Supporting Information

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SI Materials and Methods

H5N1 Turkish Cases: Bone Marrow Recovery. Six H5N1 survivors provided bone marrow and serum for this study. All were diagnosed between December 2005 and January 2006, and their diagnoses were described previously (1). Briefly, most samples were nasopharyngeal swabs tested by ELISA, rapid influenza test, and/or real-time PCR in Turkey. Results for four of the six survivors were further verified by World Health Organization laboratory testing in London. Post-recovery, bone marrow aspirates and serum from the six survivors were collected, minimally processed in RNALater (Ambion) to preserve RNA integrity, and shipped frozen on dry ice to our laboratories. The present study was reviewed and approved by both the Turkish Ministry of Health and Yüzüncü Yil University. Written guardian consent was provided for all donors.

Antibodies, Proteins, and Viruses. HA proteins were either purchased from Protein Sciences (H5 protein A/Vietnam/1203/2004, H1 protein A/ New Caledonia/20/99, H3 protein A/Wisconsin/67/05) or generated by *de novo* synthesis (H1 protein A/South Carolina/1/18) as eukaryotic codon-optimized soluble secreted HA genes (DNA 2.0) and then subcloned into pCI (Promega) for mammalian protein expression, sequence-verified, and transfected into 293 Freestyle cells (Invitrogen), in accordance with manufacturers' guidelines. Briefly, 20 μg of light chain and 10 μg of heavy chain encoding plasmid were combined with 1.0 ml of 293 fectin and incubated for 60 min. After this preincubation, the DNA mixture was combined with 3×10^7 cells in 30 ml of media, and the resulting cell suspension was grown in accordance with the manufacturer's suggestion for 7 days. After 7 days, the secreted Igs were purified from the culture supernatants by using protein A chromatography (Calbiochem). The resulting purified antibodies were buffer-exchanged into sterile PBS by using centrifugal size filtration (Plus-20; Centricon), and their protein concentrations were determined by colorimetric BCA assay (Pierce).

Recombinant viruses were genetically engineered and produced as described in ref. 2. The Indonesia, Turkey, and Egypt proteins were similarly made, except their HA genes were assembled synthetically by using eukaryotic codon-optimized sequences (DNA 2.0) and modified by the removal of nucleotides encoding the polybasic cleavage site. Inactivated viruses were made as described in ref. 2.

Serology: HA and Viral ELISA. Recombinant HA proteins and their concentrations were as follows: H5 protein A/Vietnam/1203/2004 (Protein Sciences), 10 ng/well; recombinant HA H1 protein A/ New Caledonia/20/99 (Protein Sciences), 70 ng/well; recombinant HA H3 protein A/Wisonsin/67/05 (Protein Sciences), 10 ng/well; H1N1 virus A/New Caledonia (BioSource), 70 ng/well; H3N2 virus A/Panama/2007/99 (BioSource), 10 ng/well; influenza virus vaccine reference standard for H5N1 rgA/Vietnam/1203/2004 (Food and Drug Administration, Center for Biologics Evaluation and Research), 10 ng/well

ELISA plates were coated as indicated with either recombinant HA protein or inactivated virus and incubated overnight at room temperature. The next day, plates were appropriately blocked (1% BSA in PBS, 0.05% Tween 20), and 0.1-ml serum samples, diluted in blocking buffer, were incubated, washed, and detected by using a peroxidase-conjugated anti-human Fc antibody (Jackson ImmunoResearch) and tetramethylbenzidine detection (BioFX). Absorbance at 450 nm was read, and data were recorded and are reported herein.

Donor-Specific Repertoire Recovery. Between 2 and 2.5 ml of donor bone marrow previously stored in 25 ml of RNALater (Ambion) was processed with TRI-BD (Sigma) in accordance with the manufacturer's directions and then further processed to extract purified total RNA, as described in ref. 3. Next, mRNA was purified by Oligotex spin column purification (Qiagen), and random nonamer-primed reactions and oligo dT reverse transcription reactions were performed using Accuscript (Stratagene) in accordance with the manufacturer's directions.

For each donor, the following procedures were performed. For each of the 11 λ light chain families, a single PCR amplification was performed with family-specific V_L primers and a mixture of J_L primers and primed with 75 ng of Oligo dT cDNA. κ recovery was similarly performed for each of the six κ light chain families, except that 75 ng of random nonamer-primed cDNA was used. For heavy chain recovery, $V_H1/7$, V_H3, and V_H4 were recovered individually, and V_H 2, 5, and 6 were pool-amplified with gene-specific primers and mixtures of J_H primers, and each was primed with 100 ng of random nonamer-primed cDNA. Primers and amplification conditions were essentially as described in ref. 3, using Platinum Pfx polymerase (Invitrogen). PCR products were minimally processed by PCR Cleanup (Qiagen) quantitated by A_{260}. Heavy chain reactions were gel-purified and then, if necessary, amplified again to produce quantities sufficient for cloning.

Phage Library Construction. ***Light chain cloning.*** Donor-specific barcoded vectors and equimolar pools of κ and λ light chains were digested separately with NotI and BamHI and gel-purified (Qiagen). Library ligations were performed with 200 ng of gel-purified κ or λ inserts and 1 μg of gel-purified vector. Incubation was for at least 1 h at room temperature or overnight at 14°C. Ligations were desalted using Edge BioSystems Performa spin columns. Five electroporations per library were done in 80 μl TG-1 aliquots, each recovered in 1 ml SOC, pooled, and outgrown for 1 h at 37°C. A sample of each was taken for plating and used to determine the total number of transformants. The remainder was transferred to 200 ml of 2YT + 100 μg/ml ampicillin + 2% glucose and grown overnight at 37°C. The target number of transformants per library was at least 1×10^6 per microgram of vector DNA. Light chain library plasmids were then pelleted, and the plasmids were purified by using a Qiagen high-speed Maxiprep kit.

Heavy chain cloning and phage production. Donor-specific heavy chains (V_H 1, V_H 3, V_H 4, and the V_H 2, 5, 6 pool) and light chain library collections were digested separately with 40 units per microgram of DNA with SfiI and XhoI and were gel-purified (Qiagen). Five micrograms of κ and λ light chain libraries were separately ligated overnight with 1.2 μg of an equimolar mix of the four donor-specific heavy chain preparations. The library ligations were spin-column-desalted (Edge BioSystems) and then transformed in 16–20 electroporations per library. Processing to determine the number of transformants was as described above. Phage production proceeded as described elsewhere (3). After phage production, the phage was harvested by PEG/NaCl precipitation and resuspended and stored in PBS containing 50% glycerol.

Panning. Panning was performed essentially as described in ref. 3.

Clonal ELISAs. Clonal ELISAs were performed essentially as described in ref. 3.

Microneutralization. Cross-subtype neutralization by antibodies recovered from survivors of avian influenza was performed. Indonesia and Turkey HA genes were synthetically assembled by using human codon-optimized sequences (DNA 2.0), as described above, and then used to generate recombinant engineered viruses

Recombinant influenza viruses were generated by reverse genetics, as described in ref. 2. Briefly, 1 μg each of 10 plasmids was transfected into 293 T cells in monolayer. Each transfection contained ambisense plasmids (for the expression of both vRNAs and mRNAs) for the A/Puerto Rico/8/34/PA, PB1, PB2, NP, M, and NS segments, in addition to vRNA (pPOL1 type) plasmids for the relevant HA segments and the A/Vietnam/1203/04 NA segment. [pCAGGS expression plasmid was kindly provided by J. Miyazaki, Osaka University, Osaka, Japan) (4).] Twenty hours after transfection, 293 T cells were resuspended in cell culture supernatant and used to inoculate 10-day-old embryonated eggs.

Antibodies were screened for neutralizing activity against viruses as follows. Two-fold serial dilutions of each Mab were incubated with 100 $TCID_{50}$ of virus in PBS at 37°C for 1 h. Madin–Darby canine kidney (MDCK) cell monolayers in 24-well plates were washed once with PBS and inoculated with virus–antibody mixtures. After incubation for 1 h at 37°C in 5% CO_2, the inoculum was removed and monolayers were again washed once with PBS. Opti-MEM supplemented with 0.3% BSA, 0.01% FBS, 1 μg/ml TPCK-treated trypsin was added, and cells were incubated for 72 h at 37°C. The presence of virus in cell culture supernatants was assessed by HA assays using 0.5% chicken red blood cells.

Cross-Reaction IgG ELISA. Microtiter plates were coated with 0.1 ml of the following antigens diluted in coating buffer and incubated overnight at room temperature: 100 ng/ml H5N1 Vietnam 1203/04, 250 ng/ml H5N1 Turkey/65596/06, 1 μg/ml H5N1 Indonesia/5/05, 700 ng/ml H1N1 New Caledonia/20/99, 1 μg/ml H1N1 South Carolina/1/18, and 100 ng/ml H3N2 Wisconsin/67/05. Blocking was done with 0.3 ml of blocking buffer (4% nonfat dry milk in PBS, 0.05% Tween 20). After blocking, antibodies diluted to 0.5 μg/ml in 2% nonfat dry milk blocking buffer were incubated for 2 h at 4°C, washed, and later detected using a 1:3,000 dilution of peroxidase-conjugated anti-human F_c antibody (Jackson ImmunoResearch) in 2% nonfat dry milk blocking buffer and standard TMB substrate detection (BioFX). Absorbance at 450 nm was read, and data were recorded and are reported herein. Relative ranking of antibodies is reported by their ELISA signal-to-noise ratio (–, <2; +, 2–9; ++, 9–15; +++, ≥ 15) on various proteins and minimal inhibitory concentration in microneutralization assay.

1. Oner AF, *et al.* (2006) Avian influenza A (H5N1) infection in eastern Turkey in 2006. *N Engl J Med* 355:2179–2185.
2. Fodor E, *et al.* (1999) Rescue of influenza A virus from recombinant DNA. *J Virol* 73:9679–9682
3. Barbas C, Burton DR, Scott JK, Silverman GJ (2001) *Phage Display: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
4. Miyazaki J, *et al.* (1989) Expression vector system based on the chicken β-actin promoter directs efficient production of interleukin-5. *Gene* 79:269–277.

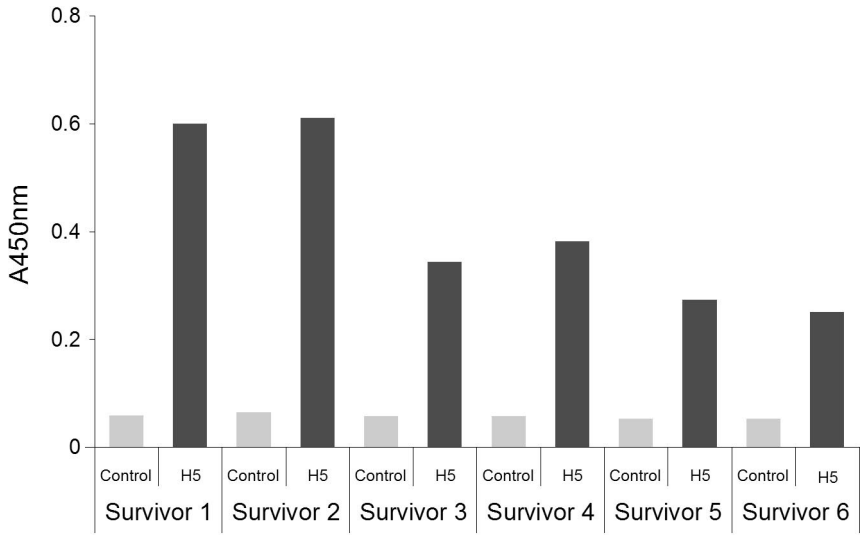


Fig. S1. Turkish avian influenza survivors have high cross-reactive titers to the H5N1 Vietnam 1203/04 HA protein. Serum ELISA analysis on purified recombinant Vietnam 1203/04 H5 HA protein at 1:10,000 demonstrates the presence of high-titer anti-HA antibodies in each of the six donors. Each survivor's serum response was of both high titer and broad reactivity because the signals resulted from binding to a heterologous H5N1 HA protein. Black bars, A/Vietnam/1203/04 HA-coated wells; gray bars, uncoated control wells.

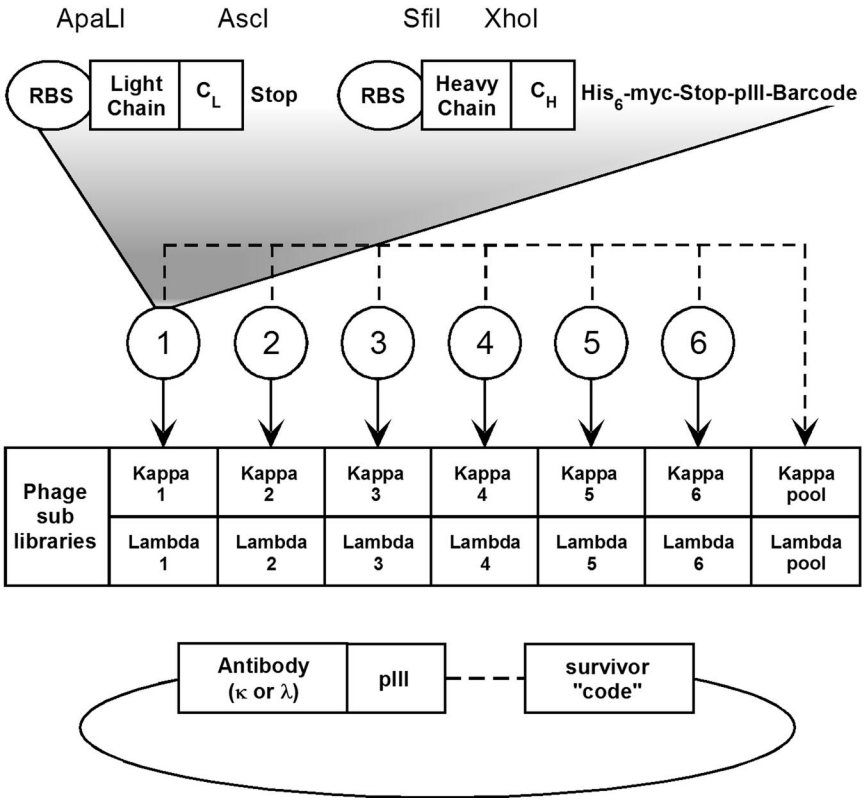


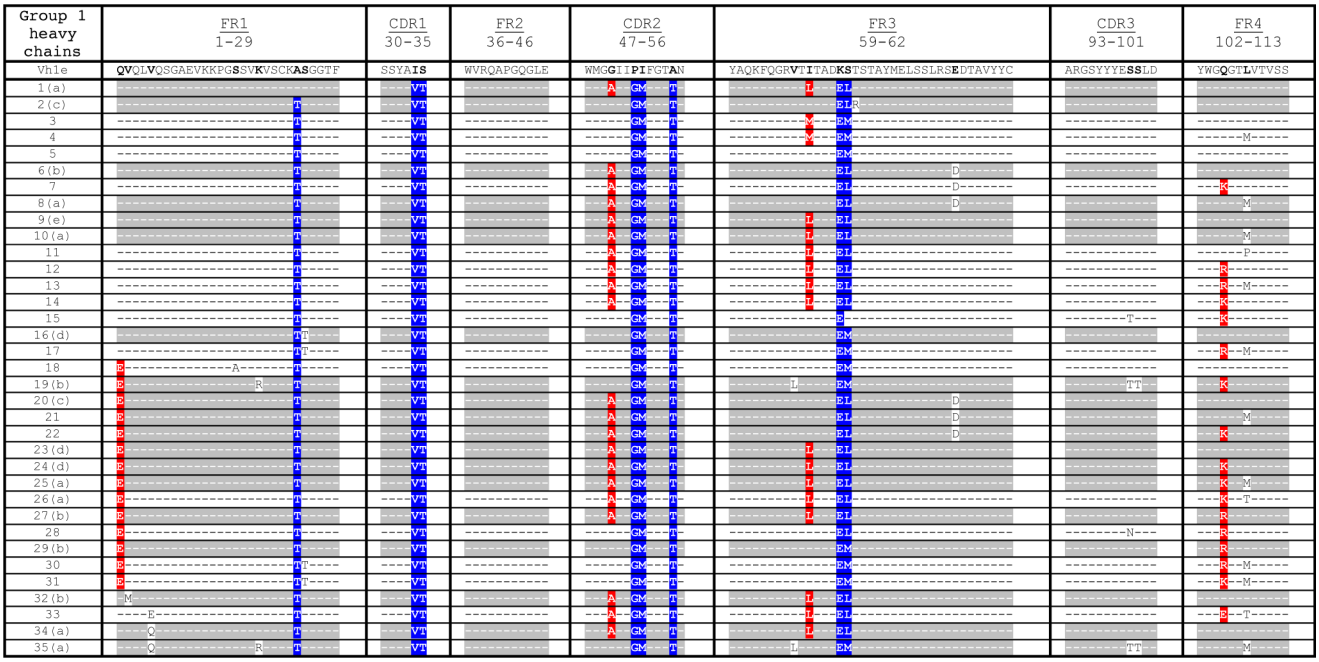
Fig. S2. Cloning and barcoding of annotated repertoires allows tracking of all clones to their sources. (*Upper*) Each donor is assigned a unique barcoded vector, and Ig repertoires are cloned via restriction sites. (*Lower*) Plasmids from any clone can be assigned to designated sublibraries on the basis of their light chain class and their survivor barcode.

Table S1. Light chain and full library total transformants in both scFv and Fab formats

	Light chains		Completed libraries	
	κ	λ	κ	λ
		scFv		
H5-1	3.00E+06	4.00E+06	1.50E+08	1.20E+08
H5-2	3.00E+06	3.00E+06	4.00E+07	1.60E+07
H5-3	8.20E+05	1.70E+06	5.30E+07	1.50E+08
H5-5	7.00E+06	5.60E+06	6.50E+08	5.60E+07
H5-6	1.50E+06	5.00E+06	ND	1.00E+07
H5 pool	ND	ND	1.80E+08	5.70E+08
Totals			1.10E+09	9.20E+08
scFv total			**2.00E+09**	
		Fab		
H5-1	1.50E+06	2.90E+06	2.90E+08	4.60E+08
H5-2	3.10E+06	9.40E+05	4.40E+08	4.30E+08
H5-3	2.80E+06	2.30E+06	3.90E+08	3.90E+08
H5-5	7.00E+06	5.60E+06	7.20E+08	1.70E+08
H5-6	1.50E+06	5.00E+06	ND	ND
H5 pool	1.90E+07		2.60E+08	
Totals			2.10E+09	1.45E+09
Fab total			**3.60E+09**	

Total diversity represented by all libraries is 5.6×10^9. ND, not determined.

Table S2. Example sequences displaying the immunochemical basis of neutralization found from survivor 5 libraries after H1N1 New Caledonia panning



The 35 unique heavy chain sequences aligned with their germ-line variable regions from the 82 unique heavy and light chain combinations. Required mutations are highlighted in blue, and predominant mutations are highlighted in red. Heavy chain sequences also discovered in H5N1 Vietnam panning are highlighted in gray. Antibody regions and Kabat numbering ranges are listed at the top of each sequence column. a, paired with 2 unique light chains; b, paired with 3 unique light chains; c, paired with 4 unique light chains; d, paired with 5 unique light chains; e, paired with 13 unique light chains.

Other Supporting Information Files

Dataset S1