

# Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors

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Investigation of the human antibody response to influenza virus infection has been largely limited to serology, with relatively little analysis at the molecular level. The 1918 H1N1 influenza virus pandemic was the most severe of the modern era<sup>1</sup>. Recent work has recovered the gene sequences of this unusual strain<sup>2</sup>, so that the 1918 pandemic virus could be reconstituted to display its unique virulence phenotypes<sup>3,4</sup>. However, little is known about adaptive immunity to this virus. We took advantage of the 1918 virus sequencing and the resultant production of recombinant 1918 haemagglutinin (HA) protein antigen to characterize at the clonal level neutralizing antibodies induced by natural exposure of survivors to the 1918 pandemic virus. Here we show that of the 32 individuals tested that were born in or before 1915, each showed seroreactivity with the 1918 virus, nearly 90 years after the pandemic. Seven of the eight donor samples tested had circulating B cells that secreted antibodies that bound the 1918 HA. We isolated B cells from subjects and generated five monoclonal antibodies that showed potent neutralizing activity against 1918 virus from three separate donors. These antibodies also cross-reacted with the genetically similar HA of a 1930 swine H1N1 influenza strain, but did not cross-react with HAs of more contemporary human influenza viruses. The antibody genes had an unusually high degree of somatic mutation. The antibodies bound to the 1918 HA protein with high affinity, had exceptional virus-neutralizing potency and protected mice from lethal infection. Isolation of viruses that escaped inhibition suggested that the antibodies recognize classical antigenic sites on the HA surface. Thus, these studies demonstrate that survivors of the 1918 influenza pandemic possess highly functional, virus-neutralizing antibodies to this uniquely virulent virus, and that humans can sustain circulating B memory cells to viruses for many decades after exposure—well into the tenth decade of life.

Recent studies suggest that the 1918 H1N1 influenza virus was of avian origin<sup>2,5</sup>, and is capable of inducing strong systemic cytokine responses that probably contribute to pathogenesis<sup>4,6</sup>. Little is known about naturally occurring adaptive immunity to this virus; however, some elderly survivors are still living. We sought to determine whether survivors showed evidence of acquired immunity to the virus. Expression of the 1918 HA antigen allowed us to identify and characterize protective antibodies induced by natural exposure of humans to the 1918 pandemic virus.

We identified a panel of 32 subjects aged 91–101 years (that is, aged from 2 to 12 in 1918), many of whom recalled a sick family member in the household during the pandemic, which suggested direct exposure

to the virus. Of the subjects tested, 100% had serum-neutralizing activity against the 1918 virus (mean titre 1:562), and 94% had serologic reactivity to the 1918 HA (as indicated by haemagglutination inhibition assay (HAI) titres of 1:40 or greater; mean titre 1:396), even though these samples were obtained nearly 90 years after the pandemic. In contrast, subjects born after the pandemic had markedly lower rates of positive serum-neutralizing tests against the 1918 virus (9 out of 10 subjects born 1926–35 had titres <1:100, 9 out of 10 subjects born 1936–45 had titres ≤1:40, 9 out of 10 subjects born 1946–55 had titres ≤1:40). Individual serologic results are shown in Supplementary Table 1.

Peripheral blood mononuclear cells from eight subjects were isolated and B lymphoblastic cell lines were generated by transformation; blood from almost all of the donors tested (7 out of 8) yielded transformed cells secreting antibodies that bound the 1918 HA protein. Supernates from 30 wells of a total of 6,578 wells tested contained 1918 HA-specific antibodies, suggesting a minimal frequency of circulating 1918 HA-specific B cells in the donors of approximately 1 in  $4.6 \times 10^6$ . We collected transformed cells from the wells corresponding to supernates showing the highest levels of specific binding to the 1918 HA (derived from five donors) and fused them to the HMM2.5 non-secreting myeloma partner<sup>7</sup> using an electrofusion technique<sup>8</sup>. We isolated 17 unique hybridoma cell lines that secreted antibodies reactive with the 1918 HA from cell lines derived from four out of five donors, and then segregated the lines by limiting dilution to yield monoclonal antibody secreting clones. Our screening identified five independent lines with HAI activity against 1918 virus from three separate donors, which we biologically cloned and designated monoclonal antibodies 1I20, 1F1 and 2B12 (donor 6), monoclonal antibody 4D20 (donor 4) and monoclonal antibody 2D1 (donor 23).

Sequence analysis of the antibody genes from the clones demonstrated that the five monoclonal antibodies were distinct and very highly mutated. Genetic features of the antibodies are shown in Table 1. It was of interest that the 1F1, 2B12 and 2D1 clones shared use of the VL1-44\*01 gene segment, suggesting a particular fitness for binding of the 1918 virus HA by the CDR1/2 light-chain loops encoded by this VL gene segment. The three clones, however, were clearly independent as they differed in the location of somatic mutations, JI segment (1F1) and in heavy-chain pairing. The numbers of somatic mutations in the variable regions were exceptionally large, almost twice the median number of 18 mutations found in class-switched memory cells in randomly selected human B cells<sup>9</sup>. These data probably suggest recurrent optimization of binding affinity through multiple rounds of somatic hypermutation and selection *in vivo*.

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**Table 1 | Genetic and binding kinetics features of 1918 HA-specific human monoclonal antibodies**

	1F1	1I20	Monoclonal antibody 2B12	2D1	4D20
Gene segments					
VH	1-2*02	3-30*02	4-30-4*01	2-70*01	2-26*01
D	5-24*01	3-10*01	3-3*01	1-26*01	4-17*01
JH	4*02	5*02	4*02	2*01	6*02
VL	1-44*01	3-15*01	1-44*01	1-44*01	3-21*01
JL	2*01	1*01	3*02	3*02	1*01
Mutations					
VH	28	26	32	19†	17
N insertions	2	8	20	15	10
P insertions	1	0	0	0	0
D	1	13	1	2	1
JH	7	2	1	1	1
VL	7	9	18	7	14
JL	3	0	2	0	2
VL-JL junction	3	6	6	2	0
Isotype/subclass	IgG1	IgG1	IgG2	IgG1	IgG1
Light chain	λ	κ	λ	λ	λ
Binding kinetics to 1918 HA					
Association rate, $K_{on}$ ( $ms^{-1}$ )	$3.1 \times 10^4$	$2.9 \times 10^5$	$1.8 \times 10^4$	$3.9 \times 10^4$	$1.7 \times 10^5$
Dissociation rate, $K_{off}$ ( $s^{-1}$ )	$1.7 \times 10^{-4}$	$1.4 \times 10^{-5}$	$1.2 \times 10^{-4}$	$1.0 \times 10^{-5}$	$1.0 \times 10^{-5}$
Affinity, $K_d$ (M)	$5.4 \times 10^{-9}$	$4.8 \times 10^{-11}$	$6.2 \times 10^{-9}$	$2.5 \times 10^{-10}$	$1.4 \times 10^{-10}$

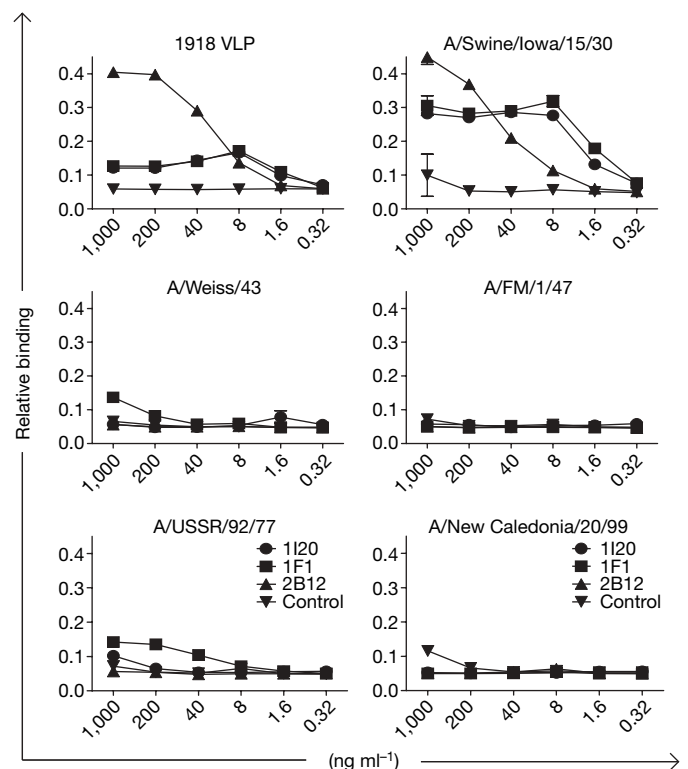
† Includes a naturally occurring nine-nucleotide insertion starting from the VH region codon 67.

Purified monoclonal antibodies were assessed by ELISA against a series of representative twentieth century H1N1 viruses including human isolates from 1918, 1943, 1947, 1977 and 1999. We also examined reactivity with influenza A/Swine/Iowa/15/30 (H1N1) virus, as the HA sequence of this virus more closely resembles the 1918 HA sequence than the sequence of any other existing isolate does. The monoclonal antibodies bound to the 1918 HA, with clear cross-reactivity with the 1930 strain, suggesting the remote origin of the antibodies (Fig. 1 and Supplementary Fig. 1). The 1F1 clone also bound to a minimal degree to the 1977 strain, a virus that is almost identical to isolates from the early 1950s<sup>10</sup>, and minimally to a 1943 isolate, but not to other post-1930 strains. The antibodies also bound to the corresponding HA expressed on the surface of mammalian cells following transfection of a complementary DNA encoding the 1918 HA as detected by immunofluorescence microscopy (Supplementary Fig. 2). The antibodies did not bind to influenza H3, B or H5 HA proteins in ELISA (data not shown). All five antibodies proved to have very high affinities for recombinant 1918 HA protein when tested by surface plasmon resonance, ranging from  $5.4 \times 10^{-9}$  to  $4.8 \times 10^{-11}$  M (Table 1).

We tested five purified monoclonal antibodies from three separate donors for inhibitory activity in an HAI assay using 1918 virus-like particles (VLP) or a panel of H1N1 viruses. The antibodies showed specific binding for 'old' viruses, including the 1918 virus or viruses that were genetically similar to the 1918 virus (Table 2 and Supplementary Table 2). Specifically, all five antibodies reacted with 1918 HA by ELISA, by HAI with VLP of the 1918 strain or with the highly genetically similar influenza A/Swine/Iowa/15/30 (H1N1) virus (Sw/30), and by neutralizing assay with reconstituted 1918 virus. Comparable HAI activities were obtained for both 1918 and Sw/30 VLP in these assays (Table 2). The five clones had specific neutralizing activities ranging from 0.32 to  $0.97 \mu g ml^{-1}$  and HAI activities of 0.18 to  $0.47 \mu g ml^{-1}$  against the 1918 virus. In contrast, the same antibodies failed to interact with, or inhibit, human H1N1 viruses isolated in 1943, 1947 or 1999 (Supplementary Table 2). The 1F1 antibody bound and neutralized the 1977 virus, albeit to a lesser degree than either the 1918 or the Sw/30 viruses (showing a specific HAI activity of  $0.16 \mu g ml^{-1}$  against the 1977 virus), and to a minimal degree the 1943 virus (Supplementary Table 2). Again, only 1F1 displayed neutralizing activity against the 1943 or 1977 viruses, with specific activities of 1.8 and  $0.88 \mu g ml^{-1}$ , respectively.

We selected antibody escape mutants for three monoclonal antibodies using the Sw/30 virus. Nucleotide sequence analysis of the HA genes from these viruses demonstrated that they had acquired

mutations in classical antigenic regions of HA. The established antigenic map of influenza A (H1N1) viruses specifies an amino acid numbering scheme and indicates five immunodominant antigenic sites, designated Sa, Sb, Ca<sub>1</sub>, Ca<sub>2</sub> and Cb<sup>11,12</sup>. The 2B12 mutants possessed mutations at residue 166 (K166Q, K166E or K166P), which lies in the Sa antigenic site (Supplementary Fig. 3), using the previous numbering scheme<sup>11</sup>. Escape mutants for 1F1 and 1I20 possessed an identical mutation, P186H, at a residue adjacent to the previously



**Figure 1 | Binding of human monoclonal antibodies to representative twentieth century H1N1 viruses.** Equivalent HA units of 1918 VLP, A/Swine/Iowa/15/30, A/Weiss/43, A/FM/1/47, A/USSR/92/77 and A/New Caledonia/20/99 influenza viruses were absorbed onto ELISA plates. An ELISA was performed using serial 1:5 dilutions of monoclonal antibodies 1F1, 1I20, 2B12 or an H5-specific control human monoclonal antibody. Relative binding ( $y$  axis) indicates optical density in ELISA binding assay to absorbed VLP or virus.

**Table 2 | Specific HAI activity of human antibodies against influenza viruses or VLP**

Antigen	Monoclonal antibody				
	1F1	1I20	2B12	2D1	4D20
Sw/30 viruses					
Wild type	0.04	0.08	1.25	0.08	0.04
P186H (site Sb)	—	—	1.25	ND	ND
K166E (site Sa)	0.04	0.04	—	ND	ND
K166Q (site Sa)	0.04	0.04	—	ND	ND
1918 VLP					
Wild type	0.08	0.16	0.31	0.01	0.04
P186H (site Sb)	—	0.63	0.31	0.01	0.04
K166E (site Sa)	<0.04	0.08	—	—	0.63
K166Q (site Sa)	<0.04	0.04	—	2.5	0.31

Specific HAI activity ( $\mu\text{g ml}^{-1}$ ) of monoclonal antibodies was calculated as the lowest concentration of antibody that displayed haemagglutinating activity. —, activity was not detected at any concentration tested, up to  $2.5 \mu\text{g ml}^{-1}$ ; ND, not determined.

defined Sb antigenic site (Supplementary Fig. 3), which encompasses the  $\alpha$ -helix of the receptor binding site in the 1918 HA structure<sup>11–13</sup>. As expected, incorporation of the Sb antigenic site mutation into VLP reduced (1I20) or eliminated (1F1) activity of the corresponding antibodies in an HAI assay without affecting binding of 2B12 (Table 2). The 2D1 and 4D20 antibodies also had reduced HAI activity against the site Sb mutant viruses and VLP, suggesting that they also bound to this site. In contrast, incorporation of the Sa mutation into VLP abolished activity of 2B12 in the HAI assay, but did not affect the activity of the other four antibodies (Table 2). The 1918 and Sw/30 virus HAs differ by only a single amino acid in each of the Sa and Sb sites (Supplementary Fig. 3), explaining why these antibodies cross-neutralize both viruses. In contrast, the 1943 isolate contains seven changes in the Sa site and seven changes in the Sb site, relative to the 1918 HA sequence, explaining the partial or complete loss of

neutralizing ability of the antibodies for these later isolates. Notably, 9 of the 12 residues at the Sb site differed between the 1918 and the 1977 virus. The ability of 1F1 to cross-neutralize the 1977 virus despite its divergence from the 1918 virus is worthy of further investigation, because these data raise the possibility that 1F1 recognizes a new, more broadly neutralizing epitope. Alternatively, the data may represent epitope recycling such that, despite significant sequence divergence, the 1F1 epitope reappeared. In either case, further characterization of this antibody may suggest strategies to elicit enhanced cross-protective immunity to influenza A viruses of a particular HA subtype.

We tested the five antibodies for therapeutic efficacy in an established mouse model of infection. Mice were inoculated by the intranasal route with the previously reconstructed 1918 virus, and morbidity (measured by weight loss), mortality and virus replication were assessed as previously described<sup>3</sup>. Each of the five 1918-specific monoclonal antibodies tested showed therapeutic efficacy when administered 1 day after infection, preventing death of animals (Table 2). Mice treated with a control (H5 HA-specific) human monoclonal antibody or human IgG did not survive. Reduced weight loss and lower levels of virus replication in the lungs of anti-1918-antibody-treated mice on day 4 after infection also demonstrated a significant protective effect that correlated well with survival data. At lower doses, the antibodies caused a statistically significant delay to death, relative to the controls.

These studies suggest that B cells responding to viral infections, or their progeny, survive for the life of the host, even nine or more decades after exposure. It is well established that a subset of plasma cells is long-lived<sup>14</sup>, and these cells contribute to durable humoral immune responses<sup>15</sup>, such as that observed after childhood smallpox vaccination<sup>16–19</sup>. Memory B cell pools also can be long-lived,

**Table 3 | Therapeutic efficacy of 1918 HA-specific monoclonal antibodies against 1918 virus in mice**

Antibody	Experiment	Dose, per mouse ( $\mu\text{g}$ )*	Weight loss (%)†	Virus in lung‡	Survival (no. protected/total no.)§
2B12	1	200	2.3	$3.8 \pm 1.4$	5/5
		20	11	ND	5/5
		2	14	ND	0/5
1F1	1	200	1.0	$3.9 \pm 1.4$	5/5
		20	12	ND	4/5
		2	10	ND	0/5
1I20	1	20	4.2	$5.4 \pm 0.2$	5/5
		2	14	ND	0/5
		0.2	16	ND	0/5
2D1	2	200	3.0	$3.3 \pm 0.5$	5/5
		20	10	$5.1 \pm 0.2$	5/5
		2	14	$5.9 \pm 0.5$	0/5
4D20	2	200	7.6	$3.5 \pm 0.6$	5/5
		20	15	$5.4 \pm 0.5$	2/5
		2	17	$5.8 \pm 0.4$	0/5
Control H5 HA antibody	1	200	13	$6.4 \pm 0.3$	0/5
		20	15	ND	0/5
		2	18	ND	0/5
Control human IgG	1	200	15	$6.3 \pm 0.2$	0/5
		2	18	$6.4 \pm 0.3$	0/5
	2	20	11	ND	0/5
		2	16	ND	0/5
	2	2	14	ND	0/5
		2	16	ND	0/5

The statistical significance of viral titre and morbidity data was determined by analysis of variance (ANOVA). The statistical significance of mortality data was determined using the null model likelihood ratio test and the Mann–Whitney *U*-test.

\* Groups of mice were infected intranasally with five LD<sub>50</sub> of 1918 virus and then treated 24 h later with graded doses of 1918-specific monoclonal antibodies or control antibodies.

† Maximum per cent weight loss (mean of five mice per group); highest antibody dose of each experimental group,  $P \leq 0.05$  (ANOVA) versus control antibody groups.

‡ Average lung titres of three mice on day 4 after inoculation, expressed as ( $\log_{10}$ ) 50% egg infectious dose (EID<sub>50</sub>) per ml  $\pm$  s.d. All 20 and 200  $\mu\text{g}$  dose experimental groups,  $P \leq 0.024$  (ANOVA) versus control antibody groups.

§ High antibody dose of each experimental group and 20  $\mu\text{g}$  of 1F1 or 2D1,  $P \leq 0.0031$  (Mann–Whitney *U*-test) versus control antibody groups. ND, not determined.

sustained in part by antigen-independent polyclonal stimuli<sup>20</sup>. It is difficult to be absolutely certain that the monoclonal antibodies isolated here were first stimulated by exposure during the 1918 pandemic. However, the clinical history of the subjects and the high functional specificity of the monoclonal antibodies for the 1918 strain strongly suggest that recent exposures do not account for this immunity. Probably, boosting by antigenically related viruses in the early decades of the twentieth century may have contributed to the ability of these subjects to sustain these B cells. The variable genes of five independent human neutralizing antibodies had a very high frequency of somatic mutations, associated with strong binding constants and high potency. The *in vivo* efficacy of treatment with these antibodies shows that the development of functional adaptive immunity to the pandemic virus did occur in survivors of the 1918 pandemic.

It has long been known that infusion of neutralizing antibodies can protect mice from lethal influenza virus infection, and transfusion of convalescent blood products to 1918 influenza victims may have had a beneficial effect<sup>21</sup>. Thus, the monoclonal antibodies described here could serve as potential therapeutics for a re-emergent 1918-like virus. The techniques described here suggest that it may be possible to recover human antibodies that display a wide array of specificities corresponding to the viruses and other pathogens that have infected an individual during their lifetime.

## METHODS SUMMARY

Recombinant 1918 virus HA (A/South Carolina/1/1918) was produced as described<sup>11</sup>. Peripheral blood mononuclear cells were obtained from volunteers born in 1915 or earlier. Hybridomas were generated from EBV-transformed B cell lines by electrofusion to the HMM2.5 cell line<sup>7,8</sup>. When hybridoma lines formed colonies in the presence of selecting drugs, lines were cloned by limiting dilution. Secreted monoclonal antibodies were concentrated and purified by fast protein liquid chromatography (FPLC). The isotype and subclass of secreted antibodies were determined by ELISA. Nucleotide sequences of variable gene segments were determined by automated sequence analysis of cloned cDNA<sup>22</sup>. The identity of the gene segments and mutations from the germline sequences were determined by alignment using the ImmunoGeneTics database<sup>23</sup>. Viruses were propagated in 10-day-old embryonated chicken eggs. Influenza A/South Carolina/1/18 virus was prepared as previously described<sup>3</sup>. Expression plasmids encoding the 1918 HA and neuraminidase (NA) proteins were described previously<sup>24,25</sup>. Binding of antibodies was determined using 1918 VLP or influenza A viruses as the coating antigen in ELISA. VLP were produced by co-transfection of 293T cells with expression plasmids for the 1918 HA and 1918 NA, consistent with a recent report<sup>26</sup>. HAI assays of sera or antibodies were performed according to standard protocols using chicken red blood cells<sup>27</sup>. For microneutralization assay, ten 50% tissue-culture infective dose units (TCID<sub>50</sub>) of virus was preincubated with dilutions of sera or monoclonal antibody and then used to infect Madin-Darby canine kidney (MDCK) cells in 96-well plates, as described<sup>28,29</sup>. The kinetic interaction of monoclonal antibodies with the 1918 HA protein was determined by surface plasmon resonance. Antibody escape mutants were isolated by treatment of Sw/30 virus with excess antibody as described<sup>12,30</sup>. Mice were inoculated intranasally with five LD<sub>50</sub> (lethal dose to 50% of animals) of the 1918 virus. At 24 h after inoculation, we administered 1918-specific monoclonal antibody or control antibodies to each mouse. Mice were observed for weight loss or death. Subsets of animals were killed for virus titre.

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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- Johnson, N. P. & Mueller, J. Updating the accounts: global mortality of the 1918–1920 “Spanish” influenza pandemic. *Bull. Hist. Med.* **76**, 105–115 (2002).
- Taubenberger, J. K. *et al.* Characterization of the 1918 influenza virus polymerase genes. *Nature* **437**, 889–893 (2005).
- Tumpey, T. M. *et al.* Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* **310**, 77–80 (2005).
- Kobasa, D. *et al.* Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* **445**, 319–323 (2007).
- Taubenberger, J. K. The origin and virulence of the 1918 “Spanish” influenza virus. *Proc. Am. Phil. Soc.* **150**, 86–112 (2006).

- Kash, J. C. *et al.* Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* **443**, 578–581 (2006).
- Posner, M. R., Elboim, H. & Santos, D. The construction and use of a human-mouse myeloma analogue suitable for the routine production of hybridomas secreting human monoclonal antibodies. *Hybridoma* **6**, 611–625 (1987).
- Yu, X., McGraw, P. A., House, F. S. & Crowe, J. E. Jr. An optimized electrofusion-based protocol for generating virus-specific human monoclonal antibodies. *J. Immunol. Methods* **336**, 142–151 (2008).
- Tian, C. *et al.* Evidence for preferential Ig gene usage and differential TdT and exonuclease activities in human naive and memory B cells. *Mol. Immunol.* **44**, 2173–2183 (2007).
- Nakajima, K., Desselberger, U. & Palese, P. Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950. *Nature* **274**, 334–339 (1978).
- Stevens, J. *et al.* Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science* **303**, 1866–1870 (2004).
- Caton, A. J., Brownlee, G. G., Yewdell, J. W. & Gerhard, W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* **31**, 417–427 (1982).
- Brownlee, G. G. & Fodor, E. The predicted antigenicity of the haemagglutinin of the 1918 Spanish influenza pandemic suggests an avian origin. *Phil. Trans. R. Soc. Lond. B* **356**, 1871–1876 (2001).
- Manz, R. A., Thiel, A. & Radbruch, A. Lifetime of plasma cells in the bone marrow. *Nature* **388**, 133–134 (1997).
- Slifka, M. K., Antia, R., Whitmire, J. K. & Ahmed, R. Humoral immunity due to long-lived plasma cells. *Immunity* **8**, 363–372 (1998).
- Amanna, I. J., Slifka, M. K. & Crotty, S. Immunity and immunological memory following smallpox vaccination. *Immunol. Rev.* **211**, 320–337 (2006).
- Crotty, S. *et al.* Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J. Immunol.* **171**, 4969–4973 (2003).
- Hammarlund, E. *et al.* Duration of antiviral immunity after smallpox vaccination. *Nature Med.* **9**, 1131–1137 (2003).
- Amanna, I. J., Carlson, N. E. & Slifka, M. K. Duration of humoral immunity to common viral and vaccine antigens. *N. Engl. J. Med.* **357**, 1903–1915 (2007).
- Bernasconi, N. L., Traggiai, E. & Lanzavecchia, A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* **298**, 2199–2202 (2002).
- Luke, T. C., Kilbane, E. M., Jackson, J. L. & Hoffman, S. L. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? *Ann. Intern. Med.* **145**, 599–609 (2006).
- Weitkamp, J. H. *et al.* Generation of recombinant human monoclonal antibodies to rotavirus from single antigen-specific B cells selected with fluorescent virus-like particles. *J. Immunol. Methods* **275**, 223–237 (2003).
- Ruiz, M. *et al.* IMGT, the international ImmunoGeneTics database. *Nucleic Acids Res.* **28**, 219–221 (2000).
- Tumpey, T. M. *et al.* Existing antivirals are effective against influenza viruses with genes from the 1918 pandemic virus. *Proc. Natl Acad. Sci. USA* **99**, 13849–13854 (2002).
- Glaser, L. *et al.* A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. *J. Virol.* **79**, 11533–11536 (2005).
- Chen, B. J., Leser, G. P., Morita, E. & Lamb, R. A. Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. *J. Virol.* **81**, 7111–7123 (2007).
- World Health Organization Collaborating Centers for Reference and Research on Influenza. in *Concepts and Procedures for Laboratory-Based Influenza Surveillance* (eds Kendal, A. P., Skehel, J. J. & Pereira, M. S.) B17–B35 (Centers for Disease Control and Prevention, 1982).
- Mozdzanowska, K. *et al.* A pulmonary influenza virus infection in SCID mice can be cured by treatment with hemagglutinin-specific antibodies that display very low virus-neutralizing activity *in vitro*. *J. Virol.* **71**, 4347–4355 (1997).
- Reed, L. J. & Muench, H. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**, 493–497 (1938).
- Yewdell, J. W., Webster, R. G. & Gerhard, W. U. Antigenic variation in three distinct determinants of an influenza type A haemagglutinin molecule. *Nature* **279**, 246–248 (1979).

**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Author Contributions** X.Y., P.A.M., M.D.H. and F.S.H. made and cloned the monoclonal antibodies, sequenced antibody genes, and performed immunofluorescence experiments. T.T. characterized the interaction of the antibodies with viruses and VLPs and selected for and characterized the escape mutants. C.J.K. performed biosensor studies. T.M.T., C.P. and L.A.P. designed and performed *in vivo* studies. O.M. sequenced the HA genes of the H1N1 viruses used in this study and performed ELISA assays with these viruses. P.V.A. assisted with HAI and neutralization assays and with cloning of recombinant HA molecules. J.S. and I.A.W. provided recombinant HA. E.L.A. led the clinical recruitment and, together with

C.F.B. and J.E.C., conceived of the experimental plan. C.F.B. and J.E.C. wrote the manuscript. All authors discussed the results and commented on the manuscript.

**Author Information** Antibody nucleotide sequences have been deposited in GenBank under accession numbers EU169674 to EU169679, and EU825947 to EU825950. Reprints and permissions information is available at [www.nature.com/reprints](http://www.nature.com/reprints). Correspondence and requests for materials should be addressed to J.E.C. (James.Crowe@vanderbilt.edu), C.F.B. (Chris.Basler@mssm.edu), or E.L.A. (altschel@umdnj.edu).

## METHODS

**Subjects.** Volunteers born in 1915 or earlier were recruited at the UMDNJ site. We obtained 50 ml of peripheral venous blood from each subject after informed written consent.

**Antigen.** Recombinant A/South Carolina/1/1918 virus HA was generated in a baculovirus expression system and purified, as described<sup>11</sup>.

**Production of monoclonal antibodies.** Peripheral blood monocytes were obtained from eight donors by density gradient centrifugation of whole heparinized blood. B cells were transformed in 384-well plates with approximately 1,000 B cells per well by *in vitro* culture in medium with CpG ODN 10103 (Coley) and Epstein–Barr virus (EBV) (supernate from cell line B95.8). Supernates from the resulting cell lines were tested for reactivity with the 1918 HA by ELISA. Hybridomas were generated using cells from wells containing reactive lines by fusion to the HMMA2.5 non-secreting myeloma cell line<sup>7</sup> using electrofusion followed by HAT (hypoxanthine, aminopterin and thymidine) and ouabain drug selection, as described<sup>8</sup>. When hybridomas formed colonies, lines were screened using an anti-1918 HA ELISA; positive lines were cloned by limiting dilution. Supernates of high-density cultures were produced in CELLline devices (BD Biosciences). Purified and concentrated preparations of each of the antibodies were prepared by FPLC using protein G conjugated resin on an AKTA instrument (GE Healthcare) followed by concentration and buffer exchange using ultra centrifugal filter devices (Millipore).

**Characterization of monoclonal antibodies.** The isotype and subclass of secreted antibodies were determined by ELISA. Nucleotide sequences of variable gene segments were determined from cloned cDNA generated by PCR with reverse transcription (RT–PCR) amplification of cellular messenger RNA using variable gene-specific primers designed to amplify antibody genes from all gene families<sup>22</sup>. Identity of the gene segments and mutations from the germline sequences were determined by alignment using the ImMunoGeneTics database (<http://imgt.cines.fr>)<sup>23</sup>.

**Viruses and plasmids.** The following viruses were propagated in 10-day-old embryonated chicken eggs: influenza A/Weiss/43, A/FM/1/47, A/USSR/92/77, A/New Caledonia/20/99, A/Swine/Iowa/15/30. The sequences of the HA genes of these viruses used were confirmed by RT–PCR and cDNA sequence analysis. Influenza A/South Carolina/1/18 virus was prepared as described<sup>3</sup>. Expression plasmids encoding the 1918 HA and NA proteins were described previously<sup>24,25</sup>. The 1918 virus was handled under biosafety level 3 enhanced (BSL3) containment in accordance with guidelines of the National Institutes of Health (NIH) and the Centers for Disease Control and Prevention (CDC) (available at <http://www.cdc.gov/flu/h2n2bsl3.htm>) and in accordance with requirements of the US Department of Agriculture (USDA)–CDC select agent program.

**ELISA.** Equivalent HA units of 1918 VLP or of influenza A viruses were diluted in coating buffer (51-2713KC; BD Biosciences) and adsorbed overnight onto ELISA plates (Nunc). Plates were washed with PBS containing 0.05% Tween-20 and blocked using blocking solution (555213; BD Biosciences) or PBS containing 5% FCS at room temperature for 1 h. Diluted monoclonal antibodies were added to the plates and incubated at 4 °C for 16 h. Plates were washed and incubated with HRP-conjugated goat anti-human IgG (H10507; Caltag) for 1 h. Washed plates were developed using TMB substrate, and the reaction was stopped using 1 M sulphuric acid. Optical density was read at 450 nm using an ELISA plate reader.

**Production of VLP.** VLP were produced by co-transfection of 10<sup>6</sup> 293T cells with 1 µg each of expression plasmids for the 1918 HA and NA. Two days after transfection, supernatants were collected and assayed for HA activity. Our ability to produce these haemagglutinating particles in the absence of other viral proteins is consistent with a recent report<sup>26</sup>.

HAI assays of sera or monoclonal antibodies were performed according to standard protocols<sup>27</sup>. In brief, sera were initially diluted 1:10 in receptor-destroying enzyme from *Vibrio cholerae* (Denka Seiken). Serial dilutions of sera or monoclonal antibodies were pre-incubated with 8 HA units of virus per well. Chicken red blood cells were added to a final concentration of 0.5% and the plate was incubated on ice for 30–60 min.

**Microneutralization assay.** Ten TCID<sub>50</sub> units of virus were preincubated with dilutions of sera or monoclonal antibody and used to infect MDCK cells in 96-well plates with six replicates, as described<sup>28</sup>. Neutralizing concentrations were defined as the reciprocal of the highest dilution of serum in which 50% of wells were infected, as calculated by the method of ref. 29. Specific neutralizing activity of antibodies was calculated as the lowest concentration of antibody that displayed activity.

**Biosensor studies.** The kinetic interaction of antibodies with recombinant 1918 HA protein was determined by surface plasmon resonance using a Biacore 2000 instrument. Purified 1918 HA protein was diluted to 30 µg ml<sup>-1</sup> in 10 mM sodium acetate, pH 4.5, and covalently immobilized at 5 µl min<sup>-1</sup> by amine coupling to the dextran matrix of a CM5 sensor chip (Biacore AB) with a target density of 1,200 response units. Unreacted active ester groups were blocked with 1 M ethanolamine. All five purified 1918 monoclonal antibodies and a human H5-specific influenza antibody (negative control) at concentrations ranging from 5 to 500 nM in HBS/Tween-20 buffer (Biacore AB) were injected over the immobilized 1918 HA protein or reference cell surface. Association rates ( $K_{on}$ ), dissociation rates ( $K_{off}$ ), and affinities or equilibrium dissociation constants ( $K_d$ ) were calculated by aligning the binding curves globally to fit a 2:1 Langmuir binding model using BIAevaluation 4.1 software. The goodness of each fit was based on the agreement between experimental data and the calculated fits, in which the  $\chi^2$  values were below 1.0.

**Selection and characterization of antibody escape mutants.** Mutants were isolated as described<sup>12,30</sup>. In brief, escape mutant viruses were selected by treatment of Sw/30 virus with excess antibody, followed by recovery of neutralization-resistant viruses in eggs. RNA was extracted from virus-infected allantoic fluid, then cDNA was generated by RT–PCR, cloned, sequenced and aligned to previously determined wild-type virus HA gene sequences.

**Animal studies.** Female BALB/c (8-week-old) mice were inoculated intranasally with 5 LD<sub>50</sub> in a 50 µl volume of the virulent reconstituted 1918 virus. At 24 h after inoculation, we administered 200, 20, 2 or 0.2 µg (approximately 10, 1, 0.1 or 0.01 mg kg<sup>-1</sup>) of 1918-specific monoclonal antibody or a similarly prepared human monoclonal antibody to H5 influenza HA (clone IE5), or an equal volume of human IgG, to each mouse, in groups of 11 (highest dose) or 5 (lower doses) mice. Mice were observed for 16 days for weight loss or death. Subsets of three animals treated with the highest dose were killed on day 2 and 4 after infection, and whole lungs were homogenized in 1 ml of sterile PBS. Virus titre in lung tissue homogenates was determined by plaque titration in MDCK cell monolayer cultures.