Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Recurring and Adaptable Binding Motifs in Broadly Neutralizing Antibodies to Influenza Virus Are Encoded on the D3-9 Segment of the Ig Gene

Highlights
- Structure of bnAb S9-3-37 bound to influenza hemagglutinin stem was determined
- D3-9-encoded region of S9-3-37 contributes majority of the interaction surface with HA
- D3-9 gene segment of S9-3-37 can engage the HA stem in two different reading frames
- D3-9 gene segment represents a recurring mechanism for antibody targeting of HA stem

Authors
Nicholas C. Wu, Seiya Yamayoshi, Mutsumi Ito, Ryuta Uraki, Yoshihiro Kawaoka, Ian A. Wilson

Correspondence
yoshihiro.kawaoka@wisc.edu (Y.K.), wilson@scripps.edu (I.A.W.)

In Brief
Identifying signature features common to broadly neutralizing antibodies (bnAbs) is key to universal flu vaccine design. Wu et al. report that the D3-9 encoded segment of an influenza hemagglutinin stem-targeting bnAb contributes the majority of the interaction surface and is a recurring motif in antibodies that target the hemagglutinin stem.
Recurring and Adaptable Binding Motifs in Broadly Neutralizing Antibodies to Influenza Virus Are Encoded on the D3-9 Segment of the Ig Gene

Nicholas C. Wu, Seiya Yamayoshi, Mutsumi Ito, Ryuta Uraki, Yoshihiro Kawaoka, and Ian A. Wilson

SUMMARY

Discovery and characterization of broadly neutralizing antibodies (bnAbs) to the influenza hemagglutinin (HA) stem have provided insights for the development of a universal flu vaccine. Identification of signature features common to bnAbs from different individuals will be key to guiding immunogen design. S9-3-37 is a bnAb isolated from a healthy H5N1 vaccinee. Here, structural characterization reveals that the D3-9 gene segment of S9-3-37 contributes most of the interaction surface with the highly conserved stem epitope on HA. Comparison with other influenza bnAb crystal structures indicates that the D3-9 segment provides a general mechanism for targeting HA stem. Interestingly, such bnAbs can approach the HA stem with vastly different angles and orientations. Moreover, D3-9 can be translated in different reading frames in different bnAbs yet still target the same HA stem pocket. Thus, the D3-9 gene segment in the human immune repertoire can provide a robust defense against influenza virus.

INTRODUCTION

Development of a universal influenza vaccine has been of long-term interest for global health. In the past decade, the discovery and characterization of broadly neutralizing antibodies (bnAbs) to the influenza hemagglutinin (HA) stem have provided invaluable insights for development of a universal influenza vaccine (Impagliazzo et al., 2015; Wu and Wilson, 2018; Yassine et al., 2015). The HA stem domain, unlike the receptor-binding head domain, is highly conserved across influenza strains and subtypes (Nobusawa et al., 1991). Therefore, antibodies that target the HA stem usually have much greater breadth than those that target the hypervariable head (Wu and Wilson, 2017), which is the location of the major antigenic sites (Gerhard et al., 1981; Wiley et al., 1981). Consequently, elicitation of stem-binding bnAbs has become a major goal for universal influenza vaccine development (Erbelding et al., 2018).

Understanding features that are common to bnAbs isolated from different individuals, whether through natural infection or vaccination, is important for immunogen design in the development of a universal influenza vaccine. As more and more influenza bnAbs are characterized, several multidonor classes of bnAbs have begun to emerge (Avnir et al., 2014; Joyce et al., 2016; Wu and Wilson, 2017). Stem-binding bnAbs that utilize V_{H}1-69 germline genes are probably the most well-characterized multidonor class (Dreyfus et al., 2012; Ekiert et al., 2009; Kasyap et al., 2008; Lang et al., 2017; Sui et al., 2009; Throsby et al., 2008), and the alleles used code for a signature motif consisting of Ile65 and Phe54 in complementarity-determining region (CDR) H2 and Tyr98 in CDR H3 (Avnir et al., 2014; Lang et al., 2017). Recently, Joyce et al. reported three additional multidonor classes of bnAbs (Joyce et al., 2016): (1) bnAbs that utilize V_{H}6-1 and D3-3 germline genes with a signature motif consisting of MIFGI in CDR H3; (2) bnAbs that utilize V_{H}1-18 and D3-9 with a signature motif of RXXLTG in CDR H3; and (3) bnAbs that utilize V_{H}1-18 and a QXXV motif in CDR H3. It is likely that more multidonor classes of bnAbs have yet to be discovered.

We describe here the structure of bnAb S9-3-37, which targets the HA stem mainly using a relatively long CDR H3 of 21 residues. Interestingly, CDR H3 of S9-3-37 has a very similar conformation as that of a pan-influenza A bnAb Fl6v3 (Corti et al., 2011), despite the huge disparity in their overall orientations with respect to the HA stem. Both S9-3-37 and Fl6v3 utilize the D3-9 gene segment and encode an LXYFXWL motif in CDR H3 that is critical for targeting the most conserved part of the stem epitope. We further performed a structural comparison between S9-3-37 and 31.b.09 (Joyce et al., 2016), which also utilizes the D3-9 gene segment. Our analysis reveals that the D3-9 gene segments of S9-3-37 and 31.b.09 are translated in different reading frames. Nonetheless, Phe in the D-3-9-encoded LXYFXWL motif of S9-3-37 and Leu in the D3-9-encoded ILTG motif of 31.b.09 targets the same pocket in the HA stem.
**RESULTS**

**Structural Characterization of S9-3-37**

S9-3-37 is a group 1-specific bnAb that was isolated from healthy volunteers who were vaccinated with the H5N1 pre-pandemic vaccine (Yamayoshi et al., 2018). S9-3-37 uses the healthy volunteers who were vaccinated with the H5N1 pre-S (Joyce et al., 2016) and can be found in the B cell repertoires of healthy donors (DeKosky et al., 2015). Overall, this study reveals that the D3-9 gene segment offers a recurrent and robust strategy for targeting the HA stem in which at least two different binding poses can be utilized depending on the reading frame.

Moreover, both reading frames of the D3-9 gene segment are utilized by influenza bnAbs from multiple vaccinated individuals (Joyce et al., 2016) and can be found in the B cell repertoires of healthy donors (DeKosky et al., 2015). Overall, this study reveals that the D3-9 gene segment offers a recurrent and robust strategy for targeting the HA stem in which at least two different binding poses can be utilized depending on the reading frame.

**Paratope Analysis Reveals the Importance of D3-9 in Binding**

Next, we compared the paratopes of S9-3-37 and Fl6v3 and computed the buried surface areas (BSAs) in the S9-3-37-HA complex and Fl6v3-HA complex (PDB 3TZN) (Corti et al., 2011) (see STAR Methods). The paratope of S9-3-37 is dominated by their variable domains of heavy chains (VH) were performed. When their D gene-encoded regions are superimposed, the base of their CDR H3s twist with respect to each other (Figure 1C). When their Vh framework regions are aligned, the CDR H3 loops of S9-3-37 and Fl6v3 point in opposite directions (Figure 1D). Together, our structural analysis reveals that the S9-3-37 and Fl6v3 antibodies engage the HA stem surface in very different overall orientations and angles of approach, but nevertheless still have very similar CDR H3 conformations, especially for the residues that are important for engaging the conserved HA stem epitope.

| HA Group | Subtype | Strain | S9-3-37 Fab (Kd) | S9-3-37 IgG (Kd) |
|----------|---------|--------|-----------------|-----------------|
| 1        | H1N1    | A/New York/1/1918 | ++++              | ++++            |
| 1        | H1N1    | A/WSN/1933       | ++++              | ++++            |
| 1        | H1N1    | A/duck/Alberta/60/1976 | +++              | ++++            |
| 1        | H1N1    | A/Beijing/262/1995 | +++              | ++++            |
| 1        | H1N1    | A/Solomon Islands/3/2006 | ++++           | ++++            |
| 1        | H1N1    | A/California/04/2009 | ++++             | ++++            |
| 1        | H2N2    | A/Adachi/2/1957  | +                | +++             |
| 1        | H5N1    | A/Vietnam/1203/2004 | +++              | ++++            |
| 1        | H5N1    | A/Indonesia/05/2005 | +++              | ++++            |
| 1        | H5N2    | A/turkey/Wisconsin/1/1966 | ++              | +++             |
| 1        | H3N2    | A/Hong Kong/1/1968 | -                | -               |
| 1        | H3N2    | A/Panama/2007/1999 | -                | -               |
| 2        | H3N2    | A/Perth/16/2009  | -                | -               |
| 2        | H4N6    | A/duck/Czechoslovakia/1956 | -            | -               |
| 2        | H7N7    | A/Netherlands/219/2003 | -             | -               |
| 2        | H7N9    | A/Shanghai/2/2013 | -                | -               |
| 2        | H10N7   | A/chicken/Germany/N/1949 | -           | -               |
| 2        | H14N5   | A/mallard/Astrakhan/263/1982 | -         | -               |
| 2        | H15N8   | A/shearw./W. Australia/2576/1979 | -      | -               |

++++, Kd <1 nM; ++++, Kd = 1–10 nM; ++, Kd = 10–100 nM; +, Kd = 100–1,000 nM; and –, no binding.
in electrostatic interactions with HA1 Lys32 and HA2 Asp57, respectively (Figure S3B). N56K, along with N58K, also hydrogen bonds with Gln99 in CDR H3 (Figure S3C). These observations suggest that somatic mutations in CDR H2 not only facilitate interactions with HA but also help stabilize the base of CDR H3. Therefore, somatic mutations in CDR H2 are likely to play a critical role during affinity maturation of S9-3-37.

**Epitope Analysis Explains the Group 1 Specificity of S9-3-37**

Many HA residues are targeted by both S9-3-37 and FI6v3 (Figure 3A), namely HA1 residue 289, and HA2 residues 18, 19, 20, 21, 38, 41, 42, 45, 46, 48, 49, 52, 53, 56, and 57 (H3 numbering is used throughout, unless indicated otherwise). In contrast, some HA residues (HA1 18, 38, 39, 40, 291, 292, and 318; and
HA1 residues 8, 28, 29, 30, 287, 290, and 316; and HA2 residues 39 and 43). Those shared epitope residues, which are targeted by CDR H3 (Figure 3A), are more conserved than those epitope residues that are unique to either S9-3-37 or Fl6v3 (Figures 3B and S4). In contrast to Fl6v3, which binds to both groups 1 and 2 HAs, S9-3-37 only binds to group 1 HAs. Natural amino acid variants in HA1 residue 38 and HA2 residue 50, both of which are epitope residues unique to S9-3-37, may explain the narrower breadth in S9-3-37 as compared to Fl6v3. Most group 1 HAs carry an Asn at HA2 residue 50, whereas HA2 residue 50 is highly conserved as Gly among group 2 HAs (Figure S3D). In our crystal structure, HA2 Asn50 forms an H-bond with Arg97 of S9-3-37 VH (Figure S3E). When Gly is present at HA2 residue 50, this H-bond interaction would be abolished, which can partially explain lack of binding of S9-3-37 to group 2 HAs. In HA1 residue 38, an N-glycosylation site is present in most group 2 HAs, but not in group 1 HAs, and can provide steric hindrance to the HA stem epitope (Figure S3F). CDR L1 of S9-3-37 is spatially proximal to HA1 residue 38 (Figure S3G), and a glycan at HA1 residue 38 in group 2 HAs would likely clash with CDR L1 of S9-3-37. In comparison, although CDR H2 of Fl6v3 occupies a similar space (Figure S3G), it is more distant from HA1 residue 38, which allows it to accommodate a glycan at HA1 residue 38.

Figure 2. Comparison between the Paratopes of Fl6v3 and S9-3-37
(A and B) Buried surface area (BSA) upon binding for each residue in (A) S9-3-37 Fab and (B) Fl6v3 Fab is plotted along the residue position in the sequence. The regions corresponding to the CDRs (based on Kabat numbering) are shaded in gray. The slight misalignment of the CDRs in the light chains is due to a two-residue deletion in the sequence of CDR L1 of Fl6v3. Of note, those contacting residues that flank the N-terminal region of CDR H1 of Fl6v3 are classified as part of CDR H1 according to the Chothia CDR structural definition (Al-Lazikani et al., 1997), but not in the Kabat CDR sequence definition.

(C) Side chains of D3-9-encoded residues in S9-3-37 are shown in stick representation. HA is shown in a white surface.

(D) The BSA for each residue in the D3-9-encoded region in S9-3-37 is indicated.

Figure 3. Comparison between the Epitopes of Fl6v3 and S9-3-37
(A) Residues that are common in the epitopes of Fl6v3 and S9-3-37 are in blue. Residues that are unique to the Fl6v3 epitope are in orange. Residues that are unique to the S9-3-37 epitope are in yellow. CDR H2, H3, and L1 of S9-3-37 are shown in tube representation.

(B) Sequence conservation of each epitope residue was quantified by sequence entropy, which was calculated based on the alignment of 20 representative strains from different types and subtypes shown in Figure S4. “Both,” residues that are common between the epitopes of Fl6v3 and S9-3-37. “Fl6v3,” residues that are unique to the Fl6v3 epitope. “S9-3-37,” residues that are unique to the S9-3-37 epitope.
In fact, the N-glycosylation site at HA1 Asn38 has limited the ability of several stem-binding bnAbs to neutralize group 2 influenza subtypes (Wu and Wilson, 2017). Subsequently, these analyses show that while both S9-3-37 and FI6v3 utilize a long CDR H3 to target the most conserved part of the epitope, other epitope residues that are unique to each antibody are less conserved and play a critical role in determining their breadth. The different angles of approach may also be critical for the difference in breadth between S9-3-37 and FI6v3, as shown for VH1-69-encoded bnAbs (Lang et al., 2017).

D3-9 Engages the HA Stem via Multiple Binding Modes

Recently, Joyce et al. isolated a number of heterosubtypic cross-reactive B cells from six individuals (subjects 1, 16, 31, 36, 54, and 56) enrolled in an H5N1 DNA/MIV-prime-boost influenza vaccine trial (Joyce et al., 2016). Their study discovered a number of bnAbs, including one called 31.b.09, that utilized VH1-18 and D3-9 (Joyce et al., 2016), which is the same as in S9-3-37. Nonetheless, when we compared the sequence of S9-3-37 to that of 31.b.09, we noticed that their D3-9 gene segments were translated in different reading frames (Figure 4A). In other words, despite the usage of the same D gene segment, the corresponding amino acid sequences in the D gene-encoded regions of S9-3-37 and 31.b.09 are totally different. The D3-9 gene segment in S9-3-37 encodes for amino acids LGYFDWL, whereas the D3-9 gene segment in 31.b.09 encodes for amino acids ILTG. When we compared the structure of S9-3-37-HA complex with that of 31.b.09-HA complex (PDB 5K9O) (Joyce et al., 2016), we found that the Phe within the LGYFDWL motif in S9-3-37 and Leu within the ILTG motif in 31.b.09 occupy the same pocket in the HA stem region. This observation demonstrates that the D3-9 gene segment can engage the HA stem with two different reading frames. Unlike the two reading frames outlined above, the third reading frame of D3-9 gene segment contains a stop codon in the middle of the translated protein sequence, making it unlikely to be utilized (Figure S5A).

Involvement of D3-9 in Anti-influenza Immunity

Five of the six subjects studied by Joyce et al. utilized the D3-9 gene segment in their cross-reactive B cells (Joyce et al., 2016), with varying frequency (Figure 4B). Based on a motif searching approach (see STAR Methods), we classified the CDR H3 sequence of each B cell clone that utilized the D3-9 gene segment into S9-3-37-like or 31.b.09-like. Briefly, those D3-9-encoded CDR H3 sequences that contained an LXYFXWL motif, where X represents any amino acid, were classified as S9-3-37-like (Figure 4C). In other words, despite the usage of the same D gene segment, the corresponding amino acid sequences in the D gene-encoded regions of S9-3-37 and 31.b.09 are totally different.
S9-3-37-like. Those D3-9-encoded CDR H3 sequences that contained an ILTG motif, with one mismatch allowed at position 1, 3, or 4 (i.e., no variation was allowed for Leu at the second position), were classified as 31.b.09-like. S9-3-37-like CDR H3 could be observed in four heterosubtypic cross-reactive B cells from subjects 31, 54, and 56, whereas 31.b.09-like CDR H3 could be observed in 78 heterosubtypic cross-reactive B cells from subjects 1, 16, and 31 (Figures 4C and 4D; Table S2). The four S9-3-37-like CDR H3 sequences identified in this analysis are relatively long (19–26 residues, Figure 4D), as also seen in S9-3-37 (21 amino acid) and FI6v3 (20 amino acid), suggesting that they may bind to HA stem in a similar manner to S9-3-37 and FI6v3. Joyce et al. indeed showed that antibodies from three of those four B cell clones (31.d.01, 54.e.01, and 56.h.01) could neutralize multiple influenza subtypes (Joyce et al., 2016). Of note, both 31.d.01 and 56.h.01 are group 1-specific bnAbs, whereas 54.e.01 can neutralize viruses from both group 1 and group 2.

In addition, we analyzed four additional datasets to examine the frequency of D3-9 usage in heterosubtypic cross-reactive B cells. These include 306 H1/H3/H7 cross-reactive B cells (Andrews et al., 2017), 55 H1/H3 cross-reactive B cells (McCarty et al., 2018), 42 HA stem-specific B cells (Andrews et al., 2015), and 198 HA stem-specific B cells (Corti et al., 2011; Pappas et al., 2014). Similar to the dataset from Joyce et al. (Joyce et al., 2016), all four additional datasets contain B cells that utilize the D3-9 gene segment (Figures S5B–S5E; 22% (140 out of 645) in Joyce et al. (2016), 7% (20 out of 306) in Andrews et al. (2017), 4% (2 out of 55) in McCarty et al. (2018), 7% (3 out of 42) in Andrews et al. (2015), and 17% (33 out of 198) in Corti et al. (2011) and Pappas et al. (2014). Overall, 16% (198 out of 1,246) heterosubtypic cross-reactive B cells utilized the D3-9 gene segment across all analyzed datasets. These findings demonstrate preferences in the use of the D3-9 gene segment in HA heterosubtypic cross-reactive B cells.

We further performed a structural comparison between S9-3-37 and a representative bnAb reported in Andrews et al. (2017), namely 27-1C08 (VH1-2+D3-9) that contains the LXYFXWL motif. Despite the vast difference in the overall conformations of the CDR H3 loops in 27-1C08 (PDB 5WCA) (Andrews et al., 2017) and S9-3-37, the conformations of their D3-9-encoded regions are highly similar (Figure S6A). While the structure of 27-1C08 in complex with HA is not available, our observations suggest that the D3-9-encoded regions from 27-1C08 would bind to the same stem pocket as S9-3-37. These results also support the notion that the D3-9 gene segment provides a recurring strategy to target the HA stem.

S9-3-37-like CDR H3s in B Cell Repertoires of Healthy Donors
We further aimed to analyze the D3-9-encoded CDR H3 sequences that contained an ILTG motif, with one mismatch allowed at position 1, 3, or 4 (i.e., no variation was allowed for Leu at the second position), were classified as 31.b.09-like. S9-3-37-like CDR H3 could be observed in four heterosubtypic cross-reactive B cells from subjects 31, 54, and 56, whereas 31.b.09-like CDR H3 could be observed in 78 heterosubtypic cross-reactive B cells from subjects 1, 16, and 31 (Figures 4C and 4D; Table S2). Both 31.b.09-like and S9-3-37-like CDR H3 sequences could be found (Figure 5A), although the frequency of 31.b.09-like CDR H3 sequences (~1%) was much higher than S9-3-37-like CDR H3 sequences (~0.01% to ~0.1%). S9-3-37-like CDR H3s with at least 20 amino acids long could be observed in two of the three healthy donors (Figure 5B). This analysis shows that B cells that utilize S9-3-37-like CDR H3, despite being less common than 31.b.09-like CDR H3, are prevalent in the B cell repertoires of healthy donors.

S9-3-37-like CDR H3s Are Uncommon in Non-HA-Targeting B Cells
To assess the relative prevalence of 31.b.09-like (ILTG motif) and S9-3-37-like (LXYFXWL motif) CDR H3s in targeting non-HA antigens, we analyzed two published datasets. The first dataset describes the memory B cell repertoires from four donors after administration of two meningococcal vaccines (Galson et al., 2015). The second dataset describes the memory B cell repertoire from three time points that are 1–2 years apart in an HIV-infected patient (Huang et al., 2016). As compared to the 1,246 HA cross-reactive antibodies that were analyzed above (Figures 4B, 4C, and SSB–S5E), the S9-3-37-like CDR H3 is rare in the...
memory B cell repertoires of the four meningococcal-vaccinated individuals and the HIV-infected patient (Figure 6). Among HA cross-reactive antibodies, the frequency of S9-3-37-like CDR H3 (2.2%, 27 out of 1,246) is 3-fold lower than that of 31.b.09-like CDR H3 (6.7%, 84 out of 1,246). In contrast, in the memory B cell repertoires of meningococcal-vaccinated individuals and the HIV-infected patient, an S9-3-37-like CDR H3 is either undetectable or >100-fold less prevalent than the 31.b.09-like CDR H3. This analysis suggests that, while 31.b.09-like CDR H3 may be involved at very low frequencies in targeting other non-HA antigens, S9-3-37-like CDR H3 is much more specific for targeting the HA stem region.

We also analyzed the germline usage of 53 HIV bnAbs from different clonotypes (Eroshkin et al., 2014), in which two of them (PGT136 and VRC-PG04b) utilized the D3-9 gene segment (Figure S7A). Nonetheless, in these two HIV bnAbs, the D3-9 gene segment only encodes for two to three amino acids (Figures S7B and S7C). Therefore, D3-9 gene segment in these two HIV bnAbs is unlikely to be as critical for engaging the epitope. Consistent with the analysis of memory B cell repertoires (Figure 6), the result here implies that the D3-9 gene segment has a specific role in bnAbs that target influenza HA, but not in other microbial antigens analyzed to date.

**DISCUSSION**

From the X-ray structure of group 1 influenza bnAb S9-3-37, we uncovered an important role of the D3-9 gene segment in targeting the HA stem region. In particular, the binding of the D3-9-encoded CDR H3 region to the HA stem is resilient to different reading (translation) frames and has minimum dependency on the $V_{H}$ and $J_{H}$ gene segments. Importantly, the D3-9 gene segment is utilized by HA stem-binding bnAbs from multiple donors (Andrews et al., 2017; Corti et al., 2011; Joyce et al., 2016; Wyrzucki et al., 2014).

An “SOS component” of the antibody repertoire has been used to describe a germline response that is immediately available upon microbial infection with minimum somatic mutations (Lerner, 2011, 2016). $V_{H}$_1-69 is perhaps the most well-known “SOS component” of the antibody repertoire (Dreyfus et al., 2012; Ekiert et al., 2009; Kashyap et al., 2008; Lang et al., 2017; Lerner, 2011, 2016; Sui et al., 2009; Throsby et al., 2008). Our results suggest that the D3-9 gene segment is another “SOS component” of the antibody repertoire. However, it seems the response from D3-9 gene segment is not as general as $V_{H}$_1-69. $V_{H}$_1-69 is frequently observed in immune response against pathogens other than influenza virus, such as hepatitis C virus (Marasca et al., 2001), HIV (Gorny et al., 2012; Luftig et al., 2006), and middle east respiratory syndrome coronavirus (Ying et al., 2015). In contrast, our analysis indicates that D3-9 gene segment, especially the reading frame that encodes the motif LXYFXWL (S9-3-37-like CDR H3), is more specific to influenza virus (Figures 6 and S7). To date, most large-scale analyses on germline usage have focused on $V_{H}$ gene segments. Future analysis should investigate whether other D gene segments and even $J_{H}$ gene segments contribute to the “SOS component” of the antibody repertoire.

Structural characterization of multidonor class bnAbs has been highly valuable for development of the universal influenza vaccine using a reverse-engineering approach. For example, two different headless HA immunogens (Impagliazzo et al., 2015; Yassine et al., 2015) were designed based on the binding modes of CR9114 (Dreyfus et al., 2012) and CR6261 (Ekiert et al., 2009; Throsby et al., 2008), which are both $V_{H}$_1-69-encoded bnAbs. Structural characterization of such bnAbs also provides templates for antiviral development (Wu and Wilson, 2018). These HA stem-binding bnAbs have also inspired the design of small proteins and peptides that display antiviral activities.
(Chevalier et al., 2017; Fleishman et al., 2011; Kadam et al., 2017; Whitehead et al., 2012). In fact, a small neutralizing peptide that binds to the HA stem was designed primarily based on CDR H3 of bnAb FI6v3 containing the D3-9-encoded LXYFXWL motif (Kadam et al., 2017) (PDB 5W6T; Figure S6B). We anticipate that future discovery and characterization of multitron class bnAbs will continue to provide insight into antiviral and vaccine design against influenza virus.

As several HA stem-binding bnAbs are currently being tested in clinical trials for both prophylactic and therapeutic usage (Sparrow et al., 2016), it is equally important to understand the potential for bnAb escape. Although it is generally more difficult for influenza virus to escape from stem antibodies compared to head antibodies (Anderson et al., 2017; Chai et al., 2016; Doud et al., 2018; Ekiert et al., 2009; Sui et al., 2009; Throsby et al., 2008; Yamayoshi et al., 2018), strong escape mutations have been identified for several HA stem-binding bnAbs (Anderson et al., 2017; Chai et al., 2016; Ekiert et al., 2011; Friesen et al., 2014; Henry Dunand et al., 2015; Yamayoshi et al., 2018). Most of the escape mutations dramatically decrease the binding affinity to bnAbs. Nonetheless, increasing the membrane fusion pH of the HA can also contribute to HA stem-binding bnAb escape (Chai et al., 2016). A previous study by some of the authors here has shown that HA1 N21S/Y (residue 11 in H1 numbering), which eliminates the highly conserved N-glycosylation site at HA1 Asn21, abolished the neutralization activity of several HA stem-binding bnAbs, including S9-3-37 (Yamayoshi et al., 2018). Interestingly, Asn21 is not part of the S9-3-37 epitope (Figures S6C and S6D), suggesting that the escape mechanism of N21S/Y is not due to a loss of a direct interaction that could decrease binding to S9-3-37. Rather, such S9-3-37 escape could be due to an increase in the membrane fusion pH, since removal of the N-glycosylation site at Asn21 has been suggested to decrease the pH stability of the virus and, hence, make it more fusion sensitive at higher pH (Yin et al., 2017). While the HA stem is a promising target for antiviral and vaccine design, future studies of potential escape mechanisms will be critical for the development of a robust anti-influenza strategy.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Cell cultures
- **METHOD DETAILS**
  - Expression and purification of S9-3-37 Fab
  - Expression, purification and biotinylation of influenza hemagglutinin
  - Biolayer interferometry binding assay
  - Crystallization and structural determination
  - Buried surface area calculation
  - Analysis of natural HA variants
  - Analysis of published antibody sequences
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
- **DATA AND SOFTWARE AVAILABILITY**
Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004). WebLogo: a sequence logo generator. Genome Res. 14, 1188–1190.

DeKosky, B.J., Kojima, T., Rodin, A., Charab, W., Ippolito, G.C., Ellington, A.D., and Georgiou, G. (2015). In-depth determination and analysis of the human paired heavy- and light-chain antibody repertoire. Nat. Med. 21, 86–91.

Doud, M.B., Lee, J.M., and Bloom, J.D. (2018). How single mutations affect viral escape from broad and narrow antibodies to H1 influenza hemagglutinin. Nat. Commun. 9, 1386.

Dreyfus, C., Laursen, N.S., Kwaks, T., Zuidgeest, D., Khayat, R., Ekiert, D.C., Lee, J.H., Mettlager, Z., Bujny, M.V., Jongeneelen, M., et al. (2012). Highly conserved protective epitopes on influenza B viruses. Science 337, 1343–1348.

Ekiert, D.C., Bhabha, G., Elsliger, M.A., Friesen, R.H., Jongeneelen, M., Throsby, M., Goudsmit, J., and Wilson, I.A. (2009). Antibody recognition of a highly conserved influenza virus epitope. Science 324, 246–251.

Ekiert, D.C., Friesen, R.H., Bhabha, G., Kwaks, T., Jongeneelen, M., Yu, W., Ophorst, C., Cox, F., Korse, H.J., Brandenburg, B., et al. (2011). A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333, 843–850.

Ekiert, D.C., Kashyap, A.K., Steel, J., Rubrum, A., Bhabha, G., Khayat, R., Lee, J.H., Dillon, M.A., O’Nei, R.E., Faynboym, A.M., et al. (2012). Cross-neutralization of influenza A viruses mediated by a single antibody loop. Nature 489, 526–532.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and construction of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501.

Erbeling, E.J., Post, D.J., Stemmy, E.J., Roberts, P.C., Augustine, A.D., Ferguson, S., Paules, C.I., Graham, B.S., and Fauci, A.S. (2018). A universal influenza vaccine: the strategic plan for the National Institute of Allergy and Infectious Diseases. J. Infect. Dis. 218, 347–354.

Eroshkin, A.M., LeBlanc, A., Weekes, D., Post, K., Lu, X., Rajput, A., Butera, S.T., Burton, D.R., and Godzik, A. (2014). bNAber: database of broadly neutralizing broadly neutralizing antibodies. Nat. Commun. 5, 86–91.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and construction of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501.

Ekiert, D.C., Bhabha, G., Elsliger, M.A., Friesen, R.H., Jongeneelen, M., Throsby, M., Goudsmit, J., and Wilson, I.A. (2009). Antibody recognition of a highly conserved influenza virus epitope. Science 324, 246–251.

Ekiert, D.C., Friesen, R.H., Bhabha, G., Kwaks, T., Jongeneelen, M., Yu, W., Ophorst, C., Cox, F., Korse, H.J., Brandenburg, B., et al. (2011). A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333, 843–850.

Ekiert, D.C., Kashyap, A.K., Steel, J., Rubrum, A., Bhabha, G., Khayat, R., Lee, J.H., Dillon, M.A., O’Nei, R.E., Faynboym, A.M., et al. (2012). Cross-neutralization of influenza A viruses mediated by a single antibody loop. Nature 489, 526–532.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and construction of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501.

Ekiert, D.C., Bhabha, G., Elsliger, M.A., Friesen, R.H., Jongeneelen, M., Throsby, M., Goudsmit, J., and Wilson, I.A. (2009). Antibody recognition of a highly conserved influenza virus epitope. Science 324, 246–251.

Ekiert, D.C., Friesen, R.H., Bhabha, G., Kwaks, T., Jongeneelen, M., Yu, W., Ophorst, C., Cox, F., Korse, H.J., Brandenburg, B., et al. (2011). A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333, 843–850.

Ekiert, D.C., Kashyap, A.K., Steel, J., Rubrum, A., Bhabha, G., Khayat, R., Lee, J.H., Dillon, M.A., O’Nei, R.E., Faynboym, A.M., et al. (2012). Cross-neutralization of influenza A viruses mediated by a single antibody loop. Nature 489, 526–532.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and construction of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501.

Ekiert, D.C., Bhabha, G., Elsliger, M.A., Friesen, R.H., Jongeneelen, M., Throsby, M., Goudsmit, J., and Wilson, I.A. (2009). Antibody recognition of a highly conserved influenza virus epitope. Science 324, 246–251.

Ekiert, D.C., Friesen, R.H., Bhabha, G., Kwaks, T., Jongeneelen, M., Yu, W., Ophorst, C., Cox, F., Korse, H.J., Brandenburg, B., et al. (2011). A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333, 843–850.

Ekiert, D.C., Kashyap, A.K., Steel, J., Rubrum, A., Bhabha, G., Khayat, R., Lee, J.H., Dillon, M.A., O’Nei, R.E., Faynboym, A.M., et al. (2012). Cross-neutralization of influenza A viruses mediated by a single antibody loop. Nature 489, 526–532.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and construction of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501.

Ekiert, D.C., Bhabha, G., Elsliger, M.A., Friesen, R.H., Jongeneelen, M., Throsby, M., Goudsmit, J., and Wilson, I.A. (2009). Antibody recognition of a highly conserved influenza virus epitope. Science 324, 246–251.

Ekiert, D.C., Friesen, R.H., Bhabha, G., Kwaks, T., Jongeneelen, M., Yu, W., Ophorst, C., Cox, F., Korse, H.J., Brandenburg, B., et al. (2011). A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333, 843–850.

Ekiert, D.C., Kashyap, A.K., Steel, J., Rubrum, A., Bhabha, G., Khayat, R., Lee, J.H., Dillon, M.A., O’Nei, R.E., Faynboym, A.M., et al. (2012). Cross-neutralization of influenza A viruses mediated by a single antibody loop. Nature 489, 526–532.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and construction of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501.

Ekiert, D.C., Bhabha, G., Elsliger, M.A., Friesen, R.H., Jongeneelen, M., Throsby, M., Goudsmit, J., and Wilson, I.A. (2009). Antibody recognition of a highly conserved influenza virus epitope. Science 324, 246–251.

Ekiert, D.C., Friesen, R.H., Bhabha, G., Kwaks, T., Jongeneelen, M., Yu, W., Ophorst, C., Cox, F., Korse, H.J., Brandenburg, B., et al. (2011). A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 333, 843–850.
Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326.

Pappas, L., Foglierini, M., Piccoli, L., Kallewaard, N.L., Turinli, F., Silacci, C., Fernandez-Rodriguez, B., Agatic, G., Giacchetto-Sasselli, I., Pellicciotta, G., et al. (2014). Rapid development of broadly influenza neutralizing antibodies through redundant mutations. Nature 516, 418–422.

Sparrow, E., Friede, M., Sheik, M., Torvaldsen, S., and Newall, A.T. (2016). Passive immunization for influenza through antibody therapies, a review of the pipeline, challenges and potential applications. Vaccine 34, 5442–5448.

Stevens, J., Blixt, O., Tumpey, T.M., Taubenberger, J.K., Paulson, J.C., and Wilson, I.A. (2006). Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science 312, 404–410.

Sui, J., Hwang, W.C., Perez, S., Wei, G., Aird, D., Chen, L.M., Santelli, E., Stec, B., Cadwell, G., Ali, M., et al. (2009). Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat. Struct. Mol. Biol. 16, 265–273.

Throsby, M., van den Brink, E., Jongeneelen, M., Poon, L.L., Alard, P., Cornelissen, L., Bakker, A., Cox, F., van Deventer, E., Guan, Y., et al. (2008). Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS One 3, e3942.

Whitehead, T.A., Chevalier, A., Song, Y., Dreyfus, C., Fleishman, S.J., DeMattos, C., Myers, C.A., Kamisettey, H., Blair, P., Wilson, I.A., and Baker, D. (2012). Optimization of affinity, specificity and function of designed influenza inhibitors using deep sequencing. Nat. Biotechnol. 30, 543–548.

Ye, J., Ma, N., Madden, T.L., and Ostell, J.M. (2013). IgBLAST: an immunoglobulin variable domain sequence analysis tool. Nucleic Acids Res. 41, W34–W40.

Yin, Y., Zhang, X., Qiao, Y., Wang, X., Su, Y., Chen, S., Qin, T., Peng, D., and Liu, X. (2017). Glycosylation at 11Asn on hemagglutinin of H5N1 influenza virus contributes to its biological characteristics. Vet. Res. (Faisalabad) 48, 81.

Ying, T., Prabakaran, P., Du, L., Shi, W., Feng, Y., Wang, Y., Wang, L., Li, W., Jiang, S., Dimitrov, D.S., and Zhou, T. (2015). Junctional and allele-specific residues are critical for MERS-CoV neutralization by an exceptionally potent germline-like antibody. Nat. Commun. 6, 8223.
### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**       |        |            |
| ExpiCHO Expression System Kit | Thermo Fisher Scientific | A29133 |
| HyClone insect cell culture medium | GE Healthcare | SH30280.03 |
| Phosphate-buffered saline (PBS) | Thermo Fisher Scientific | 14040133 |
| Ni-NTA Superflow | QIAGEN | 30450 |
| HA protein sequences | GISAID; https://gisaid.org/ | N/A |
| S9-3-37 antibody | Yamayoshi et al., 2018 | N/A |
| DH10Bac competent cells | Thermo Fisher Scientific | 10361012 |
| **Biological Samples** |        |            |
| ExpiCHO cells | Thermo Fisher Scientific | A29127 |
| Sf9 cells | ATCC | CRL-1711 |
| High Five cells | Thermo Fisher Scientific | B85502 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| DpnI | New England Biolabs | R0176L |
| Trypsin | New England Biolabs | P8101S |
| Sodium chloride (NaCl) | Sigma-Aldrich | S9888 |
| Tris Base | Sigma-Aldrich | 11814273001 |
| Concentrated hydrochloric acid (HCl) | Sigma-Aldrich | H1758 |
| Sodium azide (NaN₃) | Sigma-Aldrich | S2002 |
| Bovine Serum Albumin (BSA) | Sigma-Aldrich | A9418 |
| Tween 20 | Fisher Scientific | BP337-500 |
| **Critical Commercial Assays** |        |            |
| In-Fusion HD Cloning Kit | Takara | 639647 |
| KOD Hot Start DNA Polymerase | EMD Millipore | 71086-3 |
| PCR Clean-Up and Gel Extraction Kit | Clontech Laboratories | 740609.250 |
| QIAprep Spin Miniprep Kit | QIAGEN | 27106 |
| NucleoBond Xtra Maxi | Clontech Laboratories | 740414.100 |
| **Deposited Data** |        |            |
| X-ray coordinates and structure factors | This study | PDB: 6E3H |
| **Oligonucleotides** |        |            |
| Primers for cloning | Integrated DNA Technologies | N/A |
| **Recombinant DNA** |        |            |
| phCMV3-S9-3-37 Fab heavy chain | This study | N/A |
| phCMV3-S9-3-37 light chain | This study | N/A |
| pFast-A/New York/1/1918 (H1 HA) | Ekiert et al., 2012 | N/A |
| pFast-A/WSN/1933 (H1 HA) | Ekiert et al., 2012 | N/A |
| pFast-A/duck/Alberta/60/1976 (H1 HA) | Ekiert et al., 2012 | N/A |
| pFast-A/Beijing/262/1995 (H1 HA) | Ekiert et al., 2012 | N/A |
| pFast-A/Soledon Islands/3/2006 (H1 HA) | Ekiert et al., 2012 | N/A |
| pFast-A/California/04/2009 (H1 HA) | Ekiert et al., 2012 | N/A |
| pFast-A/Adachi/2/1957 (H2 HA) | Ekiert et al., 2012 | N/A |
| pFast-A/Vietnam/1203/2004 (H5 HA) | Ekiert et al., 2012 | N/A |
| pFast-A/Indonesia/05/2005 (H5 HA) | Ekiert et al., 2012 | N/A |
| pFast-A/turkey/Wisconsin/1/1966 (H9 HA) | Ekiert et al., 2012 | N/A |

*(Continued on next page)*
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ian A. Wilson (wilson@scripps.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell cultures
Exp2CHO cells were maintained according to the manufacturer’s instructions (Thermo Fisher Scientific). Sf9 cells and High Five cells were maintained in HyClone insect cell culture medium (GE Healthcare).

METHOD DETAILS

Expression and purification of S9-3-37 Fab
The heavy and light chains of S9-3-37 Fab were cloned into phCMV3 vector with an N-terminal secretion signal \( \text{M} \text{E} \text{T} \text{D} \text{T} \text{L} \text{L} \text{L} \text{W} \text{L} \text{L} \text{W} \text{V} \text{P} \text{G} \text{S} \text{T} \text{G} \). The phCMV3-S9-3-37 heavy chain and phCMV3-S9-3-37 light chain were produced in the Exp2CHO expression system (Thermo Fisher Scientific) according to the Max Titer Protocol as described in the manufacturer’s instructions. S9-3-37 Fab was purified by the 5 mL HiTrap Protein G HP antibody purification columns and buffer exchanged into 1x PBS.

Expression, purification and biotinylation of influenza hemagglutinin
Influenza hemagglutinin (HA) was prepared for binding studies as previously described (Ekiert et al., 2011). Briefly, the HA ectodomain, which corresponds to 11–329 (HA1) and 1–176 (HA2) based on H3 numbering, was fused with an N-terminal gp67 signal peptide and a C-terminal BirA biotinylation site, thrombin cleavage site, trimerization domain, and a His\(_6\) tag, and then cloned into a customized baculovirus transfer vector (Ekiert et al., 2011). Recombinant bacmid DNA was generated using the Bac-to-Bac system (Thermo Fisher Scientific) according to the Max Titer Protocol as described in the manufacturer’s instructions. S9-3-37 Fab was purified by the 5 mL HiTrap Protein G HP antibody purification columns and buffer exchanged into 1x PBS.
the C-terminal tag (BirA biotinylation site, thrombin cleavage site, trimerization domain, and the His6 tag) and to produce the cleaved mature HA (HA1/HA2). The trypsin-digested Viet04 HA 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.

**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 ~10-50 μg mL⁻¹ in 1x kinetics buffer (1x PBS with 0.01% BSA and 0.002% Tween 20) was loaded onto streptavidin biosensors and incubated with supernatant from transfected cells or with the indicated concentration of Fab. Streptavidin biosensors that were not loaded were used as a reference for subtracting background binding from signals. Briefly, the assay consisted of five steps: 1) baseline: 60 s with 1x kinetics buffer; 2) loading: 120 s with biotinylated HA0; 3) baseline: 60 s with 1x kinetics buffer; 4) association: 120 s with samples (supernatant from transfected cells or purified Fab); and 5) dissociation: 120 s with 1x kinetics buffer. For estimating the K_d, a 1:1 binding model was used.

**Crystallization and structural determination**

S9-3-37 Fab was incubated with the purified Viet04 HA trimer in a molar ratio of 4.5:1 overnight at 4 °C. The S9-3-37 Fab-Viet04 HA 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 9 mg mL⁻¹ in 10 mM Tris pH 8.0, 50 mM NaCl, and 0.02% Na3. Crystal screening was carried out using our high-throughput, robotic CrystalMation system (Rigaku) at TSRI. The initial crystal screening was based on sitting drop vapor diffusion method with 35 μL reservoir solution and each drop consisting 0.1 μL protein + 0.1 μL precipitant. Diffraction-quality crystals were obtained with a reservoir solution containing 20% PEG 6000, 0.1 M HEPES pH 7.0 at 20 °C.

Diffraction data were collected at Stanford Synchrotron Radiation Lightsource beamline 12-2. The data were indexed, integrated and scaled using HKL2000 (HKL Research) (Otwinowski and Minor, 1997). The structure was solved by molecular replacement using Phaser (McCoy et al., 2007), modeled using Coot (Emsley et al., 2010), and refined using Refmac5 (Murshudov et al., 2011). For molecular replacement, PDB 2FK0 (Stevens et al., 2006) was used as the model for Viet04 HA, and a homology model generated by PIGSPro (LePore et al., 2017) was used for S9-3-37. Ramachandran statistics were calculated using MolProbity (Chen et al., 2010). IgBLAST (Ye et al., 2013) was employed to identify the CDRs on S9-3-37 Fab. The Kabat numbering scheme was used.

**Buried surface area calculation**

Solvent accessibility was computed by DSSP (Kabsch and Sander, 1983). Buried surface area (BSA) was calculated by subtracting the solvent accessibility of the bound form from that of the apo form. HA residues that had a non-zero BSA were identified as epitope residues.

**Analysis of natural HA variants**

A total of 103,301 full-length HA protein sequences from different subtypes were downloaded from the Global Initiative for Sharing Avian Influenza Data (GISAID; https://gisaid.org). To avoid temporal sampling bias, we sampled at most 20 sequences per year per subtype, which resulted in a total of 6,984 HA sequences. Multiple sequence alignment of those 6,984 HA sequences was performed by MAFFT version 7.157b (Katoh and Standley, 2013). Sequence logos were generated by WebLogo (Crooks et al., 2004).

**Analysis of published antibody sequences**

CDR H3 sequences recovered from H3 and H5 cross-reactive memory B cells of six H5N1 DNA/MIV-prime-boost influenza vaccinated subjects were retrieved from Table S3 in Joyce et al. (Joyce et al., 2016). Germline usage of H1/H3/H7 cross-reactive B cells from six H7N9 DNA/MIV-prime-boost influenza vaccine subjects was retrieved from Table S7 in Andrews et al. (Andrews et al., 2017). Germline usage of H1 and H3 cross-reactive memory B cells from subjects immunized with trivalent influenza vaccine was retrieved from Figure S4 in McCarthy et al. (McCarthy et al., 2018). Germline usage of HA stem-specific memory B cells from subjects immunized with trivalent influenza vaccine was retrieved from Tables S3 and S4 in Andrews et al. (Andrews et al., 2015). Germline usage of HA stem-specific memory B cells from a single subject immunized with seasonal influenza vaccine was retrieved from Figure S1 in Pappas et al. (Pappas et al., 2014). CDR H3 sequences from memory B cells of three healthy donors and their occurrence frequency information were retrieved from Supplemental Dataset 1 in DeKosky et al. (DeKosky et al., 2015). CDR H3 sequences from the IgG* memory B cell repertoires from four donors after administration of two Meningococcal vaccines (Galson et al., 2015), and the IgG* memory B cell repertoire from three time points that were one to two years apart in an HIV-infected patient (Huang et al., 2016), were downloaded from the Observed Antibody Space database (Kovaltsuk et al., 2018). Nucleotide sequences of HIV bnAbs were retrieved from bNABer database (Eroshkin et al., 2014). Germline usages were identified using IgBlast (Ye et al., 2013). S9-3-37-like
CDR H3s were defined as those CDR H3s that utilized the D3-9 gene segment and encoded a LXYFXWL motif, where X represents any amino acid. 31.b.09-like CDR H3s were defined as those CDR H3s that utilized the D3-9 gene segment and encoded an ILTG motif with one mismatch in the non-underlined residue (i.e., no variation was allowed for the Leu at second position of the ILTG motif).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was not performed in this study.

**DATA AND SOFTWARE AVAILABILITY**

The X-ray coordinates and structure factors of S9-3-37 in complex with H5 HA have been deposited in the RCSB Protein Data Bank under accession code 6E3H. Custom python scripts for sequence analyses have been deposited to [https://github.com/wchnicholas/S9-3-37](https://github.com/wchnicholas/S9-3-37).