Supplementary data for:

RAPID CLONING OF HIGH AFFINITY HUMAN MONOCLONAL ANTIBODIES AGAINST INFLUENZA VIRUS

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Supplementary Figure Legends

Supplementary Figure 1. Determination of clonality and expression of recombinant monoclonal antibodies. a, Determination of total frequency of clonally-related B cells. Each slice of the pie chart (left panel) represents a unique clonal expansion or the proportion of variable gene sequences from a single donor that were derived from particular progenitor B cells (clonal pool). Each clonal pool represents variable genes that share identical VH, D, and JH genes and junctions between these genes, but can be distinguished based on the accumulation of somatic mutations[18]. Right panel: the phylogenic tree and individual amino acid sequences of one of the clonal pools (CP1). ELISPOT assays demonstrated that 1,300 anti-influenza ASCs were detectable in each ml of blood from this donor at day 7, totaling ~5% of all B cells. Therefore in each ml of blood, there were over 400 ASCs, or 1.5% of all B cells derived from this single clonal expansion (totaling ~2,000,000 clonal progeny in the blood of that person). Interestingly, analysis of 86 variable genes from this one clonal expansion found that most (85%) differed by individual somatic mutations, and thus mutation and expansion likely occurred together and *de novo* after vaccination. **b,** Strategy for generating recombinant mAbs from the VH and Vκ genes of single cell-sorted influenza specific ASCs. Single day 7 ASC cells ($CD19^{+}/CD20^{lo}/CD3^{-}/CD27^{hi}/CD38^{hi}$) were sorted and the VH and Vκ genes amplified by a one-step reverse transcription and PCR with primers to the variable gene leader region and constant regions. The identified light and heavy chain genes were cloned into an expression vector and then cotransfected into 293 cells. **c,** Time line for the above procedure. This protocol can progress from immunization to production of multiple human mAbs in less than thirty days.

Supplementary Figure 2. Binding curves of the influenza-specific antibodies to the five different strains of influenza. Curves are grouped by virus type to which the particular antibody had the greatest Kd. Note that heterosubtypic cross-reactivity (to both A and B strains) was limited to low affinity interactions and HAI activity was not evident for more than one type of hemagglutinin for any particular antibody (For example, antibodies that inhibited H2 could not inhibit H1 or B strain hemagglutinin). We suspect these antibodies may bind common non-protein epitopes (such as glycans) or that they are somewhat polyreactive.

Supplementary Figure 3. Identification of individual antibody specificities: Each virus or vaccine specific antibody was tested by immunoprecipitation (I.P.) for extraction of particular influenza proteins from the reactive strain (or whole vaccine for those without particular strain reactivity). Those that did not precipitate any antigen by I.P. were tested for binding to the viral denatured proteins transferred to nitrocellulose membranes by Western blot. Examples of the various specificities detected are indicated for each viral strain (**a-d**) compared. As the anti-influenza A (H3N2) antibodies each bound equally to either of the very similar A/California/7/2004 (2005/6 vaccine) and A/Wisconsin/67/2005 (2006/7 vaccine) strains, the latter strain to which most donors were vaccinated was used for IP. For five of the 53 reactive antibodies an antigen could not be determined. These antibodies were typically low affinity and somewhat polyreactive suggesting that the ASCs may have been non-specifically recruited to the immune response after vaccination. Some antibodies appeared to cross-react to both the A and B strains by ELISA (Sup. Fig . 2) and so all were tested by I.P. and Western blot for cross reactivity to the heterosubtypic influenza virus strains as well. Of the eight antibodies tested, two

(D2-8 and D4-4) were found to I.P. HA from both the Influenza B and A strains. The findings of these experiments are summarized in Fig . 3c and in Table 1.

Supplementary Figure 4. Representative ASC antibodies can neutralize influenza infection of MDCK cells. All three anti-H3N2 antibodies tested neutralized A/Wisconsin/67/2005 infectivity (D1-8 and D1-9 from donor 1, and D3-4 from donor 3) an anti-B/Shanghai/361/2002 antibody tested from donor 6 could neutralize viral infectivity. Half-maximal tissue-culture infectious dosages of virus were neutralized in the percentage of replicate wells indicated (Y axis) at various dilutions of antibody (X axis). The negative control antibody is an anti-A/New Caledonia/20/99 (H1N1) antibody that as expected does not neutralize the H3N2 or B influenza strains.

Supplementary Tables

Supplementary Table 1: The frequency of replacement (R) versus silent (S) mutations predicts selection of the ASCs.

	Region	R	S	R/S	Ratio (CDR/FWR)
IgM GC & Mem	CDR	1,367	335	4.08	1.81
	FWR	2,461	1,094	2.25	
IgG GC & Mem	CDR	2,325	626	3.71	2.14
	FWR	3,180	1,834	1.73	
IgG ASC	CDR	1,953	526	3.71	2.10
	FWR	2,210	1,252	1.77	

Supplementary Table 2: Background Mutation Frequency.

	Mutations	Total IgG Bases*	Frequency (Mut./1,000 bp)	Predicted per sequence
IgG ASC	74	69,618	1.06	0.37 (1 in 3 sequences)
IgG Mem, GC	310	124,640	2.49	0.87

*An average of 246 bases of the constant region that is not subjected to physiological somatic hypermutation was sequenced along with the VDJ rearrangement for each of: 346 IgG ASC transcripts and for 623 IgG memory and GC sequences.

Detailed Methods

Study subjects

Study subjects were all normal healthy volunteers receiving the influenza vaccine (either Fluzone 2005-06 formulation (Aventis Pasteur Inc) or Fluvirin 2006-07 formulation (Chiron). Blood sampling of all volunteers followed established NIH guidelines with institutional review board and approval from the Emory and the OMRF review boards.

Cell and serum isolation

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood using Vacutainer cell preparation tubes (CPT) (Becton Dickinson, BD). After extensive washing the PBMC were resuspended in R-10: RPMI-1640 + 10% fetal calf serum (FCS) supplemented with penicillin, streptomycin, L-glutamine, and freshly added 50 mM B-mercaptoethanol. Fresh cells were used in all assays. Plasma was isolated from the CPT tubes and was heat inactivated at 56 °C for 30'. For single cell analyses and antibody production B lymphocytes were isolated from human blood and tonsils of healthy adult volunteers as previously described[17]. Briefly, PBMCs were enriched using a lymphoprep gradient (CellGro), washed and resuspended in PBS containing 2% inactivated fetal calf serum. The cells were stored on ice until staining and sorting, always within the same day.

ELISPOT assay

Direct ELISPOT to enumerate the number of either total IgG secreting or influenza specific ASC present in the PBMC samples were essentially done as previously described[25]. Briefly, 96-well ELISPOT filter plates (Millipore, MAHA N4510) were coated overnight with either the

influenza vaccine (Fluzone 2005-06 formulation; Aventis Pasteur Inc. or Fluvirin 2006-07 formulation, Chiron) at a dilution of 1/20 in PBS or with goat anti-human Ig (Caltag). Plates were washed and blocked by incubation with RPMI containing 10% FCS at 37°C for 2 hrs. Purified and extensively washed PBMCs were added to the plates in dilution series and incubated for 6 hrs. Plates were washed with PBS followed by PBS containing 0.05% Tween and then incubated with a biotinylated anti-huIgG (gamma) antibody (Caltag) and incubated for 1.5 hrs at room temperature. After washing, the plates were incubated with an avidin-D-HRP conjugate (Vector Laboratories) and finally developed using AEC substrate (3 amino-9 ethyl-carbozole, Sigma). Developed plates were scanned and analyzed using an automated ELISPOT counter (Cellular Technologies Ltd.).

Memory B cell assay

Memory B cell assays were essentially done as previously described[14,25]. In brief, PBMC were plated in 24-well dishes at $5x10^5$ cells/well in R-10 supplemented with an optimized mix of polyclonal mitogens: pokeweed mitogen extract (PWM) (made in house), phosphothiolated CpG ODN-2006[26], and Staphylococcus Aureus, Cowan (SAC) (Sigma). 7 wells were cultured per individual for 6 days with 7 non-stimulated wells as a negative control. The stimulated cells were harvested, washed extensively and assayed using the ELISPOT assay described above. Data is represented as the percentage of IgG secreting influenza-specific cells over the total number of IgG secreting cells. Estimation of the total frequency of memory B cells that were influenza specific following vaccination was based on the finding that on average 12% of blood B cells from 25 healthy normal adults are IgG memory cells (CD19+CD27+IgG+).

Hemagglutination inhibition assay

HAI titers were determined for the three viruses making up the 05-06 vaccine (A/New Caledonia/20/99 (H1N1), A/California/7/2004 (H3N2) and B/Shanghai/361/2002; viruses kindly provided by the CDC) as previously described[27]. Briefly, serum samples were treated with receptor destroying enzyme (RDE; Denka Seiken Co.) by adding of 1 part serum to 3 parts RDE and incubating at 37^0 C overnight. The following morning, the RDE was inactivated by incubating the samples at 56^0 C for one hour. The samples were then serially diluted with PBS in 96 well v-bottom plates and 8 HAU (as determined by incubation with 0.5% turkey RBCs in the absence of serum) of either the H1N1, H3N2, or influenza B virus was added to each well. After 30 minutes at room temperature, 50 ul of 0.5% turkey RBCs (Rockland Immunochemicals) suspended in PBS with 0.5% BSA was added to each well and the plates were shaken manually. After an additional 30 minutes at room temperature, the serum titers were read as the reciprocal of the final dilution for which a button was observed. Negative and positive control serums for each virus were used for reference (data not shown; reagents provided by the CDC)**.**

Flow cytometry and cell sorting

Flow cytometry analysis was performed on whole blood. Briefly, 300-400 μl blood was incubated with the appropriate antibodies for 30 minutes at room temperature. Red blood cells were then lysed by incubation with FACS lysing Solution (Beckton Dickinson) for 4 minutes at RT. All antibodies used for determining the dynamics of ASC or memory cells production and for bulk variable gene analyses were purchased from Pharmingen with the following exceptions: CD27 APC (ebiosciences) and goat anti-huIgG FITC (Southern Biotechnologies). For these cell sorting experiments, PBMCs were stained with the appropriate markers and sorted on a FACSVantage. Analysis of data was performed using FlowJo software.

For single cell analysis and production of mAbs, B cells were bulk sorted using a Becton-Dickinson FACS Aria cytometer and then resorted into 96-well PCR plates with a Cytomation MoFlo cytometer fitted with a single-cell sorting apparatus (98-99% purity detected on sort of the single cells). Antibodies used for flow cytometry for these analyses were anti-CD3 and anti-CD20 conjugated to FITC (Caltag), anti-CD38 conjugated to APC-Cy5.5 (Caltag), anti-CD27 conjugated to PE (Caltag), and anti-CD19 conjugated to PE-Alexa 610 (Caltag). In order to improve the efficiency of the single-cell PCR by ensuring only IgG+ cells were sorted, bitoinylated anti-IgD and strepavidin-Pe-Cy7 (Pharmingen) and anti-IgG (Caltag) conjugated to in house to Alexa-647 ((Invitrogen) were used. IgG^+IgD^- ASCs were gated as $CD19^+$ $CD3^-$ $CD20^{low}$ and then subgated as $CD27^{high}$ $CD38^{high}$. Naïve B cells (IgD^+CD38^-, tonsil, or IgD^+CD27^-, blood), IgG^+ and IgM^+ GC cells ($CD38^+$ tonsil) and memory cells ($CD38^-CD27^+$ tonsil or $CD27^+$ blood) were isolated as previously described[15-17].

Single cell RT-PCR and PCR of antibody variable region genes

Single B cells were sorted into 96-well PCR plates containing 10 mM Tris-HCL with 40 units/ul of RNase inhibitor (Promega). Plates of single cells were immediately frozen on dry ice and stored at -80C. V_H and Vκ genes from each cell were amplified in a one-step RT-PCR reaction (Qiagen) using a cocktail of sense primers specific for the leader regions and antisense primers to the Cγ constant regions for heavy chains and Cκ for the light chain. One microliter from each RT-PCR reaction was amplified in separate PCR reactions for the individual heavy and light chain gene families using nested primers as previously described[15,24]. PCR products were then sequenced (ABI 3730 capillary sequencer). When recombinant antibodies were to be generated, upon identification of the variable genes, sense primers unique to the particular variable genes and antisense primers binding the particular junctional genes were used in a new nested PCR

reaction with 1 ul of the RT-PCR as template to incorporate restriction sites at the ends of the variable genes for cloning. Variable genes were amplified from bulk RNA of IgG ASC and memory cells or IgM memory cells by RT-PCR using primers specific to the V_H3 and V_H4 families as previously described[15,17,29].

Analysis of clonality and somatic mutations of variable region genes.

For analysis of clonality, variable genes were randomly cloned and sequenced from the bulk RNA of 10^4 to 10^5 ASCs amplified by RT-PCR from 10 donors (by donor, n = 22, 47, 49, 12, 16, 19, 36, 25, 34, and 63 VH genes) and verified by single cell RT-PCR analysis of single sorted ASCs from four donors (by donor, n = 65, 37, 30, and 50 VH genes). For bulk analysis, V_H3 and V_H4 family variable genes (representing 2/3s of all antibody heavy chain genes[28]) were randomly cloned and sequenced, and for the single cell PCR analyses all V_H genes were considered. Most clones from the single cell PCR analyses were verified by isolation of similar light chain (Vκ) transcripts from the same cells in a multiplex PCR reaction. All donor ASC samples were verified to be anti-influenza positive by ELISPOT (Fig . 1b) or by production of recombinant mAbs from the single cells (see below). The naïve cell variable gene libraries as well as many of the IgM and IgG GC and memory V_H gene libraries presented for comparison in Fig . 3 were from historical data previously published by our laboratory[15-17,29,30]. The n-values for clonal-relatedness include the following (Fig . 3b): from blood naïve cells of 5 donors (n = 61, 24, 15, 14, and 24 sequences), from blood IgM memory cells of eight donors (n = 28, 17, 27, 11, 23, 12, 29, and 20 VH gene sequences), and from blood IgG memory cells of six donors (n = 23, 18, 18,17, 22, and 21 sequences), tonsillar naïve B cells were analyzed from 8 tonsil donors (n = 125, 32, 16, 22, 32, 23, 46, and 81 VH genes), tonsillar IgM germinal center (GC) and memory cells from 12 tonsil donors (n = 50, 42, 35, 16, 60, 15, 50, 25, 39, 19, 55, and 58 VH genes), and

tonsillar IgG GC or memory cells from 13 donors (n = 113, 25, 14, 40, 12, 41, 11, 23, 18, 51, 15, 54, and 69 VH genes). For analysis of somatic hypermutation frequency only certain VH gene libraries with the highest quality sequences were considered. Background mutation rates were determined by analyses of a portion of the Ig-constant sequence cloned with each of the VDJ transcripts and that is not subjected to somatic hypermutation. Background mutations rates were insignificant (Sup. Table 2). As each variable gene sequence also included a portion of the IgM of IgG constant region that is not targeted by physiological mutation we could verify that the sequences were of high quality. The n-values for analysis of somatic hypermutation include the following: 357 sequences of anti-influenza ASCs from 11 donors (by donor, n = 63, 18, 33, 46, 49, 11, 36, 11, 30, 35, 25); For IgG GC and memory cells, 623 V_H gene sequences were analyzed from 14 donors (by donor n = 110, 37, 19, 28, 174, 40, 25, 15, 21, 18, 22, 24, 19, 71); for IgM GC and memory cells 638 V_H gene sequences were analyzed from 17 donors (by donor, n = 56, 158,18, 91, 17, 10, 16, 30, 19, 28, 11, 36, 29, 13, 22, 20, 64); and for naïve cells 166 sequences from 6 donor (by donor, n = 18, 42, 21, 34, 15, 36). Antibodies expressed from the clonal variants differing only by accumulated somatic mutations bound similarly to the vaccine virus strains and antigens and are included in the total enumerations but are not graphed.

Recombinant monoclonal antibody expression

Following purification and digestion of the V_H (digest: AgeI and SalI) or V_k (digest: AgeI and BsiWI) genes, the amplified cDNAs of the antibody variable genes from each single cell were cloned into expression vectors containing human IgG, or Igκ constant regions as previously described (Sup. Fig. 1b)[15,24]. Maxi prep plasmids (Qiagen) containing the heavy and light chain Ig genes were cotransfected into the 293A cell line using the Calcium Phosphate method. Transfected 293A cells were allowed to secrete antibodies in serum-free DMEM supplemented

with 1% Nutridoma SP (Roche) for 4 to 5 days. Antibodies were purified using immobilized protein A beads (Pierce). Proper antibody expression and purity were verified by polyacrylamide gel electrophoresis, and purified antibody concentrations were determined using the EZQ Protein Quantization system (Molecular Probes). Naïve cell and IgG memory cell antibodies from unimmunized donors were produced previously in the laboratory.

ELISA to determine influenza binding affinities

To screen for influenza binding ELISA, microtiter plates were coated with a cocktail of the vaccine strains totaling 8 HAU of total virus per well (2005/2006: A/New Caledonia/20/99, A/California/7/2004, and B/Shanghai/361/2002-like, 2006/2007: A/New Caledonia/20/99, A/Wisconsin/67/2005, and B/Malaysia/2506/2004). Each individual virus strain was then used in ELISA assays to characterize the affinity and specificity of antibodies reactive to the mixture. As described within the text some antibodies were also tested for binding to the actual vaccine by coating the plates with the various vaccines at a dilution of 1/20 in PBS. Influenza viruses were freshly grown in chicken eggs and purified by sucrose gradient centrifugation by standard methods[23]. Goat anti-human IgG (Goat anti-human I-peroxidase-conjugate (Jackson ImmunoResearch, West Grove, PA) was used to detect binding of the recombinant antibodies followed by development with horseradish peroxidase substrate (BioRad, Hercules, CA). Absorbencies were measured at OD415 on a microplate reader (Molecular Devices, Sunnyvale, CA). Antibody affinities (Kd or half maximum dissociation constants) were calculated by nonlinear regression analysis and SCATCHARD plots of influenza ELISA curves plotted from a dilution series of 8 concentrations of antibody ranging from 10 ug/ml to 0.125 ug/ml using the GraphPad Prism statistics software.

Viral Neutralization assays

Influenza neutralizing activity was detected using MDCK cells and $100TCID_{50}$ (50% Tissue culture infectious doses) of A/Wisconsin/67/2005 or B/Shanghai/361/2002 based on the WHO manual[23]. Briefly, the virus-antibody mixture was incubated with $100TCID_{50}$ at room temperature for 2hrs prior to the monolayers of MDCK cells. The plate was incubated at 37^0c with 5% CO2 and humidified atmosphere for 3-4 days. The cell cultures were then observed under inverted microscope and scored for viral Cytopathogenic effect.

Immunoprecipitation and Western Blot analyses

For IP, 100ul lysis Buffer (1% Triton X-100, 50mM Tris-Hcl-PH8.0, 150mM NaCl, 1% Sodium Deoxycholate, 0.1% SDS) was mixed with 8 HAU of virus and incubated at RT for 30 min. Antibody was added to 10ug/ml (1ug of mAb). The antibody and viral lysate was incubated at 37C for 40-50min. Protein A-Sepharose (Pierce) was prepared in Lysis buffer at a volume of 25ul/sample. Protein A-Sepharose was incubated with the Ab-virus lysate at RT for 1hr with constant agitation. The protein A-Sepharose was pelleted by centrifugation for 3 min at 3000 rpm and the precipitate washed with 400ul of lysis buffer. The protein-A-Sepharose was again pelleted and washed with 400ul of 20mM Tris (PH7.5). The protein-A-Sepharose was again pelleted and then resuspended into 25ul of Laemmli gel sample buffer (Bio-Rad). The samples were then boiled for 5 min at 95C. The protein A was pelleted and 15-20ul of supernatant was loaded onto 12% Tris-Glycine polyacrylamide gels. The gels were run in 1xTGS at 150V for 1 hr 30 min and then stained with sypro-orange (1X, Invitrogen) in 7.5% acetic acid for 45min-1hr. The gels were then destained with 7.5% acetic acid for 45 min. Gels were then read using a STORM840 system (Molecular Dynamics). For Western blots, virus (8 HAU) was diluted and

boiled in denaturing/reducing sample buffer, then run on denaturing polyacrylamide gels (as above) followed by electrophoretic transfer to nitrocellulose membranes. The membranes were incubated with each Ab at 5ug/ml and detected with HRP anti-human IgG (Jackson Immunoresearch) and developed with ECL plus reagent (GE health care). Membranes were analyzed using a STORM840 system (Molecular Dynamics)

Analysis of sequences and statistics

Variable region sequences were analyzed using in house software and the NCBI BLAST search engine. Clonal trees were generated using the Phylum software package. Statistical analyses (described in context) and saturation curves to determine antibody affinities were performed using GraphPad Prism. Average frequencies of clonal relatedness and somatic mutation were by non-paired, 2-tailed students t-tests (Fig . 2a and b). Chi-square tests were used to determine if summed mutation frequencies of ASCs were higher (Fig . 2c).

Supplementary references

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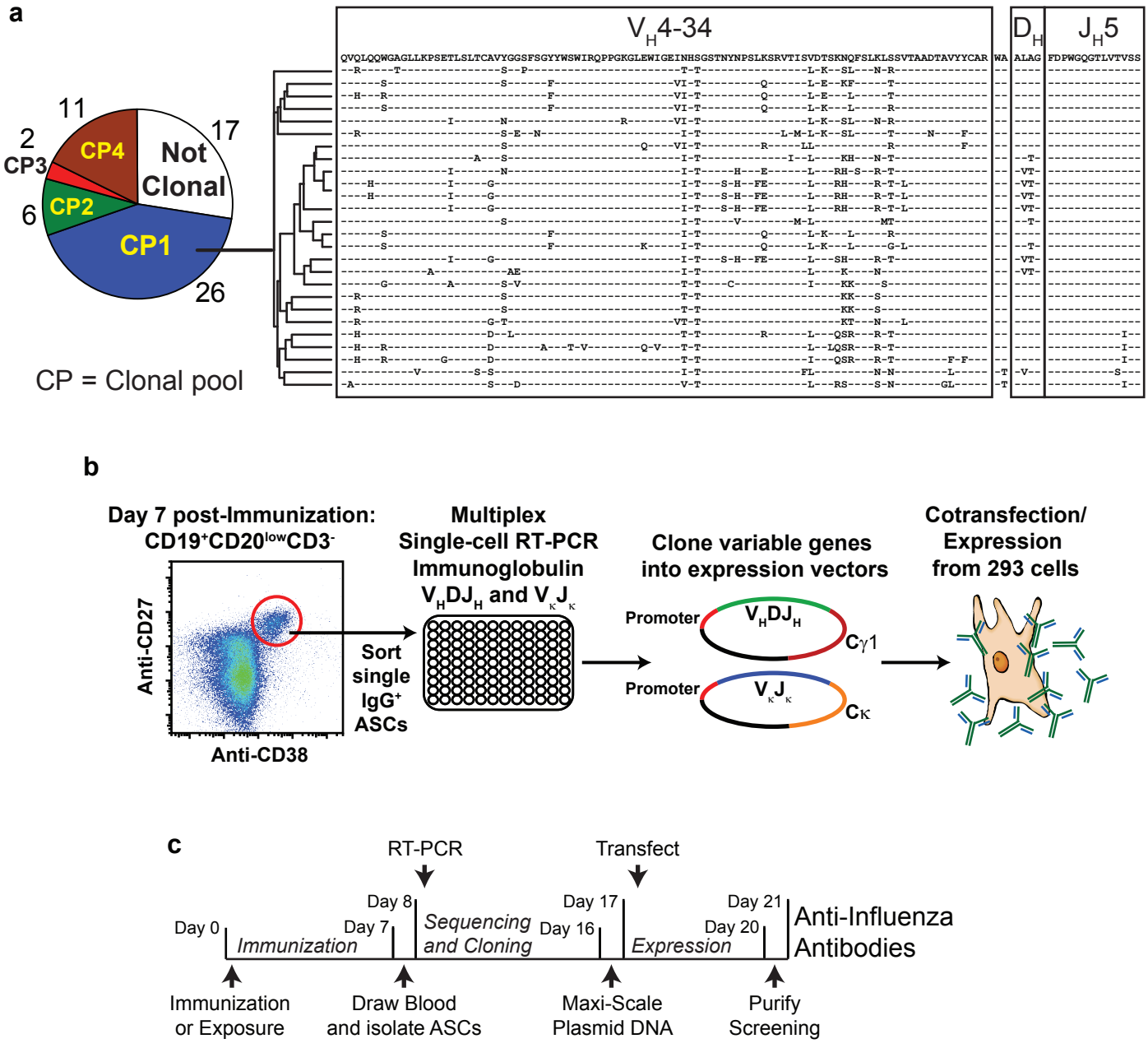
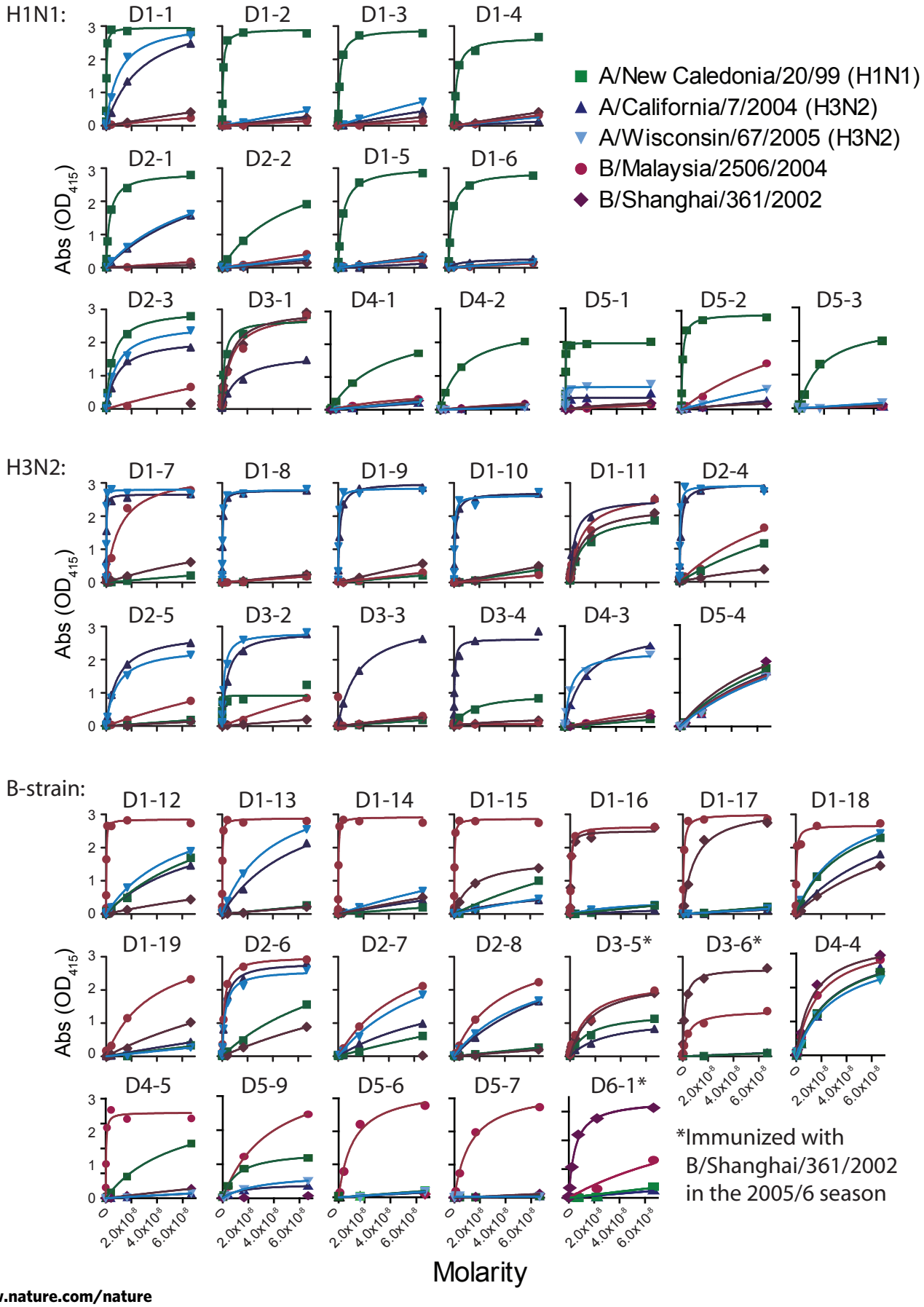
Supplementary Figure 1

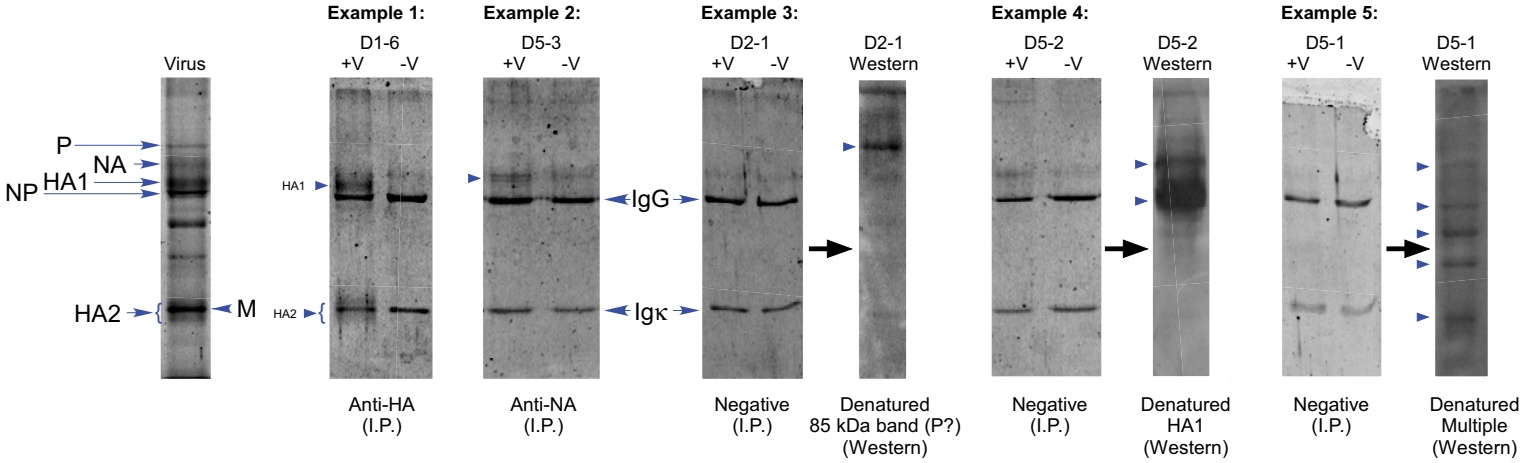


Supplementary Figure 2



Supplemental Figure 3

a A/New Caledonia/20/99 (H1N1)



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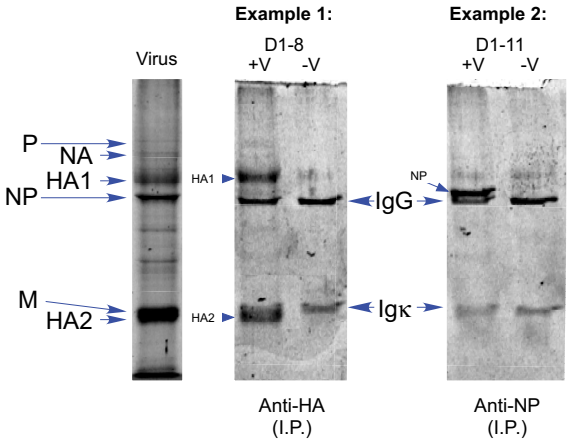
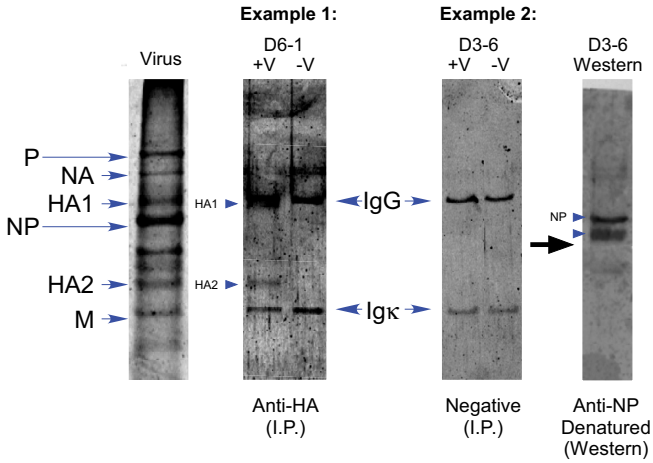
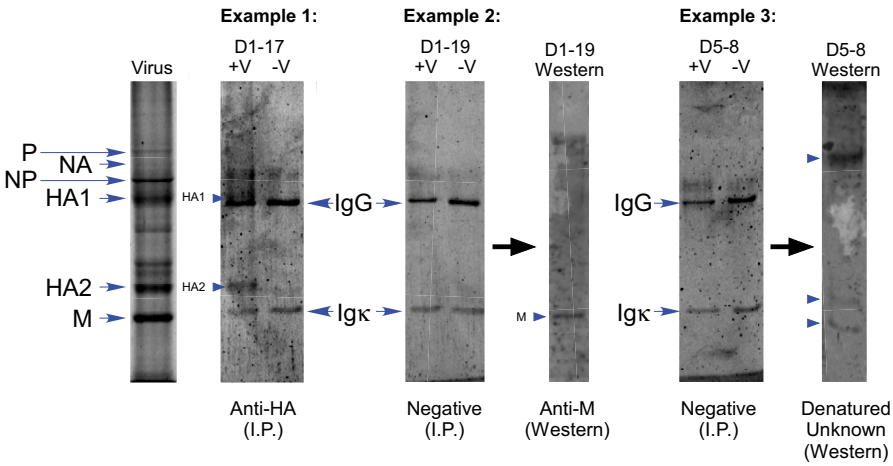


c B/Shanghai/361/2004 (2005/6 season)



d B/Malaysia/2506/2004 (2006/7 season)



Supplementary Figure 4