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Broadly protective human antibodies that target the active site of influenza virus neuraminidase

Daniel Stadlbauer[1,2,#], **Xueyong Zhu**[3,#], **Meagan McMahon**[1], **Jackson S. Turner**[4], **Teddy J. Wohlbold**[1], **Aaron J. Schmitz**[4], **Shirin Strohmeier**[1], **Wenli Yu**[3], **Raffael Nachbagauer**[1], **Philip A. Mudd**[5], **Ian A. Wilson**[3,6,*], **Ali H. Ellebedy**[4,*], **Florian Krammer**[1,*]

[1]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA.

[2]Department of Biotechnology, University of Natural Resources and Life Sciences, A-1190, Vienna, Austria.

[3]Department of Integrative Structural and Computational Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

[4]Division of Immunobiology, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA.

[5]Division of Emergency Medicine, Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA.

[6]Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

Abstract

Better vaccines against influenza viruses are urgently needed to provide broader protection against diverse strains and subtypes. Identification of novel broadly neutralizing epitopes targeted by protective antibodies aid in such efforts. Influenza vaccine development has largely focused on the hemagglutinin but the other major surface antigen, the neuraminidase, has reemerged as a potential target for universal vaccines. Here, we describe three human monoclonal antibodies isolated from an H3N2 infected donor that bind with exceptional breadth to multiple different influenza A and B virus neuraminidases. These antibodies neutralize the virus, mediate effector functions, are broadly protective *in vivo*, and inhibit neuraminidase activity by directly binding to the active site.

[*]Correspondence to wilson@scripps.edu (I.A.W.); ellebedy@wustl.edu (A.H. E.); florian.krammer@mssm.edu (F.K.).

Author contributions: DS, XZ, MM, TJW, RN, SS and WY characterized the antibodies, PAM, AJS and JST isolated the antibodies, DS, XZ, IAW, AHE and FK conceptualized the study and wrote the manuscript.

[#]These authors contributed equally to this work.

Competing interests: DS, XZ, MM, RN, JST, AJS, PAM, TJW, SS, WY, FK and IAW declare no conflict of interest. A.H.E. is a consultant for Inbios. Washington University has filed a patent application based on the findings reported here with AHE, FK and IAW as inventors.

Data and materials availability: Coordinates and structure factors have been deposited in the Protein Data Bank as entries 6Q1Z (1G04 in complex with N9 NA), 6Q20 (1E01 Fab in complex with N2 NA) and 6Q23 (1G01 Fab in complex with N1 NA). Antibody sequences are deposited on GenBank (accession nos. MN013068 (1G01 VH), MN013072 (1G01 Vκ), MN013070 (1E01 VH) MN013070 (1E01 Vκ), MN013069 (1G04 VH) and MN013073 (1G04 Vκ) available from GenBank/EMBL/DDBJ)]. All data are available in the main text or the supplementary materials. Materials used in the analysis are available from the participating laboratories under standard academic material transfer agreements.

Structural and functional characterization of these antibodies informs on development of neuraminidase-based universal vaccines against influenza virus.

One Sentence Summary:

Human monoclonal antibodies to influenza neuraminidase broadly protect against influenza A and B viruses.

Seasonal influenza virus infections cause significant global morbidity and mortality annually. In addition, pandemics occur at irregular and unpredictable intervals and can claim millions of lives. Current seasonal influenza vaccines induce narrow, strain-specific immune responses and have variable effectiveness depending on how well they match the circulating strains (1). These vaccines also do not protect against new pandemic or emerging viruses. Therefore, broadly protective or universal influenza virus vaccines are required (2). Current vaccines are designed to induce antibody responses against the major surface glycoprotein of the virus, the hemagglutinin (HA) (3). Antibodies to HA, specifically to its immunodominant globular head domain, can block virus binding to its sialic acid receptor, but such activity is often restricted to the vaccine strain. Antibodies to the less dominant HA stalk have been shown to have much greater breadth within and across influenza A groups 1 and 2, and rarely also to influenza B viruses (4–7).

The second major virus surface glycoprotein is the neuraminidase (NA), which cleaves the terminal sialic acid from N-linked glycans that also act as receptors (8). The enzymatic activity of NA is important for releasing incoming viruses trapped by glycans of natural defense proteins on mucosal surfaces and for the release of nascent viruses as they bud from infected cells. Anti-NA antibodies can block interactions between NA and its substrate (9, 10). Anti-NA monoclonal antibodies (mAbs) and NA vaccination protect against influenza virus lethal challenge in animal models (9–15). Furthermore, NA vaccination in guinea pigs can prevent virus transmission (16). Most importantly, anti-NA antibodies independently correlate with protection from infection in field studies, as well as in human challenge studies (17–19).

The dominant antigenic sites on the HA mutate at a high rate, due to immune pressure, error rate of the polymerase, and the high plasticity of its globular head domain (20). NA, on the other hand, exhibits a slower drift that is discordant with that of HA (21, 22). Therefore, antibody responses against NA typically show broader cross- reactivity, however this breadth has been assumed to be limited to the respective subtype (N1-N9 for influenza A viruses) (8). Here, we report three clonally related mAbs derived from plasmablasts isolated from an H3N2-infected individual that show broad heterosubtypic binding to NAs from influenza A group 1 (N1, N4, N5, N8), group 2 (N2, N3, N6, N7, N9) and influenza B viruses (Fig. 1A).

Single plasmablasts were sorted from peripheral blood mononuclear cells (PBMCs) on day 5 after onset of symptomatic illness and the corresponding immunoglobulin heavy and light variable (IGHV and IGLV) chain genes were cloned and expressed as previously described (23). The mAbs were then screened for binding to recombinant H3 HA, N2 NA, nucleoprotein and matrix protein 1. Three antibodies from this screening (of 45 mAbs

isolated), 1G04, 1E01 and 1G01, bound to the N2 NA of the seasonal influenza virus strain A/Hong Kong/4801/2014 (H3N2), which is presumably closely related to the strain that caused the infection. The antigen-binding fragments (Fab) of 1G04, 1E01 and 1G01 bound with low nanomolar affinities to the N2 NA of A/Hong Kong/4801/2014 (fig. S1). 1G04, 1E01 and 1G01 form a three-member clonal family where IGHV3–20 and IGKV1–9 encode the IGHV and IGLV genes, respectively (Fig. 1B). The plasmablasts producing the three mAbs most likely originated from memory B cells as evidenced by the accumulation of many somatic hypermutations, particularly in the IGHV genes. Alignment of the heavy chain amino-acid sequences of each mAb to the germline gene, IGHV3–20*04, showed that 1G04, 1E01 and 1G01 differ at 12, 14 and 19 positions, respectively (Fig. 1B).

On further characterization, the three antibodies displayed broad binding to recombinant N2 NA (group 2) from seasonal and avian influenza viruses (Fig. 1A, Table S1).

Furthermore, 1G04 showed some cross-reactivity to N3 and N6 (group 2), and N1, N5 and N8 (group 1), as well as weak binding to influenza B NA (Fig. 1C). 1E01 showed a broader binding pattern that included group 2 NAs N3, N6, N7 and N9, group 1 NAs N1, N5 and N8, as well as strong binding to influenza B NA from the B/Victoria/2/87 lineage and weak binding to the NA of the B/Yamagata/16/88 lineage. Finally, mAb 1G01 showed the broadest binding activity that covered all group 1 NAs (N1, N4, N5 and N8) and group 2 NAs (N2, N3, N6, N7 and N9), as well as NAs from both influenza B virus lineages (Fig. 1C).

We next examined the functional capacity of the three mAbs in an enzyme linked lectin assay (ELLA) that measures neuraminidase inhibition (NI) (Figs. 1D and 2A, fig. S2, Table S2). 1G04 inhibited N2, N3 and certain N1 NAs, while 1E01 inhibited group 2 NAs, N1 and B/Victoria/2/87 lineage NAs. 1G01 inhibited the activity of all A/group 2 and group 1 NAs and B/Victoria/2/87 lineage NA (Figs. 1D and 2A, fig. S2). NA activity can be inhibited by antibodies binding directly to epitopes within the enzyme active site or through steric hindrance when antibodies bind proximal to the active site. However, NI through steric hindrance is only observed in the ELLA when larger substrates, like fetuin, are used. When small molecules are used as substrate, as in an NA-Star assay, antibodies that do not bind directly to the active site might not inhibit (9, 10). When tested in the NA-Star assay, all three mAbs potently inhibited NA activity, hinting at a binding footprint within the NA active site (Fig. 2B and fig. S3). For 1G01 (50% inhibitory concentration (IC_{50}) of 1.35 nM), this was confirmed using the antigen-binding fragment (Fab) instead of the full antibody in ELLA to remove steric hindrance to inhibition. The 1G01 Fab still displayed potent inhibition (IC_{50} of 7.62 nM), which indicates that the antibody may be directly targeting the active site (Fig. 2C). Interestingly, a germline reverted version of 1G04 also potently inhibited NA activity in the ELLA assays (Fig. 2D). Importantly, all three antibodies inhibited an oseltamivir-resistant H3N2 virus at similar potency as a susceptible control virus (fig. S4). Typically, anti-NA antibodies do not show activity in *in vitro* neutralization assays (9), but all three mAbs showed strong inhibitory activity in assays against various N2 viruses (Fig. 2E and fig. S5). While this effect is not due to inhibition of viral entry into cells, it reflects the strong NI activity of the mAbs in blocking virus egress and replication necessary for a positive readout in that assay. Of note, the mAbs neutralized and inhibited

the H3N2 A/Philippines/2/1982 virus more potently than the more recent H3N2 A/Hong Kong/4801/2014 virus (Fig. 1D and 2E, fig. S5), which may be due to the patient's initial exposure/imprinting to H3N2 viruses in the 1980s when the patient was born. In addition to Fab-based antiviral activity, fragment crystallizable (Fc)-Fc receptor (FcR) mediated effector functions, such as antibody dependent cellular cytotoxicity (ADCC) are important for some broadly protective anti-HA antibodies and have also been detected for anti-NA antibodies (9, 24). All three mAbs showed activity against a panel of different NAs in an ADCC bioreporter assay, even when they exhibited weak binding (e.g., 1G04 binding to influenza B NA) (Fig. 2F and fig. S6).

To elucidate the epitopes of the three mAbs and the structural basis of their broad protection, we determined crystal structures of 1G04 with the N9 NA from the recent H7N9 epidemic isolate A/Hunan/02650/2016 (Hunan N9) at 3.45 Å (1G04 has binding affinity to this specific strain of N9), 1E01 in complex with the N2 NA of the 1957 H2N2 pandemic isolate A/Japan/305/1957 (Japan57 N2) at 2.45 Å and 1G01 in complex with the N1 NA of the 2009 pandemic H1N1 isolate A/California/04/2009 (CA04 N1) at 3.27 Å resolution (Fig. 3, Table S3). In all three Fab-NA complex structures, one Fab bound to one NA protomer of the NA tetramer. Significantly, all three Fabs bound to the NAs such that they fully blocked the NA active site (Fig. 3). 1G04, 1E01 and 1G01 recognized their epitopes using both heavy and light chains; the 21-amino acid long complementarity determining regions (CDR) H3 of the three antibodies played an especially dominant role in their NA interactions. The total buried surface areas on the NA by 1G04, 1E01 and 1G01 was 1030 Å2, 900 Å2 and 800 Å2, respectively, of which 87%, 84% and 77% arose from the heavy chain, and 67%, 66% and 77% from the extended CDR H3 loop.

Antibody 1G04 interacted with Hunan N9 NA using CDRs L1, L2, H1, H2 and H3 (Fig. 3A, fig. S7, table S4). The CDR H3 of 1G04 contributed most of the Fab- interactions to 20 N9 NA residues including 10 active site residues, which are conserved in group 1, group 2 and influenza B NAs: R118, E119, D151, R152, I222, R224, E276, E277, R371 and Y406. CDRs L1, L2, H1 and H2 contacted some non-conserved N9 NA residues. Similarly, 1E01 interacted with Japan57 N2 using the same five CDR loops as 1G04. The CDR H3 of 1E01 dominated the interaction with 16 N2 NA residues including all the conserved active site epitope residues recognized by 1G04 except for E276 (Fig. 3B, fig. S8, table S5). Interestingly, 1G01 bound CA04 N1 NA using only CDRs L1, L2 and H3, as well as one residue, Y67, from the framework region (FR) L3. The 21-residue CDR H3 of 1G01 interacted with 19 CA04 N1 NA residues, which included 13 NA-conserved active site (10 of which are conserved with 1G04) or second-shell residues, including L134, R156, W178 (Fig. 3C, fig. S9, table S6). The epitopes of these three antibodies are distinct from the structurally characterized mouse NA antibodies, which bind to epitopes distant from the active site or on the rim of the active site (12, 25–27).

Next, we assessed the protective capacity of the three mAbs *in vivo* in the mouse model. We tested the ability of the mAbs to protect against viruses expressing a human N2, an avian N2, a swine N3, and avian N6, N7 and N9 NAs (all group 2), a human N1, avian N1, N4, N5 and N8 NAs (all group 1), and an influenza B NA from the B/Victoria/2/87 lineage (Fig. 4A, C–M, table S7). All virus strains were lethal in the mouse challenge models except for avian

N2 and N6, in which severe weight loss, but no mortality, was observed in the animals that received the control antibody.

Animals received mAbs at 5 mg/kg two hours prior to infection, and morbidity (weight loss) and mortality were then monitored for 14 days. The mAbs protected from both weight loss and mortality in a manner that was consistent with their reactivity patterns, indicating the ability of the antibody to bind substrate *in vitro* is predictive of its ability to protect *in vivo*. In some cases, e.g. for 1G04 and the H4N6 and B/Malaysia.2506/2004 challenges (Fig. 4E and M) protection was observed despite the absence of strong NI activity. However, binding can mediate effector function (fig. S6) which can, in combination with residual NI, likely also provide protection from challenge. Notably, 1G01 provided full protection from lethality against all challenge viruses. Except for the N4 challenge (Figure 4J), where some transient weight loss was observed, 1G01 provided complete protection against weight loss. Furthermore, the protective effect of 1G01 was tested in a dose de-escalation study with an H3N2 challenge in both BALB/c and DBA/2J mice. A dose as low as 0.3 mg/kg was fully protective for BALB/c mice and 1 mg/kg protected 80% of the DBA/2J (fig. S10). We also monitored virus replication in the lungs of challenged animals on day 3 and day 6 post infection and did not detect any replicating virus using a plaque assay suggesting that the strong *in vitro* inhibiting and neutralizing activity of the three mAbs translates to sterilizing immunity *in vivo*, at least on the sampled days (Fig. 4B). This was further corroborated by measuring titers using a limiting dilution methods in embryonated eggs which has lower sensitivity. Only residual titers were found on day 3 post infection and no virus was detected in any treated mouse on day 6 post infection (fig. S11). To determine if the mAbs had therapeutic potential, we infected mice with a lethal dose of H3N2 virus and treated them with antibody 48- or 72-hours post-infection (Fig. 4N and O).

While transient weight loss was observed, all animals recovered when treated with a low dose of 5 mg/kg mAb.

The isolation of these three mAbs illustrates that humans can make highly potent heterosubtypic anti-NA antibodies that can inhibit influenza A and B viruses. Of note, the three mAbs represent 6.67% of the generated mAbs from this patient, indicating that at least in this individual they are relatively common. However, earlier antibody characterization and vaccination studies indicated that, while protection can be very broad within a subtype, immunity to NA is not heterosubtypic (compared to anti-HA stalk antibodies that typically bind across different subtypes) (9–15). Attempts to raise heterosubtypic anti-NA mAbs in rabbits against a conserved, linear peptide yielded mAbs with low NI activity and little protective effect (28, 29). A few human anti-NA mAbs have recently been reported that show some cross-reactive binding, but they do not have any anti-viral activity *in vitro* or *in vivo* (10). Of note, there is some serological evidence with polyclonal serum that natural infection in humans can induce broadly-reactive anti-NA antibodies (30, 31). The mAbs we report here are highly potent and mediate strong protection *in vivo*, with 1G01, which has the highest number of somatic hypermutations and the smallest footprint, exhibiting the broadest binding. Based on the reactivity profiles, it seems likely that the B cell clones encoding these mAbs were initially engaged by an H3N2 infection and have acquired affinity to N1 and influenza B virus NA antigens through subsequent exposures of this

individual to H1N1 and influenza B viruses. It is conceivable that such antigenically broad exposures allowed these mAbs to recognize other influenza A virus NA subtypes to which the individual was never exposed. The recent H3N2 infection of this individual then recalled these clones. In fact, serum from the patient taken at 5 days post symptom onset displayed cross-reactivity against various NAs and also exhibited high hemagglutination and microneutralizaion titers against the H3N2 virus A/Hong Kong/4801/2014 (320 and 2560, respectively). And NI titer of 536 (against an H6N2 virus (A/Texas/50/2012) (fig. S12). It is possible the pre-exposure serum concentrations of these antibody species in this individual were not high enough to mediate protection. In addition, the patient presented with several underlying risk factors like obesity, diabetes, hypertrophic cardiomyopathy and asthma, which could explain why the person got infected.

Neuraminidase is a validated drug target and several small molecules that inhibit its activity are licensed as influenza therapeutics (32). Of note, in this work several attempts to generate H3N2 escape mutants were unsuccessful. Like the three mAbs reported here, these small molecules target the active site of the NA. Therefore, and because of the extensive breadth of these mAbs, they could potentially be used as antivirals for treatment of seasonal, pandemic and zoonotic influenza virus infection in humans. In addition to the potential of these mAbs as therapeutics, they might also be useful for antibody- guided vaccine design. Currently licensed influenza virus vaccines, including inactivated and live attenuated vaccines, are poor inducers of anti-NA immunity (8). However, recombinant NA-based vaccines, as well as NA virus-like particles, have been shown to induce high titers of anti-NA antibodies that can broadly protect in lethal mouse and ferret challenge models (13–15). Furthermore, recombinant NA-based vaccines have been shown to inhibit influenza virus transmission in the guinea pig model (16). While broad protection within a subtype was observed in these studies, heterosubtypic immunity was not detected, although it was explicitly tested in the mouse model (13). However, sequential prime-boost regimens with recombinant NA vaccines that include different subtypes or scaffolded/stabilized constructs that represent the 1G01 epitope might result in the induction of 1G01-like antibodies.

In summary, we isolated three mAbs that are clonally related and bind to the influenza virus NA by inserting a long CDR H3 into the enzymatic active site, and hence occupying the sialic acid substrate site. All three mAbs show broad binding with 1G01 inhibiting all influenza A virus NA subtypes and influenza B virus NA, making these mAbs extremely promising candidates for therapeutic development. These antibodies are potent inhibitors of NA activity *in vitro* and provide broad protection from mortality and morbidity *in vivo*. The discovery of these mAbs raises the hope that similar antibodies can be induced in the population if the right vaccination regimen is administered. Knowledge of the binding mode and epitope of these mAbs may then guide the development of NA-based universal influenza virus vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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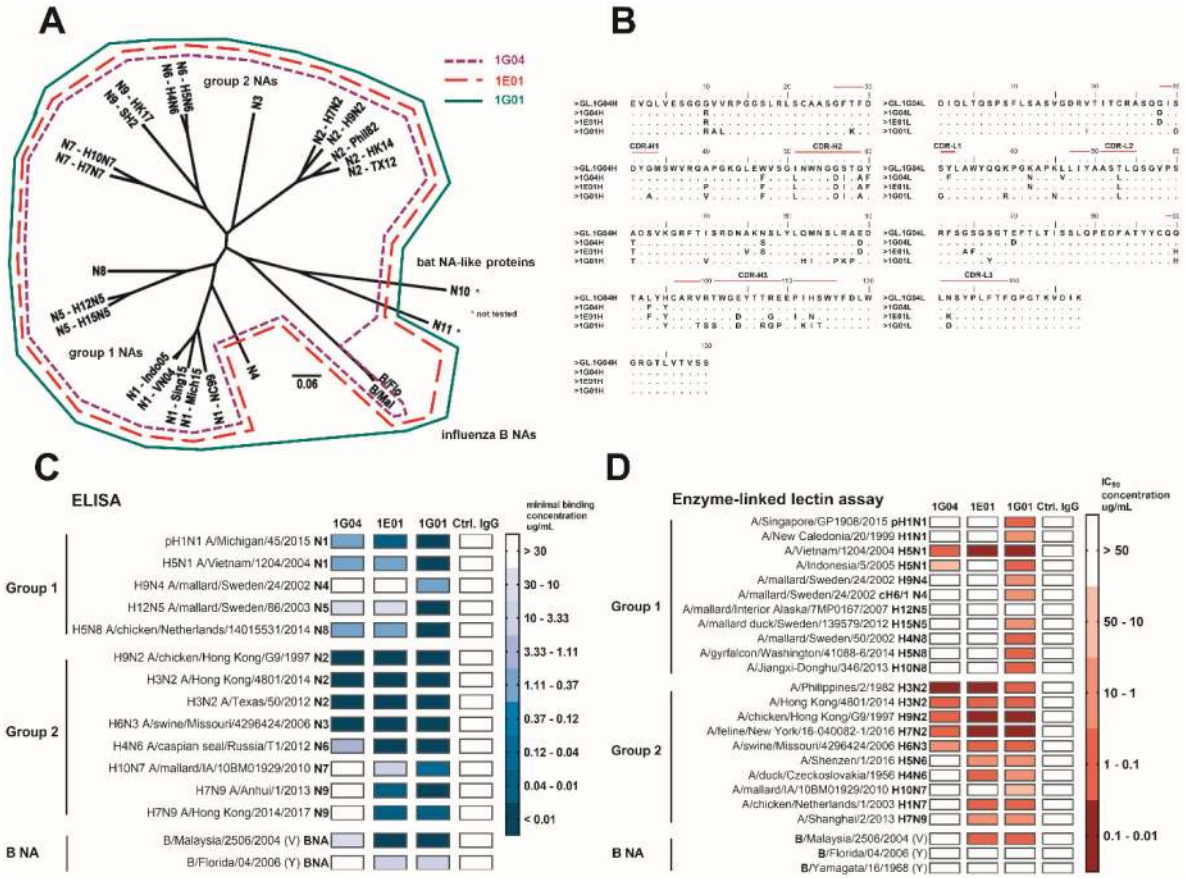

Fig. 1. Breadth of mAbs 1G04, 1E01 and 1G01.

(**A**) Phylogenetic tree of influenza A and B virus NAs. The reactivity breadth of the three mAbs is indicated. The scale bar represents a 6% change in amino acids. The tree was built using amino acid sequences in ClustalOmega and visualized in FigTree. (**B**) Alignment of the amino-acid sequences of each mAb heavy chain (top) and light chain (bottom) to their closest germline immunoglobulin genes as identified by NCBI IgBlast. (**C**) Heat map of antibody binding to recombinant protein in ELISA. The minimal binding concentration is indicated. (**D**) Heat map of antibody activity in ELLA NI assays. The IC_{50} is indicated. For re-assortant strains, virus strain names correspond to the NA of the virus used.

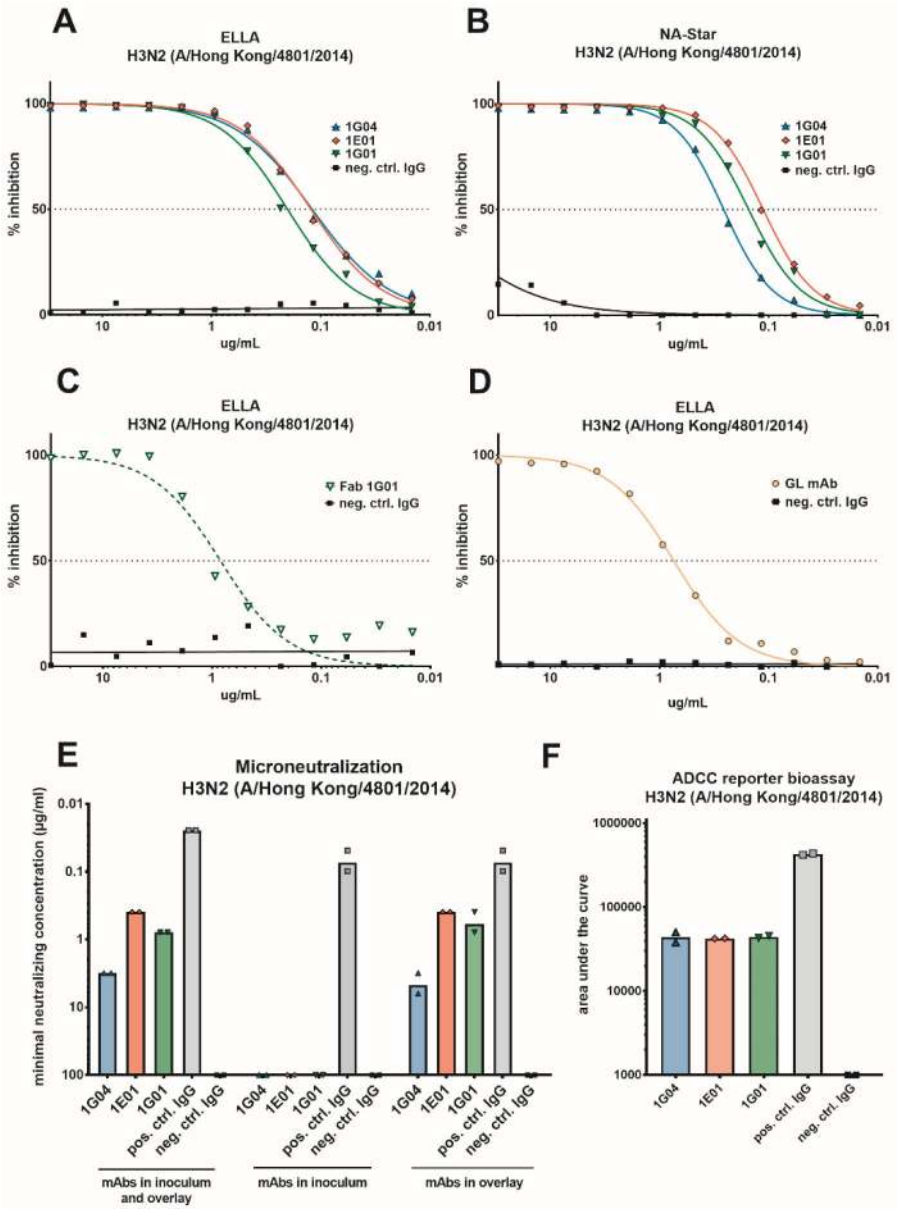

Fig. 2. *In vitro* functionality of mAbs 1G04, 1E01 and 1G01.

(**A**) NA representative inhibition curves in an ELLA assay (large substrate, steric hindrance sensitive) against H3N2 strain A/Hong Kong/4801/2014. (**B**) Activity of the same mAbs against A/Hong Kong/4801/2014 in an NA star assay (small substrate, steric hindrance insensitive). (**C**) Activity of the Fab of mAb 1G01 in an ELLA assay against A/Hong Kong/4801/2014. (**D**) A germline (GL) version of 1G04, representing all three antibodies, is also active against H3N2 strain A/Hong Kong/4801/2014. (**E**) All three mAbs are active in a microneutralization assay against H3N2 strain A/Hong Kong/4801/2014 when antibody is added to the inoculum and overlay. The NA antibodies cannot neutralize the virus when added to the inoculum only, but antibodies prevent viral egress when added to the overlay. The minimal neutralizing concentration is defined as the lowest antibody concentration at which no hemagglutination is detected. (**F**) ADCC reporter bioassay activity of the three

mAbs. MAb CR9114 which has known ADCC reporter activity against H3N2 was used as positive control.

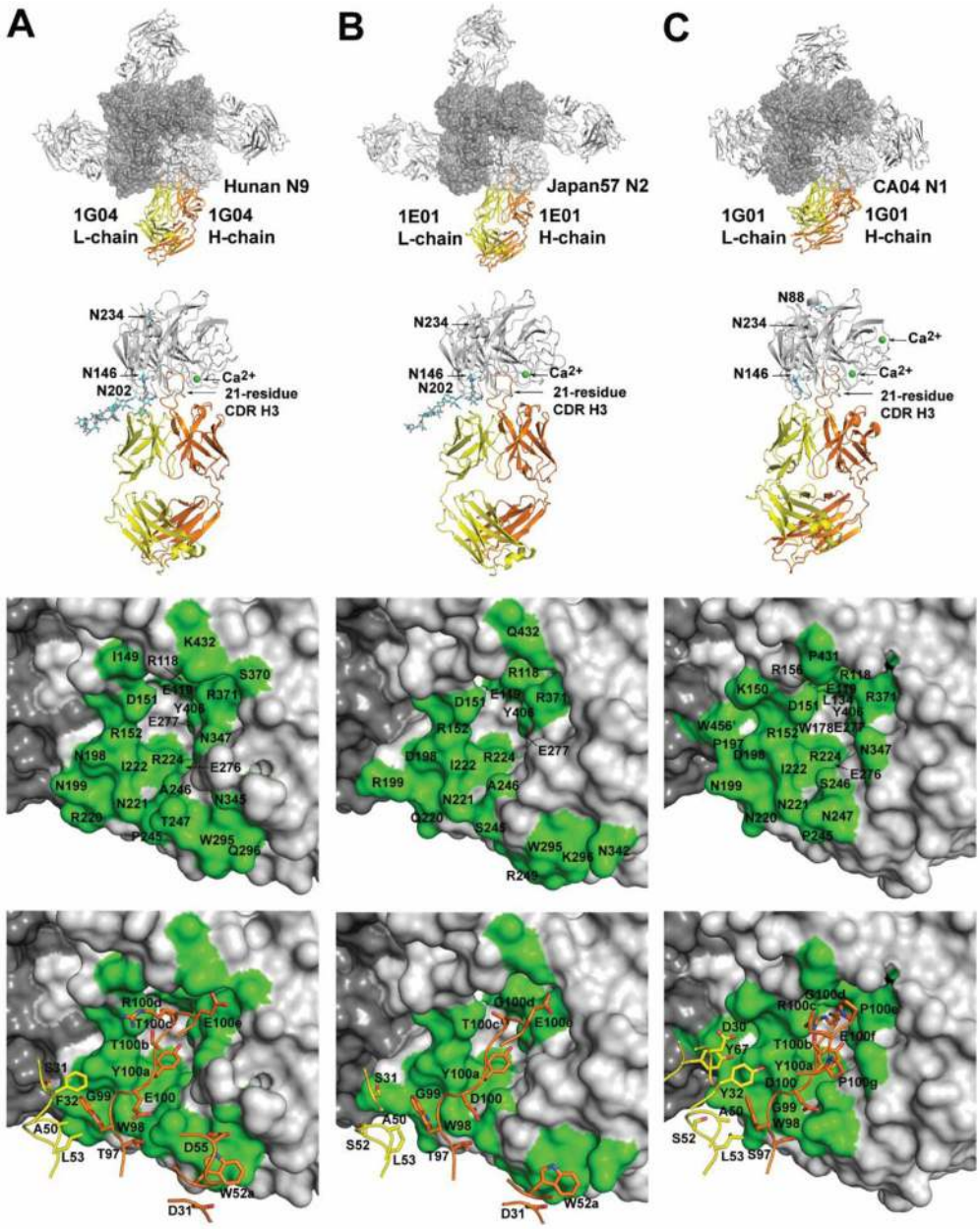

Fig. 3. Crystal structures of 1G04, 1E01 and 1G01 Fabs in complexes with NAs.

(**A**) Crystal structure of 1G04 with Hunan N9 NA at 3.45 Å resolution. From top to bottom, the NA tetramer with one Fab bound to each protomer of the NA tetramer, the NA protomer with one Fab, the epitope on the NA, and the Fab paratope on the NA. (**B**) Crystal structure of 1E01 with Japan57 N2 NA at 2.45 Å resolution. From top to bottom, as for A. (**C**) Crystal structure of 1G01 with CA04 N1 NA at 3.27 Å resolution. From top to bottom, as for A. For all panels, one NA-Fab protomer is colored with NA in light grey and Fab light chain (L-chain) in yellow and Fab heavy chain (H-chain) in orange. The NA and Fab in the other protomers are in dark grey and light grey, respectively. N-linked glycans are shown in stick representation with cyan carbon atoms. Calcium ions are shown as greens spheres. The molecular surface depicting the epitope is colored in green.

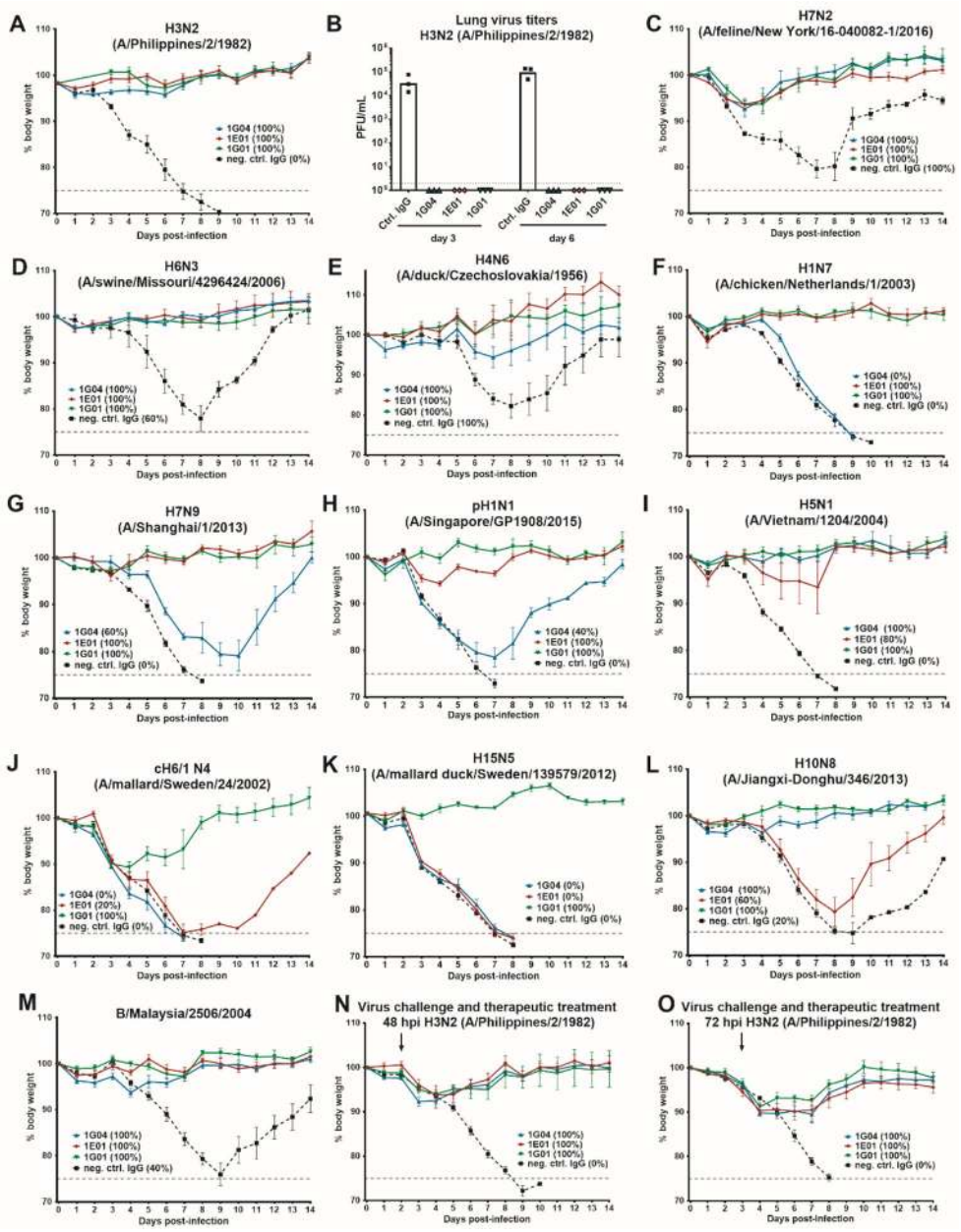

Fig. 4. *In vivo* protection by mAbs 1G04, 1E01 and 1G01.

(**A, C-M**) The efficacy of the three mAbs in a mouse model against challenge with viruses expressing group 1, group 2 and influenza B virus NAs. Animals were treated with 5 mg/kg of mAbs intraperitoneally 2 hours before intranasal virus challenge. Five animals per group were used. (**B**) Lung titers of animals treated prophylactically with mAbs (as described for A) on day 3 and day 6 post infection. Three mice per group were used. We also tested therapeutic treatment of 5 mg/kg at 48 (**N**) and 72 (**O**) hours post infection. The treatment time points are indicated with errors. Five animals were used per group. For A and C-O, percent weight loss is shown and percent survival is indicated in the respective figure legends. Weight loss was monitored daily except for the 1G01 group in (A) on days 1 and 2

due to technical malfunctioning of a cage. For re-assortant strains, virus strain names correspond to the NA of the virus used.