International Immunology, Vol. 32, No. 9, pp. 613–621
doi:10.1093/intimm/dxaa038
Advance Access publication 6 June 2020

SHORT COMMUNICATION

Stereotyped B-cell response that counteracts antigenic variation of influenza viruses

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Received 7 April 2020, *editorial decision* 1 June 2020, *accepted* 1 June 2020

Influenza A subtypes are categorized into group 1 and group 2 based on the hemagglutinin (HA) sequence. Owing to the phylogenetic distance of HAs in different groups, antibodies that bind multiple HA subtypes across different groups are extremely rare. In this study, we demonstrated that an immunization with acid-treated HA antigen elicits germinal center (GC) B cells that bind multiple HA subtypes in both group 1 and group 2. The cross-group GC B cells utilized mostly one V_H gene (1S56) and exhibited a sign of clonal evolution within GCs. The 1S56-lineage IgGs derived from GC B cells were able to bind to HA protein on the infected cell surface but not to the native form of HA protein, suggesting the cryptic nature of the 1S56 epitope and its exposure in infected cells. Finally, the 1S56-lineage IgGs provided protection against lethal infection in an Fc-dependent manner, independent of the virus-neutralizing activity. Thus, we identified 1S56-lineage antibodies as a unique stereotype for achieving cross-group influenza specificity. The antigens exposing the 1S56 epitope may be good candidates for broadly protective immunogens.

Keywords: germinal center, vaccine, non-neutralizing antibodies, cross-group, Fc-dependent

Introduction

Influenza A virus (IAV) has evolutionally acquired multiple strategies to evade antibody surveillance, one of which is to prepare antigenic variations of the major envelope hemagglutinin (HA) protein. On the basis of the similarity of HA sequence, IAV strains are categorized into 18 subtypes, which are further subdivided into two phylogenetically distant groups (group 1: H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18; group 2: H3, H4, H7, H10, H14 and H15) (1). Antibodies against IAV strains from one subtype infrequently cross-react with those from other subtypes owing to a high degree of variations in antibody epitopes. The membrane distal region of the HA, known as globular head, harbors highly antigenic epitopes but hyper-variability in the regions mitigates broad reactivity of antibodies across virus strains in different HA subtypes. There are, however, regions that are conserved across divergent virus strains within the same subtype or among different subtypes. Thus, humoral immunity can counteract the antigenically divergent IAV by generating broadly reactive antibodies against conserved HA epitopes (2).

Multiple classes of conserved HA epitopes have been identified as targets of broadly protective antibodies in humans and mice. Two classes of epitopes are present on the surface of native HA trimers—conformational epitopes of the stem region [conformational stem (CS)] (3–7) and receptor-binding site (RBS) epitopes of the head region (8–10). The antibodies to these epitopes prevent the virus replication process and exhibit broad neutralization activity against multiple strains. However, most broadly neutralizing antibodies (bnAbs) show limited breadth within group 1 or 2, and only few antibodies isolated to date exhibit cross-group specificity. Notably, many human CS antibodies acquire intra-group 1 breadth through preferential usage of a stereotyped V_H gene, V_H1-69, that exists in multiple donors (11, 12).

Two additional classes of conserved HA epitopes with distinct structural and functional features have been recently elucidated. The epitopes, denoted as head interface (13–15) and long alpha helix (LAH) epitopes (16–18), are buried within the native form of HA trimers. These conserved epitopes are

poorly antigenic, owing to steric hindrance (15, 16), and therefore need to be exposed externally to elicit specific antibodies in sufficient amounts. Antibodies to these concealed epitopes have no virus-neutralizing activity *in vitro*, but they have been found to confer protection in mice challenged with lethal doses of the viruses (13, 15, 16). These antibodies are effective against a broader spectrum of the virus strains that cause severe diseases in humans, such as H1 and H5 in group 1 and H3 and H7 in group 2 (15, 16). Moreover, the antibodies to these cryptic epitopes confer cross-group protection in an IgG Fc-dependent manner (15). Collectively, these data highlight the antibodies to such cryptic epitopes as promising targets of broadly protective influenza vaccines.

One strategy to expose the cryptic epitopes is to convert native HA into post-fusion HA that is normally generated under endosomal acidification during virus replication process (16, 19). Indeed, an immunization with acid-treated HA antigens accelerated B-cell selection towards LAH epitope and elicited IgG antibodies to the epitopes more abundantly than untreated HA antigens (16). As a result, the acid-treated HA immunogens enhanced cross-protective ability in mice against mutant virus infection within the same H3 subtype. Here, we show that the same HA immunogens induce germinal center (GC) B cells with unique cross-group reactivity that differs from the reactivity of known antibodies. Further, the cross-group reactivity is achieved by a stereotyped B-cell receptor (BCR) with mostly one V_H gene. Finding the unique cross-group antibodies further strengthens the strategy of using HA immunogens that expose cryptic but conserved epitopes for eliciting broadly protective antibodies.

Methods

Mice and viruses

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All mice were maintained under specific pathogen-free conditions and used at ages of 7–12 weeks. All animal experiments were approved by Animal Ethics Committee of the National Institute of Infectious Diseases, Japan.

The X31 (H3N2) viruses were propagated on embryonated chicken eggs and purified through a 10–50% sucrose gradient as previously described (20). HA-split antigens were prepared as previously described (16). In brief, purified live viruses were suspended in 0.1% Tween 80 and mixed with ether to isolate the HA-split vaccine, and then some of the split vaccines were fixed with formalin. To convert into the post-fusion form, HA-split antigens were treated with acidic buffer (pH5.0) before fixation.

Preparation of recombinant HA proteins

Recombinant HAs (rHAs) of X31 (H3N2), A/Uruguay/716/07 (Uruguay, H3N2), A/Narita/1/2009 (Narita, H1N1pdm09), NIBRG14 (H5N1) or A/Anhui/1/2013 (Anhui, H7N9) strain lacking C-terminal transmembrane regions were produced in a baculovirus expression system (Takara Bio Inc., Shiga, Japan) and purified as previously described (21).

For the rHA panel, 18 subtypes of trimeric HA proteins, which contain the extracellular domain of HA that is C-terminally fused to the thrombin site, the trimeric Foldon of T4 fibritin and a Strep-tag II plus a His tag, were produced in the Expi293 Expression System (ThermoFisher), according to the manufacturer's instructions and purified as previously described with some minor modification (22). In brief, 7 days post-transfection, the medium was clarified by centrifugation at 1200 × *g*, filtered and purified on a HisTrap excel column (GE Healthcare Bio-Sciences). The purified HA proteins were concentrated using Amicon Ultracell (Merck) centrifugation units with a cut-off of 30 kDa, and the buffer was changed to phosphate-buffered saline (PBS) (pH 7.4). The strains used in this study were as follows: A/South Carolina/1/1918 (H1N1), A/Japan/305/57 (H2N2), A/Hong Kong/1/68 (H3N2), A/duck/Czechoslovakia/1956 (H4N6), A/Laos/JP127/2004 (H5N1) A/mallard/Sweden/81/2002 (H6N1), A/Anhui/1/2013(H7N9), A/mallard/Sweden/24/2002 (H8N4), A/guinea fowl/Hong Kong/WF10/99 (H9N2), A/mallard/Interior Alaska/10BM01929R0/2010 (H10N7), A/northern shoveler/Netherlands/18/1999 (H11N9), A/mallard/Interior Alaska/7MP0167/2007 (H12N5), A/black-headed gull/Sweden/1/1999 (H13N6), A/mallard/Astrakhan/263/1982 (H14N5), A/shearwater/Australia/2576/1979 (H15N9), A/black-headed_gull/Sweden/5/99 (H16N3), A/little yellow-shouldered bat/Guatemala/060/2010 (H17N10) and A/flat-faced bat/Peru/033/2010 (H18N11).

Enzyme-linked immune sorbent assay

To assess binding breadth or determine avidity indices (AvIn) of cultured B cells, enzyme-linked immune sorbent assay (ELISA) plates (Nunc-Immuno plates Maxisorp, ThermoFisher) were coated with anti-mouse IgG (Fab specific) antibody (Sigma-Aldrich, Cat#: M6898, 5 μg ml^{-1}), Uruguay rHA, Narita rHA, NIBRG14 rHA, Anhui rHA or inactivated X31 HA-split vaccine. To determine the relative avidity to native or acid-treated HA antigens, non-fixed HA-split vaccine was coated on the ELISA plates, and then treated with/without acidic buffer (pH 5.0) for 30 min as previously described (16). After blocking with PBS containing 1% bovine serum albumin (Sigma-Aldrich), culture supernatants were applied to plates at a 1:10, 1:50, 1:100 or 1:500 dilution, and then incubated with goat anti-mouse IgG-horseradish peroxidase (1:5000, SouthernBiotech, Cat#: 1030-05). HRP activity was visualized with *o*-phenylenediamine dihydrochloride substrate (Sigma), and measured at 490 nm using an iMark Microplate Reader (Bio-Rad). To determine the binding of total IgG and antigen-specific IgG, the threshold optical density was set at the point representing the average plus 6 SDs from negative control wells without sorted B cells. To obtain the AvIn, the concentrations of total IgG and rHA-binding IgG or HA-split-binding IgG were determined with reference to the standard monoclonal antibodies (mAbs). The AvIn for HA-split vaccines were obtained by calculating the ratios of antigen-specific IgG per total IgG of each sample. For the control, the fold change of five CS binding antibodies (FI6, MEDI8852, 16.a.26, 31.a.83 and 56.a.09) was also determined (3, 6, 23).

For the assessment of binding breadth of constructed mAbs, a panel of 18 rHA trimers was coated on ELISA plates. The mAbs and secondary antibodies were diluted in Solution

1 (1 μg ml^{-1}) and Solution 2 (1:5000) of Can Get Signal (TOYOBO, Osaka, Japan), respectively. Area under the curve (AUC) was calculated using Prism (GraphPad).

To validate the binding of LALA-PG mutant mAbs, X31 rHA was coated and serially diluted mAbs (starting from 1 μg ml^{-1}) were applied to plates.

BCR sequence analysis

The rearranged V(D)J sequence of murine B cells was analyzed as follows. Total RNA was extracted from frozen cells after single-cell cultures using RNeasy Plus Micro Kits (Qiagen) and subjected to reverse transcription using SuperScript III CellsDirect cDNA Synthesis kits (Invitrogen) and random hexamer (Invitrogen) for cDNA synthesis. V_H and V_L genes were amplified by two rounds of nested PCR with established primers and methods (24). The V(D)J rearrangement and the number of somatic hypermutations were identified using IgBlast IMGT. Some clones sharing identical V_H/J_H gene and HCDR3 length with over 75% of identity in nucleotide sequence were clustered. Those with different Vκ/Jκ genes were excluded. Clonal lineage trees were inferred by GCtree (25) with the '-bootstrap = 100' option, using the unmutated V gene sequence of the V(D)J clonal rearrangement for outgroup rooting.

Generation of mAbs

Chimeric mAbs with mouse V(D)J and mouse IgG2c constant region were generated as described before (24). In some mAbs, LALA-PG-mutations were introduced in IgG2c (26). In brief, the V_H/V_L genes of selected B cells from single-cell cultures were cloned into mouse IgG2c expression vectors with or without LALA-PG-mutation and then subsequently produced by transfected Expi293F cells (ThermoFisher, Cat#: A14527). IgGs were purified from the culture supernatant using a protein G column (ThermoFisher) and the buffer was changed to PBS before further analysis.

Cell surface HA staining of HA-expressing EL4 cell lines

EL4 cell lines expressing HA of X31 were propagated and the cell surface HA was stained as previously described with some minor modification (15). In brief, control or EL4-expressing cells were diluted to 1 × 10^6 cells ml^{-1} with Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 2% fetal bovine serum (Biowest), 2 mM -glutamine, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 55 μM 2-mercaptoethanol (Gibco). Then, sample mAbs were added at 5 μg ml^{-1}, incubated for 30 min and detected with 1:500 diluted anti-mouse IgG allophycocyanin (APC, BioLegend, Cat#: 405308) using a FACS CantoII (BD Biosciences).

Preparation of virus-infected MDCK cell lines and cell surface HA staining

The virus-infected MDCK cell line was prepared as previously described with some minor modification (27). MDCK cells (Influenza Virus Research Center, National Institute of Infectious Diseases, Japan) were plated in a 12-well plate (3 × 10^5 cells per well) overnight and infected with X31 or A/Anhui/01/2005 (H5N1)-PR8-IBCDC-RG5 (the United States Centers for Disease Control and Prevention) strains at a multiplicity of infection of 0.1 for 42 h in 34°C incubator. Then, cells were collected by gentle pipetting and fixed with PBS containing 4% formalin. Sample mAbs were added at 1 μg ml^{-1}, incubated for 30 min and detected with 1:500 diluted anti-mouse IgG APC using FACS CantoII (BD Biosciences).

In vitro neutralization assay

Virus-neutralization antibody titers were determined by microneutralization assays using MDCK cell lines. Serially diluted mAbs were pre-incubated with X31 (100 median tissue culture infectious dose, TCID50), and then added to MDCK cells in the presence of 5 μg ml^{-1} acetyltrypsin (Sigma). After 3 days of incubation, the virus-neutralization antibody titer was determined and expressed as the minimal antibody concentrations that inhibit viral replication.

In vivo protection assay

C57BL/6 mice were intraperitoneally (i.p.) treated with 10 mg kg^{-1} body weight of either IgG2c wild-type or LALA-PG-mutant mAbs. More than 3 h later, they were anesthetized by i.p. injection with the mixture of 3% medetomidine hydrochloride (Domitor) (Zenoaq, Fukushima, Japan), 10% butorphanol tartrate (Vetorphale) (Meiji Seika Parma Co., Tokyo, Japan) and 8% midazolam (Dormicum) (Astellas Pharma Inc., Tokyo, Japan) in a volume of 200 μl. The mice were intranasally (i.n.) infected with X31 virus at a dose of 5 × median lethal dose (LD50) in a volume of 50 μl, followed by i.p. injection of 100 μl antagonist containing 2.4% atipamezole hydrochloride (Antisedan) (Zenoaq). All mice were monitored daily for survival and body weight loss during 2 wk after infection. The humane endpoint was set at 25% body weight loss relative to the initial body weight at the time of infection.

Statistics

Statistical analyses were performed with a two-tailed Mann–Whitney test or unpaired Welch's *t*-test. Statistical significance between the body weight loss was determined by two-way analysis of variance (ANOVA). Statistical significance between the survival rates was determined by Kaplan–Meier survival curves and log-rank test. All statistical analyses were performed using Prism (GraphPad). *P*-values <0.05 were considered significant and indicated by asterisks: ***$P < 0.001$, ****$P < 0.0001$.

Results and discussion

We previously observed that systemic immunization with acid-treated HA-split antigens (denoted as post-fusion-like HA antigens hereafter) elicits cross-reactive GC B cells in secondary lymphoid organs, as is the case for GC B cells in lung tissues following influenza virus infection (16, 28). The cross-reactive GC B cells were induced by post-fusion-like HA immunogen from the X31 strain (H3N2 expressing HA from the 1968 isolate), and were then detected using two H3 HA antigens, derived from the same X31 strain and drifted A/Uruguay/716/07 (H3N2 isolated in 2007) strain. A single-cell culture approach revealed that 51.7% (165/319 clones) of the cross-reactive GC B-cell clones produced IgGs that

bound a synthesized peptide encompassing the LAH in the HA C-terminal region (16, 17). About 4% of GC B-cell clones bound to the head-only H3 HA protein that exposes the RBS and head interface epitopes (8, 15). Further, about 8% of GC B-cell clones recognized CS epitopes under competitive ELISA using competitor CS IgG (MEDI8852 clone) (6). Therefore, the remaining cross-reactive GC B-cell clones (36.4%, 116/319) were not mapped to known epitopes in our previous screen and remained negative for all parameters tested (16).

In this study, the binding breadth of the unmapped B-cell clones was evaluated by ELISA using rHAs from four HA subtypes causing human infection (Fig. 1A). The majority of monoclonal IgGs from GC B-cell clones failed to cross-react with group 1 H1 and group 2 H7, but about half of them cross-reacted with H3 and H5 HAs in group 2 and group 1, respectively. There are at least four classes of epitopes for broadly protective antibodies: CS epitopes, RBS epitopes, head interface epitopes of the head region (3–10, 13–15) and LAH epitopes of the stem region (16–18). The binding property of H3 and H5-binding IgGs is quite different from those provided by four classes of broadly protective IgGs, suggesting that H3 and H5-binding IgGs target unique epitopes. Comparative analysis on the AvIn revealed a 2-fold increase in the antibody binding to post-fusion-like HA compared to native HA (Fig. 1B). Thus, these results support that

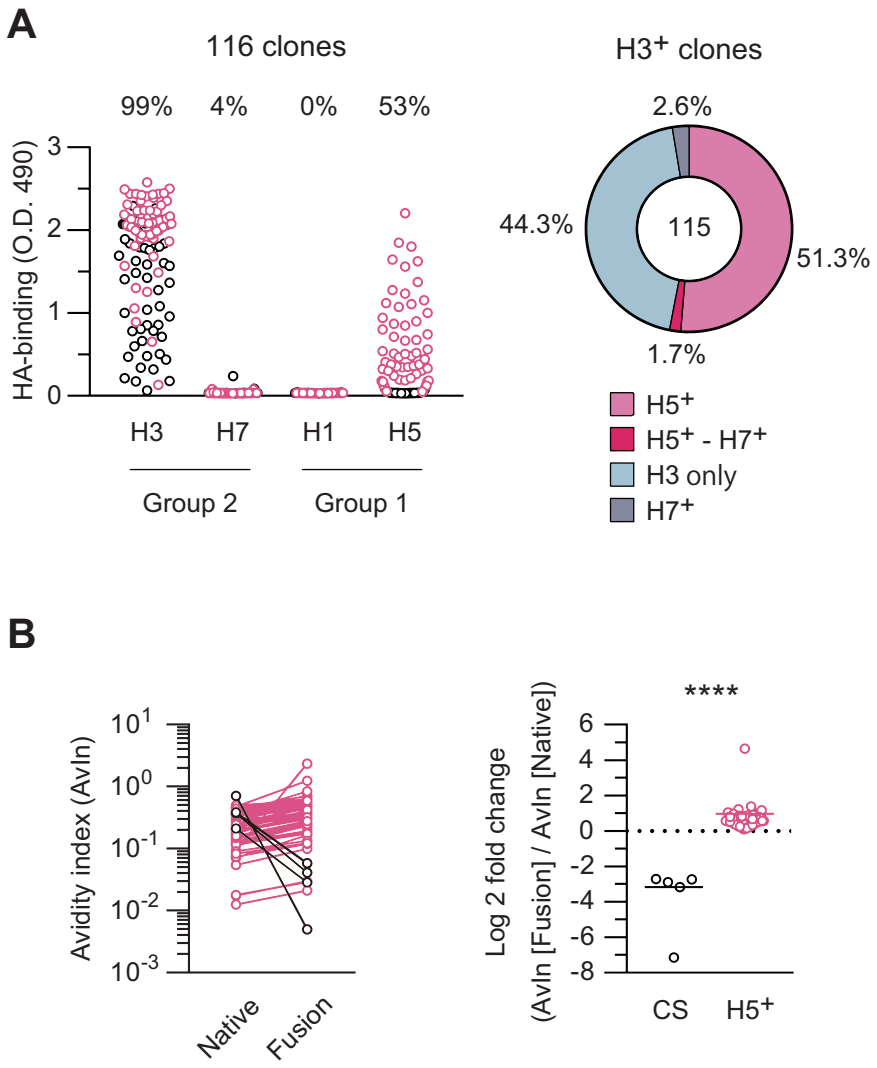

Fig. 1. Induction of cross-group GC B cells by post-fusion-like HA immunization. Culture supernatants of cross-reactive GC B cells were recovered from single-cell cultures. (A) IgG binding against four subtypes of HA causing human infection. Binding ability was measured by ELISA using HA subtypes H3 (A/Uruguay/716/07) and H7 (A/Anhui/1/2013), and H1 (A/Narita/1/2009) and H5 (NIBRG14, containing the HA gene from A/Vietnam/1194/2004). The percentages of positive clones against each HA subtype (left) and ratios of $H5^+H7^-$, $H5^+H7^+$, $H5^-H7^-$, and $H5^-H7^+$ (right) are shown. Each circle represents the result from an individual clone and H5-positive or -negative clones are colored in red or black, respectively (left). (B) AvIn toward native and post-fusion-like HA antigens were determined from supernatants of H3–H5 cross-reactive clones or five mAbs targeting the CS epitope as control. The CS antibody set was the same as in previous work (16). The strain-specific 1E11 clone was used as reference. Fold change was calculated by AvIn and comparably plotted. Each dot represents the result from an individual mAb clone or H3–H5 double-positive clone. The *P*-value was determined with the two-tailed Mann–Whitney test. ****$P < 0.0001$. (A and B) H3–H5 double-positive clones are colored in red.

the epitopes targeted by H3 and H5 cross-group epitopes are partially cryptic under native trimeric HA but are exposed in post-fusion-like HA for accelerating B-cell recognition and activation. It is important to mention that the antibodies are still accessible to the cryptic epitopes in rHAs (Fig. 1A) and HA-split antigens (Fig. 1B). Therefore, the partial crypticity of the epitope does not completely prevent antibody binding in these HA antigens that are coated on ELISA plates.

Broadly reactive IgGs in humans and mice sometimes utilize a limited V_H gene template for acquiring the breadth. For example, human CS IgGs with intra-group reactivity frequently utilize V_H1-69 as a stereotyped V_H gene (11, 12). Murine head interface IgGs also utilize V_H5-9-1 as one of the representative V_H genes (13). To dissect the clonal lineages of H3 and H5 cross-group IgGs, we determined V_H/V_L gene sequences from cross-group GC B-cell clones from two independent sets of experiments (Fig. 2A). The majority of B-cell clones in both the sets reproducibly utilized a single V_H gene, 1S56, and the remaining clones utilized closely related V_H genes (1S19 and 1S50). The GC B-cell clones in the 1S56 lineage accumulated somatic hypermutations at levels comparable to other V_H genes from infection-induced GC B cells (Fig. 2B) (28). Notably, we found multiple clones sharing the same complementary determining region 3 (CDR3), thus permitting the creation of clonal trees with a sign of B-cell evolution within the GC (Fig. 2C). Collectively, the post-fusion-like HA immunogens recruited B cells with the limited 1S56 lineage into the GC reaction and drove the clonal evolution to the epitopes that are conserved between H3 and H5 subtypes.

Next, three recombinant mAbs (13A1, 31E4 and 34A8) from the 1S56 lineage were created for further analysis. We first assessed the cross-reactivity of 1S56-lineage mAbs as well as that of control LAH (mLAH1 and F2) and CS (MEDI8852) mAbs to a panel of 18 rHA proteins (Fig. 3A). Consistent with Fig. 1, we found three 1S56-lineage mAbs bound to H3 and H5, but not to H1 and H7 (Fig. 3A). Whole HA panel analysis further revealed the broader reactivity of 1S56-lineage antibodies to total eight HA subtypes (H2, H5, H6, H8 and H18 in group 1, and H3, H4 and H14 in group 2) (Fig. 3A). It should be stressed again that the cross-group reactivity in the 1S56 lineage differed from that observed in broadly protective antibodies with known epitopes (7, 10, 13, 16–18, 29, 30). The unique breadth, in addition to the previous exclusion from four known classes of broadly reactive IgGs, strengthens the 1S56-lineage IgG as a novel class of cross-group IgG (16), though the detailed structure of 1S56 epitopes remains to be determined.

The 1S56 epitope is concealed from antibody access in native HA trimers that exist on the virus particle (Fig. 1B). Therefore, we speculated that the 1S56 epitope might be exposed externally for antibody recognition during the virus replication process. We addressed this issue by analyzing the antibody binding to virus-infected cells. We initially prepared EL4 cell lines expressing H3 HA antigens on their surface as previously reported (15). The HA antigens on EL4 cell lines were brightly labeled by two classes of IgGs (1E11 and MEDI8852) that bind neutralizing epitopes on native HA trimers (Fig. 3B) (6, 16); however, IgGs against the LAH epitope (mLAH1) (16) and 1S56 epitope failed to bind native HA trimers on the surface of EL4 cell lines. Poor binding by these IgGs confirmed the cryptic nature of LAH and 1S56 epitopes on the cell surface of native HA trimers. Moreover, the extent of crypticity appears to be promoted in HA antigens expressed on the cell surface compared to plate-bound HA antigens by unknown reasons (Fig. 1B). The antibody accessibility was further assessed under the similar conditions, except the usage of X31-infected MDCK cell lines. In stark contrast to HA-expressing EL4 cell lines, the infected cells were brightly labeled by IgGs to cryptic epitopes at levels similar to MEDI8852 and slightly lower than 1E11 (Fig. 3C), except by F2 mAb that is non-reactive to H3 HAs (Fig. 3A). Moreover, H5N1-infected cells were also labeled by 1S56-lineage IgGs (Fig. 3D), confirming the cross-group reactivity to HA antigens on infected cells. Thus, the cryptic epitopes are indeed exposed on the infected cells to allow the cross-group recognition by 1S56-lineage IgGs.

Binding of IgG to the infected cells has been suggested to confer protection via Fc-dependent pathways (independent of virus-neutralizing activity), including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) or complement-dependent cytotoxicity (CDC) (1). Next, we examined the virus-neutralizing activity using three clones of 1S56-lineage IgG2c (Fig. 4A). The IgG2c subclass was chosen as the high affinity IgG subclass for activating Fc gamma receptor (FcγR) (31). The control MEDI8852 neutralized H3N2 virus at 2.29 μg ml^{-1}, but all three 1S56 mAbs failed to neutralize viruses up to 50 μg ml^{-1}. To further explore the protective function of 1S56-lineage IgGs and their Fc dependence, we generated recombinant IgG2c with the LALA-PG mutation, which removes the binding capacity of IgG2c to both FcγR and complement (26). Then, we compared the protective ability of Fc mutants with that of wild-type IgG2c Fc. First, we confirmed the comparable HA binding between normal and mutant IgG2c by ELISA (Fig. 4B), meaning that the LALA-PG mutation did not affect HA binding affinities. All three 1S56-lineage mAbs with or without Fc mutations were infused into mice for monitoring the protective ability against lethal H3N2 virus. We observed significant improvement in body weight loss and survival of infected mice following antibody infusion, indicating the protective role of 1S56-lineage IgGs despite the absence of virus-neutralization activity (Fig. 4A and C). In contrast, all mice receiving Fc mutants drastically lost body weight at levels comparable to those of the mice treated with PBS only, resulting in lethal infection by day 9 (Fig. 4C). Together, these results demonstrate that 1S56-lineage mAbs confer Fc-dependent protection against lethal virus infection.

This study was initially motivated by our previous finding that 36.4% of cross-reactive GC B cells elicited by post-fusion-like HA immunogen remained unmapped to known epitopes for cross-protective IgGs (16). The sequence analysis revealed highly restricted clonality that was exclusively encoded by stereotyped V_H genes, V_H1S56 and its related V_H genes. The stereotyped 1S56-lineage antibodies showed cross-group specificity to multiple HA subtypes, with the specificity being different from broadly reactive antibodies with known epitopes including LAH epitope that had been previously identified from the same experimental sets (7, 10, 13, 16–18, 29, 30). Moreover, the 1S56-lineage antibodies targeted the cryptic epitopes in native HA trimers as the binding avidity increased

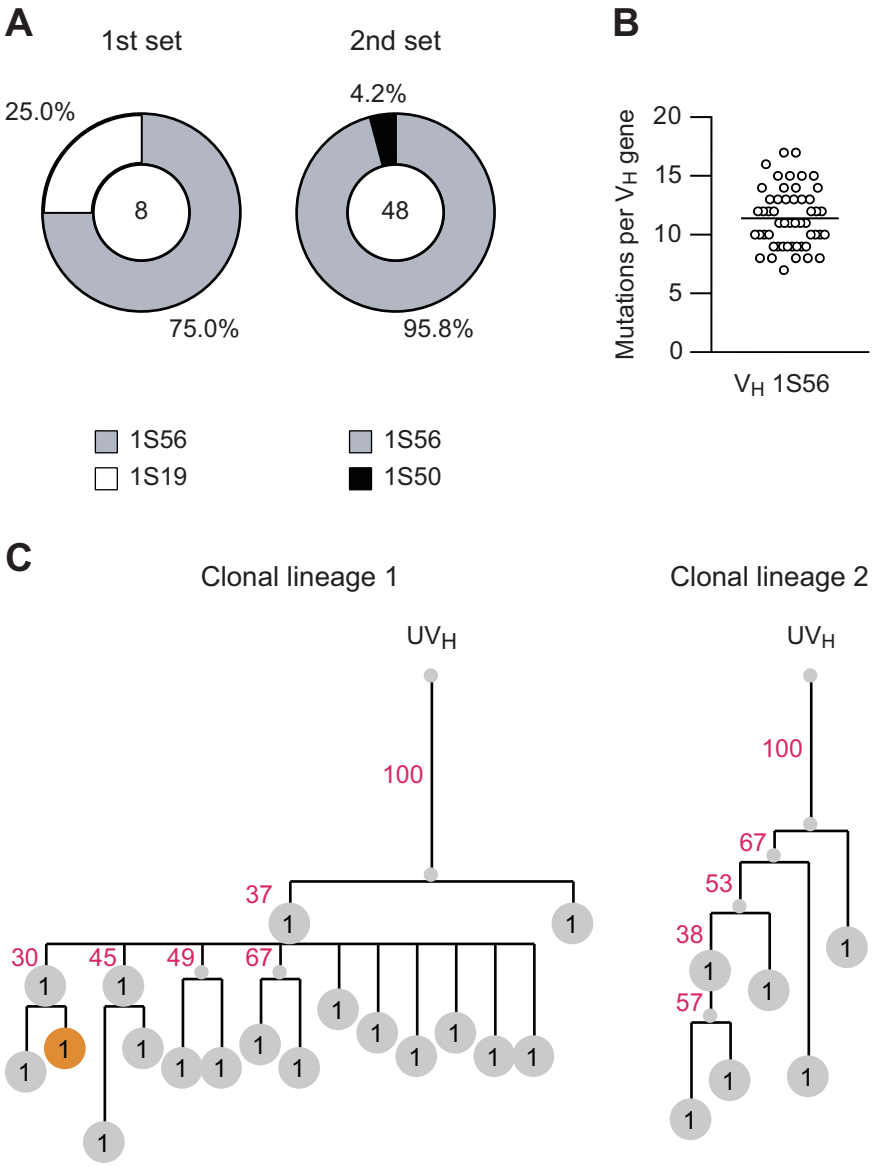

Fig. 2. V_H gene usage and clonal evolution of H3–H5 cross-reactive GC B cells. (A) V_H gene usage of H3–H5 cross-reactive B cells. The results are shown separately from two independent sets of immunization (one mouse from one set and four mice from second set, respectively). The number of cells sequenced is shown at the center of each pie chart. (B) Numbers of mutations are plotted per V_H gene. Each circle represents the result from individual cells. The horizontal line indicates the mean. (C) Clonal lineage trees of V_H 1S56 clones are shown. Two clusters with distinct J_H usage and CDR3 sequence were selected to create phylogenetic trees using GCtree (25). Individual clones are shown as the large nodes numbered with abundance of each genotype. Small unnumbered nodes indicate inferred unobserved ancestral genotypes. A colored node represents the clone chosen to generate a mAb for the following experiments (named 34A8, color is matched to following figures). UV_H; unmutated V_H gene.

in post-fusion-like HA antigens, and failed to label the cells expressing native HA trimers on the surface. Thus, the cross-group conservation and cryptic nature highlights the 1S56 epitope as a unique HA target for broadly protective IgGs, although the exact epitope structure remains to be addressed.

Despite the limited accessibility to native HA trimers, the 1S56-lineage IgGs were able to bind HA antigens on infected cells, albeit at lower intensity than an anti-head neutralizing IgG (Fig. 3C). The findings connected the gap between no neutralizing activity and significant protective function by the 1S56-lineage IgGs. The non-neutralizing IgGs do not prevent the virus infection process; however, the IgG-coated infected cells would be cleared by FcγR- or complement-mediated pathways, both of which contribute to accelerated clearance of virus *in vivo* (1). Indeed, the protective function by 1S56-lineage IgGs was more evident in survival rather than in loss of body weight (Fig. 4C), supporting the fact that the infused IgGs conferred protection without preventing the virus infection process.

One intriguing finding in this study is the identification of 1S56-lineage V_H genes as a stereotyped template for cross-group antibodies. The human germline V_H gene, V_H1-69, is

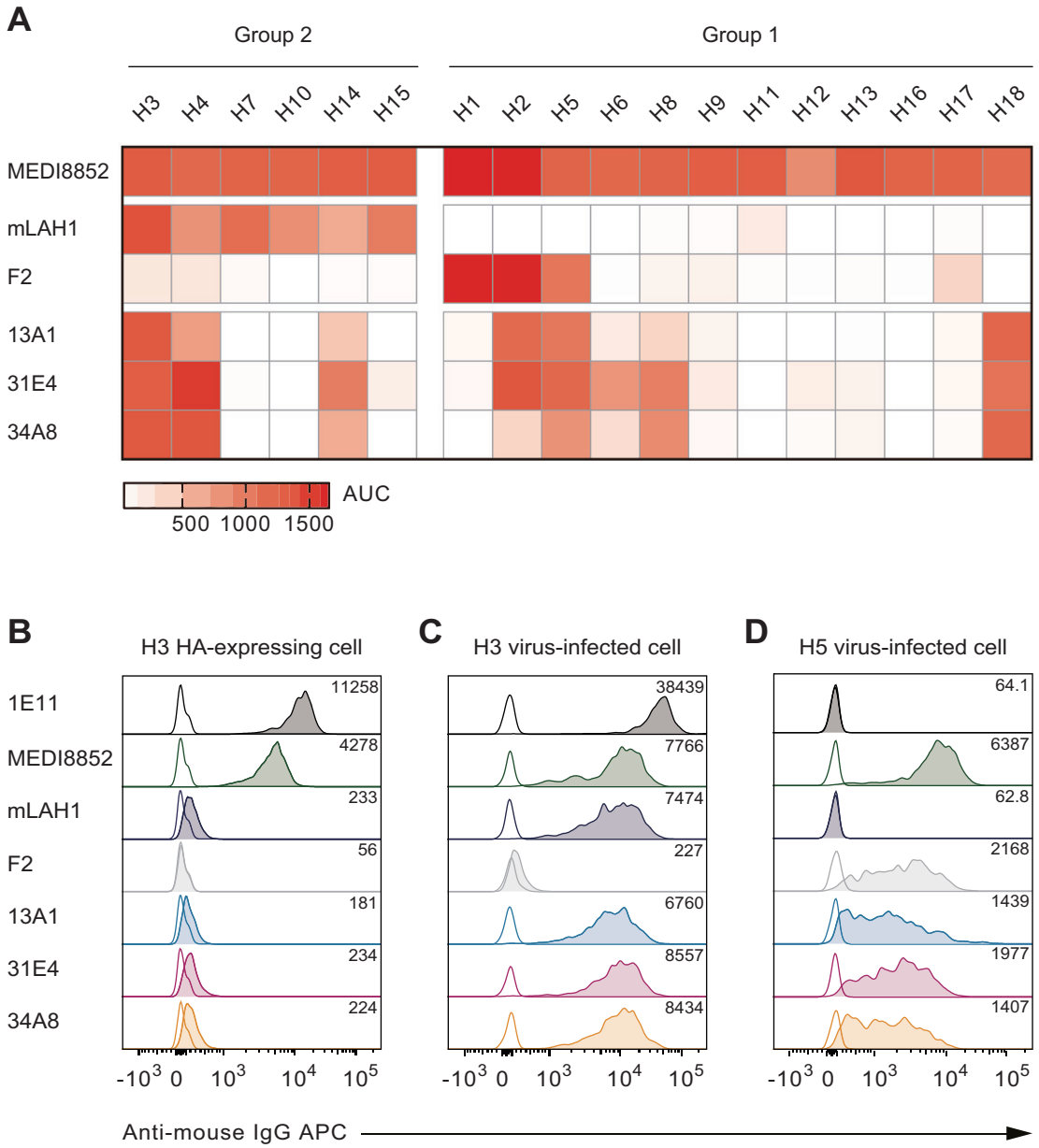

Fig. 3. Cross-group specificity and HA binding properties of V_H 1S56-linage clones. (A) Binding breadth of recombinant mAbs against 18 subtypes of rHA trimers. Full strain names of each subtype are detailed in 'Methods'. Binding of each mAb to each strain is color-coded according to the AUC of the dilution curve as indicated in the color legend. 13A1, 31E4, 34A8; 1S56-lineage clones chosen for further assays, mLAH1; group 2 LAH clone (16), F2; group 1 LAH clone, MEDI8852; group 1 and group 2 CS clone (6). (B) IgG binding to HA-transfected EL4 cell lines was determined by flow cytometry. (C, D) IgG binding to H3N2-infected (C) or H5N1-infected (D) MDCK cell lines was determined by flow cytometry. Representative histograms are shown. Geometric mean fluorescent intensity (gMFI) was calculated by FlowJo 10 and is shown at the right side of each histogram. All assays were conducted in triplicates. Representatives are shown from two independent experiments.

frequently utilized in many human CS antibodies with intra-group 1 breadth (11, 12). The V_H1-69 encoded antibodies in humans are also broadly reactive to other pathogens, including hepatitis C virus (32). The preference for the stereotyped V_H1-69 gene is speculated to be the result of host adaptation to the viruses that have interacted with the human immune system for a long period of time (11). The isolation of the stereotyped V_H1S56 gene in mice suggests the involvement of other micro-organisms for regulating the antibody responses because mice are not the natural hosts for influenza virus. The detailed epitope analysis would be a key step to gain insights on the issue. For human counterparts of V_H1S56-lineage antibodies, it is important to mention that several human monoclonal IgGs have been previously identified as better binders to acid-treated HA than to native HA (33). Such human IgGs provide Fc-dependent protection without virus-neutralization activity, similarly to murine V_H1S56-lineage IgGs.

Multiple mechanisms are suggested to be involved in the Fc-mediated protection by non-neutralizing or poorly neutralizing IgGs against influenza antigens under surrogate assays

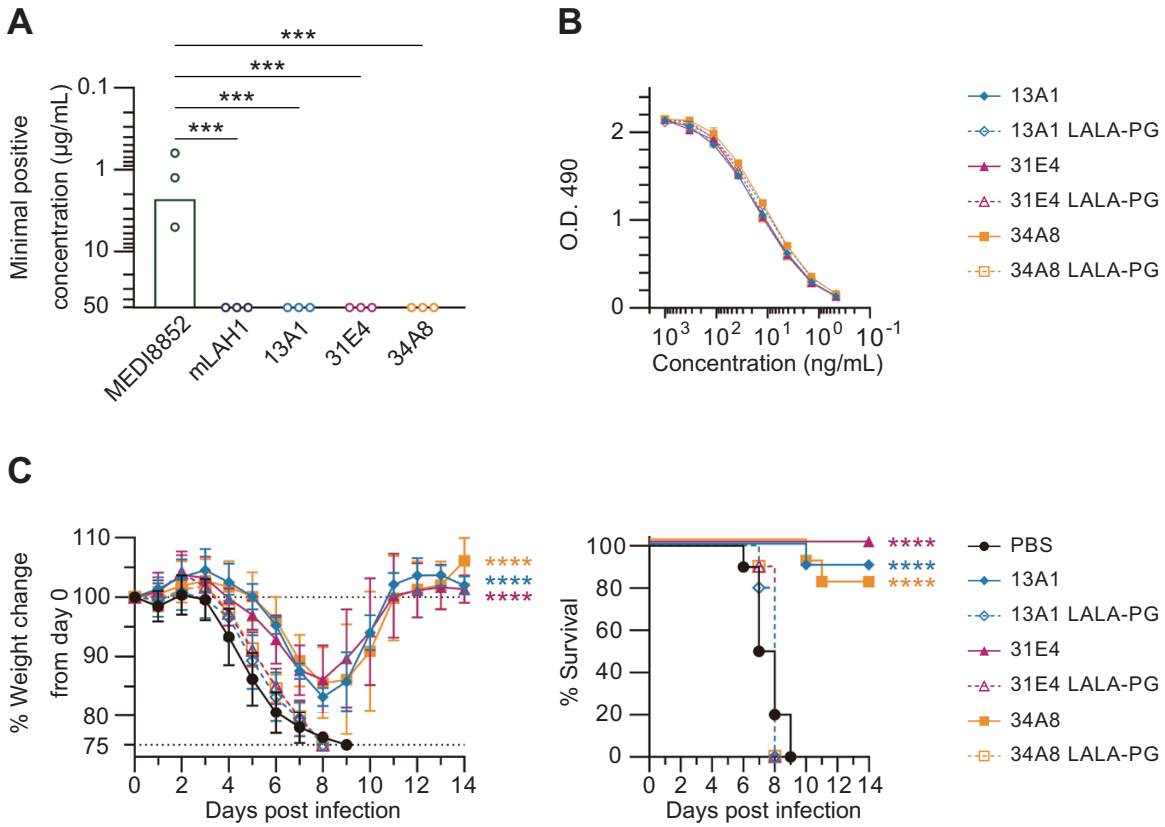

Fig. 4. Neutralizing and protective function of 1S56-lineage mAbs. (A) Neutralizing activity of purified mAbs. Serially diluted 1S56-lineage mAbs were subjected to virus-neutralization assay using X31 virus. MEDI8852 and mLAH1 were applied as positive and negative control, respectively. Minimal neutralizing titer is shown (50 μg ml^{-1} as maximum). Bars represent the mean. The *P*-values were determined with unpaired Welch's *t*-test. ***$P < 0.001$. (B) HA binding properties from wild-type and LALA-PG mutant mAbs. Binding ability was measured by ELISA using rHA of X31. Assay was conducted in triplicates. Data are shown as mean ± SD. (C) *In vivo* protection ability of 1S56-lineage mAbs. Mice were treated with 10 mg kg^{-1} body weight of each mAb and then infected with a lethal dose (5 × LD50) of X31 virus after 3 h of antibody injection. Those that lost more than 25% of initial weight were euthanized. The combined data from two independent experiments ($n = 5$ per group) are shown. Each plot indicates the data of each time points. Value represent mean ± SD. The *P*-values were determined with two-way ANOVA (body weight loss) or log-rank test (survival curve). ****$P < 0.0001$.

in vitro (6, 15, 33–37). Fc-mediated ADCC, ADCP and CDC pathways are known to be activated in correlation with the protection *in vivo*; however, the pathways detected under *in vitro* assays significantly vary among studies using different classes of IgG antibodies. The pioneer study using poorly neutralizing IgGs against CS epitopes detected ADCC activity *in vitro* and FcγR-dependent protection *in vivo* (31). Further, recent studies using non-neutralizing IgGs to head interface epitope also found ADCC activity *in vitro* and Fc-mediated protection *in vivo* (15). However, there are cross-protective IgGs to unknown epitopes that enhanced ADCP activity rather than ADCC and CDC activity *in vitro* (33). LALA-PG mutations used in this study severely attenuate all three pathways (26); hence, it is important to dissect the relative contribution of each protection pathway by genetic approaches *in vivo*, in addition to the *in vitro* surrogate assays and IgG Fc mutant assays that have been performed in previous studies.

Funding

This work was supported by Japan Society for the Promotion of Science KAKENHI (JP19K17656) and by Japan Agency for Medical Research and Development (JP19fk0108051 and JP20fk0108141).

Acknowledgements

We thank Drs Stephen C. Harrison and Aaron G. Schmidt (Harvard Medical School) for providing HA proteins. We thank Drs Garnett Kelsoe and Masayuki Kuraoka (Duke University School of Medicine) for providing HA-expressing EL4 cell lines. We thank Dr Nancy Cox (US-CDC) for providing vaccine strain. We also thank Ms E. Izumiyama for technical assistance. The super-computing resource was provided by Human Genome Center (the University of Tokyo).

Conflicts of interest statement: Y.A. and Y.T. declare that an intellectual property application has been filed regarding post-fusion-like HA immunogen. All other authors declared no conflicts of interest.

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