Unique binding pattern for a lineage of human antibodies with broad reactivity against influenza A virus

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Most structurally characterized broadly neutralizing antibodies (bnAbs) against influenza A viruses (IAVs) target the conserved conformational epitopes of hemagglutinin (HA). Here, we report a lineage of naturally occurring human antibodies sharing the same germline gene, V_{H}3-48/V_{K}1-12. These antibodies broadly neutralize the major circulating strains of IAV in vitro and in vivo mainly by binding a contiguous epitope of H3N2 HA, but a conformational epitope of H1N1 HA, respectively. Our structural and functional studies of antibody 28-12 revealed that the continuous amino acids in helix A, particularly N49_{HA2} of H3 HA, are critical to determine the binding feature with 28-12. In contrast, the conformational epitope feature is dependent on the discontinuous segments involving helix A, the fusion peptide, and several HA1 residues within H1N1 HA. We report that this antibody was initially selected by H3 (group 2) viruses and evolved via somatic hypermutation to enhance the reactivity to H3 and acquire cross-neutralization to H1 (group 1) virus. These findings enrich our understanding of different antigenic determinants of heterosubtypic influenza viruses for the recognition of bnAbs and provide a reference for the design of influenza vaccines and more effective antiviral drugs.
Influenza A virus (IAV) infection remains a serious and persistent threat to global public health. Given the rapid antigen drift and shift of influenza viruses, the current seasonal influenza vaccines are insufficient to meet broad social needs. The emergence of drug-resistant influenza viruses limits the widespread use of anti-influenza drugs. Therefore, there is an urgent medical need for a more universal solution to meet the challenge of influenza A viruses. Genetically, 16 IAV subtypes are classified into two distinct groups according to the phylogenetics of hemagglutinin (HA), with another two new analogical HAs isolated from bats (named H17 and H18). Usually, the H5 and H7 subtypes are highly pathogenic avian influenza viruses associated with sporadic severe human infection. H1 and H5 subtypes circulate annually in human society and are therefore the components of seasonal influenza vaccines. The envelope glycoprotein HA of IAV is the major target of neutralizing antibodies. The globular head domain mediates the receptor binding process, and the HA stem leads to virus-cell membrane fusion induced by the low pH in endosomes. Several human antibodies have been isolated from vaccinated or naturally infected individuals that target conserved epitopes within HA that showed different levels of cross-reactivity towards group 1 and group 2 IAVs. The majority of bnAbs target the HA stem region, thus neutralizing influenza A viruses by inhibiting the low pH-induced conformational rearrangement of HA, hence blocking membrane fusion; while a small fraction of them targets the conserved HA head region and block HA-sialic acid receptor binding. These antibodies have provided powerful tools to identify such dominant germline scaffolds, thus speeding up the development of broad-spectrum therapeutic agents. Moreover, understanding how bnAbs overcome highly diversified HA antigenic variations to broadly neutralize divergent IAVs would also contribute to the development of universal influenza vaccines.

In the current study, we report a lineage of naturally occurring human antibodies isolated from a healthy donor who was vaccinated with a trivalent seasonal split vaccine. The representative antibody 28-12 exhibited broad neutralizing activity against both group 1 and group 2 IAV viruses in vitro and protected mice against H3N2 and H1N1 virus challenge in vivo. The character of this lineage of human antibodies is unusual. These antibodies (28-2, 28-4, 28-6 and 28-12) belong to the same lineage carrying VH3-48/DH2-2/JH6 germline genes for the stem-directed bnAbs against IAVs. The characterized bnAbs have provided powerful tools to identify such dominant germline scaffolds, thus speeding up the development of broad-spectrum therapeutic agents. Moreover, understanding how bnAbs overcome highly diversified HA antigenic variations to broadly neutralize divergent IAVs would also contribute to the development of universal influenza vaccines.

Results

Isolation and characterization of $V_{H3-48}$/$V_{K1-12}$ lineage antibodies. We conducted single-cell PCR experiments to screen mAbs that might protect against heterosubtypic IAV infection from memory B cells of one healthy individual ~4 week after vaccination with seasonal trivalent influenza vaccine in 2016, which contains three components, A/California/7/2009(H1N1) pdm09-like virus, A/Victoria/361/2011(H3N2)-like virus and B/Wisconsin/1/2010-like virus (Fig. 1a). We performed memory B cell screening experiments using group 2 A/HongKong/01/1968 (HK/68) H3N2 HA protein as the bait. As the H1N1 and H3N2 subtypes are the most prevalent circulating strains, subsequent cloning and screening of antibodies for reactivity were conducted with soluble HA proteins and human infecting viruses of both HK/68 H3N2 and SC/09 H1N1 (A/Sichuan/01/2009), which led to the identification of a series of antibodies with different levels of cross-reactivity towards H1N1 and H3N2. Among these antibodies, eleven mAbs cross-neutralizes both H3N2 and H1N1. Notably, 28-4, and 28-12 showed better cross-neutralizing activity with IC50 under 2 μg/ml as compared with other isolated antibodies in this study, which is comparable with a previously reported antibody MEDI8852 (Fig. 1b).

We performed gene sequence analysis of the isolated antibodies by using IMGT and IgBLAST. A large portion of antibodies utilized the $V_{H3-48}$ germline genes (Fig. 1c), of which, 28-2, 28-4, 28-6 and 28-12, belong to the same lineage carrying $V_{H3-48}$/ $D_{H3-2}$/ $J_{H3-6}$ and $V_{K1-12}$/ $J_{K5}$ gene segments. It is worth noting that these antibodies showed different epitope determinants between different subtypes that they mainly bind a continuous epitope of H3N2 HA, while recognizing H1N1 HA in a conformation dependent pattern. The reason can be elucidated from our functional studies combined with our cryo-EM structures of 28-12 Fab in complex with HAs of H1N1 and H3N2. The binding activity of 28-12 to H1N1 is much more dependent on helix A, the fusion peptide and several HA1 residues, which are discontinuous in the primary sequence. Nevertheless, the continuous epitope within the helix A of H3N2 HA makes the major contribution to the binding of 28-12. We further revealed that N49H1A is the critical residue to determine the epitope feature of H3N2 HA by 28-12; however, the equivalent residue is substituted by T49HA2 in H1N1. We further revealed that 28-12 was likely first primed by group 2 viruses (H3) and further enhanced the reactivity to H3 and acquired cross-neutralization against group 1 viruses (H1). We described the putative key somatic hypermutations of 28-12 to achieve broad and potent neutralization. This study improves our understanding of the significant flexibility of bnAbs in recognizing distinct antigenic determinants among different IAV subtypes. Our finding supports 28-12 as a potential therapeutic reagent for both prophylaxis and treatment of circulating H1N1 and H3N2 IAV infection in humans and provide a reference for the design of broad influenza vaccines and more effective antiviral drugs.

Broad binding and neutralization profile of $V_{H3-48}$/ $V_{K1-12}$ lineage antibodies. To verify the reactive breadth of the $V_{H3-48}$/ $V_{K1-12}$ lineage antibodies, BLI-based $K_D$ values were measured within each mAb to purified soluble HA proteins from divergent subtypes of group 1 and group 2 IAVs. Each $V_{H3-48}$/ $V_{K1-12}$ lineage mAb displayed comparable binding constant ($K_D$ value) to each HA protein from different subtypes that belong to group 2 (the human infecting H3, H7 strains and the avian H4, H14 strains) and group 1 (the human infecting H1, H9 strains and the avian H6, H8 strains) viruses with $K_D$ values ranging from 0.001 to 32.5 nM (Fig. 2a).

To extend the evaluation, we directly compared the in vitro neutralization activity and breadth of $V_{H3-48}$/ $V_{K1-12}$ lineage antibodies with those of MEDI8852 and 39.29 using a diverse panel of influenza strains from group 1 and group 2 viruses. All $V_{H3-48}$/ $V_{K1-12}$ lineage antibodies neutralized the tested H3N2 (A/HongKong/01/1968 and A/Jiangxi-Donghu/312/2006) and H1N1 (A/Sichuan/01/2009 and A/California/07/2009) viruses. Most of the antibodies tested failed to neutralize the A/Shanghai/02/2013 H7N9 virus, except for MEDI8852 and 28-12, with IC50 values of 7.615 and 36.64 μg/ml, respectively. Antibody 39.29 failed to neutralize the A/duck/Hunan/8-19/2009 H4N2 virus at the highest concentration tested (50 μg/ml). 28-12 exhibited neutralizing potency against group1 H1 strains with an average IC50 values of 1.53 μg/ml, compared to values of 6.38, 3.02 and 11.02 μg/ml for 28-2, 28-4 and 28-6, respectively. They also neutralize group 2 H3 strains with average IC50 values of 3.61, 4.19, 6.72 and 2.256 μg/ml for 28-2, 28-4, 28-6 and 28-12, respectively. MEDI8852 and 39.29 were included as positive controls with comparable IC50s to H1 and H3 strains (Fig. 2b).
To further define whether the binding of the VH3-48/VK1-12 lineage antibodies to HA proteins was conformation dependent, we denatured the H3 and H1 HA proteins and performed ELISA. We found the VH3-48/VK1-12 lineage antibodies showed comparable reactivity with both untreated and denatured H3 HA. In contrast, they lost the ability to bind the denatured H1 HA, while reacting to a conformational epitope in H3 clade subtypes, but a conformational epitope in H7 clade and some group 1 subtypes, indicating a rare antibody treatment was delayed for 3 days (Fig. 3g, h). Overall, these results indicate that 28-12 could broadly protect mice against both H1N1 and H3N2 viruses in vivo.

Prophylactic and therapeutic efficacy of 28-12 in vivo. We further carried out in vivo protection studies of 28-12 in mice against lethal influenza challenges of two different strains, group 1 SC/09 H1 and group 2 HK/68 H3. In the prophylaxis assay, the mice were first administered different doses of 28-12 (30, 10, 3, or 1 mg/kg) and then challenged with a lethal dose of H1N1 and H3N2 viruses 24 h later. The administration of a lower dose of 28-12 (1 or 3 mg/kg) fully protected the mice from H1N1 (Fig. 3e, f) or H3N2 (Fig. 3a, b) infection. To evaluate the therapeutic efficacy of 28-12, we administered 28-12 (25 mg/kg) at different time points following infection with SC/09 H1 or HK/68 H3 viruses. The data showed that all the mice survived when treated with 28-12 at day 2 post infection with HK/68 H3, and 80% survival was achieved when inoculation was delayed for 3 days, with slightly reduced body weight, which recovered starting on day 3 or day 4 (Fig. 3c, d). In the challenge model of SC/09 H1, 80% or 100% of the mice survived when treated with 28-12 1 or 2 days' post infection, while only 40% survival was observed when antibody treatment was delayed for 3 days (Fig. 3g, h). Overall, these results indicate that 28-12 could broadly protect mice against both H1N1 and H3N2 viruses in vivo.

Cryo-EM structures of 28-12 Fab-HA complexes. Our biochemical and functional data suggested that VH3-48/VK1-12 lineage antibodies neutralize H3N2 mainly by targeting a continuous epitope of HA, while reacting to a conformational epitope
to H1N1 (Fig. 2c). To provide the structural basis of the unique recognition pattern, we determined the cryo-EM structures of the 28-12 Fab in complex with the trimeric HAs from HK/68 H3 and WA/11 H1 at 3.7-Å- and 3.5-Å-resolutions, respectively, with imposed C3 symmetry (Fig. 4a, b and Supplementary Figs. 4–5). To overcome the preferred orientation problem (preferred top-view orientation) associated with the 28-12-H3 complex, also seen in the H3N2 Hong Kong HA trimer (HK/68 H3)22, we adopted the stage-tilt strategy for data collection22. We then built an atomic model for the 28-12-H3 and 28-12-H1 complexes, respectively, and the model fits in the corresponding map well (Supplementary Figs. 4e, f and 5d, e).

The structural and functional basis for the unique recognition pattern of 28-12. Our two cryo-EM structures showed that the 28-12 Fab specifically recognizes the conserved HA stem region for both H3 and H1 (Fig. 4a, b). Consistently, 28-12 inhibited cell–cell fusion by blocking low-pH induced HA conformational change (Supplementary Fig. 6). We divided the amino acids that constitute the epitope of 28-12 Fab on H3 or H1 into two parts: amino acids within helix A of HA2, and amino acids within the fusion peptide and HA1 (Fig. 4c, f).

Interestingly, our structural comparison revealed that the binding pattern of 28-12 to H1 and H3 HAs is distinct to some extent. Overall, 28-12 appears to form more contacts with the fusion peptide and the HA1 residues adjacent to helix A in H1 than with those in H3 (Fig. 4c, f, m). For H1, the D19HA2 within the fusion peptide forms hydrogen bonds with S54HA2C, N56HA2C, T57HA2C, and Y112HA2C; S299HA1 forms hydrogen bond with Y108HA2C (Fig. 4d and Supplementary Table 3). Moreover, W21HA2 on the fusion peptide forms hydrophobic interactions with V109HA2C. Another two amino acids from HA1, including V40HA1 (which is a hydrophilic amino acid N40 in H3) and L300HA1, also form hydrophobic interactions with V109 and Y108 both from HCDR3 (Fig. 4d, i, j and Supplementary Table 2). In contrast, H3 contributes only two epitope residues outside helix A to interact with 28-12. Specifically, H3 D19HA2 on the fusion peptide forms hydrogen bonds with T57HA2C and Y112HA2C (Fig. 4g and Supplementary Table 3), and H3 W21HA2 on the fusion peptide forms hydrophobic interactions with V109HA2C (Fig. 4g, k, l and Supplementary Table 2).

Besides, we also calculated the atom-to-atom contacts between 28-12 and the fusion peptide/HA1 of H1 and H3, respectively, under a distance cut-off value of 4.5 Å (Fig. 4m and Supplementary Tables 4 and 5). It appears that 28-12 forms more contacts with H1 (48 contacts) than that with H3 (17 contacts). On the basis of our structural analysis, we performed further site-directed single mutation of the epitope residues in the fusion peptide and HA1 and determined the binding activity between HA and 28-12. The mutations D19HA2, W21HA2, and V40HA1 in H1 significantly decreased H1 HA reactivity to 28-12 (Fig. 4p), while the mutations D19HA2 and W21HA2 in H3 did not affect H3 HA binding activity to 28-12 (Fig. 4o). Collectively, these results indicate that the epitope in the fusion peptide and HA1 is indispensable for 28-12 to react with H1.

Furthermore, helix A is another critical element for 28-12 to recognize both H3 and H1. Our structural analysis suggested that there are epitope amino acids shared by both H3 and H1, including Q42HA2, D46HA2, N55HA2, and E57HA2 forming hydrogen bonds with HCDR3, and K39HA2 forming salt bridge with D92HA2 (Fig. 4c–h and Supplementary Table 3). In addition, we found that A36HA2, L38HA2, I45HA2, I46HA2, I56HA2, and W21HA2 form hydrophobic interactions with HCDR2 or HCDR3 (Fig. 4i–l and Supplementary Table 2). Notably, H3 has a unique epitope residue, N49HA2, which forms strong hydrogen bonds with C102, N106, Y108, and V109 on HCDR3 (Fig. 4h and
Supplementary Table 3). Overall, the atom-to-atom contact numbers between 28-12 and H1 or H3 helix A are 144 and 191, respectively (Fig. 4n and Supplementary Tables 4 and 5). It is worth noting that the H3 residue N49HA2 contributes much more contact numbers (32) with 28-12 than the substitute residues T49HA2 (10) of H1 (Fig. 4n and Supplementary Tables 4 and 5). The site-directed mutational binding assay also confirmed that N49HA2 is critical for H3 HA binding to 28-12, while T49AHA2 did not affect the binding between H1 HA and 28-12 (Fig. 4o, p). Collectively, these results indicate that the epitopes within helix A are critical for 28-12 to react with both H1 and H3, and form more interactions with H3.

Interestingly, antibody 28-12 harbors a long HCDR3 with 23 amino acids compared with many of the reported bnAbs9,12,13,23 (Supplementary Fig. 1 and 7), in line with our structural observation that HCDR3 heavily contributes to the interactions with HA proteins, which form multiple hydrogen bonds or hydrophobic interactions with antigens (Fig. 4c–n). We also compared the HCDR3 of 28-12 with 3I14 which also has a long HCDR3 (23aa) in length. For one single protomer (on the primary protomer), 3I14 HCDR3 mainly makes hydrophobic and hydrogen bonds with helix A and the fusion peptide, which is similar with 28-12 HCDR3 in recognizing H3 HA. Additionally, 28-12 HCDR3 also contacts with several HA1 residues of A/HongKong/01/1968 H3N2 (d.p.i.) and A/Sichuan/01/2009 H1N1 (d.p.i.).

Fig. 3 Prophylactic and therapeutic efficacy of 28-12 in mice. a, b Prophylactic efficacy of 28-12 against a lethal challenge with 68/HK H3. Mice were treated with 30, 10, 3 or 1 mg/kg 28-12 or PBS 24 h before intranasal inoculation with the influenza virus. The weight loss and survival data were collected daily for 14 days after inoculation (day 0). n = 5. c, d Therapeutic efficacy of 28-12 against a lethal challenge with 68/HK H3. Mice were treated with PBS buffer (at day 0) or 25 mg/kg 28-12 immediately after or 1, 2, or 3 days after intranasal inoculation with influenza virus. The weight loss and survival data were collected for 14 days after inoculation with viruses (day 0). n = 5. e, f Prophylactic efficacy of 28-12 against a lethal challenge with SC/09 H1. n = 5. g, h Therapeutic efficacy of 28-12 (25 mg/kg) against a lethal challenge with SC/09 H1. n = 5. Error bars represent the mean ± S.D (a, c, e, g). All data show a representative experiment from two independent experiments.
H1, but not H3, for the primary protomer. It seems that the HCDR3 of 28-12 is more significant than that of 3I14 to recognize HA as it contributes more amino acids to interact with H3 HA (12) and H1 HA (11), respectively, while 3I14 devotes 7 HCDR3 amino acids to interact with H3 or H6 (Supplementary Fig. 7).

We also found that the cleavage of HA0 (the activated HA) doesn’t affect the binding activity of 28-12 to this epitope on H3N2 HA, but obviously impairs the reactivity to H1N1 HA, as compared with the binding to HA0 protein (Supplementary Fig. 8). These data further indicate the distinct intrinsic properties of HA proteins from different HA subtypes within 28-12 epitope.

**N49HA2 is critical for 28-12 binding to the continuous epitope within H3 helix A.** To further confirm the critical residues composing the epitope for 28-12 binding to H3, we conducted sequence alignment of HA2 residues 36-57 between H1 and H3 and identified 6 different residues (Fig. 5a). We synthesized 6 peptides with single substitution mutations from H3 to H1 and
found that the binding ability decreased significantly when there was a mutation from N to T at position 49 of HA2 compared with the wild-type peptide. Nevertheless, other single mutations did not affect binding (Fig. 5b). This result is also consistent with our structural data that H3 N49HA2, but not H1 T49HA2, strongly interacts with HCDR3 residues Y108 and V109 of 28-12, with hydrogen bonds and more atom-to-atom contacts (Fig. 4e, h, n and Supplementary Table 3). Sequence alignment of 36-57HA2 within divergent subtypes showed that all H3 clade IAVs (H3/H4/H14) share a residue N at position 49HA2 but a residue T in other subtypes (Fig. 5c). Similarly, the VH3-48/VK1-12 lineage antibodies reacted well to the peptides 36-57HA2 of H3/H4/H14 subtypes, but not other subtypes (Fig. 5d–f). In brief, these data demonstrated that the residue N49HA2 of helix A is critical for 28-12 binding to the continuous antigenic epitope of H3, and further indicate different epitope features of HA proteins between H1 and H3 subtypes for antibody binding.

Role of somatic mutations in shaping antibody 28-12. To determine how the unmaturated common ancestor (UCA) of 28-12 evolved to achieve cross-group neutralization with distinct recognition patterns, we analyzed the contribution of somatic mutations of 28-12 carrying 8 amino acid substitutions in V\(\text{H}1\) and 9 amino acid substitutions in V\(\text{L}1\) (Fig. 6a). We first expressed 28-12 germline versions (GHGLs) and versions formed by 28-12 light chains paired with germline heavy chains (GHLs) and vice versa (HGLs). The fully germline version (GHGL) of 28-12 reacted with a panel of group 2 H3N2 isolates but failed to recognize a panel of group 1 H3N2 HA, indicating that naive B cells that generated 28-12 could recognize H3N2 and that successional somatic mutations enhanced the reactivity to H3N2 and concurrently evolved the cross-reactivity to H1N1.

Discussion

H1N1 and H3N2 IAVs circulate annually. Recently, coinfection with IAV was reported to enhance SARS-CoV-2 infectivity, thus posing another threat to public health during the COVID-19 pandemic.24–26. HA stem region-directed bnAbs provide an option for antibody-based therapeutics against novel emerging IAVs from zoonotic origin and seasonal circulating IAV strains. In addition, universal vaccine design has also been inspired by the epitopes and recognition patterns of bnAbs. Structural analysis of antibody/HA complexes enhances our understanding of cross-group heterosubtypic binding features.

In this study, we identified a lineage mAbs, 28-2, 28-4, 28-6 and 28-12 belonging to the same lineage VH3-48/DI\(\text{H}1\)2-2/H3K1-12/f1-5, which is rarely reported. In addition to VH3-48, broad neutralizing antibodies using VH1-18, VH1-69, VH6-1 and VH13-30 germline genes have also been reported, which indicates the diverse germline selection of broad-spectrum antibodies against IAVs.

Their recognition pattern is also unique, as they mainly bind a continuous epitope within H3N2 HA, but a fully conformational epitope within H1N1 HA. The antigenic determinant differences, especially the N49HA2 (H3) /T49HA2 (H1) revealed distinct antigenicity of HA proteins between H1 and H3. The epitope dependency on the distant HA1 residues and the fusion peptide in the primary sequence probably makes the epitope in H1 HA more conformational by 28-12. Another reason might be attributed to the biophysical features of VH3-48/VK1-12 lineage antibodies. The long and flexible variable regions, especially the 23 amino acids of HCDR3, might adopt different conformations to fit structurally divergent antigens. HCDR3 residues 108Y and 109V make hydrophobic interaction with L52HA2 (H3) or V52HA2 (H1). 108Y and 109V also makes hydrophobic contacts with some HA1 residues V40, S299 and L300 of H1, but not H3. 106NCDR3 forms hydrophobic bond with N53HA2 in H1, and all N53HA2, N49HA2 and E57HA2 in H3. In addition, the observations of the differential binding of 28-12 to H1 and H3 cleaved HA s also indicate the distinct intrinsic properties of HAs within this epitope. We hypothesis that cleavage of H1 HA0 may cause conformational change and destroy the integrity of epitope in H1 HA, thus...
resulting in reduced binding activity to antibody 28-12. However, cleavage of H3 HA0 may not change the overall epitope structure, therefore the cleaved H3 HA still maintains the reactivity to 28-12. Thus, the epitope feature differences among distinct IAV subtypes should be carefully evaluated in the design of HA stem-based immunogens during universal influenza vaccine development.

Comparison of 28-12 with previously reported cross-group anti-HA stem bnAbs revealed that the helix A-targeting bnAbs bind HAs with overlapping but distinct binding sites and multiple approaching angles towards the HA stem region. The epitopes of antibodies CR6261, CT149, 39.29, CR9114 and FI6v3 all span HA1 and HA2 regions on one single protomer (for the primary protomer). 3I14 only binds HA2 of the primary protomer. However, here 28-12 shows different binding dependency on HA1 epitope residues, which is important for binding to H1 (HA1 residues V40, S299 and L300), but not H3 (Supplementary Figs. 9, 11 and Fig. 4c–e). The cross-binding on HA1 and HA2 regions on one protomer probably makes the epitope more conformation dependent. Consistent with the structural analysis, both the non-HA1/HA2 spanning mAbs (for one single protomer) 28-12 and 3I14 showed binding activity to the continuous epitope of H3 peptide 36-57_HA2, but not to H1 (Supplementary Fig. 10). A critical reason for this unique binding feature might be attributed to the critical residue N49_HA2 in H3 for both 28-12 and 3I14, which is T49_HA2 in H1 (Fig. 6a). Of noting, 28-12 displayed different germline with 3I14 (VH3-30) and engaged less epitopes in the adjacent protomer (Supplementary Fig. 9). 28-12 is also unique as it has a long HCDR3 (23aa), which makes more extensive interaction with HA protein than other bnAbs. Although 3I14 also has a long HCDR3 (23aa), it seems that the HCDR3 of 28-12 is more significant than 3I14 to recognize HA as it contributes more amino acids to interact with H3N2 HA (12) and H1N1 HA (11), respectively, while 3I14 devotes 7 HCDR3 amino acids to interact with H3 and H6 HAs (Supplementary Fig. 7). Thus, 28-12 represents a unique example among the helix A targeting bnAbs. The HCDR3 of 28-12 might be developed as a potential template to produce small proteins or peptide-based antivirals as recently reported.

We quantified the contribution of 28-12 lineage in broad-reactive B cells by analyzing four reported HA specific human antibody datasets. Generally, 1.619% (17 out of 1050) cross-reactive B cells utilized V_{3-48} germline genes among the four analyzed datasets. However, antibodies using both V_{13-48} and V_{2-12} germline genes are not found in the four analyzed datasets. From B cell repertoires of healthy donors with paired heavy chain and light chain using a dataset from DeKosky et al., which contained 134,345 sequences in total from three healthy donors, B cells engaging V_{3-48} and V_{2-12} concurrently are rare with frequency ranging from 0.060% to 0.078%. We thus consider this as a special case in our study. We also found the 28-12 epitope-targeting antibodies in the serum of vaccinated human donors could be detected but exist as a relatively low frequency (Supplementary Fig. 12). The recognition pattern of the antibodies to group 1 or group 2 IAVs enables the flexibility of bnAbs against influenza A viruses: the continuous encounter of HA antigens with similar but slightly different B cell epitopes results in the in vivo evolution of V_{4-HA1}/V_{K-12} lineage antibodies. Similar to previously reported models of V_{16-1} and other lineages of bnAbs that developed from group 2-specific germline precursors, we revealed that 28-12 UCAs showed group 2 viruses (H3) tendentious and subsequently enhanced their reactivity to group 2 viruses and acquired cross-reactivity to group 1 viruses (H1) by somatic mutations. These findings also reaffirmed the possibility of inducing cross-reactive antibodies via rationally designed sequential immunization with different HA immunogens.

In summary, the human antibody 28-12 itself is of high neutralizing potency and not polyreactive, therefore could serve as a potential antiviral drug for both prophylaxis and treatment of circulating H1N1 and H3N2 IAV infection. Additionally, the unique epitope targeted by 28-12 and the paratope of 28-12, especially the HCDR3 sheds some lights on the design of potential universal vaccines and more effective antiviral drugs against IAVs.

**Methods**

**Ethics statement.** The procedures in this study involving in the isolation of HA-specific memory B cells from the volunteer and the influenza virus-related experiments were approved by the Ethics Review Committee of Institut Pasteur of Shanghai, Chinese Academy of Sciences. The animal experiments in a biosafety level 2 (BSL-2) facility were approved by Institutional Animal Care and Use...
Fig. 6 Somatic hypermutations of 28-12. a Sequence alignment of 28-12 and its UCA. Dots indicate identical residues. CDR regions according to IMGT are shown in red. b The reactivity of 28-12 germline variants to H3N2 (b) and H1N1 HAcs (c). GHGL, VH germline paired with VL germline. HGL, 28-12 VH paired with its VL germline. d Heatmap showing the EC50 values of 28-12 variants with single somatic mutations to H3N2 and H1N1 HAs. e For both 28-12-H3 and 28-12-H1 complexes, VL D92 forms hydrogen bonds with the main chain of VH S103 in HCDR3, which also contacts the residue D46HA2 through hydrogen bonds. f For both 28-12-H3 and 28-12-H1 complexes, VH D92 of 28-12 forms salt bridges with K39HA2 of both H3N2 and H1N1 HAs. The colors of HA1 and HA2 and the heavy chain and light chain of 28-12 are shown as in Fig. 4. The salt bridge and hydrogen bond are shown as red and black dashed lines, respectively. Data are presented as mean values of duplicates (b, c). Representative data are shown from two independent experiments (b, c).

Committee at Institut Pasteur of Shanghai. The collection and study of the 50 vaccinated donors’ sera were approved by the Ethical Review Committee of National Institute for Viral Disease Control and Prevention, China CDC. All manipulations were strictly conducted in compliance with animal ethics guidelines and approved protocols.

Viruses and cells. The wild-type influenza viruses A/Sichuan/1/2009 (H1N1), A/California/07/2009 (H1N1), A/duck/Hunan/8-19/2009 (H4N2), and A/Jiangxi-Donghu/312/2006 (H3N2) and the recombinant influenza viruses A/Shanghai/02/2013 (HA, NA) x A/Puerto Rico/8/34 (H7N9) and A/HongKong/01/1968 (HA, NA) x A/Puerto Rico/8/34 (H5N1) were all grown on Madin-Darby canine kidney (MDCK) cells, while A/canine/Beijing/362/2009 (H2N2) was amplified in embryonated eggs. These viruses were used in the in vitro microneutralization assay. The A/Hong Kong/01/1968 (HA, NA) x A/Puerto Rico/8/34 (H3N2) and A/Sichuan/1/2009 (H1N1) viruses were used in the in vivo assay. MDCK, HeLa and HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with 10% foetal bovine serum (FBS). CHO cells (Thermo Fisher Scientific) were cultured in ExpiCHO expression medium.

Recombinant HA proteins. The gene encoding the trimeric ectodomains of the HK/68 H3 and WA/11 H1 HA proteins with a T4 fibrin trimerization motif in the C-terminus were expressed in a Bac-to-Bac Baculovirus Expression System (Invitrogen) and used for structural study. HA proteins were purified by affinity chromatography using a His trap Excel column (GE, EDTA-resistant) and size-exclusion chromatography using a Superdex 200 10/300 column (GE Healthcare).

The following recombinant HAS expressed either from baculovirus or mammalian expression systems were all utilized for the binding affinity assay: A/Hong Kong/01/1968 (H3N2) HA (Sino Biological, cat. no. 40161-V08B), A/Netherlands/219/2003 (H7N7) HA (Sino Biological, cat. no. 11082-V08B), A/Brevig Mission/1/1918 (H1N1)
HA (Sino Biological, cat. no. 11055-V08B). Antibodies were purified from the culture supernatant. In brief, the HA head antibody 139/S (H3) or 5j8 (H1) was coated to a 1:1 ratio of HA/AB in 0.1 M carbonate buffer (pH 9.6). After washing twice, the cells were treated with anti-HA antibody 139/S (H3) or 5j8 (H1) was coated to a 1:1 ratio of HA/AB in 0.1 M carbonate buffer (pH 9.6). After washing twice, the cells were treated with anti-HA antibody (Sigma-Aldrich, cat. no. A0170), followed by incubation with tetramethylbenzidine (Beyotime, cat. P0209), and the absorbance was read at 450 nm.

To evaluate the reactivity between antibodies and peptides, peptides (0.5 mg/ml in 100 µl/well) were captured onto 96-well plates, and ELISA was performed as described above.

The antibody binding activity to untreated or denatured HA was measured as described above. The HA protein was denatured with 0.1% SDS, 50 mM DTT and a metal bath at 100 °C for 10 min.

Membrane fusion inhibition assay. HeLa cells in 12-well plates were transfected with FMG plasmids encoding the HA gene fragment of the HK/68 H3 or SC/09 H1 strain using Lipofectamine 2000 transfection reagent. Twenty-four hours after transfection, the HeLa cells were incubated with 28-12, 50% (IC50) was analyzed with GraphPad Prism 7.0. A specific CR9114 (4FQY13), CT149 (4UBD15), 39.29 (4KVN14), FI6v3 (3ZTJ16), 3I14 (3C1116), and 2F9 (4F4F16) antibodies were purified from the CHO cell supernatant. In brief, the HA head antibody 139/S (H3) or 5j8 (H1) was coated to a 1:1 ratio of HA/AB in 0.1 M carbonate buffer (pH 9.6). After washing twice, the cells were treated with anti-HA antibody (Sigma-Aldrich, cat. no. A0170) was used to detect the binding affinity, followed by incubation with tetramethylbenzidine (Beyotime, cat. P0209), and the absorbance was read at 450 nm.

To evaluate the reactivity between antibodies and peptides, peptides (0.5 mg/ml in 100 µl/well) were captured onto 96-well plates, and ELISA was performed as described above.

The antibody binding activity to untreated or denatured HA was measured as described above. The HA protein was denatured with 0.1% SDS, 50 mM DTT and a metal bath at 100 °C for 10 min.

Biolayer interferometry (BLI) analysis. To test the binding affinity between 28-12 and different HA proteins, 28-12 (15 µg/ml) was immobilized on an anti-human IgG-Fc-coated biosensor (AHCh) surface for 300 s. The baseline interference phase was then read for 180 s in kinetics buffer (KB: 1 x PBS), followed by subsequent association phase in KB for 1800 s. Antigen was then serially diluted in blocking buffer (starting from a concentration of 10 µg/ml) and were incubated in the wells of 37 °C for 2 h. The samples were washed three times, and an anti-human Fc HRP antibody (Sigma-Aldrich, cat. no. A0170) was used to detect the binding affinity, followed by incubation with tetramethylbenzidine (Beyotime, cat. P0209), and the absorbance was read at 450 nm.

To evaluate the reactivity between antibodies and peptides, peptides (0.5 mg/ml in 100 µl/well) were captured onto 96-well plates, and ELISA was performed as described above.

The antibody binding activity to untreated or denatured HA was measured as described above. The HA protein was denatured with 0.1% SDS, 50 mM DTT and a metal bath at 100 °C for 10 min.

HA conformational change inhibition assay. To activate HA0 into HA1 and HA2, HK/68 H3 HA-histidine protein (Sino Biological) was mixed with trypsin in PBS buffer at room temperature for 2 h. Trypsin activity was stopped by adding aprotinin (Sigma, cat. A1153). The above mixture was divided into 3 groups and incubated with 28-12, the anti-HCV antibody 8D6, or no antibody in PBS buffer at 37 °C for 2 h. Samples were treated with PBS or buffer with lower pH (100 mM sodium acetate, 1% n-dodecyl β-D-maltoside (Sigma-Aldrich, cat. no. D4641), pH = 4.8) at 37 °C for 1 h through a buffer exchange using Sephacryl S-200 Spin Desalting Columns (Thermo Fisher Scientific, cat. no. 89882). Samples were neutralized with Tris-HCl (pH 9.0) or PBS to a pH of 8.0. The above samples were mixed with 0.0025 mg trypsin individually and incubated at room temperature for 1 h. Samples were then run on a 10% SDS-polyacrylamide gel electrophoresis gel under nonreducing conditions and blotted using an HRP-conjugated 6x His mouse monoclonal antibody (Proteintech, Cat No. 11692-04, 1:1000 dilution) or no antibody at 37 °C for 1 h. The supernatants were removed, and the cells were treated with trypsin (2.5 mg/ml) for 10 min at 37 °C to cleave HA0 into HA1 and HA2. After washing twice, the cells were incubated with 28-12, 8D6 or no antibody at 37 °C for 1 h. Afterwards, the supernatants were removed, and the cells were treated with citric acid (pH 4.8) at 37 °C for 10 min. The acidic medium was replaced with DMEM supplemented with 10% FBS, and the cells were incubated for 3 h, followed by 4% polyoxymethylene fixation and staining with 0.5% crystal violet. Synctium formation was observed with the Olympus IX73 inverted microscope and the images were collected by Olympus cellSens Entry software.

HA mutagenesis and binding measurements. The DNA sequences encoding the extracellular domain of HA proteins of HK/68 H3 or WA/11 H1 were fused to a C-terminal RX His tag. Site-directed mutagenesis was performed with a commercialized KOD-Plus mutagenesis kit (TOYOBO). The plasmids were transfected into CHO cells in 12-well plates. The supernatants were harvested 96 h after transfection. ELISA was performed to measure expression of individual HA proteins in the cell supernatant. In brief, the HA head antibody 139/S (H3) or 5j8 (H1) was coated onto a plate, and 100, 500, 1000, 2000, and 5000-fold dilutions of cell supernatant were then added and captured by the anti-6 X His antibody. Serially diluted purified HA-8 X His was used as a standard, followed by detection with an HRP-conjugated mouse anti-His antibody (Proteintech). The concentration of the sample was calculated according to a standard curve. Another ELISA was performed to analyze the relative binding activities between these HA mutants and 28-12. HA mutants at 200 ng/ml were incubated with plates precoated with 28-12, followed by detection with an HRP-conjugated mouse anti-6 X His antibody. The binding activity of the mutants to 28-12 was compared to that of wild-type HA.

Preparation of 28-12 Fab. Papain (Sigma-Aldrich, cat. no. P4762) was dissolved in PBS with 20 mM L-cysteine to activate papain. Antibody 28-12 was digested with papain at an antibody-to-papain ratio of 200:1 at 37 °C for 6 h. Protease activity was stopped by iodoacetamide (Sigma-Aldrich, cat. no. I1625). 28-12 was purified using Protein A to remove the Fc region and undigested IgG antibody, followed by 2–3 rounds of dialysis using PBS at 4 °C for 6 h. Size-exclusion gel filtration chromatography was performed to further purify 28-12 Fab.

Cloning, expression and purification of antibodies. The amino acid sequences of heavy and light chain variable regions of MDIE885 (5W4A), CR8020 (5D3Y), CR9114 (4FYI13), CT149 (4UBD15), 39.29 (4KVN14), F6w3 (3ZTJ16), 314 (6W6G16), S139/144 (4GMS) and 58J (GenBank: JF971168) and JF971169 were downloaded from PDB or NCBI GenBank, codon optimized in mammalian cells and synthesized by Shanghai GeneRay Biotech. The cloning of the IgG1 Fc and VH chain expression plasmids was previously described36. The plasmids encoding the Fc and VH chain expression plasmids was previously described36. The optical density at 50 nm (OD540) was recorded with a plate reader (Bio-Tek Epoch 2), and the data were analyzed with GraphPad Prism 7.0 software.

Protective efficacy of 28-12 in mice. The animal experiments described in this study were approved and in compliance with the BSL-2 laboratory guidelines of the PubMed Communications. The HA protein was denatured with 0.1% SDS, 50 mM DTT and a metal bath at 100 °C for 10 min.
Preparation of 28-12 Fab/trimeric HA complexes. Trimeric HK/68 H3 and WA/11 H1 HAs were expressed in the insect baculovirus expression system, while 28-12 was expressed in the E. coli expression system previously. Fab 28-12 was mixed with trimeric HA at a Fab-to-monomer HA molar ratio of 2:1 and incubated at 4°C overnight for complex formation. The Fab-trimeric HA complex was purified by size-exclusion gel filtration chromatography in elution buffer (20 mM Hepes, 50 mM NaCl, pH 8.0), followed by concentrating the complex to 5–6 mg/ml.

Cyto-EM sample preparation and data acquisition. To prepare vitrified samples, the purified 28-12-H1 or 28-12-H3 complex was diluted to 0.5 mg/ml, and a 2 µl aliquot of sample was applied to a plasma-treated holey carbon grid (Quantifoil, R1/2/1.3, 200 mesh). The grid was blotted with a Vitrobot Mark IV (Thermo Fisher Scientific) and then plunged into liquid ethane cooled by liquid nitrogen.

For the 28-12-H3 dataset, 240,827 and 180,686 particles remained after 2D classification, with particles coordinating the tilt relationship between the 28-12-H1 and 28-12-H3 trimer features remaining for further processing. After re-classification, the map obtained was 3.7-Å-resolution 28-12-H3 map. For this and the following 28-12-H1 cryo-EM maps, the post-process was carried out by DeepEMHacer42, and the resolution in CryoSPARC using uniform refinement was estimated to determine their defocus, and these particles were re-extracted with correction-defocus41. Then we combined the particles together and performed one round of refinement. After that, we re-extracted the particles coordinating the refinement result to recenter the particles. We then performed 3D classification, and a class of 191,540 particles with better structural features was selected. After refinement, CTF refinement and Bayesian polishing, the map was refined to 4.1-Å-resolution with imposed C3 symmetry. We then carried out 3D classification, generating a better class with 105,383 particles. After refinement, we obtained a 7.6 s, leading to a total accumulated dose of 38 e/Å2 for the specimen.

Cyto-EM data processing and 3D reconstruction. Single-particle analysis was mainly executed in Relion19 unless otherwise specified. All images were aligned and summed using MotionCor2 software39. After CTF parameter determination using CTFFIND4, particle autopicking, manual particle checking, and reference-free classification, particles with 28-12-H3 trimer features remained for further processing. The sequences used in Fig.5 for analysis were: H3 A/HongKong/01/1968 (UniProtKB/Swiss-Prot: Q91MA7.1), H7 A/Netherlands/219/2003 (GenBank: AAR26040.2), H4/ A/swine-ontario/01911-1/1999 (GenBank: AAG17429.1), H14 A/Alaskan/263/1982 (UniProtKB/Swiss-Prot: P26136.1), H15 A/duck/AUS/341/1983 (GenBank: AB088132.1), H1 A/California/962008 (GenBank: ACP1105.1) and H10 A/TangGu-Donghu/346/2013 (GISAID Accession:EP149747, https://platform.epicov.org/epi3#frontend#142009). Source data are provided with this paper.

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Affinity depletion of anti-H3 peptide 36-57_HA1 antibodies. The collection and study of the 30 vaccinated donors sera were approved by the Ethical Review Committee of National Institute for Vital Disease Control and Prevention, China CDC. The informed consent was obtained by the participants. Selected synthetic biotinylated H3 peptide 36-57_HA1 (Genescript) were added at 150 ng/well to streptavidin-coated plates and incubated at room temperature for 1 h in PBS containing 0.1% Tween-20. The sera were added into the plates and incubated for 20 min at RT for absorption. The unbound antibodies were collected after 24 h by washing the plates with PBS. The results were expressed in the ELISA expression system, as described previously. For ELISA-based detection of polyreactivity, plates were coated with poly IC (15 mg/ml) (Life Technologies), LPS (15 mg/ml) (Sigma), or recombinant human insulin (10 mg/ml) (Sigma Biological) in carbonate buffer. Plates were blocked with PBS with 0.5% Tween. Antibodies were serially diluted and incubated on the plate for 1 h at 37°C followed by detection with goat anti-human IgG Fc at 1:8000. Absorbances were measured at OD450. An influenza antibody CR914 and an anti-dsDNA antibody CR92 was used as a positive control. For immunofluorescence-based detection of polyreactivity, Hep-2 cells were plated the day before experiment. Hep-2 cells were fixed with 4% PFA for 10 min followed by permeabilization with 0.3% Triton X-100 in PBS for 15 min. Biotinylated antibodies were incubated with cells for 1 h at the concentration of 50, 10 and 2 µg/ml, respectively and a FITC-linked streptavidin was then incubated with the cells for 1 h. The immunofluorescence was read and analyzed by Operetta (PerkinElmer).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data presented in this study are available within the figures and in the Supplementary Information. Cyto-EM maps for the H3-28-12 complex and H1-28-12 complex have been deposited at the Electron Microscopy Data Bank with accession codes EMD-33023, and EDL-33024, respectively. Associated atomic models have been deposited in the Protein Data Bank with accession codes 7X6L and 7X6O for H3-28-12 and H1-28-12, respectively. The sequences used in Fig. 5 for analysis were: H3 A/HongKong/01/1968 (UniProtKB/Swiss-Prot: Q91MA7.1), H7 A/Netherlands/219/2003 (GenBank: AAR26040.2), H4/ A/swine-ontario/01911-1/1999 (GenBank: AAG17429.1), H14 A/Alaskan/263/1982 (UniProtKB/Swiss-Prot: P26136.1), H15 A/duck/AUS/341/1983 (GenBank: AB088132.1), H1 A/California/962008 (GenBank: ACP1105.1) and H10 A/TangGu-Donghu/346/2013 (GISAID Accession:EP149747, https://platform.epicov.org/epi3#frontend#142009). Source data are provided with this paper.

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Author contributions

B.S., Y.C., D.Y.W. and Z.Y.L. initiated and coordinated the research and participated in the experimental design and discussion. X.Y.S., C.X.L., Z.Y.L. and C.Y.Y. designed and carried out all the experiments. C.X.L. and F.F.W. performed the NS-EM. C.X.L. collected cryo-EM data, performed the cryo-EM reconstructions and the model building with the involvement of M.L.J. Z.Z., W.W.S. and J.P.D. performed the trimeric HA protein expression. S.F.C., L.L., Z.W., S.L., L.Y.M. and Y.G.Z. participated in the antibody- and virus- associated experiments. X.P.D., Z.K.Q. Z.Y. and S.B.T. provided valuable suggestions. B.S., Y.C., Y.S.Y., C.X.L. and Z.Y.L. wrote the manuscript and all authors contributed to the preparation of the manuscript.

Competing interests

B.S., X.S., X.L.L. and Z.L. are inventors on the patent application of antibody 28-12. The other authors declare no competing interests.

Additional information

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