Supporting Information

Galli *et al.* 10.1073/pnas.0813390106

SI Materials and Methods

Study Protocol. This was a phase-II, randomized, controlled, observer-blind, single-center study, carried out in Italy from 2006 to 2008. The study protocol (registered at Clinical Trials.gov as NCT 00382187) was in accordance with the Helsinki Declaration and Good Clinical Practice principles and approved by the local Ethics Committee. The vaccine was a monovalent H5N1 subunit from the A/Vietnam/1194/2004 influenza virus obtained by reverse genetics (NIBRG-14) and grown in hens' eggs. Forty healthy adults (63% males, 38% females, mean age 34.8 years, range 24–50 years) were enrolled and randomized in a 1:1:1 ratio to 3 groups. All groups were similar in respect to age, sex, and ethnicity. All enrolled subjects gave their written informed consent and none of them had serious health problems or a history of allergies to vaccines' components or was under immune-suppressive therapy. Vaccines were administered at days 1, 22, and 202 in the deltoid muscle in a volume of 0.5 mL, using coded prefilled syringes. One group received 15 μg of plain H5N1 (Non-Adj-15, $n = 13$), another group received 7.5 μg of H5N1 adjuvanted with MF59 (MF59–7.5, $n = 14$; Aflunov), and the third group received 15 μg of H5N1 adjuvanted with MF59 (MF59–15, $n = 13$). As in previous studies (1–5), MF59 was mixed with the antigen at a 1:1 ratio (vol:vol).

Immunogenicity assays were carried out on coded specimens collected at baseline, 3 weeks after the first and the second immunization (days 1, 22, and 43; $n = 13$, 14, and 13 from the Non-Ad-15, the MF59–7.5, and the MF59 group, respectively), 3 and 6 months after the second immunization (day 130, $n = 13$, 14, and 12; and day 202, $n = 12$, 13, and 12), and 3 weeks and 6 months following the booster dose (day 223, $n = 11$, 13, and 12; and day 382, $n = 11$, 12, and 10).

Peripheral Blood Mononuclear Cells Preparation. PBMC were isolated by Ficoll gradient (Amersham Pharmacia) centrifugation of heparinized blood within 6 h after bleeding and either used immediately or frozen at −150 °C. PBMC were thawed in PBS containing 20 μg/ml DNase (Sigma-Aldrich), washed, and diluted in complete medium (RPMI with 100 units/mL penicillin, 100 μg/mL of streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids; Invitrogen). Average viability of thawed PBMC samples was >90%.

Analysis of Antigen-Specific T Cell Response. The antigen-specific T cell response was assessed by stimulating PBMC with H5N1 or the following pools of peptides (18-mers overlapping by 10): (*i*) a pool of 70 peptides spanning the whole H5 A/Vietnam/1194/2004 protein; (*ii*) a pool of 50 peptides spanning the regions of H5 conserved among the strains A/Vietnam/1194/2004, A/Indonesia/5/2005, and A/Duck/Singapore/97; (*iii*) a pool of 18 peptides spanning the regions of H5 unique to A/Vietnam/1194/2004; (*iv*) a pool of 18 peptides spanning the regions of H5 unique to A/Indonesia/5/2005; and (*v*) a pool of 29 peptides spanning the regions of H5 unique to A/Duck/Singapore/97. All peptides were synthesized at the Institute of Biochemistry (University of Lausanne, Switzerland) and purified to 90% by HPLC.

PBMC were stimulated with the different peptide pools (final concentration of each individual peptide: 2.5 μg/mL) or H5N1 (final concentration: 1 μg/mL) in the presence of 1 μg/mL of anti-CD28 and anti-CD49d mAbs (Becton Dickinson). Brefeldin A was used at 5 μg/mL (Sigma-Aldrich). PBMC cultures in medium alone or in the presence of 1 μg/mL of an anti-CD3 mAb (Becton Dickinson) were included in each assay as negative and positive controls. PBMC were stained with the LIVE/DEAD aqua viability marker (Invitrogen), fixed and permeabilized with the cytofix/cytoperm kit (BD Biosciences), and incubated with Pacific blue-anti-hCD3, PerCP-Cy 5.5-anti-hCD4, FITC-anti-hIFN-γ, APC-anti-hIL-2, and PE-anti-h-IL-13. For each sample 1×10^6 events were acquired using a LSRII instrument (BD Bioscience) and analyzed with FlowJo software (TreeStar). Response to medium was subtracted from responses in stimulated samples for each of the response patterns.

Enumeration of H5N1-Specific Memory B Cells (MBC). Frequencies of MBC were determined by the ELISA-coupled limiting dilution assay. PBMC were plated in 0.2 ml of RPMI with 5% FBS in serial 2-fold dilutions, 6 replicates per dilution, starting from 8×10^5 PBMC/well, in 96-well U-bottom plates containing 2.5 μg/ml of a phosphorothioate CpG oligonucleotide (tcg tcg ttt tgt cgt ttt gtc gtt; Primm), 1:10,000 fixed *Staphylococcus aureus* Cowan Antigen (SAC, Calbiochem), 1:100,000 pokeweed mitogen extract (Sigma), and 1,000 units/mL rhIL-2 (Proleukin, Novartis). Parallel control cultures of PBMC were run in medium alone. On day 10 individual supernatants were collected and kept at −20 °C until tested in ELISA for their content in H5N1-specific and total IgG. ELISA assays were run on Maxisorp plates (Nunc) precoated with either H5N1 (A/Vietnam/1194/2004, 5 μg/ml in PBS, pH 7.5) or a polyclonal sheep IgG fraction against whole human IgG (Sigma) (2 μg/mL in PBS, pH 7.5) and developed with an alkaline-phosphatase-conjugated anti-human γ-chain polyclonal sheep antiserum (1:4,000 in PBS, 0.05% Tween, 3% BSA), followed by incubation with the substrate *p*-nitrophenylphosphate (Sigma). Wells displaying at 405 nm an OD $\geq$0.4 (total IgG) or an OD $\geq$0.45 (H5N1-IgG) (5-fold higher than the blank OD) were considered positive. The PBMC dilution containing one antibody-secreting cell precursor was derived by applying the Reed and Muench algorithm (6) to the distribution of antibody-positive and -negative wells among replicates. Frequencies of H5N1-IgG secreting cell precursors (H5N1-IgG MBC) were expressed as percentage of the total IgG MBC precursors measured. Numbers of subjects analyzed were as follows: 13, 14, and 13 at day 1; 11, 14, and 13 at day 22; 11, 11, and 12 at day 43; 8, 8, and 9 at day 202; 11, 12, and 12 at day 223; and 11, 11, and 10 at day 382 for the Non-Adj-15, the MF59-H5N1 7.5, and the MF59-H5N1 15 group, respectively.

Titration of Neutralizing Antibodies. Pre- and postvaccination sera were heated at 56 °C for 30 min. Titers of H5N1-specific antibodies were determined by microneutralization assay, applying an ELISA endpoint reading (1) and using the homologous A/Vietnam/1194/2004 NIBRG-14 recombinant vaccine strain.

Sera were tested in duplicate, in serial 2-fold dilutions. Titers are expressed as reciprocal value of the highest dilution giving $\geq$50% neutralization of virus growth. Control sera from humans and sheep immunized with the vaccine A/Vietnam/1194/2004 strain were included in each assay. A titer of 10 was assigned to sera that gave a negative result at the first (1:20) dilution.

Descriptive Statistics. Descriptive statistics were calculated by vaccine group, using the Statistical Analysis System (SAS) software version 9.1 (SAS Institute). MN antibody titers were Log_{10} transformed. For the 3 vaccine groups, GMTs, GMRs, and their 95% CIs were computed by exponentiation (base 10) the least-squares means and 95% CIs of the Log_{10} titers. These were obtained from a 1-way ANOVA with a factor for vaccine group.

Comparisons within each vaccination group between values at day_0 and at day_x were done by Wilcoxon's test for dependent variables (WinSTAT for Excel 2002). Comparisons between groups were done by 1-factor ANOVA, applying the least significant difference post hoc test for multiple comparisons (WinSTAT for Excel 2002). Differences in the contribution of each cytokine subset to the total population H5-specific and H5N1-specific $CD4^+$ T cells were analyzed by the Kruskal–Wallis *H*-test, followed by Wilcoxon's test for pairwise comparisons (WinSTAT for Excel 2002).

1. Nicholson KG, *et al.* (2001) Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. *Lancet* 357(9272):1937–1943.
2. Atmar RL, *et al.* (2006) Safety and immunogenicity of nonadjuvanted and MF59-adjuvanted influenza A/H9N2 vaccine preparations. *Clin Infect Dis* 43(9):1135–1142.
3. Stephenson I, *et al.* (2005) Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. *J Infect Dis* 191(8):1210–1215.
4. Stephenson I, *et al.* (2003) Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3 A/Duck/Singapore/97 vaccine in a primed human population. *Vaccine* 21(15):1687–1693.
5. Podda A, Del Giudice G (2003) MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile. *Expert Rev Vaccines* 2(2):197–203.
6. Reed LJ, Muench H (1938) A simple method of estimating fifty per cent endpoints. *Am J Epidemiol* 27(3):493–497.

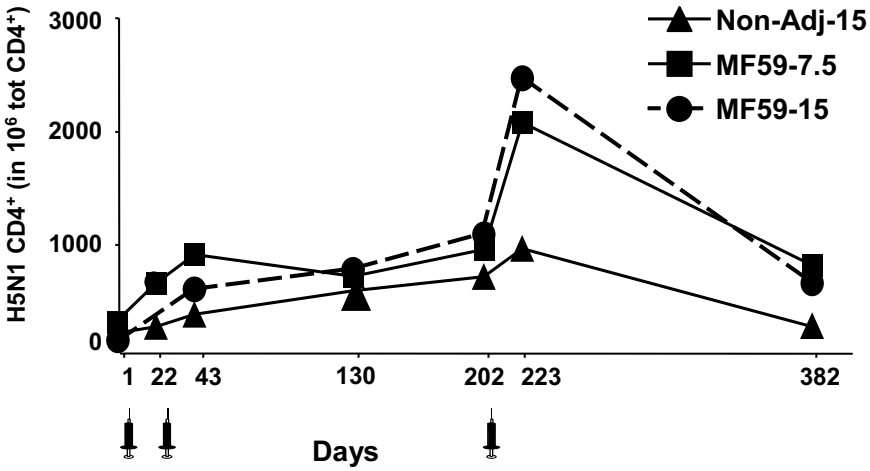

Fig. S1. One dose of MF59-H5N1 induces a stable pool of H5N1-specific $CD4^+$ T cells. Shown is the mean frequency of cytokine$^+$ $CD4^+$ T lymphocytes following in vitro stimulation of PBMC with H5N1, the antigen preparation of the vaccine (H5N1-$CD4^+$ T). At all time points frequencies of H5N1-$CD4^+$ T cells in the groups of subjects vaccinated with MF59-adjuvanted H5N1 at 7.5 μg/dose (squares) or with MF59-adjuvanted H5N1 at 15 μg/dose (circles), but not in subjects vaccinated with the nonadjuvanted H5N1 at 15 μg/dose (triangles), were significantly higher than at baseline ($P < 0.05$; Wilcoxon's test for dependent variables).

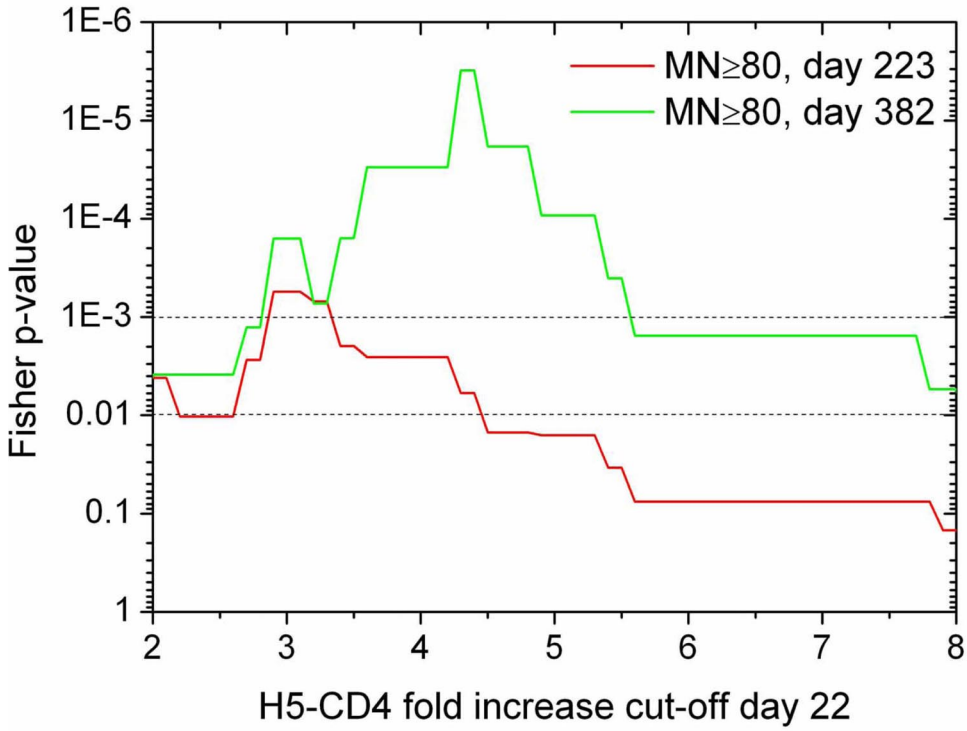

Fig. S2. Association between expansion of H5-CD4$^+$ T cells after the first dose and MN titers ≥80 at later time points. Shown are the Fisher's exact test 2-tailed *P* values for the association between the whole range of fold increases over preimmune values in H5-CD4$^+$ T cells at day 22 (≥cutoff values depicted on the *x*-axis) and MN titers ≥80 at days 223 (red line) and 382 (green line). The association was considered significant for $P < 0.01$ and highly significant for $P < 0.001$ (dotted lines). A ≥3-fold increase of H5-CD4$^+$ T cells after the first dose significantly associates with MN titers ≥80 at all time points investigated.

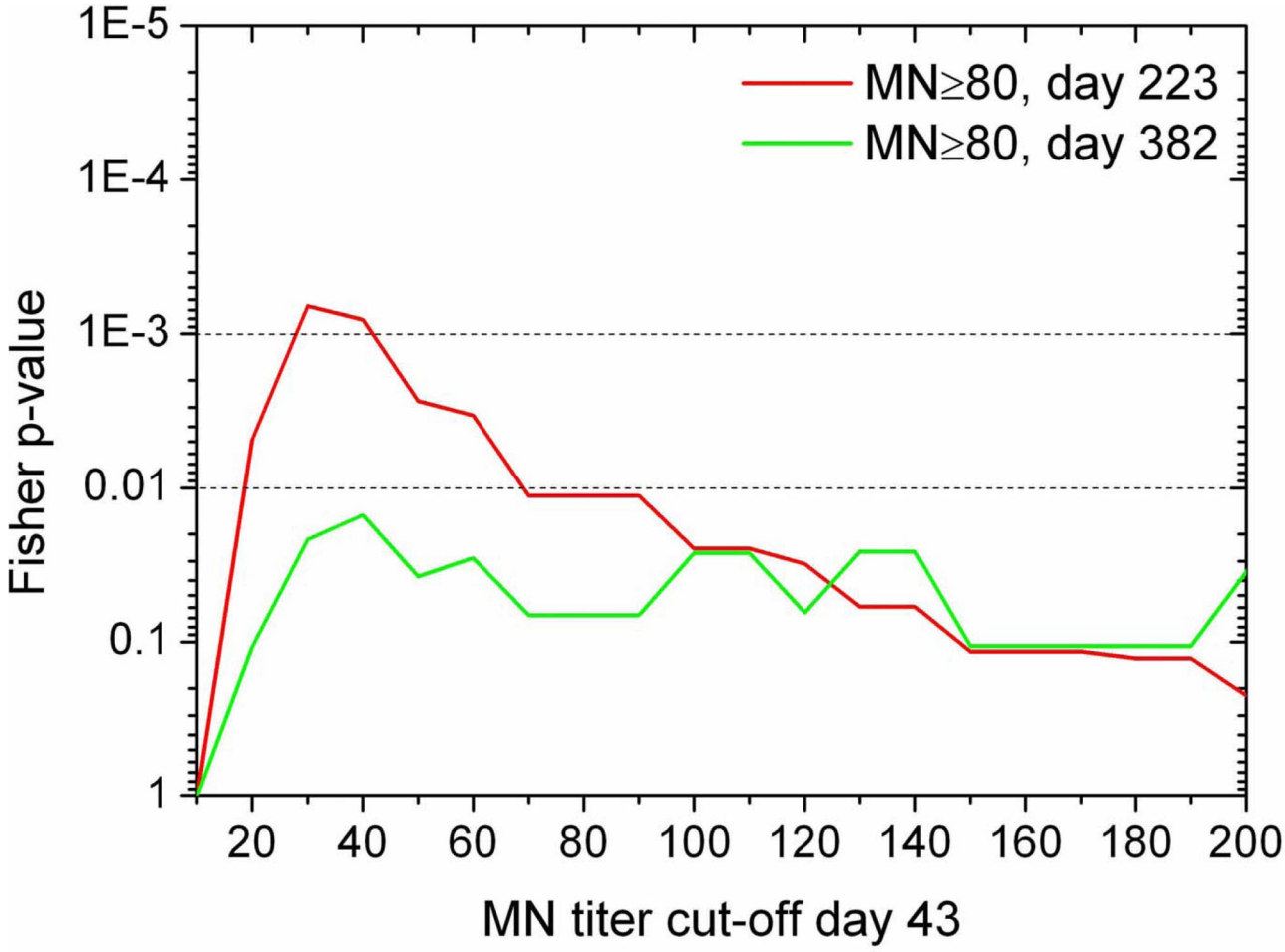

Fig. S3. Association between MN antibody titers at day 43 and MN titers ≥80 at later time points. Shown are the Fisher's exact test 2-tailed *P* values for the association between MN antibody titers at day 43 (equal to or greater than cutoff values depicted on the *x*-axis) and MN titers ≥80 at days 223 (red line) and 382 (green line). The association was considered significant for $P < 0.01$ and highly significant for $P < 0.001$ (dotted lines). MN titers ≥40 at day 43 are significantly associated with MN titers ≥80 at day 223, but no cutoff for MN titers at day 43 is associated with MN titers ≥80 at day 382.

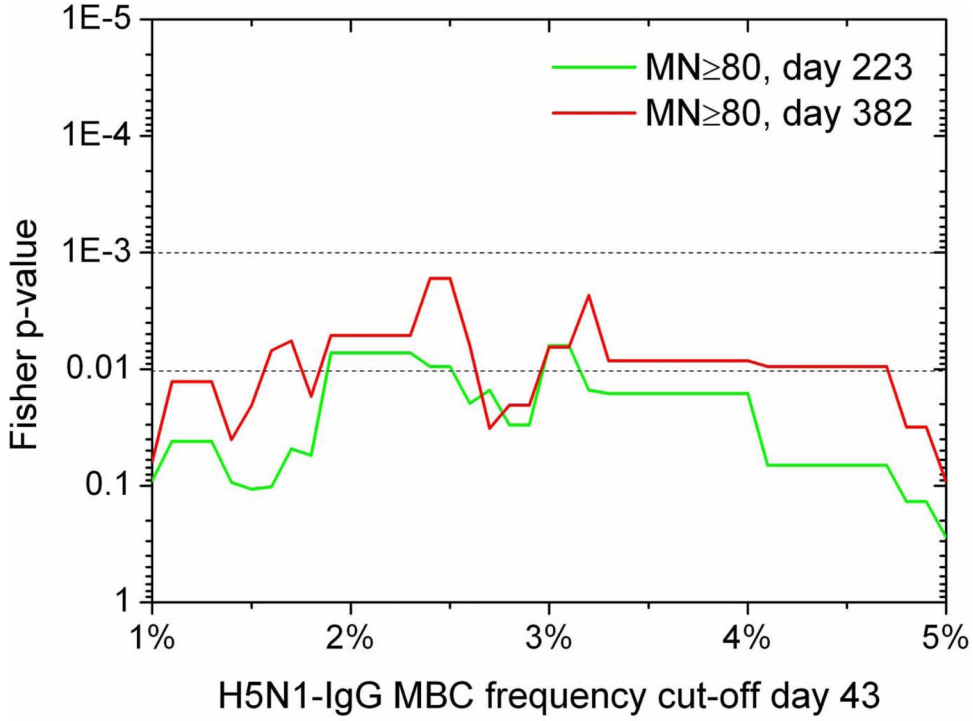

Fig. S4. Association between frequency of H5N1-IgG MBC at day 43 and MN titers ≥80 at later time points. Shown are the Fisher's exact test 2-tailed *P* values for the association between frequency of H5N1-IgG MBC at day 43 (depicted on the *x*-axis) and MN titers at days 223 (red line) and 382 (green line).

Table S1. MN titers ≥40 at day 43 predict MN titers ≥80 at day 223 but not at day 382

		≥80	<80	
		MN day 223		
MN day 43	≥40	20	4	PPV 83%
	<40	5	11	NPV 69%
P value = 0.0007		Sens. 80%	Spec. 73%	Accuracy 78%
		MN day 382		
MN day 43	≥40	11	13	PPV 46%
	<40	2	14	NPV 88%
P value = 0.04		Sens. 85%	Spec. 52%	Accuracy 63%

In each 2 × 2 contingency table sensitivity (Sens.), specificity (Spec.), positive predictive value (PPV), negative predictive value (NPV), accuracy, and 2-tailed Fisher's exact association test *P* value are shown (see *Materials and Methods*).

Table S2. At day 43 frequency of H5N1-IgG MBC ≥2.5% predicts MN titer ≥80 at days 223 and 382

		≥80	<80	
		MN day 223		
MBC %, day 43	≥2.5	14	2	PPV 88%
	<2.5	11	13	NPV 54%
P value = 0.009		Sens. 56%	Spec. 87%	Accuracy 68%
		MN day 382		
MBC %, day 43	≥2.5	10	6	PPV 62%
	<2.5	3	21	NPV 88%
P value = 0.002		Sens. 77%	Spec. 78%	Accuracy 78%

In each 2 × 2 contingency table sensitivity (Sens.), specificity (Spec.), positive predictive value (PPV), negative predictive value (NPV), accuracy, and 2-tailed Fisher's exact association test *P* value are shown (see *Materials and Methods*).

Table S3. Significance of associations between predictors and efficacy endpoints

Predictors		Endpoint: MN ≥80	
		Day 223	Day 382
$CD4^+$ T cells	Day22	0.006	0.001
	Day43	0.96	0.04
MBC	Day22	1	1
	Day43	0.11	0.02
MN titer	Day22	1	1
	Day43	0.008	0.48

Shown is the Bonferroni-corrected Fisher's exact test *P* value for the association between different predictors ("$CD4^+$ T cells," ≥3-fold expansion of H5-$CD4^+$ T cells over baseline; "MBC," frequency of H5N1-IgG MBC ≥2.5%; "MN titer," MN titer ≥40) and the efficacy endpoint of MN titer ≥80 at days 223 and 382.