Structural and genetic basis for development of broadly neutralizing influenza antibodies

Daniel Lingwood1*, Patrick M. McIntamney1*, Hadi M. Yassine1*, James R. R. Whittle1, Xiaoti Guo1, Jeffrey C. Boyington1, Chih-Jen Wei1 & Gary J. Nabel1

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 ancestors1,2, 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 defined1,4. 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 chance2,5. 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 μg 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 complexes1,4, 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

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1Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-3005, USA.

*These authors contributed equally to this work.

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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 JOINSVLVER2* predicted germline precursor (Kabat29 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 (gHgL, 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 (Pro28, Arg30, Lys38 and Phe74) 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-ligated 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 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 receptors8,9.

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 V1-1-69 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 BCR10,11, a Ramos cell clone whose endogenous IgM is not
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.

Table 1 | HA binding and pseudovirus neutralization analysis

| Germline revertants | Mutations | H1 1999 NC |
|---------------------|-----------|------------|
| Somatic             | Full      |            |
| Germline            | None      |            |
| sCDR H1             | T28P S30R |            |
| sCDR2 H2            | A57T N58K Q61P | 0.00133 |
| sFR3                | D73E F74S A75T G76S V78A | 0.00133 |
| sCDR H3             | V100L     |            |
| sCDR H1/sCDR H2     | T28P S30R A57T N58K Q61P | 0.00133 |
| sCDR H1/sFR3        | T28P S30R D73E F74S A75T G76S V78A | 0.00133 |
| sCDR H2/sFR3        | A57T N58K Q61P | 0.950 |
| sCDR H2/sCDR H3     | A57T N58K Q61P V100L | 0.150 |
| sFR3/sCDR H3        | D73E F74S A75T G76S V78A V100L | 0.150 |
| sCDR H1/sCDR H2/sFR3| T28P S30R A57T N58K Q61P D73E F74S A75T G76S V78A | 0.00133 |

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.

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 (ARBS) (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, α = 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 V\textit{\textalpha} 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 CR2621. 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\textsuperscript{13–18}, these interactions differ. For example, 17b antibody binding to the CD4-induced site on HIV-1 Env\textsuperscript{14} 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 site\textsuperscript{14}. 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 V\textit{\textalpha} 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 grooves\textsuperscript{3}. As no other heavy-chain V\textit{\textalpha} genes have a hydrophobic CDR H2 (ref. 14), we speculate that influenza may have exerted selection pressure leading to retention of this specific protein fold that is highly conserved on the stem of diverse influenza virus HAs.

The majority of current vaccine-elicted 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\textsuperscript{21–22}. Antibodies targeting the highly conserved HA stem epitope represent the vast majority of all broadly neutralizing antibodies isolated against influenza so far\textsuperscript{1–4}. We have previously demonstrated that it is possible to elicit CR2621-like stem-directed antibodies by vaccination\textsuperscript{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 mice is associated with a relatively small number of somatic mutations. Antigens recognized by broadly neutralizing antibodies evolve with a relatively small number of somatic mutations. Despite sequence variations in the CDRs and FR regions, IGHV1-69 antibodies evolve with a relatively small number of somatic mutations.

METHODS SUMMARY

The genes encoding wild-type HA proteins (H1 A/New Caledonia/20/1999 (H199 NC) and H5 A/Indonesia/05/2005 (2005 IND)) and somatically mutated and inferred germline antibodies of CR2621, FE53, and 1009-3B05 were synthesized. Somatically mutated CR2621 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 described\textsuperscript{24}. Soluble Fab, IgG and IgM antibodies were purified using Hitrap, 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\textsuperscript{-4}–100 µg ml\textsuperscript{-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× background absorbance. CR2621 variants (0.39–25 µg ml\textsuperscript{-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 described\textsuperscript{25}.

The crystal structure of CR2621 sHgl Fab at 2.85 Å resolution was determined by molecular replacement (Methods). For membrane presentation of antibody, IGHV1-69 germ line 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 line 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 described\textsuperscript{26}.

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

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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. (gnabel@nih.gov).
resulting avi-tagged trimer was biotinylated, purified by size-exclusion chromatography at $532\,\text{nm}$; BD LSR II, BD Biosciences).

containing $0.5\%\ \text{PFA}$. ViViD-negative cell surface PE intensity was quantified 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 \times 10^7\ \text{IgM-negative cells were transfected (Cell Line Nucleofector Kit V, Lonza Group) with wild-type or Ile53Ala and Phe54Ala (CRD H2 mutated) versions of germline CR6261 receptor IgM. Twenty-four hours after transfection, cells were exposed to either $0.5\,\mu\text{g}\,\text{mL}^{-1}$ mouse anti-IgM F(ab')$_2$ (catalogue no. 9023-01, SouthernBiotech) in HBS as a positive control or HA proteoliposomes (Thermo Fisher diluted to $2.5\,\mu\text{M}$ in HBS). After $15\ \text{min}$ exposure at room temperature, cells were placed at $4\,\text{°C}$, washed two times with HBS and then lysed for $10\ \text{min}$ 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 phosphorysine-SLP-65 (p65) and phosphorysine HS1 (p75) as described$^{2,3,11}$. Total phosphorysine 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 $0.5\,\mu\text{g}\,\text{mL}^{-1}$ mouse anti-IgM F(ab')$_2$.

**Crystallography.** The Fab fragment of CR6261 sHgL was concentrated to $7\,\text{mg}\,\text{mL}^{-1}$ in PBS and crystallized in a hanging drop over a reservoir containing $100\,\text{mM}$ imidazole pH $6.5$, $17.5\%\ \text{polyethylene glycol}\ 8000$, and $3\%\ 2$-methyl-2,4-pentanediol. Crystals were cryoprotected by transfer through paratone-N and vitrified in liquid nitrogen. Data to $2.8\,\text{Å}$ 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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