Supporting Information

Valkenburg et al. 10.1073/pnas.1403684111

SI Materials and Methods

ELISA and Microneutralization. Sera were treated with Receptor Destroying Enzyme II (RDE II, Accurate) before ELISA and microneutralization assay, according to manufacturer's instructions.

ELISA plates (Nunc MaxiSorp) were coated with H3N2 virus for influenza-specific isotyping ELISA. Virus particles were disrupted by 0.5% Nonidet P-40 (Sigma) in the presence of protease inhibitor (Roche) at 37 °C for 1 h. Serum samples were diluted (PBS, 0.1% BSA, 0.05% Tween-20), and anti-mouse Ig HRP conjugates (Imgenex) were used as the secondary antibody. TMB/peroxide was used as substrate and the reaction was stopped by addition of sulfuric acid (R&D Systems), and absorbance read at 450 nm.

Neutralization assay was performed using RDE-treated sera serially diluted in virus culture medium (1:50–1:6,400 of original sera) using standard protocol (1).

HA Yeast Surface Display Epitope Mapping (HA-YSD). H3N2 A/Hong Kong/1/68 HA-YSD library was constructed as described previously (2). In brief, HA amplicions amplified from viral cDNAs were randomly digested to 50-bp fragments by DNaseI, and reassembled by PCR to 100–500 bp and cloned into pCTCON2 vectors (gifts from K. D. Wittrup, Massachusetts Institute of Technology, Cambridge, MA). The HA plasmid library was transformed into yeast (EBY100), and incubated with RDE-treated sera. After extensive washing, the antibody–yeast complexes were stained with anti-IgG AF488 (Invitrogen). IgG-positive yeast were isolated by FACS sorting (FACS Aria, BD Biosciences), grown overnight, plasmids extracted (Zymoresearch), and transformed in bacteria and sequenced.

1. Webster RG, Cox N, Stöhr K (2002) *WHO Manual on Animal Influenza Diagnosis and Surveillance* (World Health Organization, Geneva, Switzerland).

2. Xu W, Han L, Lin Z (2011) Screening of random peptide library of hemagglutinin from pandemic 2009 A(H1N1) influenza virus reveals unexpected antigenically important regions. *PLoS ONE* 6(3):e18016.

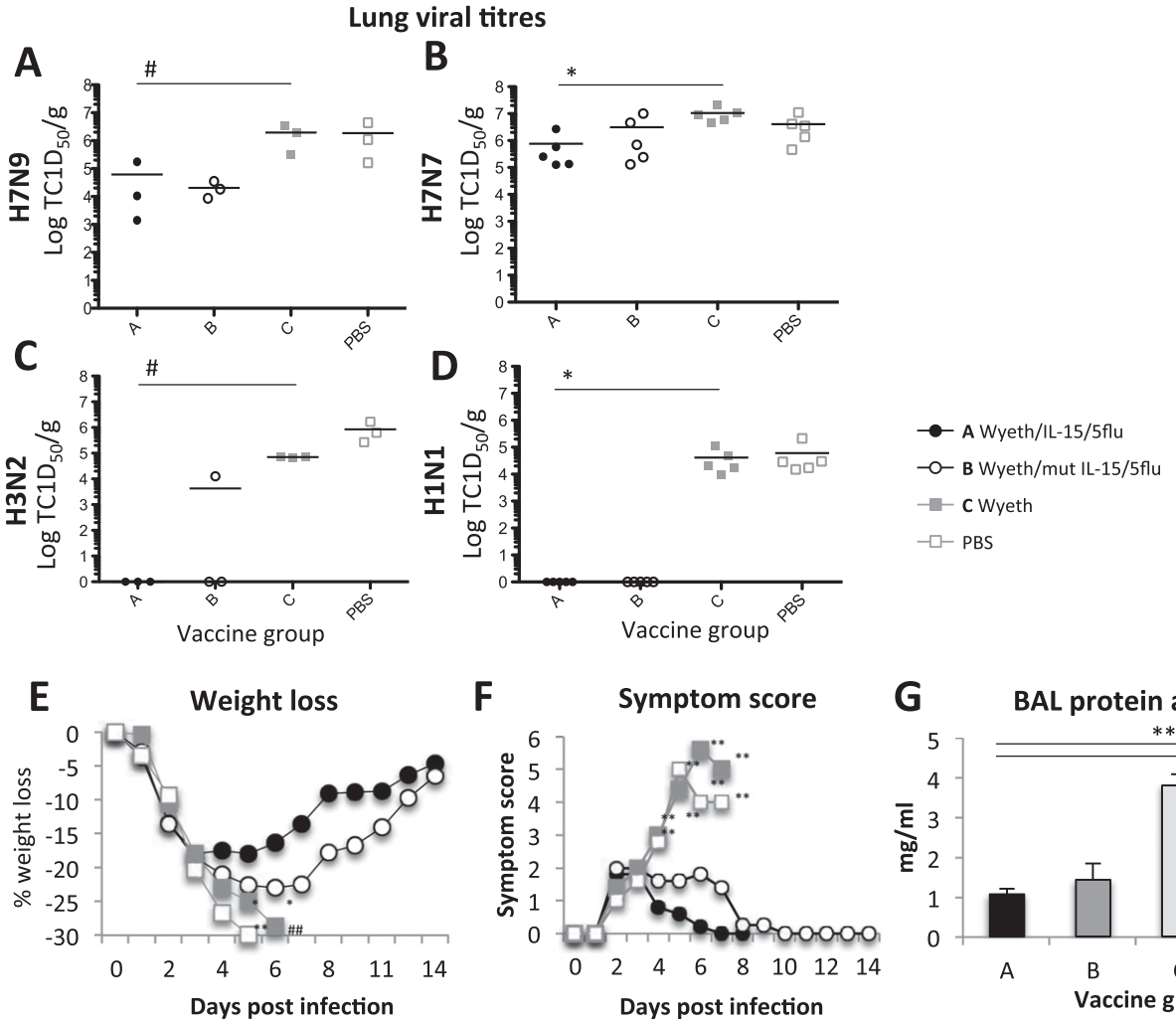


Fig. S1. Multivalent H5 vaccine reduces lung viral titers and morbidity following a lethal influenza challenge. Vaccinated mice were challenged with influenza viruses, (*A*) H7N9 (nonlethal, 1×10^5 $TCID_{50}$), (*B*) H7N7 (10 LD_{50}, $1 \times 10^5 TCID_{50}$), (*C*) H3N2 (40 LD_{50}, 4.72×10^5 $TCID_{50}$), or (*D*–*G*) H1N1 (40 LD_{50}, 9.5×10^5 $TCID_{50}$). (*A*–*D*) At day 7, lung viral titers were determined by standard $TCID_{50}$ assay on Madin-Darby canine kidney (MDCK) (data represent the results of individual mice, $n = 3$–5). (*E*) Mice were monitored daily for weight loss and (*F*) symptoms of influenza infection (combined scored from 0 to 6, ruffling, hunching, demeanor, activity). (*G*) Total protein concentration in the bronchoalveolar lavage (BAL) at day 7 postinfection was measured using a standard BCA assay at 560 nm. (*E* and *F*) Data represent mean from five mice. (*G*) Data represent mean ± SD from three mice. $^{\#}P < 0.05$, $*P < 0.01$, $^{\#\#}P < 0.005$, $**P < 0.001$ versus vaccine group A immunized with Wyeth/IL-15/5Flu.

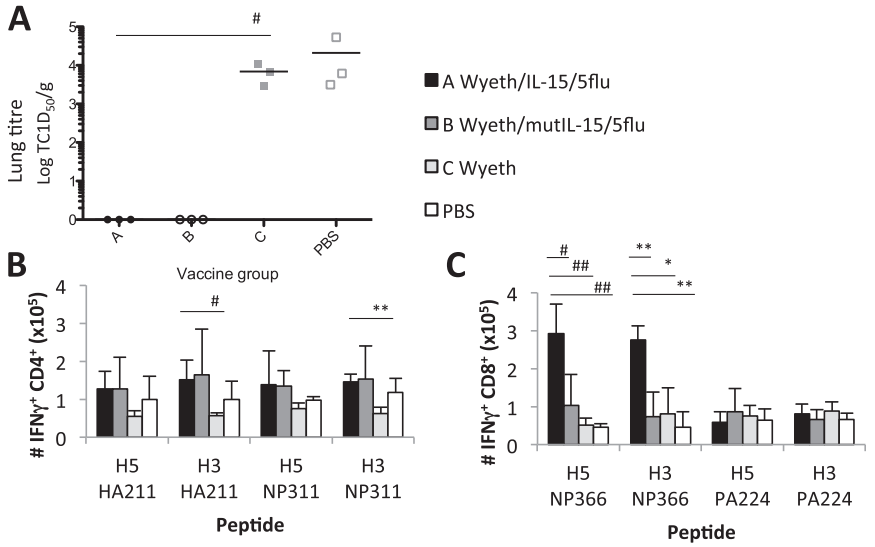


Fig. S2. Multivalent H5 vaccine is effective in B6 mice against H3N2 challenge. B6 mice were vaccinated with Wyeth/IL-15/5Flu, Wyeth/mut-IL-15/5Flu, control vaccine vector, Wyeth, or PBS, twice 3 wk apart and then challenged with 40 LD_{50} H3N2 virus. (*A*) Lungs were harvested at day 7 postchallenge from infected mice, and the viral titers determined by a standard $TCID_{50}$ assay on MDCK (data represents the results of individual mice, $n = 3$). Peptide-specific $CD4^+$ (*B*) and $CD8^+$ (*C*) T-cell responses in the spleens were measured by IFN-γ ICS assay following stimulation with HA_{211} (H5: YVKSNRLVLATGLRN or H3:----TKLR-------(- denotes conserved residues), NP_{311} (H5: QVFSLIRPNENPAHK or H3:--V--M---------), NP_{366} (H5:ASNENMEAM versus H3:-----V-I-) or PA_{224} (conserved). In *B*, and *C*, the cells were gated on $DAPI^+$, $CD3^+$, $CD4^+$ or $CD8^+$, $CD44^{hi}$, $CD69^+$, $Ki67^+$. Data represent mean ± SD from three mice. $^{\#}P < 0.05$, $^{**}P < 0.001$ versus vaccine group A immunized with Wyeth/IL-15/5Flu.

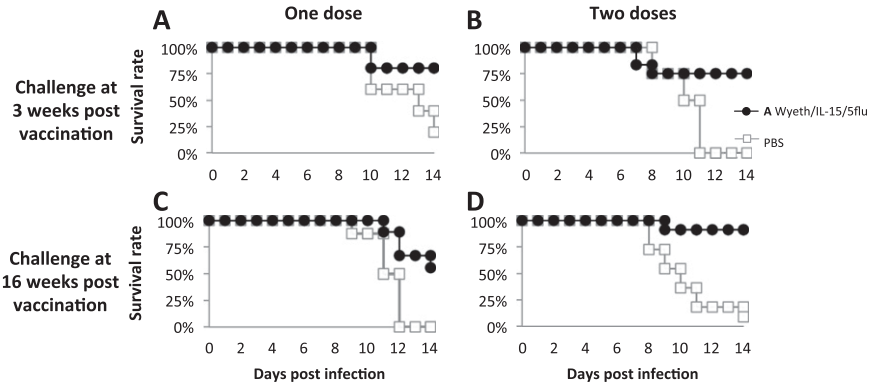
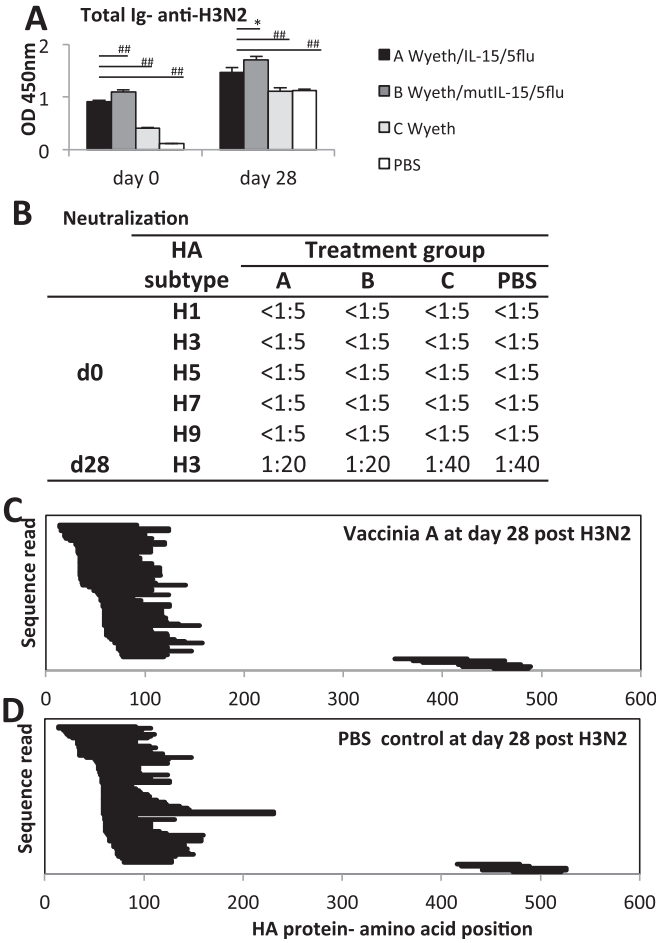


Fig. S3. The vaccine is effective with one dose and provides long term protection. BALB/c mice were vaccinated with Wyeth/IL-15/5Flu or PBS with a single dose (*A* and *C*) or two doses (*B* and *D*) and challenged 3 wk later (*A* and *B*) or 16 wk later (*C* and *D*) with 10 LD_{50} H7N7 virus. Mice were monitored daily for survival for 14 d after influenza challenge. Data represent the total percentage of mice surviving the lethal challenge ($n = 6$–11 mice).



	HA subtype	Treatment group A	B	C	PBS
d0	H1	<1:5	<1:5	<1:5	<1:5
	H3	<1:5	<1:5	<1:5	<1:5
	H5	<1:5	<1:5	<1:5	<1:5
	H7	<1:5	<1:5	<1:5	<1:5
	H9	<1:5	<1:5	<1:5	<1:5
d28	H3	1:20	1:20	1:40	1:40

Fig. S4. The vaccine induces high-titer antibody responses that are nonneutralizing and predominantly HA1 specific. Mice were vaccinated twice and then challenged with 40 LD_{50} H3N2 virus. At day 0 and day 28 postinfection, anti-influenza antibodies in the sera were measured by an ELISA with whole H3N2 virus (*A*). $^{*}P < 0.01$, $^{\#\#}P < 0.005$. To detect neutralizing antibodies, a panel of influenza viruses was used in a microneutralization assay (*B*). Data represent (mean ± SD) three mice per group from pooled sera. (*C* and *D*) H3-HA-YSD library map of day 28 postchallenge sera from mice vaccinated with Wyeth/IL-15/5Flu and PBS groups. Binding profile differences are not significant between sera from Wyeth/IL-15/5Flu and PBS vaccinated groups (χ^2 analysis). The experimental procedures for binding antibody characterization with H3-HA-YSD library are given in *SI Materials and Methods*.

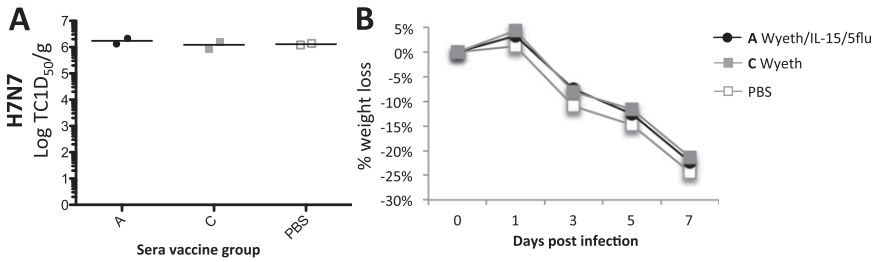


Fig. S5. Immune vaccine sera are unable to mediate passive heterosubtypic protection. Immune sera were harvested and pooled from mice vaccinated with Wyeth/IL-15/5Flu, Wyeth, or PBS (two doses of vaccine and sera collected 28 d later, $n = 16$). Sera were heat inactivated at 56 °C for 1 h, and 400 μL of sera were given intraperitoneally at days −3, −2, −1, 0, and +1 (relative to the day of influenza challenge). Mice were challenged with 10 LD_{50} of HPAI H7N7 (NL/219 challenge). In *A*, lung viral loads were measured at day 7 by standard $TCID_{50}$ assays on MDCK cells. Weight loss in recipient mice were also monitored (*B*) ($n = 2$).

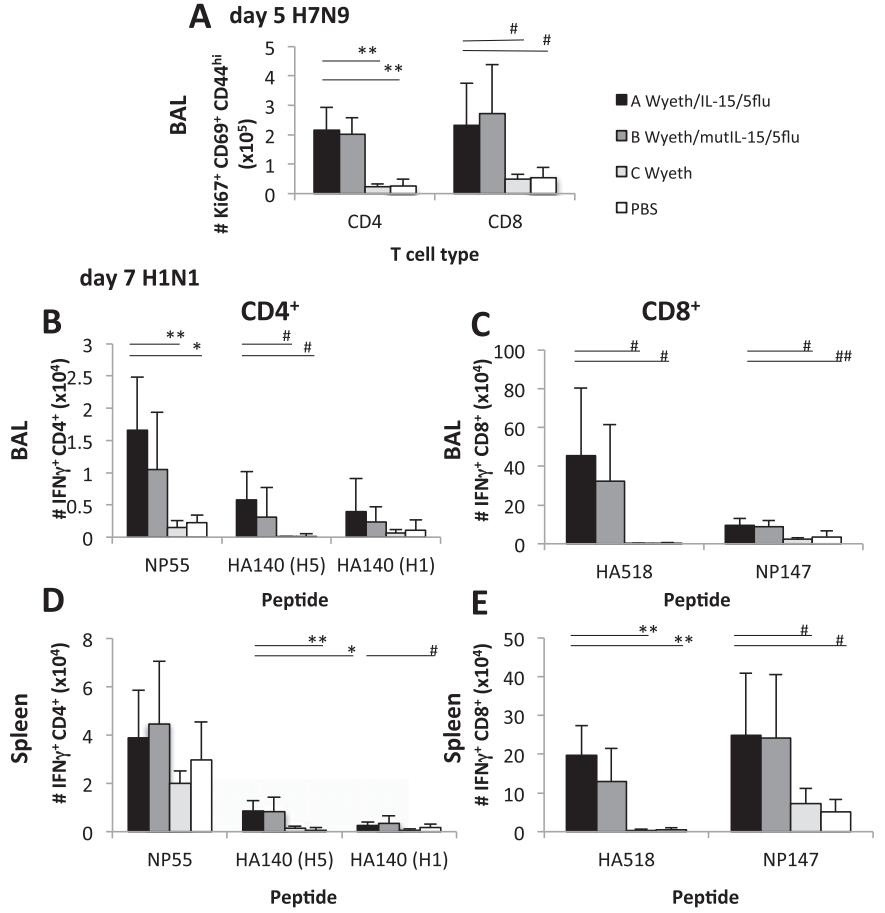


Fig. S6. Vaccination recalls activated-proliferating and influenza peptide-specific $CD4^+$ and $CD8^+$ T-cell responses after H7N9 and H1N1 challenges. Vaccinated BALB/c mice were challenged, (*A*) with H7N9 (A/SH/2/2013, nonlethal 1×10^5 $TCID_{50}$), or (*B*–*E*) with H1N1 virus (A/California/4/2009, 40 LD_{50}). (*A*) Activated and proliferating $CD4^+$ and $CD8^+$ T-cell responses were measured in the BAL. Peptide-specific $CD4^+$ (*B* and *D*) and $CD8^+$ (*C* and *E*) T-cell responses were measured by IFN-γ intracellular cytokine staining (ICS) assay following stimulation with NP_{55} or HA_{140} peptides [HA_{140} H5: KSSFFRNVVWLIKKN or H3: G-G–SRLN–T-SG (- denotes conserved residues)]. In *A*, cells were gated on $DAPI^+$, $CD3^+$, $CD4^+$ or $CD8^+$, $CD44^{hi}$, $CD69^+$, $Ki67^+$. In *B* through *E*, cells were gated on FSC/SSC, $CD4^+$ or $CD8^+$, IFN-γ^+. Data represent mean ± SD; $n = 3$–5, and the H1N1 experiment was repeated at least twice. $^{\#}P < 0.05$, $^{*}P < 0.01$, $^{\#\#}P < 0.005$, $^{**}P < 0.001$ versus Wyeth/IL-15/5Flu–vaccinated group.

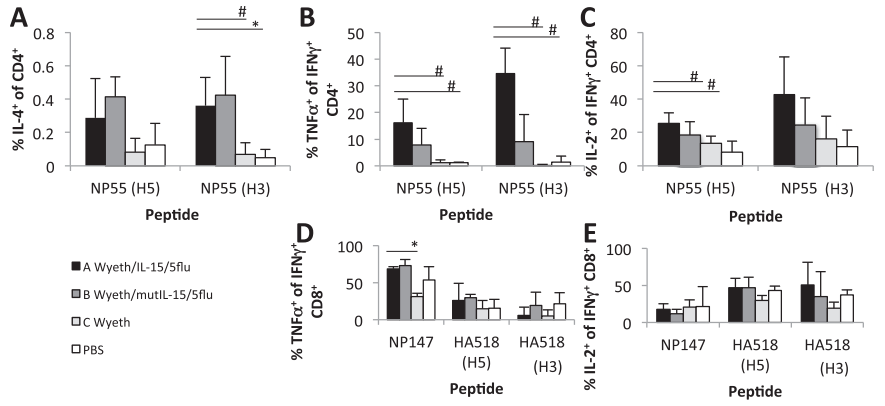


Fig. S7. Vaccination increases $CD4^+$ T-cell polyfunctionality after H3N2 challenge. Vaccinated BALB/c mice were challenged with H3N2 and at day 7 splenocytes were assessed for multiple cytokines by standard ICS. (*A*) IL-4 production by $CD4^+$ T cells that were IFN-γ^-; (*B*) IFN-γ^+ $CD4^+$ cells that were also positive for TNF-α, and (*C*) IFN-γ^+ $CD4^+$ cells that were also IL-2^+ are shown. IFN-γ^+ $CD8^+$ T cells producing TNF-α (*D*), and IL-2 (*E*) were also assessed. Data represent mean ± SD from three mice. $^{\#}P < 0.05$, $^{*}P < 0.01$ versus Wyeth/IL-15/5Flu–vaccinated group.

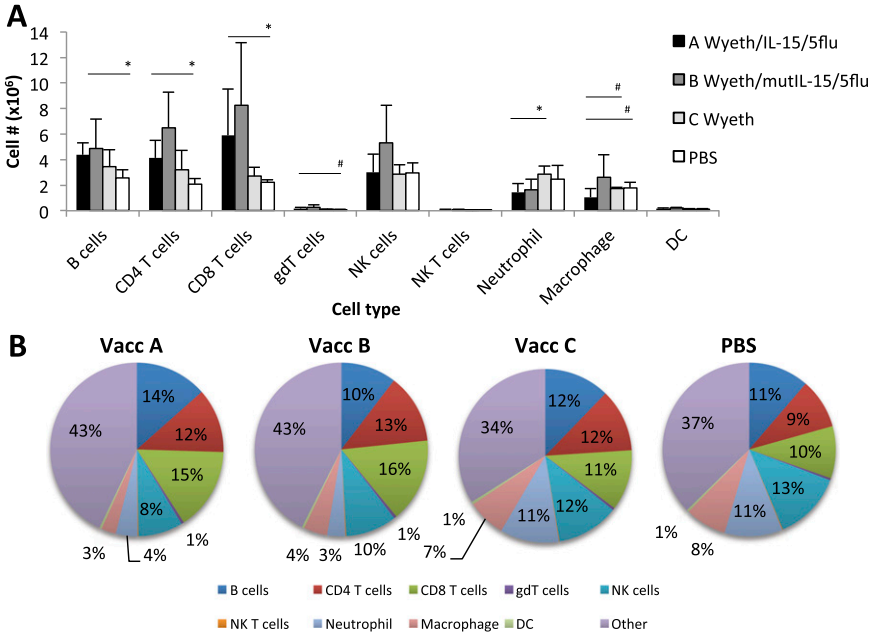


Fig. S8. Immune cell profiles in the lungs after H1N1 challenge in vaccinated mice show increased T cells and reduced neutrophils. Vaccinated BALB/c mice were challenged with 40 LD_{50} H1N1virus. Lungs were sampled at day 7, stained with a mixture of antibodies for innate and adaptive immune cells, and analyzed by flow cytometry. (*A*) Data represent total cell numbers (mean ± SD) from three mice. (*B*) Pie charts represent average cell type percent frequency at day 7 after H1N1 challenge ($n = 3$). Experiments were repeated at least twice. $^{\#}P < 0.05$, $^{*}P < 0.01$, versus Wyeth/IL-15/5Flu–vaccinated group.

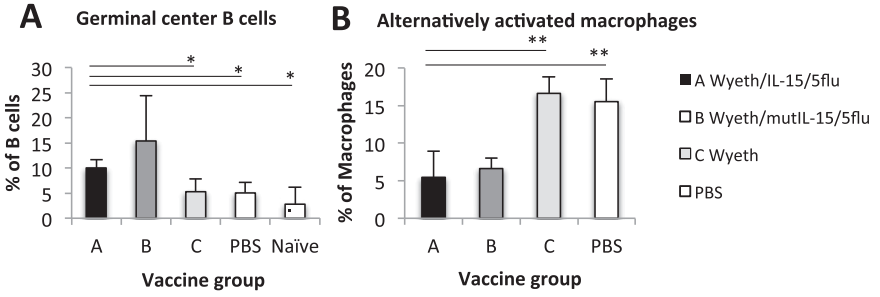


Fig. S9. Vaccination increases germinal center B cells but alternatively activated macrophages are diminished in the lungs after influenza challenge. Vaccinated mice were challenged with 40 LD_{50} H3N2 (*A*) or H1N1 (*B*) virus. In *A*, at day 7 postchallenge, mediastinal lymph node cells were stained with a mixture of mAb for germinal center B cells ($DAPI^{+}$, $B220^{+}$, $CD19^{+}$, PNA^{+}, Fas^{+}, $Bcl6^{+}$). In *B*, at day 7 after H1N1 challenge, the cells harvested from the lungs were stained with a mixture of mAb for alternatively activated macrophages ($F4/80^{+}$, MHC-$II^{int/hi}$, $CD11b^{+}$, IL-$4Ra^{+}$, $iNOS^{+}$, MMR^{+}). Data represent mean ± SD from three to five mice; $^{*}P < 0.01$, $^{**}P < 0.001$; experiments were repeated at least twice.