In vitro evolution of an influenza broadly neutralizing antibody is modulated by hemagglutinin receptor specificity

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The relatively recent discovery and characterization of human broadly neutralizing antibodies (bnAbs) against influenza virus provide valuable insights into antiviral and vaccine development. However, the factors that influence the evolution of high-affinity bnAbs remain elusive. We therefore explore the functional sequence space of bnAb C05, which targets the receptor-binding site (RBS) of influenza haemagglutinin (HA) via a long CDR H3. We combine saturation mutagenesis with yeast display to enrich for C05 variants of CDR H3 that bind to H1 and H3 HAs. The C05 variants evolve up to 20-fold higher affinity but increase specificity to each HA subtype used in the selection. Structural analysis reveals that the fine specificity is strongly influenced by a highly conserved substitution that regulates receptor binding in different subtypes. Overall, this study suggests that subtle natural variations in the HA RBS between subtypes and species may differentially influence the evolution of high-affinity bnAbs.
Seasonal and pandemic influenza A viruses have a global impact on human health and the world economy. Despite decades of research, forecasting the genetic evolution of the virus remains extremely difficult due to rapid antigenic drift and antigenic shift. Cross-species transmission further increases the unpredictability of influenza genetic dynamics in nature. Annual vaccination is the only available prophylactic measure, but its efficacy is far from perfect and can vary based on the accuracy of vaccine strain prediction. In terms of therapeutics, there are currently two classes of anti-influenza drugs, namely neuraminidase inhibitors and M2 protein inhibitors. However, drug-resistant mutants for both inhibitor classes have emerged and are present in circulating strains, suggesting an urgent need for new therapeutic agents.

The recently developed discovery of human influenza broadly neutralizing antibodies (bnAbs) against the stem region of haemagglutinin (HA)5–14 has provided significant insights for antiviral and vaccine development. For example, the stem-binding antibody CR6261 (refs 5,6) and CR8020 (ref. 7) are in clinical trials (NCT02371668 and NCT01938388) as antivirals. In addition, small protein binders, that were computationally designed15,16 based on the epitope information of a stem-binding bnAb CR6261 (refs 5,6), provided in vivo protection from influenza challenge17. These stem-binding antibodies have also guided the development of immunogens that confer heterosubtypic protection18–20. Recently, another class of influenza bnAbs that target the HA receptor-binding site (RBS) has been identified and characterized21–31. Each of these HA RBS-targeted bnAbs features a long hypervariable loop that inserts into the HA RBS. While some HA RBS-targeted bnAbs, including 8M2 (refs 26,27), CH65 (ref. 28) and 5J8 (refs 29,30), are subtype-specific due to the higher sequence variability in the RBS and its proximal regions as compared to the stem region, others, including C05 (ref. 21), S139/1 (refs 22,23), FO45-092 (refs 24,25) and 2G1 (refs 26,27), display heterosubtypic activity. Extrapolating from the success in harnessing information from stem-binding bnAbs, these HA RBS-targeted bnAbs offer an unprecedented opportunity to develop new influenza antivirals and potentially a more universal vaccine against HA RBS.

The HA RBS is a shallow pocket in the globular HA head33, and is framed by four structural elements: 130-loop, 150-loop, 190-helix and 220-loop, named after their positions on the HA primary sequence. Although a large portion of the HA RBS is extremely conserved across subtypes, some minor but very important sequence and structural variations exist that affect receptor-binding preferences and interaction mode among different subtypes and across species. For example, residues 190 and 225 (H3 numbering) are Asp in H1 human influenza strains, but Glu and Gly in most H2 and H3 human as well as avian influenza strains. The amino-acid sequences of the 220-loop also differ across subtypes and species, where residues 226 and 228 are generally Leu and Ser in human H2 and H3 subtypes, but Gln and Gly in human H1 and avian subtypes. However, it is unclear how these amino-acid variations shape the evolution of HA RBS-targeted bnAbs in sequential infection by the same or different subtypes.

Understanding the natural evolution of influenza bnAb is important towards development of a universal influenza vaccine. It is evidenced that original antigenic sin strongly influences the immune response against influenza virus33,34. For example, influenza vaccination efficacy is higher in people with no prior influenza vaccination history than in those with frequent influenza vaccination35. Immune history may also prevent influenza bnAbs from being elicited36. In addition, while many antibodies induced during the 2009 pandemic H1N1 influenza season exhibited broad neutralization activity against the HA stem37, this response was not sustained in later years36,38. These observations indicate that our understanding of the evolution and maintenance of influenza bnAbs is far from complete.

Here we aimed to study the evolution of an HA RBS-targeted bnAb C05 (ref. 21) by examining its functional sequence space (that is, the sequence requirements for binding). C05 was originally discovered from a human donor with confirmed influenza virus exposure using phage display library technology21. C05 neutralizes strains from the pandemic subtypes H1, H2, H3, as well as an H9 virus, using primarily a long, single complementarity determining region loop (CDR H3)21 that inserts into the RBS. Specifically, we performed saturation mutagenesis on the six-residue paratope region of C05 CDR H3 that interacts within the heart of the HA RBS. Subsequently, yeast display was used to independently enrich for C05 variants that bound to H1 HA (A/Solomon Islands/3/2006), and H3 HA (A/Perth/16/2009). Several C05 variants retained binding to H3 HA but lost affinity against H1 HA. We also identified C05 variants that increased binding to H1 HA, but not against H3 HA. Further analysis suggests that the amino-acid preference of the paratope region of interest becomes subtype-specific despite targeting the conserved RBS using affinity maturation in this selection process. To provide structural insights, we determined the crystal structure of a C05 Fab variant that was enriched to high frequency in selections both against H1 and H3 HAs in complex with the HA1 subunit of the HA trimer to 1.97 Å resolution. Together, our results univocally demonstrate that the HA subtype preference of the increased affinity C05 is, at least partly, attributed to the amino-acid identity at residue 190 in the HA RBS. In particular, Ser is favoured at position 100d of the C05 heavy chain when residue 190 of HA1 is an Asp and is disfavoured when residue 190 of HA is a Glu. This implies that even highly conservative substitutions that dictate HA receptor preference and mode of binding also modulate the amino-acid preference in the paratope of the antibody, which has important implications in the evolution of bnAbs to the RBS and in the development of more universal vaccines that target the RBS.

Results

**Yeast display screening of C05 Fab functional variants.** In the past few years, a number of neutralizing antibodies that target the influenza HA receptor-binding site (HA RBS) have been identified21–31. Notably, one such antibody, C05, uses a single loop on heavy-chain complementarity-determining region 3 (CDR H3) to target the HA RBS (Supplementary Fig. 1a) and is able to neutralize several, although not all, strains within pandemic subtypes from both group 1 (H1, H2) and group 2 (H3) influenza viruses21. Six consecutive amino acids at the tip of C05 CDR H3 (positions 100a to 100f) directly contact the HA RBS, which is largely conserved across influenza subtypes (Supplementary Fig. 1b). This scaffold provides an opportunity to comprehensively examine the functional sequence space of the HA RBS-targeted residues in C05, which should provide valuable information in understanding the evolution of HA RBS-targeted broadly neutralizing antibodies (bnAb) and in antiviral development against the HA RBS.

To examine the functional sequence space of the HA RBS-targeted residues in C05, we constructed a mutant library and performed screening and selection by yeast display. The amino-acid sequence of the six residues in CDR H3 in wild-type (WT) C05 that most intimately interact with the RBS is VYVSAGW (100a–100f). Saturation mutagenesis was applied to the first five amino acids (position 100a to 100e), whereas mutagenesis of the tryptophan at position 100f was restricted to the first row of the codon table, which included Phe, Leu, Ser, Tyr, Cys and Trp.
The rationale of applying a more restricted mutagenesis scheme at position 100f as compared to other positions was because: (1) bulky hydrophobic residues were suggested to be critical for HA RBS-targeted neutralizing antibodies at this spatial location; and (2) compared to saturation mutagenesis, this mutagenesis scheme would reduce the sequence diversity by more than threefold, which would increase the oversampling of each sequence variant during the screening process. The resultant mutant library has an amino-acid sequence diversity of \( 2^{20} \times 6 = 19,200,000 \), within the estimated throughput limit (\( \sim 100 \) million) for yeast display library. Different variants of C05 Fab were displayed on yeast cells. This yeast surface display library was then subjected to selection for HA-binding affinity. We used fluorescence-activated cell sorting (FACS) to enrich yeast cells that were able to interact with PE-conjugated influenza HA. The post-selection pool was then expanded and subjected to another round of selection. For each of H1, H3, and H5 HAs, three rounds of selection was performed. Variants that were able to bind to the HA would enrich in occurrence frequency throughout the screening process. Variants with higher affinity would enrich to a higher frequency. The mutant plasmid library and each of the post-selection mutant libraries were next-generation sequenced to monitor the frequency change of each variant.

This mutant library was then screened by yeast display for affinity against an H1 HA (A/Solomon Islands/3/2006, also referred to as SI06 below), an H3 HA (A/Perth/16/2009), and an H5 HA (A/Vietnam/1203/2004; Fig. 1b). Wild-type (WT) C05 is able to bind to H1 and H3 HAs, but not H5 HA due to a steric clash outside of the paratope region of interest. Therefore, H5 HA was utilized as a negative control for this screening process. For each of H1, H3, and H5 HAs, three rounds of selection was performed. Variants that were able to bind to the HA would enrich in occurrence frequency throughout the screening process. Variants with higher affinity would enrich to a higher frequency. The mutant plasmid library and each of the post-selection mutant libraries were next-generation sequenced to monitor the frequency change of each variant.
enriched to >80% by round 3 against H1 HA and H3 HA (Supplementary Fig. 3). Due to the high occurrence frequency relative to all the variants in the mutant library, WT C05 Fab from incomplete digestion of vector was excluded from our downstream analysis (see Methods). Throughout the manuscript, the identity of a given variant is denoted by the amino-acid sequence of the six residues in CDR H3 that were probed by mutagenesis and selection (for example, WT C05 Fab is VVSAGW).

CDR H3 amino-acid preference and top variants. To understand the amino-acid preference at each position of the six amino-acid residues at the tip of the CDR H3 loop, the occurrence frequency of each amino acid at individual positions was examined (Fig. 2a). We observed that Trp at position 100f was significantly enriched throughout the selection against both H1 and H3 HAs. Enrichments were also observed for Val at position 100a, Pro at position 100b, and Gly at position 100e, and were more prevalent in the selection against H1 HA compared to H3 HA. In addition, Ser at position 100d was enriched in selection against H1 HA. The detection of amino-acid enrichment indicates the success of the selection against the H1 and H3 HAs. In contrast, no enrichment was observed in selection against H5 HA (negative control). We further analysed the enrichment of individual variants (Supplementary Fig. 4). The top 10 variants after three rounds of selection against H1 HA reached a frequency ranging from 1.3% to 11.5% (from highest to lowest: VPGSGW, VPGAGW, VPGAW, VASSGW, IPGSGW, VPGGW, VPGGW, VPGGW, VPGGW, and WPEIGF). The top 10 variants after three-round selection against H3 HA reached a frequency of ranging from 0.7% to 2.1% (from highest to lowest: VPGAGW, WYVHLW, LPGGGW, YDPGGW, VPGGW, VPGGW, VPGGW, VPGGW, VPGGW, VPGGW, VVSAGW, VEPAGW). The top 10 variants after three-round selection against H5 HA reached a frequency of ranging from 0.1% to 0.5% (from highest to lowest: YVHPQF, NPQEEL, RVLVRL, VVPFWEW, NGGGRW, ARELAY, GHLHNW, DRPLAW, KLWNLW, LDAGDL, where ‘_’ indicates a stop codon). A sequence logo was created for the top 10 variants from each round of selection (Fig. 2b). Overall, the results from next-generation sequencing suggest that the paratype region of interest in C05 can tolerate a number of amino-acid substitutions without abolishing affinity against HA, and indeed can increase affinity in some cases.

HA subtype-dependent amino-acid preference of C05 Fab. We aimed to experimentally validate the next-generation sequencing results from yeast display screening. We therefore compiled a list of 43 variants from the top 20 variants by occurrence frequency in each of the round 3 selections against H1 HA and against H3 HA, and the top eight variants by occurrence frequency in round 3 selection against H5 HA. Of note, the same five variants, one of which is WT, appeared in the top 20 variants of round 3 selections against H1 HA and H3 HA (VPGAGW, VPGGW, VPGGW, VPGGW, and VVSAGW) and VEPAGW. We were able to express 40 out of 43 candidates in HEK293T cells and measured their relative affinities against H1 HA and H3 HA using unpurified cell culture supernatants (see Methods, Table 3a). Variants with improved affinity against HA were observed. From the top 20 variants in the selection against H1 HA, five variants have increased affinity against H1 HA. For the top 20 variants in the selection against H3 HA, two variants had higher affinity against H3 HA. While variants that bound strongly to H1 HA maintained a reasonable affinity towards H3 HA, many variants that bound strongly to H3 HA exhibited a large reduction in affinity against H1 HA. For example, VVDAGW and VVEAGW
had WT-like affinity against H3 HA, but their affinities against H1 HA were >100-fold less than WT. This H3-specific binding may be partially attributed to the amino-acid identity at position 100c. Variants that had an acidic residue (Asp or Glu) at position 100c tended to exhibit this property (for example, VEEAGW, VVDAGW, VDSSGW and VVEAGW).

In fact, this subtype-dependent amino-acid preference could also be observed in variants with high affinity against H1 HA. Given the strong enrichment in the yeast display screening against H1 HA, Ser at position 100d became the focus of our analysis. From the panel of 40 candidates (Fig. 3a), four pairs of variants that differed by a single amino-acid substitution from Ala to Ser at position 100d were analysed (Fig. 3b). By comparing the binding kinetics between variants in each of these pairs, we found that this Ala to Ser substitution at position 100d dramatically improved the $k_{\text{off}}$ against H1 HA, but not against H3 HA. To confirm this result, the WT C05 Fab, which contained an Ala at position 100d, and three C05 Fab variants with a Ser at position 100d (VPGSGW, VVSSGW and VTGSGW), which had the highest affinity against SI06 H1 HA, were expressed in insect cells, purified and their affinities against HA from a panel of human influenza strains were measured. These three variants had a much higher affinity against HAs from certain H1 strains than from H2 and H3 strains (Table 1; Supplementary Figs 5 and 6). These results further suggest that the affinity improvement from introducing a Ser at position 100d is H1-specific. Of note, the results in Fig. 3a and Table 1 were obtained from slightly different experimental systems (see Methods) and, therefore, some variation was expected.

HA subtype-specific binding could also be inferred from the next-generation sequencing data from the yeast display screening. Many variants were enriched in the selection against either H1...
HA or H3 HA, but not both (Fig. 3c). These variants are denoted as ‘specialists’. Consistent with the results described above, a Ser at position 100d was enriched in H1 specialists and negatively charged residues (Asp and Glu) are enriched at position 100c in H3 specialists. Collectively, our results demonstrate that the charged residues (Asp and Glu) are enriched at position 100c in both H1 and H3 HA, but not both (Fig. 3c). These variants are denoted as ‘specialists’. Consistent with the results described above, a Ser at position 100d was enriched in H1 specialists and negatively charged residues (Asp and Glu) are enriched at position 100c in H3 specialists. Collectively, our results demonstrate that the charged residues (Asp and Glu) are enriched at position 100c in both H1 and H3 HA, but not both (Fig. 3c).

### Structural characterization of VPGSGW in complex with HA1.

To facilitate the atomic understanding of the subtype-dependent amino-acid preference in C05, we determined the structure of HA1 subunit from A/Hong Kong/1/1968 (H3N2) in complex with a variant that carried a Ser at position 100d, namely VPGSGW, to 1.97 Å resolution (Table 2). VPGSGW was one of the most enriched variants in the yeast display screening against both H1 and H3 HA1 subunits (Fig. 3c; Supplementary Fig. 4). WT C05 and VPGSGW had a similar angle of approach and overall conformation (Fig. 4a), which is consistent with the negative-stain EM model of VPGSGW bound to the HA1 trimer from A/Solomon Islands/3/2006 (H1N1; Supplementary Fig. 7). Two VPGSGW–HA1 complexes were observed in the asymmetric unit.

In both complexes, the electron density for the six-amino-acid residues of interest was well defined (Supplementary Fig. 8). Interestingly, the side chain of Ser100d exhibited different conformations in the two VPGSGW–HA1 complexes (Fig. 4b), either facing outward from the six-residue loop (towards the HA1 RBS), conformation 1), or facing inward within the CDR H3 loop (conformation 2). Two main-chain–main-chain hydrogen bonds are present in the six-residue loop—between V100a and W100f, and between V100a and S100d. When the side chain of the Ser at position 4 faces inward, it forms two additional hydrogen bonds—one with the main-chain carbonyl of V100a and the other with the main-chain amide of W100f. These extra hydrogen bonds in conformation 2 would likely help further rigidify the six-residue loop.

Although two less intra-loop hydrogen bonds are made when C05 points towards the HA RBS (conformation 1), the Ser hydroxyl takes part in an ion–dipole network with E190 and S228 in the HA RBS (Fig. 4c). The short bond distances suggest that a sodium ion, instead of a water molecule, mediates the interaction among HA1 S228, HA1 E190, and VPGSGW S100d. Our high-resolution crystal structure also reveals that the main-chain carbonyl of G100c interacts with G225, S227 and E190 via water-mediated hydrogen bonds. In addition, similar to WT C05 (ref. 21), the main-chain carbonyls of S100d and G100c hydrogen bond with Y98 and S136, respectively. As described previously35, it is common for HA RBS-targeted bnAbs to mimic some aspects of the binding mode of sialic acid, the natural receptor for influenza A virus. Besides utilizing one face of the pyranose ring, sialic acid interacts with HA RBS via three moieties, namely the acetamide, carboxylate, and glycerol groups (Supplementary Fig. 9a). As seen in WT C05, VPGSGW employs the indole of W100f to bind in the same pocket as the acetamide group and the main-chain carbonyl of G100c to partially mimic the carboxylate group. However, unlike WT C05, which only partially mimics the glycerol group using the main-chain carbonyl of residue 100d, VPGSGW extends this mimicry, where the S100d hydroxyl now takes part in an ion–dipole network that involves S100d is tightly packed and likely strongly stabilizes the six-residue loop to reduce the entropic cost in binding and provides an explanation for the enrichment of ‘PG’ motif in top variants from the screen (Figs 2b and 3c).

### Interaction between S100d in VPGSGW and residue 190 in HA.

As compared to WT C05, VPGSGW had higher affinity against certain H1 strains but lower affinity against H2 and H3 strains (Table 1), despite appearing to be a better mimic of sialic acid. We postulated that the reduction of affinity was partly attributed to Glu at residue 190. VPGSGW had higher affinities against most tested HAs from H1 strains, except for A/WSN/1933, which differed from other tested H1 strains by a D190E substitution. Similarly, the fold reduction in affinity against HA from H2 and H3 strains was larger when residue 190 was Glu rather than Asp. There also was an approximately threefold reduction in affinity against HAs from A/Japan/305/1957 (H2N2) and A/Hong Kong/1/1968 (H3N2), which have Glu at residue 190, but only an approximately twofold reduction or less against A/Panama/2007/1999 (H3N2) and A/Perth/16/2009 (H3N2), which have Asp at residue 190. On the basis of the crystal structure, the ion–dipole network that involves S100d is tightly packed and likely strongly contributes to the binding (Fig. 5a). When an Asp was modelled at residue 190, S100d may adopt a different conformation that would relieve the steric constraints encountered with Glu at position 190 (Fig. 5b).

We then measured the affinity of VPGSGW against HA from A/Hong Kong/1/1968 (HK68) that was engineered with an E190D substitution (Supplementary Fig. 11). The affinity of VPGSGW improved ~23-fold when the E190D substitution was introduced into HK68 HA (from $K_d = 959$ nM to $K_d = 42$ nM; Fig. 5c). In contrast, WT C05 only had an approximately twofold

### Table 1 | Binding affinity of C05 variants against hemagglutinins from different influenza strains.

| $K_d$ in nM | Residue 190 | VVSGW (WT) | VPGSGW | VVSSGW | VTGSGW |
|-----------|-------------|------------|--------|--------|--------|
| A/Beijing/262/1995 (H1N1) | V | 23.1 ± 0.6 | 12.6 ± 0.7 | 259.3 ± 28 | 182.3 ± 0.5 |
| A/New Caledonia/20/1999 (H1N1) | E | 169.4 ± 1.0 | 719.6 ± 2.9 | 727.7 ± 20.5 | 1,539 ± 42 |
| A/Japan/305/1957 (H2N2) | E | 1,701 ± 46 | >5,000 | >5,000 | >5,000 |
| A/Hong Kong/1/1968 (H3N2) | E | 327.8 ± 12.0 | 958.8 ± 45.6 | 1,100 ± 94 | 1,860 ± 83 |
| A/Panama/2007/1999 (H3N2) | D | 926.3 ± 12.1 | 1,306 ± 12 | 1,296 ± 71 | 4,335 ± 207 |
| A/Perth/16/2009 (H3N2)* | D | 23.4 ± 0.1 | 43.6 ± 1.0 | 43.7 ± 0.5 | 125.1 ± 1.2 |

* C05 is able to make additional interaction with A/Perth/16/2009 (H3N2) via CDR H1 (ref. 21).

#### Supplemental Information

- **Table 1**: Binding affinity of C05 variants against hemagglutinins from different influenza strains.
- **Figures**: 2b, 3c, 4a, 4b, 5a, 5b, 5c
- **Supplementary Figures**: 8, 9a, 9b, 10, 11
- **Methods**: For details on the experimental procedures and data analysis.

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improvement in affinity when E190D substitution was introduced into HK68 HA (from $K_d = 328 \text{nM}$ to $K_d = 146 \text{nM}$). We further performed a neutralization assay using the IgG format of WT C05 and VPGSGW against two influenza strains, namely SI06-HA/WSN and A/Aichi/2/68. SI06-HA/WSN and A/Aichi/2/68 is a recombinant H1N1 strain with Asp at residue 190 (see Methods), whereas A/Aichi/2/68 is an H3N2 strain with Glu at residue 190. When tested against SI06-HA/WSN, VPGSGW (EC$_{50} = 1.7 \mu g \text{mL}^{-1}$) is more potent than WT C05 (EC$_{50} = 5.4 \mu g \text{mL}^{-1}$). In contrast, when tested against A/Aichi/2/68, WT C05 (EC$_{50} = 15.5 \mu g \text{mL}^{-1}$) is more potent than VPGSGW (EC$_{50} = > 100 \mu g \text{mL}^{-1}$). Overall, these results suggest that the amino-acid identity at residue 190 of the HA RBS, at least partially, modulates the binding affinity of the C05 CDR H3 loop and especially in the variant VPGSGW, illustrating how a highly conserved and functionally important substitution in the RBS shifts the amino-acid preference in the paratope of a broadly neutralizing antibody.

### Discussion

Most influenza antibodies target the globular head of haemagglutinin (HA), for which the majority of its surface is readily mutable$^{42,43}$. The HA receptor-binding site (RBS) is a highly conserved region on the HA globular head. Recently, a number of antibodies have been shown to target the HA RBS with relatively high breadth$^{21-25,28-31}$. However, it is unclear how this breadth is acquired and sustained during the evolution of HA RBS-targeted broadly neutralizing antibodies (bnAbs). In this study, we investigated the functional sequence space of C05 (ref. 21), a prototypic HA RBS-targeted bnAb, by focusing on that six amino-acid residues on the apex of its long CDR H3 that inserts into the HA RBS and make the most intimate contacts with the RBS.

Our results reveal that certain amino-acid substitutions in CDR H3 can bias the specificity of C05 towards H1 HA or H3 HA. For example, an Ala to Ser substitution at position 100d of wild-type (WT) C05 (from VVSAGW to VVSSGW) improves the specificity towards H1 HA, whereas a Ser to Asp substitution at position 100c (from VVSAGW to VVDAGW) biases the specificity toward H3 HA. The high-resolution structure of a C05 Fab variant, VPGSGW, in complex with an H3 HA1 revealed a tightly packed, ion–dipole network involving S100d of wild-type (WT) C05 (from VVSAGW to VVSSGW) improves the binding affinity towards HA that possesses an Asp at residue 190 is much higher than HA with a Glu at residue 190, likely due to more optimal steric packing. Asp predominates at residue 190 of HAs from human H1 strains, whereas Glu is prevalent in other human and all avian subtypes. E190D is a key substitution for avian-to-human receptor specificity switch in H1 strains$^{44}$. There is also evidence showing E190D can modulate the receptor specificity in H3 strains$^{45}$ as it completely abolished viral replication in cell culture that only expresses α2,3-sialic acid$^{46}$. In fact, perhaps somewhat surprisingly, Asp predominates at residue 190 of HAs in human H3 strains in the past two decades (Supplementary Fig. 12a), but not in avian H3 strains (Supplementary Fig. 12b). Asp and Glu only differ by a methylene group (CH$_2$), and yet impact the receptor specificity as well as the amino-acid preference of C05. Besides residue 190, substitutions at residues 225 (in H1)$^{47}$, 226 and 228 (in H2 and H3)$^{48,49}$, and a minor shift (<1 Å) in the width of HA RBS$^{19,50}$ also play a role in receptor specificity. This study suggests that such subtle structural variability among strains of different subtypes and receptor specificities will likely differentially influence the amino-acid preference of the paratope during antibody evolution. At the same time, RBS-targeted antibodies may also influence the evolution of HA RBS. Some residues in the HA RBS of human influenza virus continue to mutate over time, as exemplified by residue 190 (as mentioned above, Supplementary Fig. 12a) and residue 225 in H3 viruses (Supplementary Fig. 12c), which may impact the receptor-binding ability$^{51}$. Together, these observations permit us to speculate that there is a constant evolutionary interplay between human immunity and ongoing variation in the influenza HA RBS.

Many influenza bnAbs, including C05, have been isolated from the immune repertoires of people vaccinated or naturally infected with influenza virus$^{5,8,10,21,25,28,30,52}$. The breadth of these bnAbs may change when undergo further affinity maturation. This study
suggests that, at least in the case of HA RBS-targeted bnAbs, a tradeoff exists between affinity and breadth. As bnAbs continue to evolve during the natural process of affinity maturation, they may gain affinity by exploiting subtle structural features that are unique to the specific subtype that originally stimulated them. Group-specific or subtype-specific structural features are also known to present in the epitopes of stem-binding bnAbs.

It is possible that such subtype-dependent amino-acid preferences can be observed in other HA RBS-targeted and in stem-binding bnAbs. The breadths of these bnAbs may be unsustainable during repeat challenge of the same subtype, as they would become ‘specialized’. However, this tradeoff between affinity and breadth is unlikely to be universal to all bnAbs, as the affinity maturation of certain stem-binding bnAbs is characterized by both increased breadth and potency.

While our results provide valuable insight into the interaction between a peptide scaffold and HA RBS, complete optimization of C05 affinity and breadth would likely also involve to some extent CDR H1 and the rest of CDR H3, which make additional contacts with neighboring regions of HA RBS that are less evolutionarily conserved, as well as CDR H2, which may be important for stabilizing CDR H3 (ref. 21). Nonetheless, the rest of the C05 epitope that is not examined in this study has higher sequence diversity in naturally circulating strains (Supplementary Fig. 1b), which may cause a severe tradeoff between affinity and breadth. The influence of these factors on C05 binding affinity and breadth deserves further investigation.

As compared to influenza virus, the evolution of bnAbs is better characterized in the human immunodeficiency virus (HIV) field because it is more clinically practical to follow a chronically infected patient over a long period of time. It is known HIV bnAbs usually emerge only after a few years of infection with a large amount of somatic mutations. In comparison, the number of somatic mutations in influenza bnAbs is much fewer. It is likely that our immune system have optimized the germline sequences to respond to pathogens that circulate in human for centuries. Compared to influenza virus, HIV is a relatively new virus to humans, which may explain the difference in the number of somatic mutations required for developing bnAbs against these two viruses. This also highlights the role of human-virus interaction history in the evolution of bnAbs. In addition, the mode of infection may play a role in how bnAbs evolve. While HIV infection is chronic, influenza infection is not. HIV bnAbs co-evolve with antigen within the same host, whereas influenza viruses co-evolve with host immunity at a population level. The distribution of antigen on the virus surface is another critical factor to be considered. It is evidenced that HA RBS-targeted bnAbs often have much lower Fab affinity than would be expected for an effective neutralizing antibody, but can make up for this lower affinity via the bivalent avidity in the IgG to achieve high neutralization breadth. Such an avidity effect is attributed to the relatively close proximity of neighboring HAs on influenza virus. In contrast, envelope proteins on HIV are much more sparse, which allows HIV to escape from the antibody avidity effect.

Methods

Purification and biotinylation of influenza hemagglutinin. Influenza HA was prepared for binding studies as previously described. Briefly, the HA ectodomain was fused with an N-terminal gp67 signal peptide and a C-terminal BirA.
biotinylation site, thrombin cleavage site, trimerization domain, and a His tag and cloned into a customized baculovirus transfer vector. Recombinant bacmid DNA was generated using the Bac-to-Bac system (Life Technologies, Carlsbad, CA). Baculovirus was generated by transfecting purified bacmid DNA into Sf9 cells using FuGene HD (Promega, Madison, WI). HA was expressed by infecting suspension cultures of High Five cells (Life Technologies) with baculovirus at an MOI of 5 to 10 and incubating at 28 °C shaking at 75 r.p.m. for 72 h. The supernatant was concentrated. HA0 was purified by Ni-NTA and buffer exchanged into 20 mM Tris-HCl pH 8.0 and 150 mM NaCl. Biotinylation was performed by incubating 25 μg of BirA enzyme per 1 mg of HA in 100 mM Tris pH 8.0, 10 mM ATP, 10 mM MgOAc, 50 μM biotin and 50 mM NaCl as described previously. Biotinylated HA were purified by size exclusion chromatography.

**Construction of C05 Fab mutant library.** The insect cell expression plasmid that encodes WT C05 Fab was used as the template to generate the C05 Fab mutant library. PCR was performed using KOD DNA polymerase (EMD Millipore) with 1.5 mM MgSO4, 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP), and 0.5 μM each of the primers for HA (5’-AGCGTAGTCTGGAACGTCGTATGGGTACAGGC-3’ (C05HC-NTD1-F) and 5’-AGGTTAGTCTGGAGATCTGTGACAGC-3’ (C05HC-NTD1-R)). Another PCR was performed using the same condition except that the forward primer was 5’-AGGTTAGTCTGGAGATCTGTGACAGC-3’ (C05HC-NTD1-F) and the reverse primer was 5’-TTGGCTGATGACAGC-3’ (C05HC-NTD1-R). The PCR products from these two PCRs were purified by PureLink PCR Purification Kit (Life Technologies) according to the manufacturer’s instructions and digested with SfiI (New England Biolabs). The ligated product was transformed into MegaX DH10B (New England Biolabs) according to the manufacturer's instructions. Yeast transformation was performed by following the LiAc/SS-DNA/PEG protocol described in (http://mbc.berkeley.edu/labs/koshland/Protocols/YEAST/LiAc.html). Four micrograms of the SfiI-digested dual promoter yeast expression vector and 8 μg of the C05 library heavy-chain insert were used for the transformation. Transformants were incubated at 30 °C for 2 days. At least 105 colonies were collected, resuspended in YPD with 15% glycerol, and stored at −80 °C until used.

For each round of enrichment, ~107 yeast cells from the frozen stock were cultured in 250 ml SDCAA (2.0% glucose, 0.67% yeast nitrogen base, 0.5% casamino acids, 0.54% disodium phosphate and 0.86% monosodium phosphate) for 18 h at 28 °C with shaking at 250 r.p.m. The initial OD600 was ~0.3 and the final OD600 was ~1.7. Yeast cells were spun down at 4 °C with 4,750 r.p.m. for 20 min and resuspended in 100 ml SGR-CAA (20 g l⁻¹ galactose, 20 g l⁻¹ raffinose, 1 g l⁻¹ dextrinose, 6.7 g l⁻¹ yeast nitrogen base, 5 g l⁻¹ casamino acids, 5.4 g l⁻¹ NaHPO4 and 8.56 g l⁻¹ Na2HPO4) with an OD600 of ~0.6. Yeast cells were cultured for 24 h at 18 °C with shaking at 250 r.p.m. to reach an OD600 of ~1.5. 15 ml of the yeast culture was spun down, washed twice with PBS, and resuspended in 5 ml PBS. Biotinylated trimeric HA was incubated with Streptavidin PE (Ebioscience, San Diego, CA) at a molar ratio of 1:4 for 15 min. Of note, Streptavidin PE was buffer exchanged into PBS before use to remove NaN3, which is toxic to the yeast cells. The biotinylated trimeric HA-streptavidin PE complex was added to the yeast cells in PBS with a final concentration as indicated in Supplementary Table 1. After incubating at 4 °C overnight with head-to-head rotation, the yeast cells were spun down, washed twice with PBS, resuspended in (New England Biolabs). The ligated product was transformed into MegaX DH10B T1R cells (Life Technologies). Around 105 colonies were collected. C05 Fab plasmid mutant library were purified from the bacteria colonies using MaxiPrep Plasmid Purification (Clontech Laboratories).

**Yeast display screening and FACS.** The coding sequence of the heavy chain of the C05 Fab plasmid mutant library was amplified by PCR using KOD DNA polymerase (EMD Millipore) with 1.5 mM MgSO4, 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP), and 0.5 μM each of the primers for HA (5’-AGCGTAGTCTGGAACGTCGTATGGGTACAGGC-3’ (C05HC-NTD1-F) and 5’-AGGTTAGTCTGGAGATCTGTGACAGC-3’ (C05HC-NTD1-R)). Another PCR was performed using the same condition except that the reverse primer was 5’-TTGGCTGATGACAGC-3’ (C05HC-NTD1-R). The PCR product (C05 library heavy-chain insert) was purified by gel extraction using PCR Clean-Up and Gel Extraction Kit (Clontech Laboratories) according to the manufacturer’s instructions. Yeast transformation was performed by following the LiAc/SS-DNA/PEG protocol described in (http://mbc.berkeley.edu/labs/koshland/Protocols/YEAST/LiAc.html). Four micrograms of the SfiI-digested dual promoter yeast expression vector and 8 μg of the C05 library heavy-chain insert were used for the transformation. Transformants were incubated at 30 °C for 2 days. At least 10⁵ colonies were collected, resuspended in YPD with 15% glycerol, and stored at −80 °C until used.

For each round of enrichment, ~10⁷ yeast cells from the frozen stock were cultured in 250 ml SDCAA (2.0% glucose, 0.67% yeast nitrogen base, 0.5% casamino acids, 0.54% disodium phosphate and 0.86% monosodium phosphate) for 18 h at 28 °C with shaking at 250 r.p.m. The initial OD600 was ~0.3 and the final OD600 was ~1.7. Yeast cells were spun down at 4 °C with 4,750 r.p.m. for 20 min and resuspended in 100 ml SGR-CAA (20 g l⁻¹ galactose, 20 g l⁻¹ raffinose, 1 g l⁻¹ dextrinose, 6.7 g l⁻¹ yeast nitrogen base, 5 g l⁻¹ casamino acids, 5.4 g l⁻¹ NaHPO4 and 8.56 g l⁻¹ Na2HPO4) with an OD600 of ~0.6. Yeast cells were cultured for 24 h at 18 °C with shaking at 250 r.p.m. to reach an OD600 of ~1.5. 15 ml of the yeast culture was spun down, washed twice with PBS, and resuspended in 5 ml PBS. Biotinylated trimeric HA was incubated with Streptavidin PE (Ebioscience, San Diego, CA) at a molar ratio of 1:4 for 15 min. Of note, Streptavidin PE was buffer exchanged into PBS before use to remove NaN3, which is toxic to the yeast cells. The biotinylated trimeric HA-streptavidin PE complex was added to the yeast cells in PBS with a final concentration as indicated in Supplementary Table 1. After incubating at 4 °C overnight with head-to-head rotation, the yeast cells were spun down, washed twice with PBS, resuspended in

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**Figure 5 | E190D in the HA RBS favors binding against VPGSGW.** The hydrogen bond network involving S100d of the CDR H3 of VPGSGW is shown for (a) binding against HA RBS of HK68/H3, and (b) binding against HA RBS of HK68/H3 that carried mutation E190D, which is modelled based on the crystal structure of VPGSGW-HK68/H3 HA1. A putative sodium ion is represented by the purple sphere. For visual clarity, only G100c, S100d, G100e, and W100f on the Fab are displayed. (c) The affinities of WT C05 Fab (VVSAGW) and a C05 Fab variant (VPGSGW) against the HA from A/Hong Kong/1/1968 (wild type; E190; E190D mutant) are shown as a bar chart. (d,e) The neutralizing activity of WT C05 and VPGSGW in IgG format against (d) SI06-HA/WSN virus, and (e) A/Aichi/2/68 virus were measured by cell viability assay. SI06-HA/WSN virus was generated based on WSN, in which the HA ectodomain was replaced by that from SI06 (see Methods). Colour code is the same as that of c. Mean value across three replicates is shown and the error bar represents the S.D. The large error bar in VPGSGW at 100 μg ml⁻¹ is due to complete protection in one but not in the other two replicates.
5 ml PBS, and subjected to fluorescence-activated cell sorting at TSRI Flow Cytometry Core Facility. The sorted yeast cells were recovered by plating on the SDCAA agar plates. Yeast colonies were collected after 2 days of incubation at 30 °C, resuspended in YPD with 15% glycerol, and stored at −80 °C until use.

Sequencing library preparation. Plasmid was extracted from at least 10 yeast cells per sample using Zymoprep Yeast Plasmid Miniprep II (Zymo Research, Irvine, CA) according to the manufacturer’s instructions. The mutated region on the original plasmid was amplified by PCR using KOD DNA polymerase (EMD Millipore) with 1.5 mM MgSO4, 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP), and 0.5 μM each of the primers 5′-CACTTTTCCCCATACAGGA CGCTCTTCGATCCTCGGTGTGACTACGTACTACGCGCTAAGCG-TCGAC-3′ and 5′-GACCTGGAGTTCAAGTGCTCTCCCGATTCCACATGCAAGGGCG TAACACTCTTCCTCCTACGGACGCT-3′. The PCR product was purified with a Purelink PCR Purification Kit (Life Technologies, Carlsbad, CA) according to manufacturer’s instructions. The purified product was used as a template for a second PCR performed under the same conditions except that primers 5′-AATGATACGCGGGCAACCGAGATTC TACACTCTTCCTCCTACGGACGCT-3′ and 5′-CAACAGGAGAAGGCGCTA CGAGATNNNNNGTGCAGCTGACAGGCGTGCTCGTCT-3′ were used instead. The underlined ‘N’s indicate the position of the barcode for multiplex sequencing. The sequences of the barcodes are indicated in Supplementary Table 2. The product from the second PCR was subjected to next-generation sequencing using Illumina MiSeq 2 × 75 bp paired-end reads at the TSRI Next Generation Sequencing Core.

Sequencing data analysis. The sequencing data were demultiplexed using the barcode reads. For each paired-end read, the nucleotide sequence corresponding to the randomized region was extracted. If the nucleotide sequence at the randomized region was inconsistent between forward and reverse reads, the paired-end read would be discarded. In other words, at the randomized region, the reverse-complement of forward read must perfectly match the reverse read. The nucleotide sequence was translated into the amino-acid sequence. The occurrence of each amino-acid sequence was counted, with each paired-end read as one count. WT C05 Fab from incomplete digestion of the vector during mutant library construction can be distinguished from the WT C05 Fab in the mutant library due to differences in codon usage. Those counts corresponding to WT C05 Fab from incomplete digestion of the vector were filtered from downstream analysis unless otherwise stated. Custom python scripts were used for sequencing data processing. All scripts have been deposited to https://github.com/wchnicholas/C05mut.

Construction and purification of C05 Fab variants. Individual mutants for the validation experiment were constructed using the QuikChange XL Mutagenesis kit (Stratagene, San Diego, CA) according to the manufacturer’s instructions. Primers for Quikchange were designed such that they matched 18 bp flanking each side of the mutated region. The nucleotide sequence of the mutated region on the primers was designed to minimize nucleotide mismatch with the WT C05 Fab. For the expression of C05 Fab variants in mammalian cells, both light and heavy chains were cloned into the pFluse-Fc vector (Invivogen, San Diego, CA). The light chain and heavy chain were transfected into 293T cells in a 2:1 molar ratio. The plasmids were under control of EF1a promoter and HTLV enhancer. The plasmids were co-transfected into Expi293F cells at a 2:1 ratio (light:heavy). The supernatant was collected at 72 h post transfection. Full-length IgG proteins were purified from the supernatant using protein G column on AKTAexpress (GE Healthcare).

Biolayer interferometry binding assay. The binding assay was performed by biolayer interferometry (BLI) using an Octet Red instrument (Fortebio, Menlo Park, CA). Biotinylated HA0 at approximately 10–50 μM was incubated with 1 μM VPGSGW in the negative-stain data (Supplementary Fig. 7). The CR9114-VPGSGW complex was purified by size exclusion chromatography on a Hiload 16/90 Superdex 200 column (GE Healthcare) in 20 mM Tris pH 8.0, 150 mM NaCl, and 0.02% NaN3, and concentrated to ~10 mg/ml in 10 mM Tris pH 8.0, 50 mM NaCl, and 0.02% NaN3. The resulting VPGSGW-HA complex was characterized by mass spectrometry. The HA ectodomain of WSN (H3 numbering: HA1 residue 30–309) from A/Hong Kong/1/1968 (HK68/H3) was expressed in insect cells as a Fc chimera containing the C05 Fab variants were collected 3 days after transfection. Expression of the VPGSGW-HA complex was purified by size exclusion chromatography. CR9114 Fab was then incubated with the purified VPGSGW-HA complex in a molar ratio of 4.5:1. Of note, additional VPGSGW was not supplied for this incubation and may explain the low occupancy of the VPGSGW–HA complex in the negative-stain data (Supplementary Fig. 7). The CR9114–VPGSGW–HA complex was purified by size exclusion chromatography. All size exclusion chromatography were performed on a Hiload 16/90 Superdex 200 column (GE Healthcare). The binding assay was performed by biolayer interferometry using an Octet Red instrument (ForteBio, Menlo Park, CA). Biotinylated HA0 at approximately 10–50 μM was incubated with 1 μM VPGSGW in the negative-stain data (Supplementary Fig. 7). The CR9114–VPGSGW complex was purified by size exclusion chromatography. CR9114 Fab was then incubated with the purified VPGSGW-HA complex in a molar ratio of 4.5:1. Of note, additional VPGSGW was not supplied for this incubation and may explain the low occupancy of the VPGSGW–HA complex in the negative-stain data (Supplementary Fig. 7). The CR9114–VPGSGW–HA complex was purified by size exclusion chromatography.

Crystallography. Construction of crystals. The CR9114–VPGSGW–HA complex was crystallized using the sitting drop vapour diffusion method with 500 μl reservoir solution containing 0.1 M sodium citrate pH 5.5 and 9% PEG 8000. Drops consisting of 0.8 μl protein + 0.8 μl precipitant were set up at 20 °C and crystals appeared within a week. The resulting crystals were cryoprotected by soaking in a reservoir solution containing 15% PEG 400, flash cooled, and stored in liquid nitrogen until data collection.

Diffraction data for the VPGSGW-HK68/H3 HA1 complex were collected at the Stanford Synchrotron Radiation Lightsource beamline 12-2. The data were indexed in space group P43, and integrated and scaled using HKL2000 (HKL Research, Charlottetown, PE, Canada). The structure was solved by molecular replacement at 1.97 Å resolution using Phaser68 with PDB 4F88 (ref. 21) as the molecular replacement model, modelled using Coot69 and refined using Refmac5 (ref. 62). Ramachandran statistics were calculated using Molprobity70.

Electron microscopy reconstruction. HA0 from A/Solomon Islands/3/2006 (S06/H1) was treated with trypsin (New England Biolabs) to remove the C-terminal tags and cleave to produce mature HA. The trypsin-digested HA0 was then purified by size exclusion chromatography on a Hiload 16/90 Superdex 200 column (GE Healthcare) in 20 mM Tris pH 8.0, 150 mM NaCl, and 0.02% NaN3, and concentrated to ~10 mg/ml in 10 mM Tris pH 8.0, 50 mM NaCl, and 0.02% NaN3. Cryo-crystals were collected at −150 °C using liquid nitrogen, and the box was loaded into a vitrified 200 mg/cm3 Agarose Low Ice grid at 4 °C. The vitrified grid was loaded into the cryo-jar of a FEI Tecnai Spirit with camera at 1.35 Å resolution.

IgG expression and purification and neutralization assay. The heavy chains and light chains of C05 (WT or VPGSGW) were cloned into pFluse-Fc vector separately, under control of EF1a promoter and HTLV enhancer. The plasmids were co-transfected into Expi293F cells at a 2:1 ratio (light:heavy). The supernatant was collected at 72 h post transfection. Full-length IgG proteins were purified from the supernatant using protein G column on AKTAexpress (GE Healthcare).

Sequence logo. Sequence logos were generated by WebLogo (http://weblogo.berkeley.edu/logo.cgi)70.

Data availability. Raw sequencing data have been submitted to the NIH Short Read Archive under accession number: BioProject PRJNA332669. The x-ray coordinates and structure factors have been deposited in the RCSB Protein Data Bank under accession code 5UMN. The EM map has been deposited in the EMDB.
under accession number EMD-8578. All the other data that support the conclusions of the study are available from the corresponding author upon request.

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Author contributions

N.C.W., J.X., A.B.W., R.A.L. and I.A.W. conceived and designed the experiments, N.C.W., G.G. and J.X. designed and performed the yeast display screening and validation experiments, N.C.W. performed the X-ray data collection, structure determination and refinement, H.L.T. and A.B.W. performed the EM experiments, N.C.W., J.X., A.B.W., R.A.L. and I.A.W. analysed the data and wrote the manuscript. All authors edited the paper.

Additional information

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