

Structural and genetic basis for development of broadly neutralizing influenza antibodies

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Influenza viruses take a yearly toll on human life despite efforts to contain them with seasonal vaccines. These viruses evade human immunity through the evolution of variants that resist neutralization. The identification of antibodies that recognize invariant structures on the influenza haemagglutinin (HA) protein have invigorated efforts to develop universal influenza vaccines. Specifically, antibodies to the highly conserved stem region of HA neutralize diverse viral subtypes. These antibodies largely derive from a specific antibody gene, heavy-chain variable region *IGHV1-69*, after limited affinity maturation from their germline ancestors^{1,2}, but how HA stimulates naive B cells to mature and induce protective immunity is unknown. To address this question, we analysed the structural and genetic basis for their engagement and maturation into broadly neutralizing antibodies. Here we show that the germline-encoded precursors of these antibodies act as functional B-cell antigen receptors (BCRs) that initiate subsequent affinity maturation. Neither the germline precursor of a prototypic antibody, CR6261 (ref. 3), nor those of two other natural human *IGHV1-69* antibodies, bound HA as soluble immunoglobulin-G (IgG). However, all three *IGHV1-69* precursors engaged HA when the antibody was expressed as cell surface IgM. HA triggered BCR-associated tyrosine kinase signalling by germline transmembrane IgM. Recognition and virus neutralization was dependent solely on the heavy chain, and affinity maturation of CR6261 required only seven amino acids in the complementarity-determining region (CDR) H1 and framework region 3 (FR3) to restore full activity. These findings provide insight into the initial events that lead to the generation of broadly neutralizing antibodies to influenza, informing the rational design of vaccines to elicit such antibodies and providing a model relevant to other infectious diseases, including human immunodeficiency virus/AIDS. The data further suggest that selected immunoglobulin genes recognize specific protein structural 'patterns' that provide a substrate for further affinity maturation.

Antibodies to the conserved stem region of HA block membrane fusion and prevent productive infection by diverse influenza viruses. The structural basis of HA stem recognition of two such monoclonal antibodies, CR6261 and F10, has been defined^{3,4}. These antibodies bind with nanomolar affinity to a highly conserved hydrophobic groove at the interface of HA1 and HA2, the two polypeptides that constitute HA, and neutralize several influenza group 1 subtypes including H1, H5, H6, H8 and H9 (ref. 3). Among the anti-stem antibodies isolated so far, the *IGHV1-69* gene segment is observed more frequently than expected by chance^{2,5}. To understand the development of these antibodies, we studied the prototypic *IGHV1-69*-derived broadly neutralizing antibody CR6261, isolated by phage display of human immunoglobulin genes, as well as two others cloned from single human cells, FE53 and 1009-3B05 (refs 1–3). Influenza *IGHV1-69*-based broadly neutralizing antibodies undergo a relatively low degree of somatic mutation (an average of 14 amino acids in the

heavy chain, $n = 9$)^{1–4} (Fig. 1a). We first asked whether their germline antibody precursors might recognize HA with measurable affinity. Notably, the *IGHV1-69* germline ancestors of CR6261, FE53 and 1009-3B05 failed to bind HA as soluble IgG, even at concentrations as high as 100 $\mu\text{g ml}^{-1}$ (Fig. 1b).

To define the molecular basis for affinity maturation of these antibodies, we analysed the respective contributions of heavy and light chains to antigen recognition. We compared chimaeric antibodies that consisted of somatic heavy (sH) and germline light (gL) chains to the mature antibody (sHsL). The chimaeric sHgL of all three antibodies bound to a recombinant H1 HA with affinities similar to their respective matured sHsL (Fig. 1b). Maturation of the light chain thus does not affect binding to H1 HA. Rather, somatic mutation of the *IGHV1-69* heavy chain gene alone mediates the increase in binding affinity. This finding is consistent with the lack of light-chain interaction observed in the crystal structures of both CR6261–HA and F10–HA complexes^{3,4}, suggesting that *IGHV1-69* light-chain somatic mutation may be incidental and is not required for heavy–light-chain pairing or improved neutralization function.

We next investigated the minimum requirements of heavy-chain loop maturation that lead to somatic activity. We chose to focus this

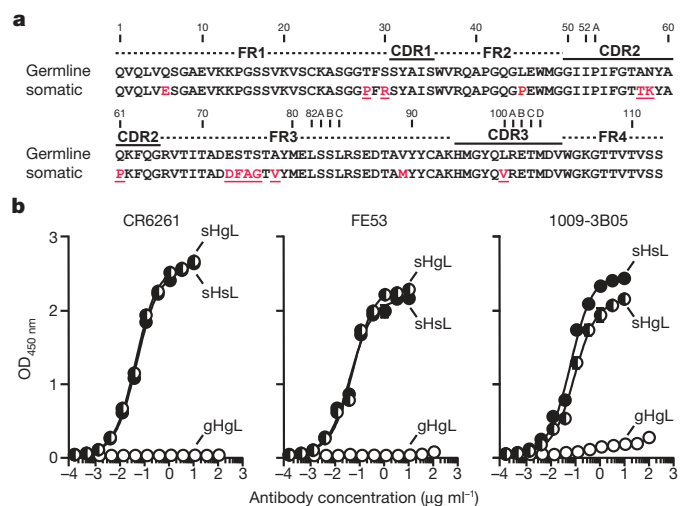


Figure 1 | Somatic maturation of heavy chain confers HA reactivity. **a**, Amino acid alignment of somatic CR6261 heavy-chain variable region (including the D and J regions) with JOINSOLVER²⁵ predicted germline precursor (Kabat²⁶ convention). Somatic mutations are shown in red. Those incorporated into germline variants are underlined. Mutations T28P and S30R are in close proximity to the Kabat-defined CDR H1 and are CR6261 contact residues, and therefore defined as CDR H1 somatic mutations in the remaining text. **b**, ELISA binding of somatic (sHsL, filled circles), respective germline (gL, open circles), and chimaeric (sHgL, half-filled circles) indicated HA-stem-specific antibodies to H1 1999 NC. OD, optical density. Error bars represent standard deviations of the mean for each antibody concentration.

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analysis on CR6261, the first IGHV1-69 anti-stem broadly neutralizing antibody discovered, for which the structure is known. The sH of CR6261 differs from the germline heavy (gH) chain by only 14 amino acids (11.6%) (Fig. 1a). Four of these amino acids (Pro 28, Arg 30, Lys 58 and Phe 74) contact the HA stem in the crystal structure (Fig. 2a). We generated several CR6261 germline variants by introducing residues from sH, including CDR H1, CDR H2, CDR H3 or FR3, into gH, singly or in combination. The resulting antibodies were analysed for their ability to bind HA and neutralize virus in an HA-pseudotyped lentiviral system⁶ (Table 1). Tested individually, only the somatic CDR H1 (sCDR H1: Thr28Pro/Ser30Arg) increased binding, but it was more than 100-fold less than sH. Furthermore, sCDR H1 alone did not confer detectable activity in our neutralization assay (Table 1). Although sFR3 alone did not improve the potency of germline CR6261, sCDR H1 and sFR3 together restored full activity for both binding and neutralization against H1N1 and H5N1 viruses (Table 1, Fig. 2b and Supplementary Fig. 1). This finding suggests that the

mutation of only a small number of germline residues enables potent neutralization.

Because CDR H1 and FR3 sit adjacent to one another in the folded protein, we examined the protein structure further. In most IGHV1-69 antibodies with no CDR H1 mutations, Phe 29 is buried in the 'canonical' conformation of the CDR H1 (ref. 7). In contrast, the somatically mutated CDR H1 of CR6261 flips the hydrophobic residue Phe 29 out, placing this residue in contact with HA. To determine whether the position of Phe 29 in CR6261 is due to maturation or binding, we solved the crystal structure of unliganded CR6261 (sHgL), and compared it to both a IGHV1-69 antibody with no mutations in CDR H1, and HA-liganded CR6261 (Fig. 2c). In the unliganded structure, Phe 29 is exposed on the surface, suggesting that the somatic mutations Thr28Pro and Ser30Arg lead to its placement there—a hydrophobic residue from the interior of the antibody is made available to contribute to the interface by mutation of adjacent residues. The CDR H1 of IGHV1-69 in both germline and affinity-matured states is not well ordered unless bound to antigen. However, the CDR H1 loop in CR6261 favours the non-canonical conformation, with Phe 29 exposed, whether or not HA is bound. The solved structure suggests that the main consequence of somatic CDR H1 mutation is to favour this non-canonical state. Comparison of these structures also explains the synergistic effect of mutations in CDR H1 and FR3; somatic mutation in FR3 introduces Phe 74 on the surface of CR6261 adjacent to Phe 29 in CDR H1, and these two sets of mutations increase the hydrophobicity of the contact surface more than either alone.

The minimal somatic mutation of the CR6261 germ line required to confer full activity made it surprising that the germline antibody did not recognize HA with any measurable affinity in solution. We proposed that antibody recognition by the low-affinity germline IGHV1-69 revertant requires a more physiological presentation, for example, on the cell surface where such antibodies would normally be expressed. A naturally bivalent transmembrane IgM form of the CR6261 germ line was transfected into human embryonic kidney 293F cells. Cell surface expression was confirmed by staining with an anti-lambda-chain antibody. Fluorescent-labelled HA stained CR6261-germline-expressing cells but not mock transfected cells, as measured by flow cytometry (Fig. 3a). In contrast, no binding was seen in solution with CR6261 germline Fab monomer, bivalent IgG, or decameric IgM antibody derivatives (Supplementary Fig. 2). The specificity of CR6261 germline binding to the HA stem was further confirmed by its minimum reactivity to a mutant HA probe that blocks anti-stem-antibody binding (Supplementary Fig. 3). FE53 and 1009-3B05 each showed similar membrane-dependent recognition (Fig. 3a). Our results suggest that membrane presentation of antibody provides a mechanism by which low-affinity germline B cells achieve sufficient binding to recognize antigens before affinity maturation, and are consistent with experiments showing that two-dimensional confinement and clustering at the plasma membrane can increase the apparent affinity of cell surface receptors^{8,9}.

Two amino acids at the tip of CDR H2, Ile 53 and Phe 54, seem to be an anchor by which germline IGHV1-69 might attach to HA (Fig. 2a). Indeed, mutation of these two amino acids to alanines abolished HA germline binding for all V_H-1-69 antibodies (Fig. 3a). Individual mutation of Ile 53 and Phe 54 also abolished binding of unmodified/native HA trimer (binding in the presence of 6'-sialyllactose to prevent sialic acid mediated cell adhesion; Fig. 3b and Supplementary Fig. 4). In the CR6261-HA co-crystal structure, CDR H2 inserts into a hydrophobic pocket between HA1 and HA2 (ref. 3) (Fig. 3c). These data suggest that this interaction of the germline CDR H2 has a central role in recognition and engagement of the HA stem.

To determine whether binding of antigen to germline antibodies displayed on the cell surface could induce BCR activation and signalling, the transmembrane IgM version of the CR6261 germline was transfected into a transformed human B cell capable of expressing a functional BCR^{10,11}, a Ramos cell clone whose endogenous IgM is not

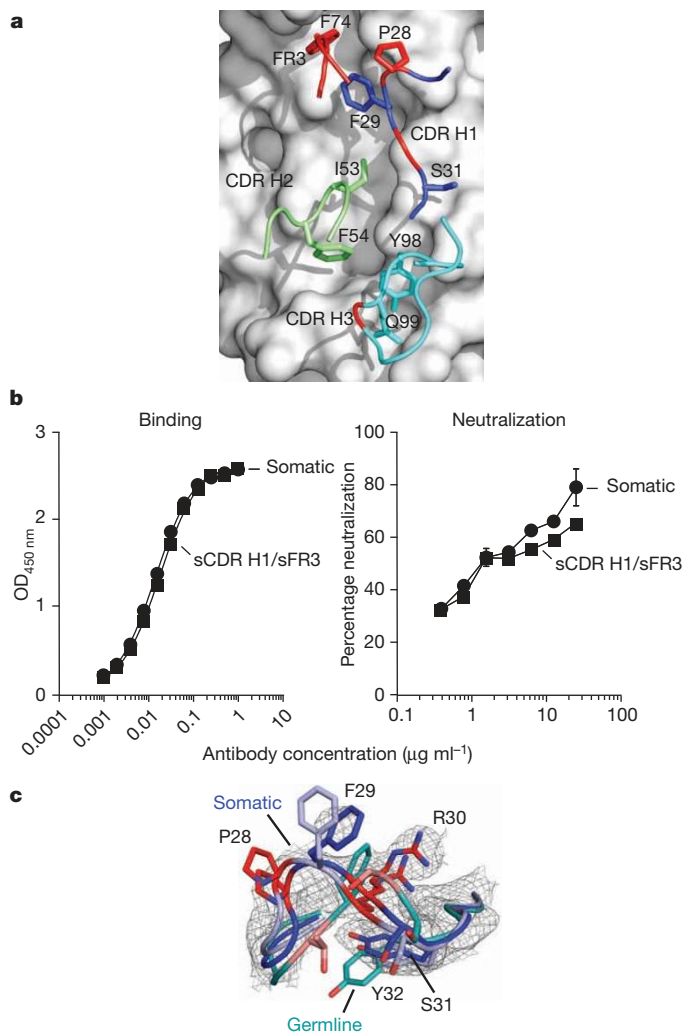


Figure 2 | CDR H1 maturation confers CR6261 neutralization activity.

a, CR6261 interaction with H1 A/South Carolina/1/1918 (1918 SC) HA stem (grey, PDB accession 3GBN). Side chains with at least 10 Å² of interaction are depicted as sticks, with somatically mutated residues coloured red. **b**, ELISA binding and neutralization of H1 1999 NC by mature (filled circles) and sCDR H1/sFR3 germline-mutated CR6261 (filled squares). Error bars represent standard deviations of the mean for each antibody concentration. **c**, CDR H1 of CR6261 uncomplexed (blue) or with HA (light blue, PDB accession 3GBN) compared with unmutated CDR H1 from 47e (cyan, PDB accession 1RZI); mutated residues are in red (CR6261) or pink (47e). A non-crystallographic symmetry (NCS)-averaged electron density map (grey) is contoured at 1.0σ.

Table 1 | HA binding and pseudovirus neutralization analysis

Germline revertants	Mutations	H1 1999 NC	
		Titre ($\mu\text{g ml}^{-1}$)	IC ₅₀ ($\mu\text{g ml}^{-1}$)
Somatic	Full	0.00133	1.9
Germline	None	>100	>25
sCDR H1	T28P S30R	0.150	>25
sCDR2 H2	A57T N58K Q61P	>100	>25
sFR3	D73E F74S A75T G76S V78A	63.2	>25
sCDR H3	V100L	>100	>25
sCDR H1/sCDR H2	T28P S30R A57T N58K Q61P	0.150	>25
sCDR H1/sFR3	T28P S30R D73E F74S A75T G76S V78A	0.00133	2.0
sCDR H1/sCDR H3	T28P S30R V100L	0.250	>25
sCDR H2/sFR3	A57T N58K Q61P D73E F74S A75T G76S V78A	0.950	>25
sCDR H2/sCDR H3	A57T N58K Q61P V100L	0.150	>25
sFR3/sCDR H3	D73E F74S A75T G76S V78A V100L	>100	>25
sCDR H1/sCDR H2/sFR3	T28P S30R A57T N58K Q61P D73E F74S A75T G76S V78A	0.00133	1.4

Binding was determined by ELISA endpoint dilution titres. Half-maximum inhibitory concentration (IC₅₀) values represent 50% neutralization of pseudotyped lentiviruses by the respective antibodies. Mutations incorporated into germline-revertant antibodies correspond to the variable regions indicated.

expressed (Methods). We found that proteoliposome-arrayed HA (Supplementary Fig. 5) selectively triggered tyrosine phosphorylation of BCR effector proteins HS1 and SLP-65 (ref. 12) (Fig. 3d). Signalling by HA was comparable to that induced by IgM cross-linking. Furthermore, mutation of Ile53Ala/Phe54Ala in CDR H2 abolished the response to

HA stimulation, consistent with the binding data and confirming the importance of the germline CDR H2 structure in naive B-cell activation. These findings indicate that engagement of low-affinity germline IGHV1-69 antibody can lead to BCR activation, thus triggering further maturation and the subsequent humoral immune response.

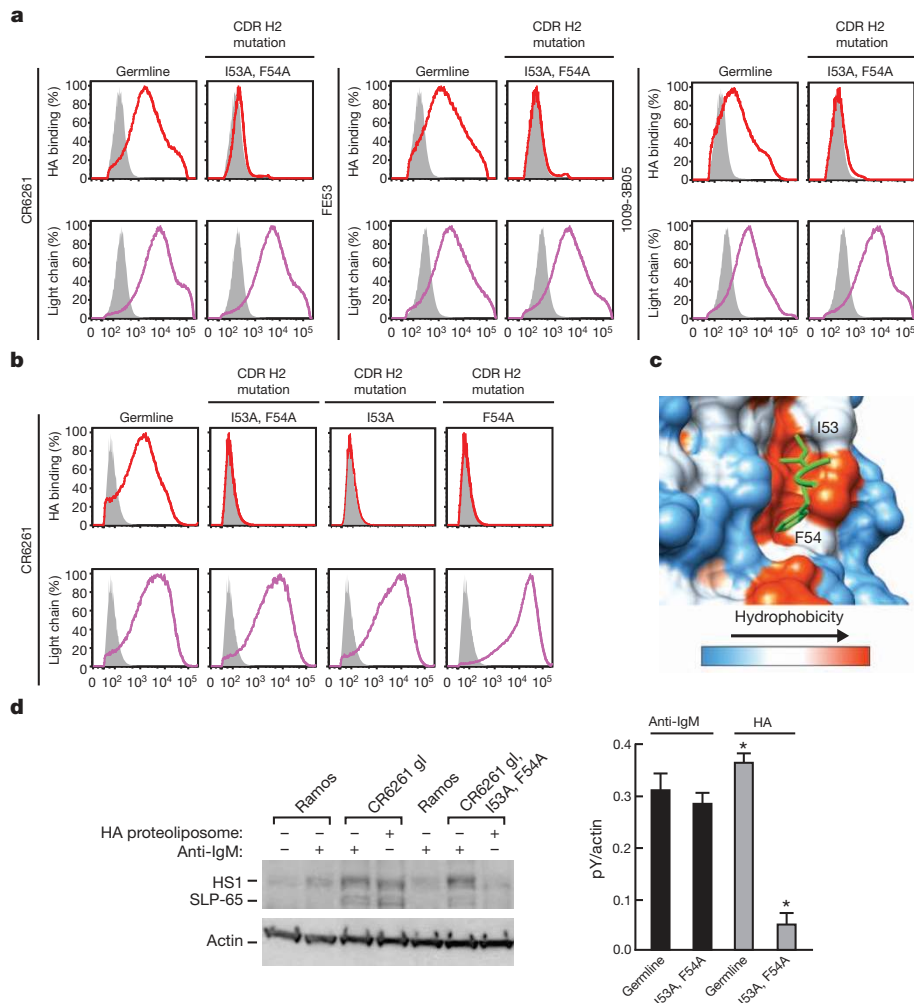


Figure 3 | HA engages and activates membrane-presented germline antibody. **a**, 293F cells expressing membrane IgMs (VRC01 (grey), IGHV1-69 germ lines (wild-type and Ile53Ala/Phe54Ala mutants, coloured lines)) exposed to HA with an N-linked glycan introduced to block the sialic-acid-binding site (Δ RB5) (red) and anti-light-chain expression controls (purple) analysed by flow cytometry. **b**, Unmodified HA trimer also engages germline

(30 mM 6'-sialyllactose present) by flow cytometry. **c**, CR6261 CDR H2 interactions with 1918 SC HA stem (PDB accession 3GBN). **d**, HA proteoliposomes selectively activate tyrosine phosphorylation (pY) by CR6261 germline BCR ($P < 0.0002$, ANOVA); wild-type and mutant CDR H2 receptor activity differed for HA (asterisk) but not for anti-IgM (Tukey's HSD, $\alpha = 0.05$). Presented are the mean values and standard errors of pY intensity.

We have shown in this study that IGHV1-69 antibodies, with no mutation of the germline-encoded VH sequence, engage influenza HA with sufficient affinity to trigger B-cell activation. In all cases, engagement depends on membrane presentation of antibodies with the same structural determinant—two specific hydrophobic residues in the CDR H2. Mutational analysis further revealed that just a few mutations convert a germline IGHV1-69 into an antibody with the full activity of CR6261. Taken together, these results suggest that the IGHV1-69 germ line is poised to form, with a varied set of CDR H3 sequences, broadly neutralized antibodies directed against the highly conserved stem of influenza HA.

Having an antibody with the inherent potential to recognize a common feature of influenza virus would seem to offer obvious evolutionary advantages. Although neutralizing antibodies against other viruses such as human immunodeficiency virus (HIV), SARS and hepatitis C virus also use the *IGHV1-69* gene for recognition^{13–18}, these interactions differ. For example, 17b antibody binding to the CD4-induced site on HIV-1 Env¹⁴ and 8066 antibody binding to HIV-1 gp41 (ref. 19) orient the CDR H2 into a hydrophobic cleft whereas others such as the SARS neutralizing antibody M396 orient CDR H2 into a more hydrophilic site¹⁶. In each case, the actual fold recognized by the CDR H2 is distinct from that recognized on the HA stem. Together, the data suggest that the IGHV1-69 CDR H2 motif is particularly well adapted to recognize the specific protein fold that is highly conserved on the stem of diverse influenza virus HAs.

This germline VH gene, although expressed as part of the adaptive immune response, may therefore serve as a primordial pattern recognition receptor, structurally adapted to participate in recognition of such hydrophobic grooves³. As no other heavy-chain V genes have a hydrophobic CDR H2 (ref. 14), we speculate that influenza may have exerted selection pressure leading to retention of this VH gene. It is also conceivable that the adaptive immune system retains this innate capacity for hydrophobic contact to facilitate the generation of high-affinity antibodies after only a limited degree of somatic mutation. This situation contrasts with the considerable somatic mutation (31%) observed in broadly neutralizing antibodies directed to the CD4-binding site of HIV²⁰, a more recently evolved virus for which there has been less opportunity to select for protective antiviral genes.

The majority of current vaccine-elicited influenza antibodies are directed to the globular head region of viral surface glycoprotein HA, which undergoes considerable antigenic drift to evade the human immune system. Thus, influenza vaccination requires annual assessment of the strains likely to circulate in the coming year to generate protective immunity for the world population. Consequently, there is great interest in the development of a universal influenza vaccine^{21,22}. Antibodies targeting the highly conserved HA stem epitope represent the vast majority of all broadly neutralized antibodies isolated against influenza so far^{1–4}. We have previously demonstrated that it is possible to elicit CR6261-like stem-directed antibodies by vaccination^{22,23}. Despite sequence variations in the CDRs and FR regions, IGHV1-69 antibodies evolve with a relatively small number of somatic mutations. At the same time, robust induction of these neutralizing antibodies in humans remains a challenge. Analysis of current genomics data (1000 Genome Project) reveals a polymorphism in the *IGHV1-69* allele at the contact site in CDR H2 where Leu replaces Phe 54 at a predicted homozygous frequency of 13% in the general population. This substitution abolishes HA recognition by all three germline antibodies (Supplementary Fig. 6). This finding underscores the remarkable specificity in the CDR H2 contact and raises the possibility that some vaccine recipients may not mount a IGHV1-69 response. At the same time, ~30% of stem antibodies isolated until now derive from other VH gene segments^{1,2}, and it remains possible that such individuals might develop responses from other IgG loci. Taken together, knowledge of the initial engagement of the HA stem with the *IGHV1-69* V gene, its subsequent BCR stimulation and somatic mutation will aid in the rational design of universal influenza vaccines.

METHODS SUMMARY

The genes encoding wild-type HA proteins (H1 A/New Caledonia/20/1999 (1999 NC) and H5 A/Indonesia/05/2005 (2005 IND)) and somatically mutated and inferred germline antibodies of CR6261, FE53, and 1009-3B05 were synthesized. Somatic mutated CR6261 germline revertants and germline CDR H2 Ala mutations of Ile and Phe were constructed by introducing mutations to germline heavy chains by site-directed mutagenesis. Plasmids encoding these proteins were transfected into the human embryonic kidney cell line 293F and isolated from expression supernatants 72–96 h after transfection. HA trimeric proteins were purified as previously described²⁴. Soluble Fab, IgG and IgM antibodies were purified using HisTrap, Protein G and IgM affinity columns, respectively, with additional gel-exclusion chromatography performed on IgM samples (GE Healthcare).

The purified antibody variants (1.7×10^{-4} – $100 \mu\text{g ml}^{-1}$) were assayed for binding to H1 1999 NC and in some cases to H5 2005 IND by enzyme-linked immunosorbent assay (ELISA) with purified trimeric HA proteins. The various antibodies were detected by peroxidase-conjugated goat anti-human IgG unless otherwise noted. Endpoint dilutions were determined from nonlinear fit dose-response curves using a detection limit of $4 \times$ background absorbance. CR6261 variants (0.39 – $25 \mu\text{g ml}^{-1}$) were also assayed for neutralization of pseudotyped recombinant lentiviruses expressing wild-type HA with the corresponding neuraminidase (NA) with a luciferase reporter gene as previously described²².

The crystal structure of CR6261 sHgL Fab at 2.85 Å resolution was determined by molecular replacement (Methods).

For membrane presentation of antibody, IGHV1-69 germ lines were expressed in membrane IgM format in 293F cells, which were then subject to a FACS-based HA cell surface binding assay 72 h after transfection (Methods). For BCR triggering, membrane IgM germ lines were transiently expressed in an IgM-negative Ramos B cell line and exposed to proteoliposome-arrayed H1 1999 NC (Methods). Activation was assessed by extent of tyrosine phosphorylation using the 4G10 pY antibody as described¹².

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Structure factors and coordinates for CR6261 sHgL Fab were deposited with the Protein Data Bank under accession 4EVN. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to G.J.N. (glabel@nih.gov).

METHODS

Plasmid construction. Genes encoding HA proteins A/New Caledonia/20/1999 (H1 1999 NC; GenBank AY289929) and A/Indonesia/05/2005 (H5 2005 IND; GenBank ABW06108.1), the corresponding neuraminidase proteins, monoclonal antibodies CR6261, FE53, 1009-3B05 and the respective inferred germline antibodies³ were synthesized using human preferred codons as described²⁷ by GeneArt (Regensburg, Germany). The germ lines were predicted using JOINSOLVER²⁵ and were created from the following segments: *IGHV1-69*01*, *IGHD2-02*02*, *IGHJ6*03* (CR6261); *IGHV1-69*01*, *IGHD2-08*02*, *IGHJ4*02* (FE53); and *IGHV1-69*06*, *IGHD2-08*01*, *IGHJ3*02* (1009-3B05). The genes were cloned into a CMV/R vector for efficient expression in mammalian cells²⁷. Soluble and membrane IgM and Fab genes were generated by overlapping PCR (IgG and IgM class switch region was identified through sequence alignment of GenBank available sequences for cloning of soluble and membrane-bound IgM antibodies). Mutations to germline antibodies were introduced using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies). The following mutations of germline CR6261 heavy chain were generated to probe maturation: CDR H1 (Thr28Pro and Ser30Arg), CDR H2 (Ala57Thr, Asn58Lys and Gln61Pro), CDR H2-61P that lacks the mutation at amino acid 61 (Ala57Thr and Asn58Lys), CDR H3 (Val100Leu) and FR3 (Asp73Glu, Phe74Ser, Ala75Thr, Gly76Ser and Val78Ala). Germline variant antibodies constructed to probe engagement correspond to the following mutations: CDR H2 mutations Ile53Ala, Phe54Ala and Phe54Leu.

Cell surface binding. To evaluate the role that cell surface presentation has in the recognition of low-affinity antigens at the initial contacting germline stage, soluble germline antibodies were expressed as receptor IgMs on the surface of 293F cells (293fectin Reagent, Invitrogen). For the HA ligand, an N-linked glycan (HA1 Arg192Thr, H3 numbering) was introduced into the receptor binding site (Δ RBS) of wild-type or HA stem binding mutant (Ile45Arg/Thr49Arg mutations in HA2, Δ stem) H1 1999 NC to prevent sialic-acid-mediated cell binding. The resulting avi-tagged trimer was biotinylated, purified by size-exclusion chromatography, and labelled with a streptavidin-linked PE fluorophore²⁰. Two days after transfection, 2×10^6 cells expressing membrane IgM versions of CR6261 germ line (gl), FE53 gl, 1009-3B05 gl (all \pm CDR H2 mutations Ile53Ala and Phe54Ala), or VRC01 (mock) were placed on ice, stained with violet fluorescent reactive dye (ViViD, catalogue no. L34955, Invitrogen) for 30 min in PBS, washed, and then incubated (1 h, 4 °C, in PBS containing 1% FBS) with either: PE-HA Δ RBS ($4 \mu\text{g ml}^{-1}$); PE-HA Δ RBS Δ stem ($4 \mu\text{g ml}^{-1}$); PE-anti-human kappa chain (catalogue no. 12-9970-42, eBioscience) (FE53 gl, 1009-3B05 gl); PE mouse anti-human lambda chain (catalogue no. 555797, BD Biosciences) (CR6261 gl). For unmodified/native HA binding, cells were incubated with $4 \mu\text{g ml}^{-1}$ trimer along with 30 mM 6'-sialyllactose to prevent sialic-acid-mediated cell binding (Supplementary Fig. 4) (1 h, 4 °C, in PBS). Cells were washed twice and then fixed in PBS containing 0.5% PFA. ViViD-negative cell surface PE intensity was quantified by flow cytometry (ViViD excitation at 407 nm, bandpass filter 450/50 nm; PE excitation at 532 nm; BD LSR II, BD Biosciences).

HA proteoliposome preparation. This platform was used to provide both the multivalent antigen display needed to oligomerize and activate the BCR²⁸ and the synaptic engagement architecture that underscores naive B-cell stimulation by antigen-presenting cells in the lymph node germinal centre²⁹. One gram of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine:1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)] succinyl (Avanti Polar Lipids) in a 1:1 molar ratio was evaporated under a stream of nitrogen for 1 h. The dry lipid film was then rehydrated in 1,000 μl of liposome buffer (50 mM HEPES, 150 mM NaCl, pH 7.25 (HBS)) and shaken for 40 min, all the time being heated above the membrane melting temperature of the lipid mixture. The resulting homogeneous suspension was subjected to ten freeze-thaw cycles and then extruded 21 times through a 100-nm pore polycarbonate membrane using the Avanti mini-extruder (Avanti Polar Lipids). HA proteoliposomes were produced by incubating the resultant liposomes with His-tagged Δ RBS H1 1999 NC trimer for 1 h at room temperature (HA trimer:lipid molar ratio of 1:900). The sample was then adjusted to 15% iodixanol (in 1.25 ml HBS) and overlaid with 1.75 ml, 0.5 ml and 0.5 ml of 10%, 2.5% and 0% iodixanol in HBS, respectively. Samples were then centrifuged at

200,000g in a TH660 rotor (Sorvall) for 2 h. The proteoliposome fraction, which concentrated at the 2.5–0% iodixanol interface (Supplementary Fig. 5), was collected and dialysed overnight (Slide-A-Lyzer Dialysis Cassette, 10000 MWCO, catalogue no. 66380, Thermo Scientific) to remove density gradient material. After dialysis, proteoliposomes were pelleted (200,000g, 2 h, TH660 rotor), resuspended in HBS and measured by the Pierce BCA protein assay (catalogue no. 23227, Thermo Scientific).

B-cell activation. Ramos B lymphocytes (ATCC) were initially stained with fluorescently conjugated anti-lambda chain and anti-IgM monoclonal antibodies (mouse PE-anti-human lambda chain, catalogue no. 555797, BD Biosciences; APC-anti-human IgM, catalogue no. 314510, BioLegend) to isolate a B-cell clone with no functional BCR at the cell surface. Such a clone was identified by flow cytometry (BD FACSAria, BD Biosciences), expanded and re-sorted for IgM and light-chain negativity for a further six generations before use. For activation studies, 2×10^6 IgM-negative cells were transfected (Cell Line Nucleofector Kit V, Lonza Group) with wild-type or Ile53Ala and Phe54Ala (CDR H2 mutated) versions of germline CR6261 receptor IgM. Twenty-four hours after transfection, cells were exposed to either $0.5 \mu\text{g } \mu\text{l}^{-1}$ mouse anti-human IgM F(ab')₂ (catalogue no. 9023-01, SouthernBiotech) in HBS as a positive control or HA proteoliposomes (HA trimer diluted to $2.5 \mu\text{M}$ in HBS). After 15 min exposure at room temperature, cells were placed at 4 °C, washed two times with HBS and then lysed for 10 min in lysis buffer (Cell Lysis Buffer, Cell Signaling Technology) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail, catalogue no. 13352700, Roche Applied Science). After SDS-PAGE, BCR activation was assessed by western blot analysis of the cell lysate: 4G10 pY (catalogue no. 05-0321, Millipore) reactivity to phosphotyrosine-SLP-65 (p65) and phosphotyrosine HS1 (p75) as described^{12,30,31}. Total phosphotyrosine intensity from three independent experiments was measured by densitometry (Image Processing in Java (Image J) software with curve area density calculation performed in Microsoft Excel). Phosphotyrosine intensity was standardized to the level of cell lysate actin (monoclonal anti- β -actin, catalogue no. A5316, Sigma), with the background value for BCR activation being defined as the extent of stimulation in untransfected IgM-negative Ramos exposed to $0.5 \mu\text{g } \mu\text{l}^{-1}$ mouse anti-human IgM F(ab')₂.

Crystallography. The Fab fragment of CR6261 sHgL was concentrated to 7 mg ml^{-1} in PBS and crystallized in a hanging drop over a reservoir containing 100 mM imidazole pH 6.5, 17.5% polyethylene glycol 8,000, and 3% 2-methyl-2,4-pentanediol. Crystals were cryoprotected by transfer through paratone-N and vitrified in liquid nitrogen. Data to 2.85 Å resolution were collected at Southeast Regional Collaborative Access Team (SER-CAT) 22-ID beamline at the Advanced Photon Source, Argonne National Laboratory. The structure was determined by molecular replacement using the program PHASER with the structure of CR6261 bound to influenza HA (3GBN) as the search model, and refined using the program PHENIX (Supplementary Table 1). The asymmetric unit contains eight copies of the Fab. The CDR H1 was deleted from the model before refinement. An electron density map calculated before modelling the CDR H1 showed only weak density corresponding to this loop; however, an NCS-averaged map calculated in COOT, which is shown in Fig. 2c, indicated clearly that the CDR H1 loop adopts a non-canonical conformation similar to that observed in the co-crystal structure with HA.

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