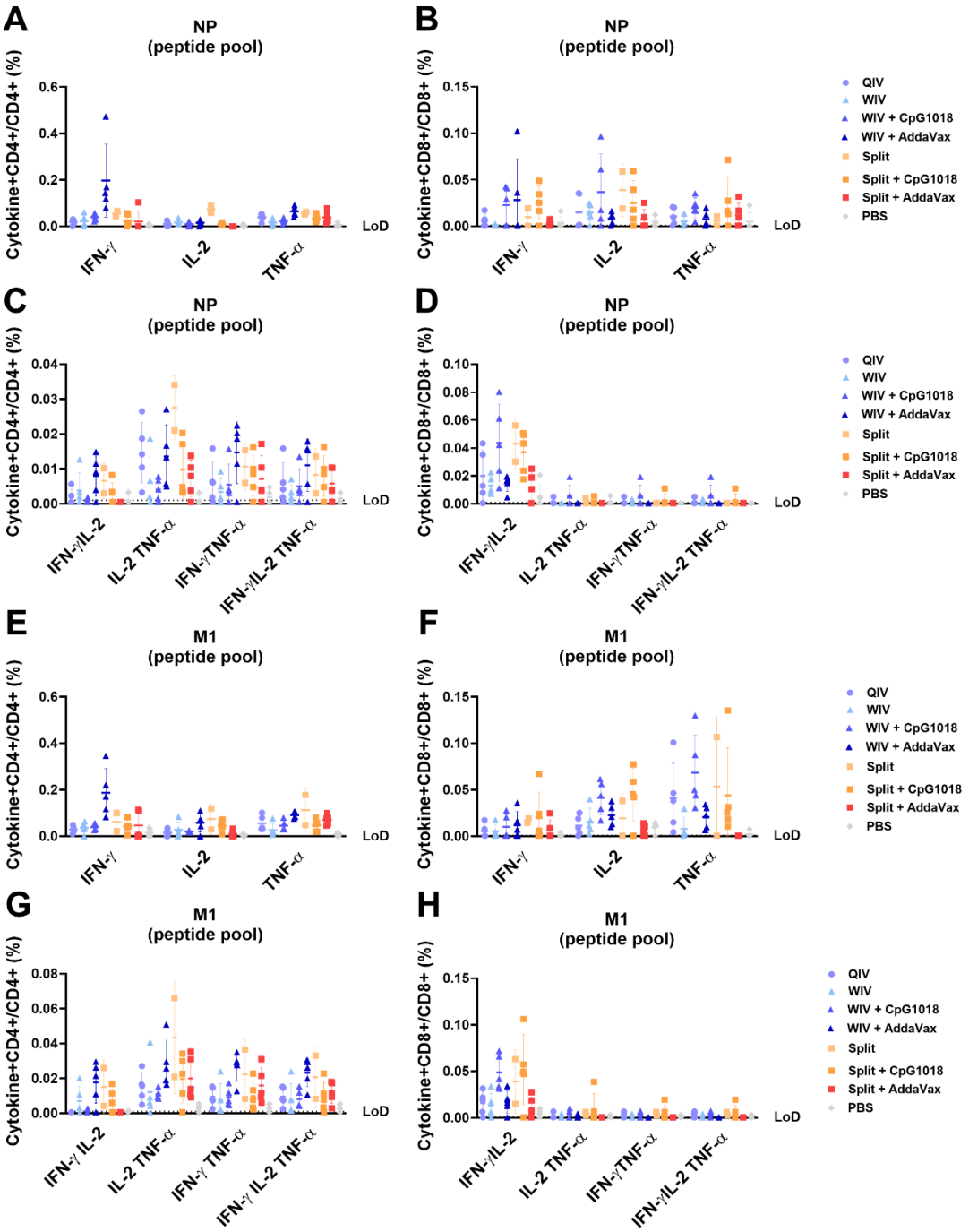


Supplementary Figure 5. Cross-reactivity, Th1/Th2 immunogenicity and germinal center B cell reaction of mHA vaccines in a low pre-existing immunity model. (A) Vaccination regimen and experimental workflow. (B) Vaccination groups. BALB/c mice were vaccinated in a two-dose vaccination scheme after a low priming vaccination with 1 µg of mH8/B_{Yam} protein followed by two doses of 1 µg HA of the different vaccines in a 3–4-week interval. WIV or split mHA vaccines were tested without adjuvant or with the addition of CpG 1018 (30 µg) or AddaVax (1:1 v:v). A QIV (FluLaval Quadrivalent) vaccinated group and an unvaccinated group (PBS) were included as controls. (C)

Binding of serum antibody titers against mH8/B_{Yam} protein from only prime and PBS group (D-F) Binding of serum antibody titers against subdominant and cross-reactive epitopes as previously described in Fig. 1 and S2. IgG2a/IgG1 ratio (G), IgG1 (H) and IgG2a (I) serum antibody titers against B/Lee/40 HA recombinant protein. (J) Frequency of germinal center B cells. Inguinal lymph nodes were collected 4 weeks after second boost and frequency of germinal center B cells (live $CD3^{-}B220^{+}CD19^{+}IgD^{-}GL7^{+}CD38^{low}$) was measured by FACS. Kruskal-Wallis test corrected using Dunn's test for multiple comparisons is depicted in these graphs (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P≤0.0001).

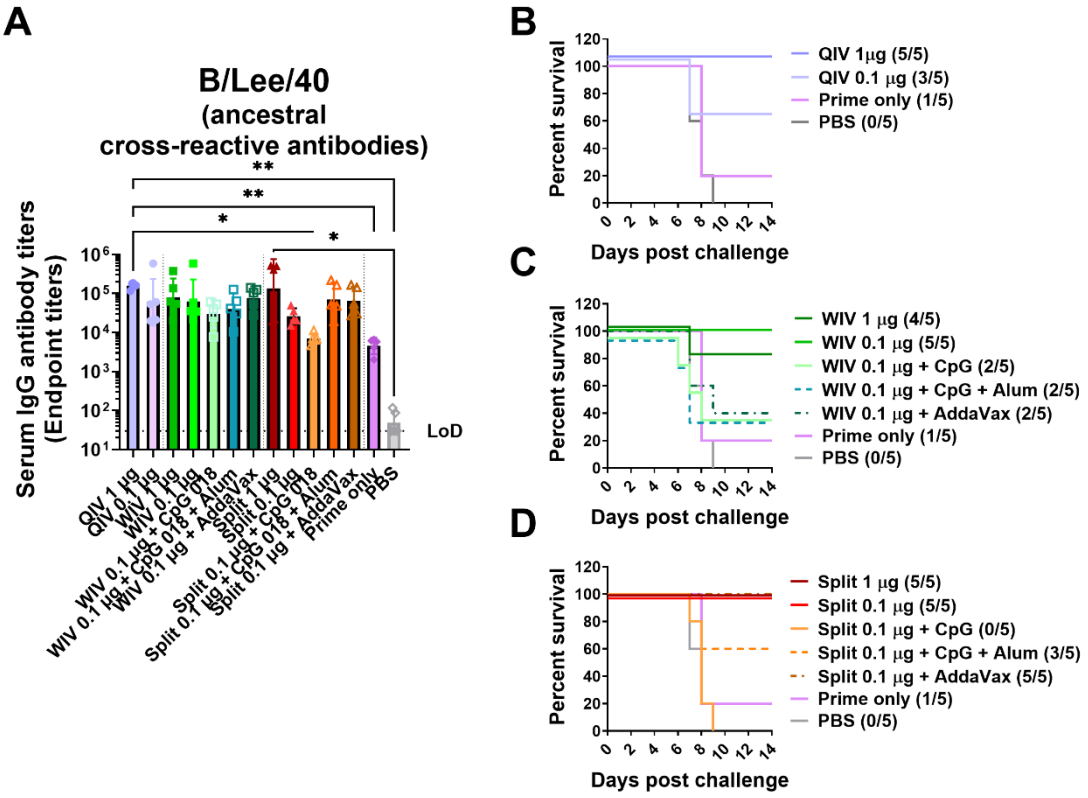


Supplementary Figure 6. In vivo protection of mHA vaccines in a low pre-existing immunity model. BALB/c mice were vaccinated in a two-dose vaccination scheme after priming with 1 µg of mH8/B_{Yam} protein as explained in Fig. 4. (A-C) Direct virus challenge (D-F) and serum passive transfer challenge with 5×mLD50 of the B/Lee/40 virus. Weight loss (A & D), survival of vaccination groups (B & E) and minimum weight (C & F) are reported for each experiment. For direct virus challenge, BALB/c mice were challenged intranasally in a total volume of 30 µL 4 weeks after second boost. In the serum passive transfer experiment, BALB/c mice received 100 µL of pooled sera intraperitoneally and 2 hours later were challenged IN as previously described. Weight loss and survival of mice were monitored for 2 weeks with a humane endpoint of ≥25% loss of the initial weight. Mean minimum weight ± SD for each group and statistical significance by Kruskal-Wallis test corrected using Dunn's test for multiple comparisons against PBS group are shown (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).



Supplementary Figure 7. Antigen-specific effector memory T cell immunity after vaccination with mHA inactivated vaccines measured by intracellular cytokine staining. BALB/c mice were vaccinated in a two-dose vaccination regime after high priming with 1 x 10^5 PFU of mH8/B_{Yam} virus with a dose of 1 µg of HA of the different vaccines in a 3-4 week interval. WIV or split mHA vaccines were tested without adjuvant or with the addition of CpG 1018 (30 µg) or AddaVax (1:1 v:v). A QIV (Flulaval Quadrivalent) vaccinated group and an unvaccinated group (PBS) were included as controls. One week after the last boost, mice were euthanized and splenocytes were

harvested. Splenocytes were *ex vivo* stimulated with NP (A-D) or M1 (E-H) peptide pools and intracellular cytokine staining was performed and analyzed by FACS. Intracellular cytokine staining was used to measure antigen-specific production of IFN-γ, TNF-α and IL-2, by $CD4^+$ **(A,C,E,G)** or $CD8^+$ T cells (**B,D,F,H)**.



Supplementary Figure 8. ***In vivo*** **cross-protection of direct virus challenge and serum passive transfer/challenge of mHA vaccines at a low dose in mice.** (A& B) Vaccination regimen and experimental workflow: BALB/c mice were vaccinated in a two-dose vaccination regime of 1 or 0.1 µg of HA of the different vaccines in a 3-4 week interval after high priming with 10^5 PFU of mH8/B_{Yam} virus. WIV or split mHA vaccines were tested without adjuvant or with the addition of CpG 1018 (10 µg), CpG 1018 (10 µg) + Alum (50 µg) or AddaVax (1:1 v:v). A QIV (Flulaval Quadrivalent) vaccinated group and an unvaccinated group (PBS) were included as controls. mH8/B_{Yam} virus prime infection was given intranasally in a total volume of 30 µL 6 months prior the two-dose immunization. Binding of serum antibody titers against B/Lee/40 HA (A) and survival from serum passive transfer challenge (B-D) with 5 mLD50 of B/Lee/40 virus was performed as previously described.