Broadly inhibiting anti-neuraminidase monoclonal antibodies induced by trivalent influenza vaccine and H7N9 infection in humans

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Abstract

The majority of antibodies induced by influenza neuraminidase (NA), like those against hemagglutinin (HA), are relatively specific to viruses isolated within a limited time-window as seen in serological studies and the analysis of many murine monoclonal antibodies (mAbs). We report three broadly reactive human mAbs targeting N1 NA. Two were isolated from a young adult vaccinated with trivalent influenza vaccine (TIV), which inhibited N1 NA from viruses isolated from humans over a period of a hundred years. The third antibody isolated from a child with acute mild H7N9 infection inhibited both group 1 N1 and group 2 N9 NAs. In addition, the antibodies cross-inhibited the N1 NAs of highly pathogenic avian H5N1 influenza viruses. These antibodies are protective in prophylaxis against seasonal H1N1 viruses in mice. This study demonstrates that human antibodies to N1 NA with exceptional cross-reactivity can be recalled by vaccination and highlights the importance of standardizing the NA antigen in seasonal vaccines to offer optimal protection.

Importance

Antibodies to the influenza NA can provide protection against influenza disease. Analysis of human antibodies to NA lags behind that for HA. We show that human monoclonal antibodies against NA induced by vaccination and infection can be very broadly reactive with the ability to inhibit a wide spectrum of N1 NAs on viruses isolated between 1918 and 2018. This suggests that antibodies to NA may be a useful therapy, and that efficacy of influenza vaccines could be enhanced by ensuring appropriate content of NA antigen.

Background

H1N1 virus entered the human population from birds in 1918. It is thought to have jumped from humans to pigs in that epoch, and it was from the pig that influenza virus was first isolated in 1931 (1), then from humans in 1933 through infection of ferrets (2). H1N1 viruses circulated continuously in humans until 1957, when newly emerged H2N2 viruses replaced them. H1N1 virus reappeared in 1977 and continued to circulate until 2009. During this

whole period, it underwent independent but continuous genetic and antigenic drift in humans and pigs. In 2009, a novel swine-origin H1N1 virus re-entered the human population and caused a pandemic. The accumulated sequence disparity between these independent descendants of the 1918 H1N1 virus had resulted in sufficient loss of cross-immunity to render most humans susceptible to infection by the porcine H1N1 virus in 2009.

Antibodies to the hemagglutinin (HA) and neuraminidase (NA) can independently provide protection from influenza disease (3-6).The study of antibodies targeting NA has been under the shadow of those against HA, although there exists an extensive amount of evidence in support of protective immunity against NA. Previous work by Schulman, Webster, Kilbourne and colleagues showed the protective effects of anti-NA antibodies in mice and ferrets. Mice inoculated with virus or purified NA protein elicited protective immunity against NA (7, 8). The anti-NA antibodies were shown to inhibit NA activity *in vitro* and reduce virus plaque size (9). Anti-NA immunity protected mice from infection, presumably by abrogating the release of virus from infected cells. Many groups subsequently elaborated the protective effects of antibodies against NA in animal models (10-12) [Reviewed by (13-15)].

Kilbourne and colleagues also showed that protective anti-NA antibodies are elicited in humans following natural infection (16, 17) and exposure to inactivated whole-virus vaccine (18). Current challenge studies in humans also confirm the independent protective effect of antibodies against NA (5). Finally, several groups have recently established the anti-NA antibody titre in human sera to be a correlate of protection in large clinical trials (3-5).

Compared to a considerable literature on human mAbs against HA, the majority of mAbs targeting NA described to date are from mice and rabbits and they show relatively limited cross-reactivity. Among the first murine mAbs against NA - NC10 and NC41, specific to the N9 NA, were analysed for functional and structural characteristics (19, 20). The murine antibody CD6, which was protective against a limited range of N1 subtype viruses including seasonal H1N1, H1N1pdm09 and avian H5N1, was found to make several contacts with

adjacent NA monomers. However, this antigenic epitope (D451G) underwent amino acid substitution in clade 6A H1N1pdm09 in 2012 viruses and prevented CD6 binding (12, 21).

Antibodies against NA act mainly through steric hindrance to block interaction of the active site of the enzyme with sialic acid templates, but may also invoke Fc-dependent protective mechanisms *in vivo* (22-24). Antibody HCA-2, which was induced in rabbits by immunisation with a 9-mer conserved peptide from the NA active site (residues 222-230), is known to bind to the active site (11, 25). This antibody reacts in western blots with a very wide range of NAs, and cross-inhibits multiple viruses of different influenza A and influenza B lineages, but only at high concentration. HCA-2 offers only partial protection, even at the high antibody dose of 60 mg/kg, and can be affected by amino acid substitutions in the active site that lead to reduced susceptibility to NA inhibitors (11). The requirement for such a high concentration of HCA-2 is probably because it reacts with a linear epitope exposed predominantly after denaturation of NA. Thus, there is scope for potent and broadly reactive human mAbs against NA that confer better protection and could be used therapeutically.

Owing to high sequence diversity in the globular head of HA, humans produced broadly reactive antibodies to the conserved stalk of HA after exposure to H1N1pdm09 virus, targeting shared epitopes in the stalks of earlier seasonal H1N1 and H1N1pdm09 viruses (26, 27). Antibodies against NA are less well studied in this context, but recently broadly reactive anti-NA antibodies have been isolated from humans after infection (28, 29). The NA of H1N1pdm09 viruses may have reactivated B cell memory for rare epitopes shared with the N1 of earlier human seasonal viruses. The authors found that 14-35% of influenza A specific mAbs induced by natural infection bound NA, whereas only 0-2% did so after vaccination. They confirmed that the NA antigen is poorly represented in many sub-unit vaccines, and that the quality and quantity of NA in different vaccines varies (30, 31).

Despite this variability, we report a panel of anti-NA mAbs with exceptionally broad reactivity, isolated from human donors after influenza vaccination or infection. Two broadly reactive human mAbs to N1 NA, isolated from a vaccinated individual, inhibited the enzymatic activity of N1 NAs from viruses circulating in the course of the last 100 years. In addition, both mAbs cross-inhibited many N1 NAs from highly pathogenic avian influenza H5N1 viruses. The antibodies were effective prophylactics protecting a commonly used mouse strain against the highly lethal Cambridge variant of H1N1 virus A/PR/8/1934 and the DBA/2 mouse strain, highly susceptible to influenza, challenged with a H1N1pdm09 virus. We also describe an antibody induced by acute H7N9 infection that cross-reacts between the human seasonal and avian N1 (group 1) and avian N9 (group 2) NAs. These exceptionally broadly reactive anti-NA mAbs offer the hope of developing vaccines that could induce them.

Results

Anti-neuraminidase mAbs from human donors

Two antibodies AG7C and AF9C were isolated from an adult (aged 23; donor C) vaccinated with 2014/15 northern hemisphere TIV containing A/California/7/2009 (Reassortant NYMC X-179A) (H1N1); A/Texas/50/2012 (Reassortant NYMC X-223) (H3N2), B/Massachusetts/2/2012 (Reassortant NYMC BX-51B); all at 15 µg/0.5 mL (AdimFlu-S produced by Addimmune Corporation, Taiwan) (Table 1). A third antibody, Z2B3, was isolated from a Chinese male child (donor Z) with a mild H7N9 infection in 2013; two more antibodies Z2C2 and Z1A11 were isolated from this donor. Similarly, three more N9 mAbs were isolated from donors W and K who were hospitalized with H7N9 virus infection (Table 1, 2). Antibodies to H7 HA from donors Z and K are already reported (32).

Inhibitory breadth of anti-N1 NA mAbs against human H1N1 viruses

We focused our analysis on three mAbs: AG7C, AF9C and Z2B3 since other antibodies were either of limited specificity or weaker in inhibition of NA. These three mAbs were tested for the inhibition of NA activity of H1N1 viruses isolated between 1934 to 2018, in an Enzyme Linked Lectin Assay (ELLA) (Figure 1, 2), and for inhibition of the enzyme activity of

the 1918 pandemic H1N1, and avian N1 and N9 NAs as recombinant proteins (Figure 3, Table 3).

The mAbs were titrated by ELLA and the concentrations required to give 50% inhibition (IC_{50}) of NA activity were calculated by linear interpolation. The titres yielded by a 1 mg/ml solution were then calculated and plotted for comparisons to control hyper-immune sheep sera obtained from the National Institute for Biological Standards and Controls (NIBSC) (Figure 2, 3). On the secondary Y-axis, IC_{50} titres are shown in ng/ml.

AF9C inhibited the NA activities of all H1N1 viruses tested which were representative of those that have circulated in humans for over 100 years (Figure 1-3). AG7C showed a slightly different specificity as it weakly inhibited or failed to inhibit the NAs from A/Brisbane/59/2007 and A/USSR/90/1977 (Figure 2). mAb Z2B3, cross-reactive with N9 NA, also showed a broad recognition of N1 NAs but again weakly inhibited A/Brisbane/59/2007 and failed to inhibit A/USSR/90/1977 NAs (Table 1; Figure 1, 2). Unlike AG7C and AF9C, Z2B3 had greatly reduced activity against recent clade 6B.1 H1N1pdm09 viruses isolated after 2014 (Figure 2).

Figure 2 shows that AG7C and AF9C titrate predominantly between 1:4,000 and 1:40,000 (IC_{50} ~250-25 ng/ml) on the set of viruses shown, with the exception that AG7C fails to inhibit N1 NA from A/Brisbane/59/2007. By contrast Z2B3 gave similar titres on A/PR/8/1934, A/England/195/2009 and A/England/621/2013 but had drastically reduced titres on A/USSR/90/1977 and the representative recent clade 6B.1 H1N1pdm09 viruses A/Serbia/NS-601/2014 and A/Switzerland/3330/2017, indicating that the genetic and associated antigenic drift in these viruses had resulted in a major alteration in the epitope recognised by Z2B3. The control hyper-immune sheep serum to A/California/07/2009 N1 showed limited cross-reactivity on recently drifted or older (former seasonal) viruses with only weak activity against N1 NA from A/PR/8/1934. The sheep anti-H7N9 (A/Anhui/1/2013)

serum contained anti-N9 NA antibodies that did not cross-react with any NAs expressed by these H1N1 viruses.

The inhibitory activity of broadly reactive anti-N1 mAbs against NAs of avian H5N1 viruses

To avoid handling avian influenza viruses, we titrated the mAbs for inhibition of recombinant N1 NAs from a range of H5N1 viruses isolated from infected humans representing several HA-clades, from pandemic virus A/Brevig Mission/1/1918 and N9 NA from H7N9 virus A/Anhui/1/2013, produced in HEK293 cells, with N1 NA from A/California/07/2009 as a positive control (Figure S1, S2 and Table 3).

AG7C inhibited all of the N1 NAs representing H5N1 viruses between 2004 and 2015 and the N1 NA from the 1918 pandemic virus A/Brevig Mission/1/1918. AF9C showed similar activity against N1 NAs from A/California/07/2009 and A/Brevig Mission/1918 but reacted less well with N1 NAs from H5N1 viruses (Figure 3). Neither AG7C nor AF9C inhibited the N9 NA. By contrast Z2B3 inhibited the H1N1pdm09 NA, the 2013 N9 NA and most of the avian N1 NAs at moderate IC_{50} values that were in general weaker than for mAb AG7C; it inhibited the 1918 N1 NA weakly. The control hyper-immune sheep serum against H1N1pdm09 NA showed a titre >1:400 with A/California/7/2009 N1 NA, with minimal cross-reactivity against avian N1 NAs, 1918 N1 NA and the 2013 N9 NA. The control sheep serum against N9 NA inhibited N9 but not N1 NAs.

Anti-N9 NA mAbs cross-reactive with N1 NA

Among six anti-N9 NA mAbs isolated from three donors exposed to H7N9 virus and tested by ELLA, three inhibited recombinant N9 NA (Figure 4). Two N9 NA-inhibiting mAbs were isolated from donor Z, where Z2B3 was a strong inhibitor and Z2C2 was a weak inhibitor (Figure 4A). All three mAbs from donor Z were cross-reactive with N1 NA (Figure 4C) and strongly inhibited the H1N1pdm09 (A/England/195/2009) N1 NA (Figure 4B). This suggests

194 that 6-year old donor Z may have made a primary antibody response to the H1N1pdm09 N1
195 NA, and subsequent infection with H7N9 stimulated the memory B cells to an epitope
196 conserved between N1 and N9 NAs. Notably, Z2B3 and Z2C2 have longer heavy chain
197 CDR3 domains than other mAbs and although Z2B3 and AF9C are both encoded by the
198 same VH gene (VH1-69), the CDR3 amino acid sequences are significantly different.
199
200 Antibodies from donors W (W1C7) and K (P17C, F4C) were found to bind N9 NA in an
201 indirect immunofluorescence screen (not shown). W1C7 and F4C were specific for N9 NA,
202 and W1C7 had a weak inhibitory effect in ELLA on N9 (Figure 4). P17C cross-reacted with
203 N1 NA with a low level of binding and showed weak inhibition by ELLA (Figure 4B, C).
204
205 Antibodies from donor Z have higher numbers of amino acid substitutions in the variable
206 regions of heavy and light chains, compared to those in mAbs from other donors (Table 2).
207 The number of substitutions in VH of mAbs Z2B3, Z2C2 and Z1A11 are 8, 13 and 17
208 respectively, whereas there are none, 1 and 1 respectively in mAbs W1C7, P17C and F4C
209 (Table 2). This suggests the mAbs from donor Z are of memory B cell origin while those from
210 donors W and K resulted from de-novo responses to acute H7N9 infection.
211
212 **Anti-NA mAbs provide prophylactic protection *in vivo***
213 All three of the anti-N1 NA mAbs, AG7C, AF9C and Z2B3, protected 100% of mice from
214 challenge with 10^4 $TCID_{50}$ (Median Tissue Culture Infectious Dose) of A/PR/8/1934 virus
215 (equivalent to 1000 LD_{50} (Median Lethal Dose) when given at a dose of 10 mg/kg 24 hours
216 before infection ($p<0.001$; Figure 5A, B). They prevented any weight loss whereas mice that
217 received an anti-N2 NA mAb (M6B12) succumbed to ≅20% weight loss by day 5 and were
218 humanely culled. An antibody to the H1 stem T1-3B (33) provided a positive control for
219 protection.
220

In another experiment, DBA/2 mice, that are highly susceptible to influenza infection (34) were treated with AG7C and AF9C antibodies 24 h before infection with 10^4 $TCID_{50}$ of X-179A (equivalent to 150 LD_{50}) virus, a reassortant containing the H1N1pdm09 vRNAs from A/California/07/2009 (Figure 5C,D). Treated mice were protected from ≅20% weight loss ($p<0.001$), whereas mice receiving a non-specific antibody had to be culled on days 5 or 6. One of 6 mice in the AG7C group was sacrificed on day 11 after losing ≅20% weight. In these prophylactic protection experiments, anti-NA mAbs were as protective as T1-3B, the positive control anti-HA stalk mAb (33).

Next, we compared the prophylactic protection by N1 mAbs with their IgG1 LALA-PG variants. These substitutions abrogate FcϒR dependent ADCC (Antibody Dependent Cell Cytotoxicity) and complement binding and fixation (35). We confirmed that these mAbs showed no difference in their inhibition of N1 NAs of X-179A and A/PR/8/1934 viruses (Figure 6). BALB/c mice were given 10 mg/Kg antibody 24 hours before intranasal infection with 10^4 $TCID_{50}$ A/PR/8/1934 virus. We found that Fc abrogation made no difference for mAb AG7C. Both the native and LALA-PG variant protected 6/6 mice without any weight loss or clinical sign (Figure 5E, F). However, with mAb Z2B3 there was up to 10% weight loss in mice treated with the LALA-PG variant, even though 6/6 mice were protected. This may indicate that for some antibodies to NA FcR mediated function may contribute to the protection.

Discussion

We show in this paper that broadly reactive and protective antibodies to N1 NA can be isolated from vaccinated and infected individuals, presumably due to the conservation in surface structure between N1 NAs (Figure 7A). The two N1 subtype specific mAbs AG7C and AF9C were isolated from the same donor who had been vaccinated in 2014 with AdimFlu-S TIV in Taiwan. AG7C inhibits N1 NAs from H1N1 viruses isolated between 1918-

2018. Although previous investigations of subunit vaccines have found varying and usually low levels of NA antigen (14, 28, 30), in this case there was clearly enough to induce a response.

The very broad reactivity of these mAbs with N1 NAs, covering the complete period of H1N1 virus circulation in humans, may have been induced by exposure to the significantly different NA derived from the H1N1pdm09 virus. Both mAbs show significant sequence divergence (Table 2) suggesting that they originated from a memory population which went through multiple rounds of selection in germinal centres following previous exposures to influenza. Both mAbs provided prophylactic protection in mice against the highly virulent variant of A/PR/8/1934 (the Cambridge strain) (36) and, in DBA/2 mice, against infection with H1N1pdm09 X-179A (A/California/7/2009). In an earlier paper Chen et al. described similar anti-N1 NA antibodies that reacted with viruses spanning the period 1918-2009 (28).

The third antibody Z2B3 was isolated from a child who experienced a mild infection with H7N9 virus in 2013. It was unusual in being cross-reactive with group 1 (N1) and group 2 (N9) NAs (Figure S1). Two similar antibodies were isolated from this donor, both of which inhibited N1 NA with some level of cross-reaction with N9 NA (Figure 4), which we interpret to imply that they were selected from a subpopulation of memory cells induced previously by N1 NA. Examination of the structure of the N1 and N9 NAs reveals a region of conserved surface around and within the active site of the enzyme, as a possible binding site for Z2B3 (Figure 6B).

mAb Z2B3 showed good reactivity with the H1N1pdm09 virus A/England/621/2013, but poor reactivity with a later clade 6B virus, A/Serbia/NS-601/2014. These two viruses showed non-conservative amino acid substitutions of only N386K and K432E in the head of NA (Figure 7C). The former site is similarly substituted in the N9 NA that Z2B3 recognizes, which suggests that K432 is within the footprint of mAb Z2B3. K432 falls within a known epitope

277 recognized by anti-N9 NA antibodies (19, 37). The crystal structure of a N9 NA-mAb
278 complex, N9-NC10, involved a contact between D56 of the antibody H-chain and K432 of N9
279 NA (GRP**K**EDK; PDB 1NMB).
280
281 K432 was conserved prior to 2013 but underwent substitution in 2014, K432E, which
282 became dominant thereafter. We suggest that N1 NA has been under strong evolutionary
283 pressure from broadly cross-reactive antibodies induced by the H1N1pdm09 NA, that were
284 selected from memory B cells raised against NA(s) of earlier virus(es). Just as the
285 conserved stalk of HA has shown a capacity for evolution under pressure from antibody
286 selection (38), the NA may similarly be forced to drift antigenically by broadly cross-reactive
287 antibodies induced by the H1N1pdm09 viruses (39).
288
289 With this in mind we examined the region of the NA surface recognized by broadly reactive
290 antibodies described by Chen et al. that inhibited or bound N1 NAs of viruses isolated
291 between 1918-2009 but not clade 6B H1N1pdm09 viruses (28, 39). Some of these
292 antibodies lost binding to N1 NAs with substitutions in a set of site-specific mutants (21, 39).
293 Many of these antibodies also did not inhibit A/Brisbane/59/2007. mAb AG7C showed a
294 similar reactivity profile and may have been affected by substitutions G249K and Q250P that
295 are common to the non-reactive NAs. These residues are exposed on the periphery of the
296 catalytic site (Figure 7D). The preceding residue N248 was substituted (N248D) in the
297 H1N1pdm09 viruses isolated post 2009 and caused a loss of recognition by some mAbs
298 described by these groups. However, this substitution is tolerated by mAb AG7C. There are
299 rare natural isolates that have substituted these residues (G249E/R and Q250R) indicating
300 that even the broadly reactive mAbs can be thwarted by virus antigenic drift (Figure S2).
301 mAb AG7C significantly lost inhibition titre against viruses with substitutions G249E/R
302 confirming that these residues are parts of an epitope (Figure 2I, 2J). However, it does not
303 seem to be affected by the Q250R substitution (Figure 2H). 11/1944 H1N1pdm09 viruses
304 sampled between 2008 and Sept 2019 had acquired the G249E/R substitution

(nextstrain.org). Substitutions of Q250 are rare; Q250L was seen in 1/1944 viruses analyzed.

Similarly, the comparison of NA sequences between A/Switzerland/3330/2017 (inhibited strongly by AF9C) and A/Luxembourg/2489/2019 (inhibited weakly) inferred the substitution D199N to be involved in the loss of recognition and therefore D199 to be a part of the epitope recognized by AF9C (Figure S2). 7/2056 NA sequences from H1N1pdm09 viruses detected between 2013-2019 had N1 D199N/A substitutions. Further structural work to define the epitopes recognised by Z2B3, AG7C and AF9C is in progress.

We found that the broadly reactive human IgG1 anti N1 mAb AG7C did not require Fc engagement for complete protection of mice. By contrast, the LALA-PG variant of mAb Z2B3 provided protection of 100% animals but with some weight loss, unlike the unmutated IgG. This contrasts with a broadly reactive mAb (3C05) containing human variable regions linked with murine IgG2a Fc, for which protection was abrogated by the Fc mutation DA265 (23). Some NA mAbs are known to protect in vivo via Fc mediated functions even in the absence of neuraminidase activity (40). However, we haven't tested non-inhibiting mAbs for in vivo protection in this paper.

It has become clear that exposure to viruses that differ significantly from those circulating, can select responses to epitopes in both HA and NA that are shared between the incoming virus and the seasonal viruses in circulation, derived from the memory B cell population (41, 42). While antibodies against new epitopes can also be generated, even in the elderly (32), it appears that they are initially at a disadvantage but may overtake and become dominant with time (43, 44). It would be wise to assume that all of these epitopes, both new and conserved, can drift under pressure from antibody selection. The inevitable implication is that updating influenza vaccines may have to continue, but broadening the memory B cell population by vaccination with as wide a range of groups 1 and 2 HAs and NAs as possible

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(45) might be a logical way of preparing the ground for a strong response to an unknown future pandemic virus.

Materials and Methods

Media, Reagents and Tissue Culture

MDCK-SIAT1 cells and adherent 293T cells (ECACC) were grown in D10 - DMEM medium (Sigma, D5796) supplemented with 10% v/v foetal calf serum (Sigma, F9665), 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all from Sigma, UK). 293F suspension cells were grown in Freestyle 293 expression medium (Life Technologies, 12338-018) on a shaker incubator. Cells were grown at 37°C, 5% CO_2 in a humidified incubator. Viruses were diluted and grown in Virus grown medium (VGM), which is DMEM with 0.1% bovine serum albumin (Sigma, A0336), 10 mM HEPES, and glutamine, Penicillin and Streptomycin as in D10.

Influenza Viruses and control sera

H1N1 viruses from the years 1977 - 2019 and H3N2 viruses were obtained from the Worldwide Influenza Centre (WIC) at The Crick Institute (London, UK). Other reassortant viruses and control sheep sera were obtained from the National Institute for Biological Standards and Controls (NIBSC), UK.

Ethics and Study Approval

The study was in compliance with good clinical practice guidelines and the Declaration of Helsinki. The protocol was approved by the Research and Ethics Committee of Chang Gung Memorial Hospital, Beijing Ditan Hospital and the Weatherall Institute of Molecular Medicine. All subjects provided written informed consent. The list of donors with their details and isolated antibodies are included in Table 1.

360 **Isolation of human mAbs**

361 mAbs were isolated from individual humans who either received seasonal influenza vaccine
362 or were naturally infected with H7N9 virus in China or Taiwan. Peripheral Blood
363 Mononuclear Cells (PBMC) were collected from individual donors either a week after
364 vaccination against influenza or from confirmed influenza infected cases 10 days after onset
365 of clinical symptoms. Antibodies were isolated from PBMC using single cell isolation and
366 cloning methods as described in detail previously (33, 46-48). Briefly, plasmablasts in PBMC
367 were stained (CD3neg, CD19pos, CD20lo/neg, CD27hi, CD38hi) and sorted as single cells
368 using flow cytometry. mRNA from single plasmablasts was reverse transcribed to DNA and
369 VH and Vk/λ genes were amplified using gene specific primers, then cloned into expression
370 vectors containing IgG1 Heavy and Vk and Vλ constant regions. Heavy and light chain
371 plasmids were co-transfected into 293T or ExpiCHO cells (Life Technologies, A29133) for
372 antibody expression.

373

374 **LALA-PG Antibody Variants**

375 The L234A, L235A, P329G (LALA-PG) amino acid substitutions (35) that abrogate Fc
376 mediated functions, were engineered into the human IgG1 Fc regions of antibodies AG7C
377 and Z3B2 by standard procedures and confirmed by sequencing.

378

379 **Antibody Screening**

380 mAbs were initially screened for binding to MDCK-SIAT1 cells infected with either H1N1 or
381 H3N2 viruses, and for lack of binding to HA protein expressed in stably transfected MDCK-
382 SIAT1 cells. Binding to NA was confirmed by immuno-precipitation with infected cells or
383 binding to 293T cells transfected with the NA gene of interest.

384

385 **Production of NA proteins**

386 Tetrameric neuraminidase proteins were expressed from constructs based on the design of
387 Xu et al. (49). In our version the signal sequence from A/PR/8/1934 HA was followed by a

388 human vasodilator-stimulated phosphoprotein (VASP) tetramerization domain and thrombin
389 site, followed by the NA sequence amino acids 69-469 (N1 numbering) (Table 3).
390 Sequences were synthesised as human codon optimised cDNAs by Geneart and cloned into
391 pCDNA3.1/- for transfection. HEK293F cells were transiently transfected using PEI-pro as a
392 transfection reagent. Protein supernatant harvested 5-7 days post-transfection was titrated
393 for NA activity in an ELLA and stored in aliquots at -80°C.
394
395 N9 NA protein (A/Anhui/1/13) was kindly provided by Donald Benton (The Francis Crick
396 Institute) (50). The expression construct consisted of ectodomain residues 75-465 with a N-
397 terminal 6x His tag, a human VASP tetramerization domain (49) and a TEV (Tobacco Etch
398 Virus) cleavage site under the control of promoter with a gp67 secretion signal peptide. The
399 protein was expressed in Sf9 insect cells using a recombinant baculovirus system (Life
400 Technologies). The protein was purified on a cobalt resin column and further purified by gel
401 filtration to ensure the removal of monomeric and aggregated protein.
402
403 For antibody inhibition measurements a dilution of the NA containing supernatant was
404 chosen that had just reached plateau activity in the ELLA. The sequences of all the
405 constructs with their identification numbers are shown in Table 3.
406
407 **NA inhibition assay: Enzyme-Linked Lectin Assay (ELLA)**
408 ELLA assay was adapted from the methods described by Schulman et al. (7) and Sandbulte
409 et al. (51). This assay detects the inhibition of NA enzymatic activity, cleavage of sialic acid,
410 by anti-NA antibodies. Viruses or recombinant NA proteins were used as the source of NA.
411 Virus growth medium was used to dilute antibodies and viruses. A Nunc Immunoassay
412 ELISA plate (Thermo Scientific, 439454) was plated overnight with 25 μg/mL fetuin (Sigma,
413 F3385). Two-fold serial dilutions of sera or mAbs performed in duplicates were incubated
414 together with a fixed amount of titrated NA source. Column 11 of a plate was used for NA
415 source only control, and column 12 was used for medium only control. After 2 h incubation,

antibody/NA mix were transferred to the PBS washed fetuin plate and incubated for 18-20 h at 37 ºC buffered by CO_2 as for tissue culture. Next day, the contents of the plate were discarded, and the plate washed 4 times with PBS. HRP conjugated peanut agglutinin (PNA-HRP, Sigma, L7759) at 1 μg/mL was added to the wells. PNA binds to the exposed galactose after cleavage of sialic acid by NA. After 1 h incubation and PBS wash, signal was developed by adding OPD (o-Phenylenediamine Dihydrochloride) solution (Sigma, P9187) and the reaction stopped after 5-15 min using 1 M H_2SO_4. Absorbance was read at 492 nm in a Clariostar plate reader (BMG Labtech).

The antibodies were titrated by doubling dilution and the concentrations required to give 50% inhibition (IC_{50}) of NA activity were calculated by linear interpolation. For comparison with the positive control sheep sera, titers of antibodies, starting concentration transformed to 1 mg/mL, were compared with the serum titers in the same graphs.

In vivo prophylaxis protection

All animal procedures were approved by an internal University of Oxford Ethics Committee and the United Kingdom Home Office. The experiments were carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals', the recommendations of the Institute for Laboratory Animal Research, and Association for Assessment and Accreditation of Laboratory Animal Care International standards. Principle of the 3Rs were applied in design of experiments.

Mice used in protection studies, DBA/2OlaHsd mice (n=6/group) for X-179A and BALB/cOlaHsd (n=6/group) for PR8 viruses were purchased from Envigo, UK and housed in individually vented cages in a special unit for infectious diseases. Mice were anesthetised by isofluorane (Abott) and 50 μL of virus was administrated intranasally 24 hours after the intraperitoneal administration of 10 mg/Kg antibody (500 μL). Mice were under regular observation and weighed. Mice with weight loss ≅20 percent or morbid clinical scores were euthanized by raising concentration of CO_2. Non-specific IgG antibody was used as a

444 negative control. Known HA-specific antibodies were used as positive controls. Mice were
445 infected intranasally with lethal doses of viruses: X-179A (150 LD_{50}, 10^4 $TCID_{50}$) and PR8
446 (1000 LD_{50}, 10^4 $TCID_{50}$).
447
448 **Sequence Analysis**
449 Amino acid substitutions in H1N1pdm09 viruses were analysed by downloading sequences
450 from the EpiFlu database of the Global Initiative on Sharing All Influenza Data (GISAID) or
451 on NextStrain.org. The viruses were randomized for geography and year during analysis,
452 and sequence alignment was done using BioEdit version 7. The sequences of all viruses
453 used experimentally were determined/confirmed at the WIC.
454
455 **Data and Statistical analysis**
456 Graphs were generated using GraphPad Prism (version 9) and Microsoft Excel 2010.
457 Protein structures were viewed using Pymol2 (Schrodinger, LLC).
458
459 The ELLA titres were expressed as half maximal inhibitory concentrations (IC_{50}: midpoint
460 between negative and plateau positive controls) derived by linear interpolation from
461 neighbouring points in the titration curve. Kaplan Maier tests were performed to analyse the
462 difference in mortality between experimental and control group mice. P values of <0.05 were
463 considered to be of significant statistical difference.
464
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Author Contributions

Conceptualisation: A.R.T., P.R., K.-Y.A.H, J.W.M., T.D.; Methodology: A.R.T., P.R., K.-Y.A.H., R.S.D.; Investigation: P.R., A.R.T., K.-Y.A.H, B.B.W., R.S.D., L.S., T.K.T., P.J.; Writing – Original Draft: P.R. and A.R.T.; Writing – Review & Editing: P.R., A.R.T., K.-Y.A.H., and R.S.D.; Funding and Supervision: A.R.T., K.-Y.A.H., R.S.D., J.W.M., and T.D.

Declaration of interest

Authors declare no conflicts of interest.

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663 **Figure Legends**

664

665 Figure 1. **Inhibition of H1N1 viruses by mAbs targeting N1 NAs**. Percentage inhibition of
666 activity by mAbs, at 20 μg/ml, targeting N1 NAs are shown. H3N2 virus (X-31) was used as
667 a negative control virus and a mAb targeting Ebolavirus glycoprotein was used as a negative
668 control antibody.

669

670

671 Figure 2. **ELLA titrations of mAbs against selected H1N1 viruses.** AG7C and AF9C are
672 N1 NA-specific antibodies. Z2B3 is a N9 and N1 NA-cross-reactive antibody. Sheep anti-
673 H1N1pdm09 N1 (A/California/07/2009) serum was used as a positive anti-N1 NA control.
674 A) ELLA IC_{50} values of anti-N1 mAbs shown as titrating from 1 mg/ml on left Y-axis to
675 compare with sheep sera and the 50% inhibitory concentration as ng/ml shown on right Y-
676 axis. Each point represents an independent measurement. Geometric mean and standard
677 deviation are shown.
678 B-J) NA Inhibition curves for H1N1 viruses from year 1934 to 2019. Note the loss of titre of
679 mAbs: mAb Z2B3 on viruses isolated after 2014 (F, G, I, J) most likely due to K432E; mAb
680 AG7C likely due to G249E/R (I, J); and mAb AF9C due to D199N (J). Experiments were
681 done at least three times. Representative graphs are shown, with mean and standard
682 deviation (n=2) of each point.

683

684

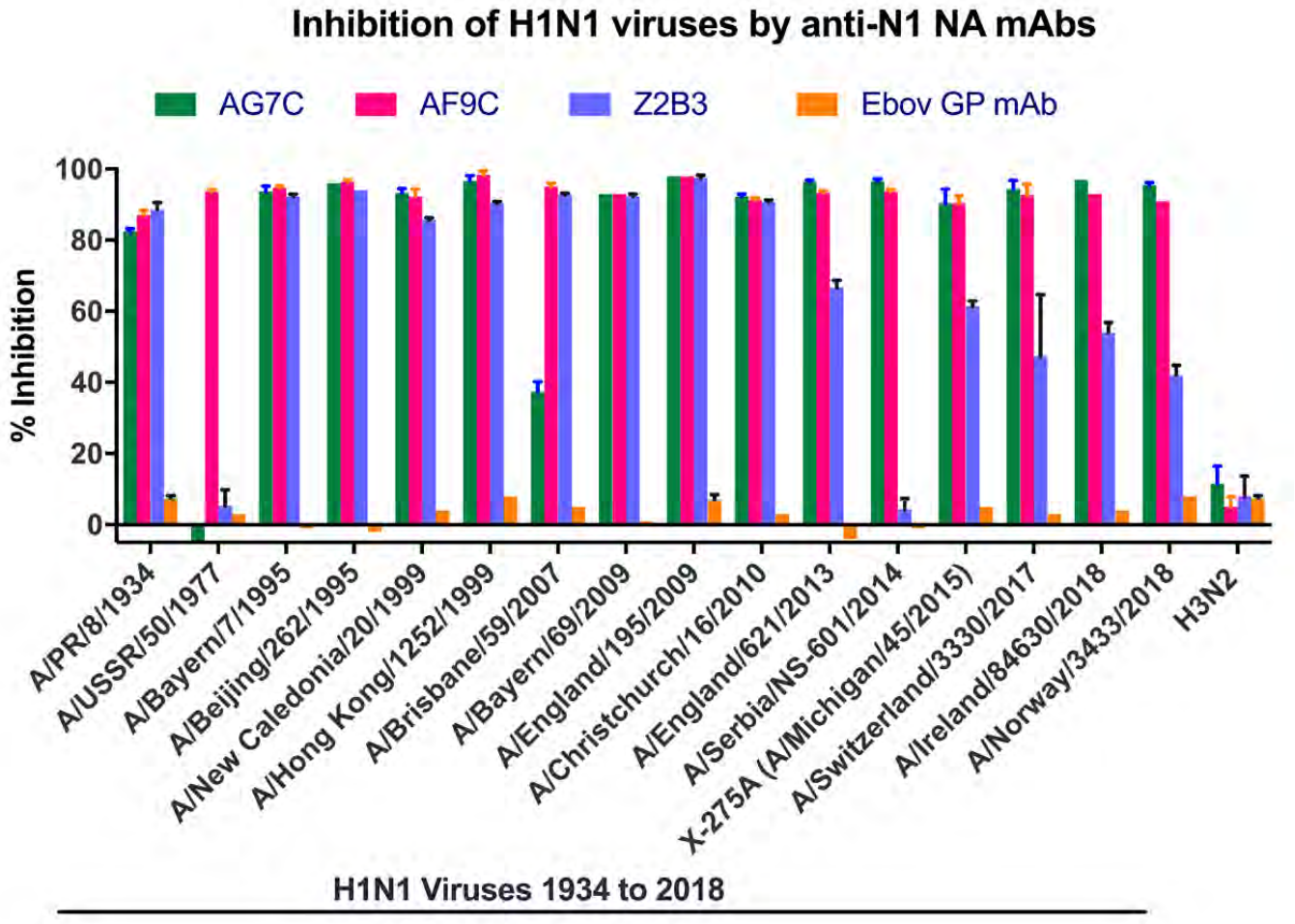
685 Figure 3. **Antibodies (1mg/ml) titrated against recombinant NA proteins by ELLA**.
686 Sheep sera raised against H1N1pdm09 (A/California/07/2009) and H7N9 (A/Anhui/1/2013)
687 viruses were used as controls. Each point represents an independent measurement.
688 Geometric mean and standard deviation are shown.

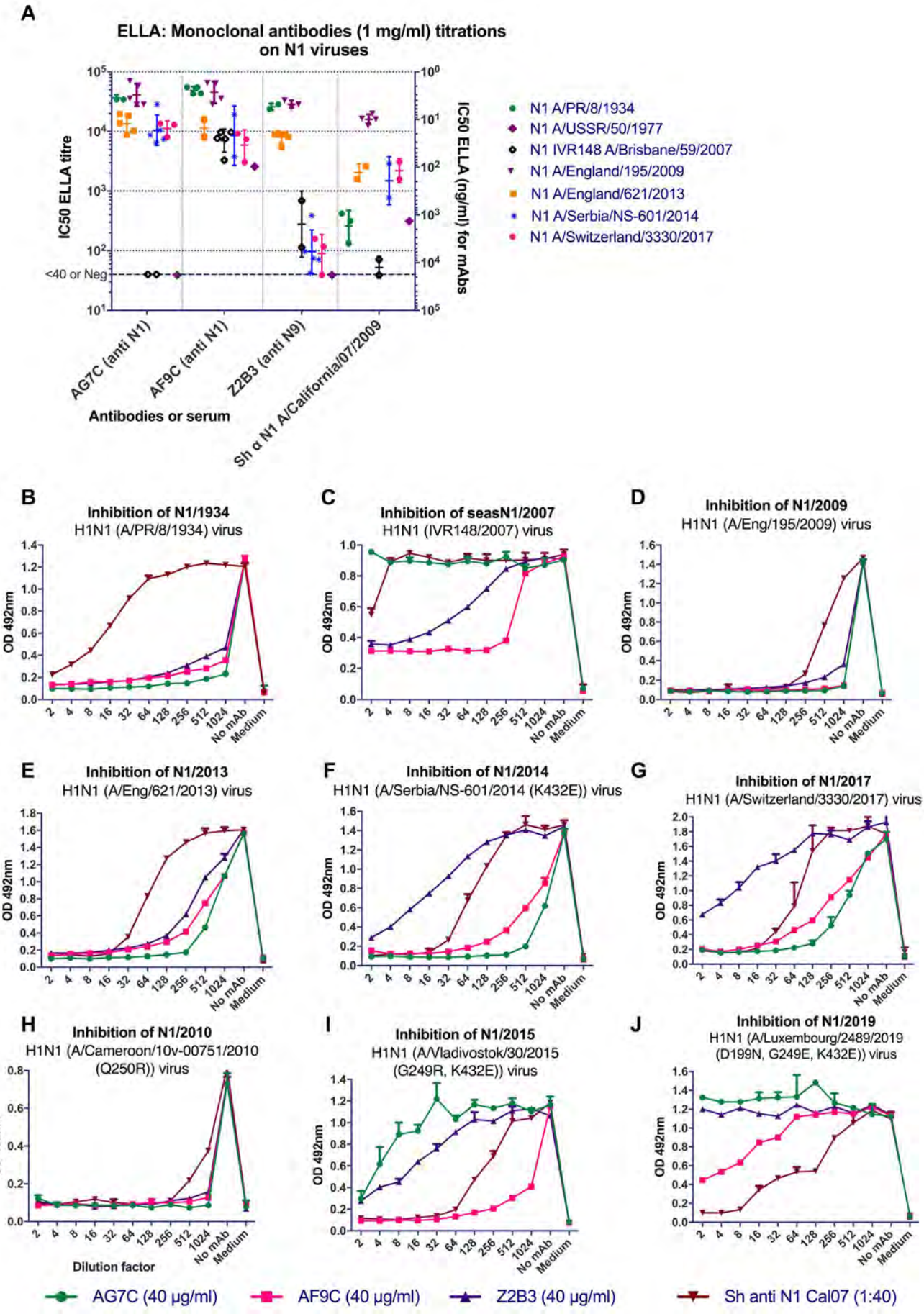
689

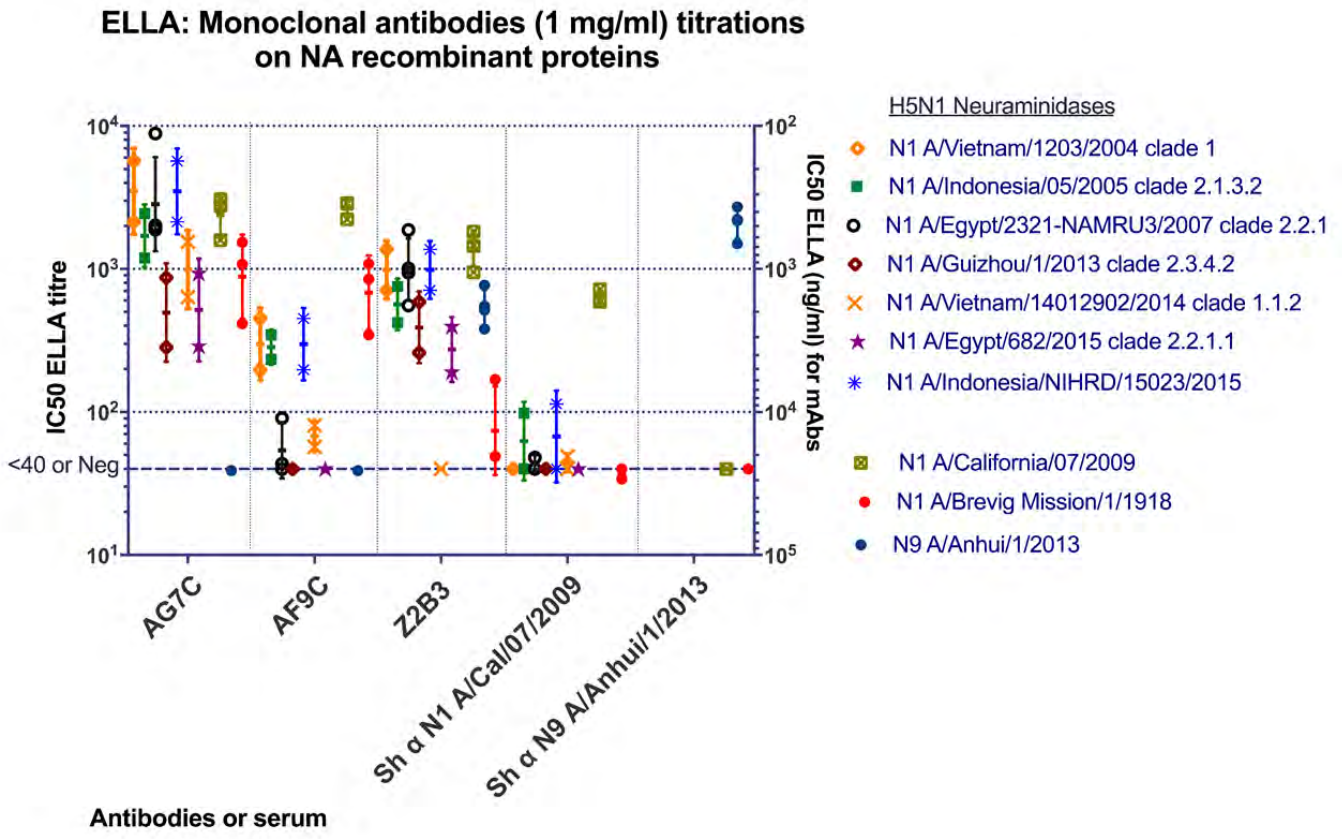
690

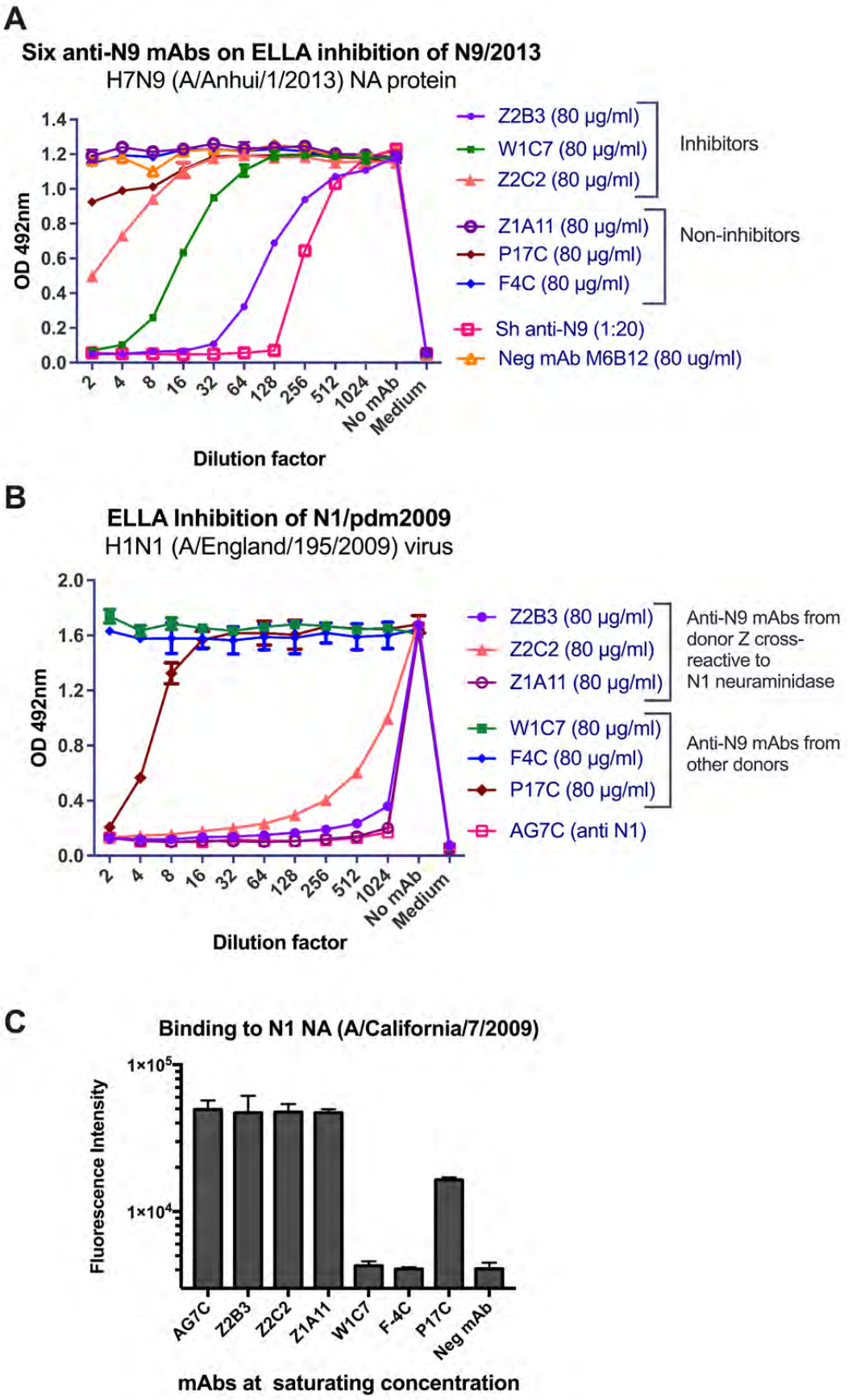
691 Figure 4. **Inhibition of NA activity by mAbs isolated from donors exposed to H7N9**
692 **virus**. A) ELLA activity of six anti-N9 antibodies on N9 NA (A/Anhui/1/2013). Sheep serum
693 raised against H7N9 virus (A/Anhui/1/2013) acts as a positive control. Anti-N2 NA mAb
694 M6B12 was used as a negative control. Experiments were performed at least twice, and
695 representative graphs are shown, with mean and standard deviation (n=2) of each point.
696 B) Cross-inhibition of N1 NA by some anti-N9 NA mAbs. Anti N1-NA mAb (AG7C) is a
697 positive control. Experiments were performed at least twice, and representative graphs are
698 shown, with mean and standard deviation (n=2) of each point.
699 C) Binding of anti-N9 NA mAbs to H1N1 (X-179A A/California/7/2009) infected MDCK-SIAT
700 cells. Experiments were performed at least twice, and a representative graph is shown with
701 mean and standard deviation.
702
703
704
705 Figure 5. **In vivo prophylactic protection by anti-N1 NA mAbs.** Mice (n=6/group) were
706 administered AG7C and AF9C mAbs at 10 mg/Kg. Weight loss following infection was
707 measured and ≅20% loss was considered as the predefined endpoint. Anti-H1 HA mAb (T1-
708 3B) cross-reactive with X-179A and A/PR/8/1934 viruses, is a positive control and an anti-N2
709 NA specific mAb (M6B12) a negative control. (A, B) Anti-N1 NA mAbs protect BALB/c
710 female mice completely against 10^4 $TCID_{50}$ of A/PR/8/1934 virus, without any weight loss
711 ($p<0.001$). Experiments were performed at least twice and representative data from
712 individual experiments are shown here.
713 (C,D) Anti-N1 NA mAbs protect DBA/2 female mice completely against a lethal dose (~150
714 LD_{50}) of X-179A virus (A/California/7/2009), with only 5-10% weight loss ($p<0.001$). One
715 mouse treated with AG7C relapsed on day 7 and was culled after losing ≅20% weight.
716 (E,F) Prophylactic protection against A/PR/8/1934 virus with N1 mAbs was compared with
717 their LALA-PG variants in BALB/c mice (n=6).
718

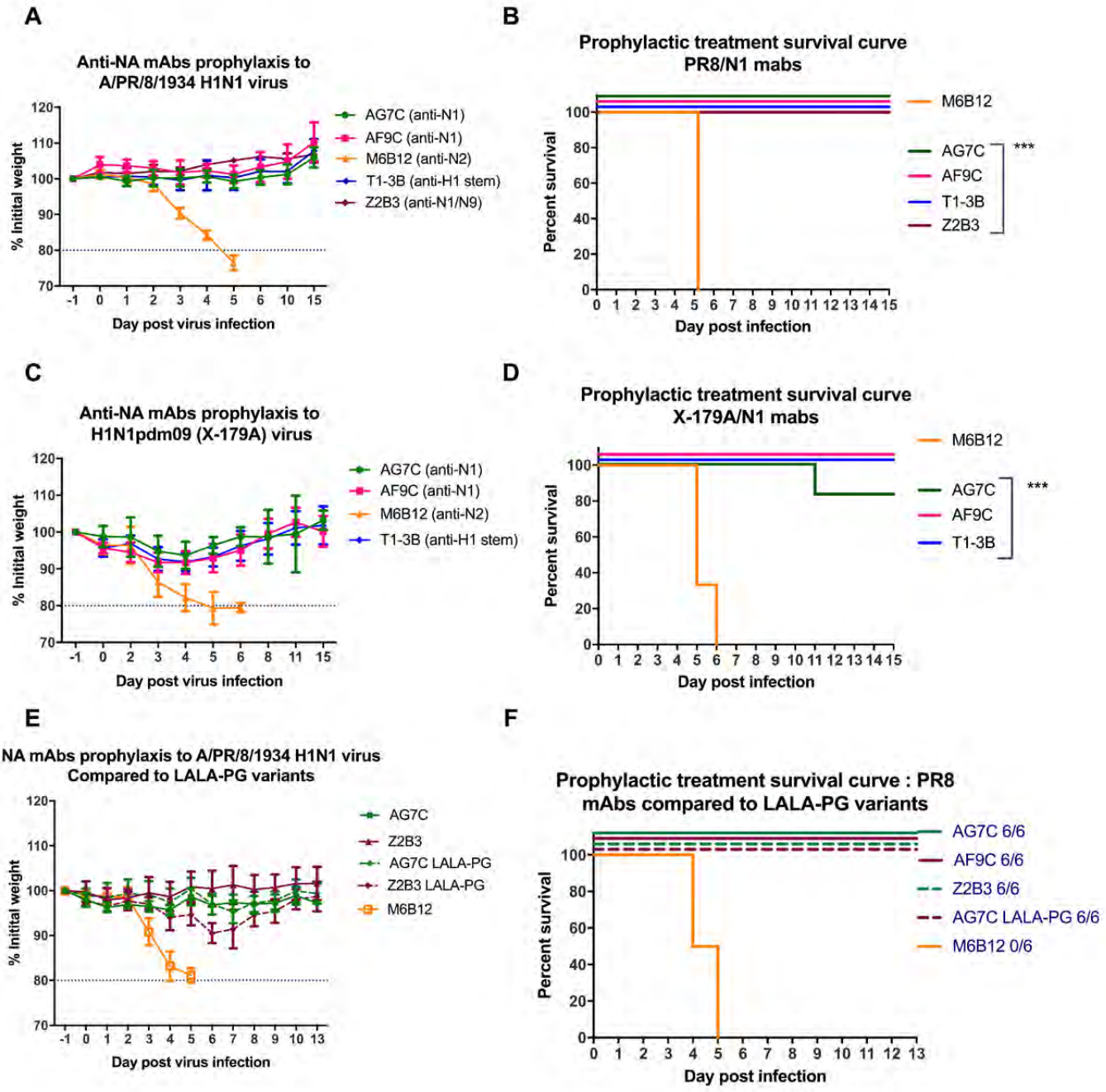
719 Figure 6. **Inhibition of N1 NAs A/England/195/2009 (A) and A/PR/8/1934 (B)** by anti-N1
720 mAbs and their LALA-PG variants on ELLA assays. Experiments were performed at least
721 twice, and a representative graph is shown with mean and standard deviation.
722
723
724 Figure 7. **Comparisons of conserved and variable surface residues between NA**
725 **subtypes.**
726 A) Conserved molecular surface shown in green between H1N1pdm09 A/California/07/2009
727 and H1N1 A/PR/8/1934 (PDB 4B7J).
728 B) Conserved molecular surface shown in green between H1N1pdm09
729 (A/California/07/2009) and H7N9 (A/Anhui/1/2013) (PDB 4B7J).
730 C) Difference in molecular surface (shown in white) between H1N1pdm09 viruses
731 A/England/621/2013 and A/Serbia/NS-601/2014
732 D) Key amino acid substitutions in two H1N1 virus NAs that mAb AG7C inhibits poorly -
733 A/USSR/50/1997 (shown in blue) and A/Brisbane/59/2007 (shown in red) compared to NA of
734 H1N1pdm09. These amino acid positions were inferred from the NA sequences alignment in
735 Figure S2. G249 and Q250P are likely to form part of the binding footprint of mAb AG7C.
736 Images were generated with Pymol2 (Schrodinger LLC).

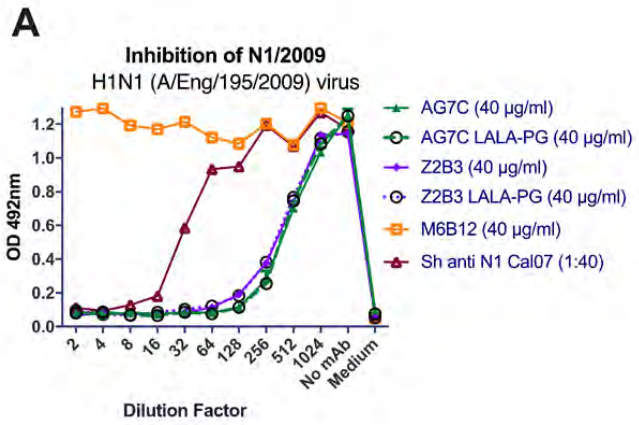


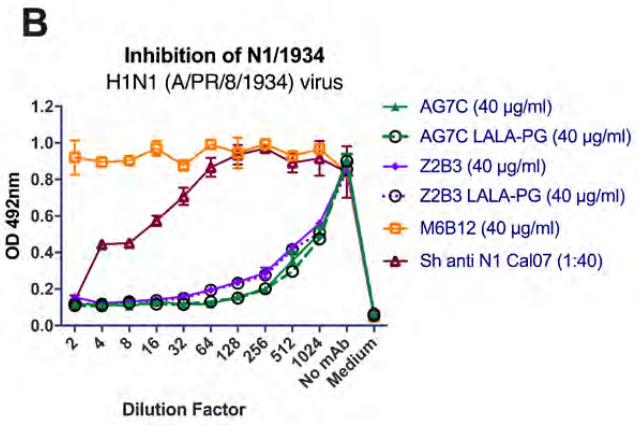






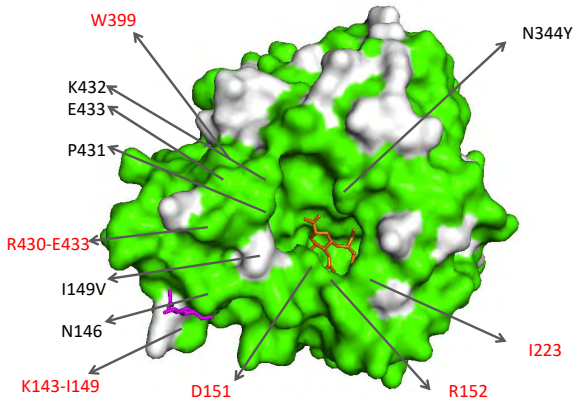






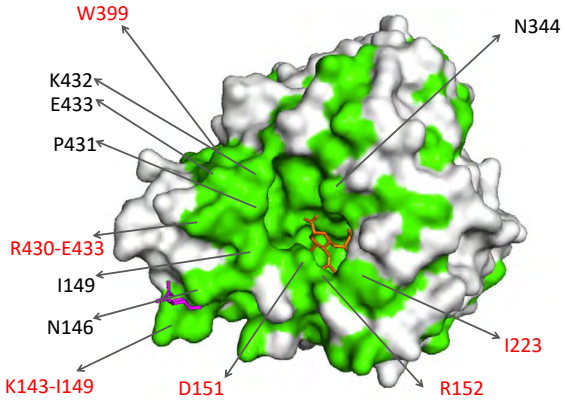
A

Conservation between
N1 (A/California/07/2009) and N1 (A/PR/8/1934)



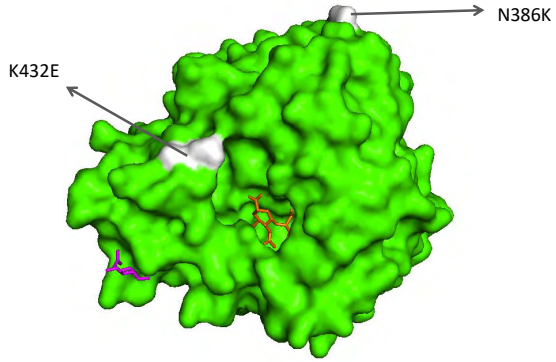
B

Conservation between
N1 (A/Cal/07/2009) and N9 (A/Anhui/1/2013)



C

pN1 PDB 4B7J showing substitutions in recent Clade 6B.1 Viruses
A/England/621/2013 v A/Serbia/NS-601/2014



D

pN1 PDB 4B7J showing substitutions in A/USSR/50/97
(blue) and A/Brisbane/59/07 (red)

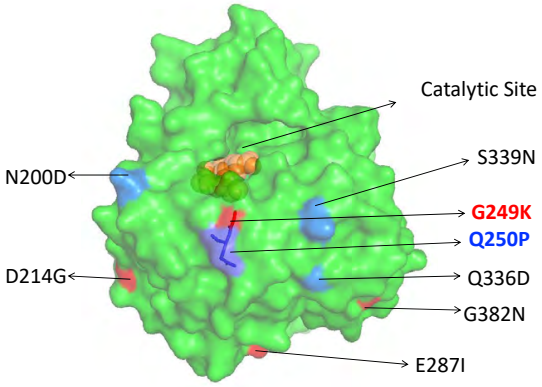


Table 1. List of donors and anti-NA antibodies isolated

Donor	**Age, Gender & Collection year**	**Antigen exposure**	**Antibodies isolated**	**mAb Specificity**
Donor C	23, Male, 2014	2014/15 inactivated TIV (AdimFlu-S)	AG7C, AF9C	Specific to N1 NA
Donor Z	6, Male, 2013	Mild H7N9 infection	Z2B3, Z2C2, Z1A11	Cross-reactive to N1 and N9 NAs
Donor W	7, Female, 2013	Severe H7N9 infection 2013	W1C7	N9 NA
Donor K	39, Male, 2014	Severe H7N9 infection 2013	P17C, F4C	N9 NA, Weak N1

Table 2. Encoding gene analysis of antibodies

		Heavy Chain							Light Chain						
Mab	**Donor; Age**	**V-GENE and allele**	**J-GENE and allele**	**D-GENE and allele**	**V-REGION identity %**	**V-REGION Nb of AA changes**	**CDR-lengths**	**AA JUNCTION**		**V-GENE and allele**	**J-GENE and allele**	**V-REGION identity %**	**V-REGION Nb of AA changes**	**CDR lengths**	**AA JUNCTION**
AG7C	C; 23	4-31*03, *06	4*01, or 4*02	5-24*01	89.0	15	10.7.11	CARDLEGHTFHDW	κ	1-39*01, or 1D-39*01	2*01	88.5	17	6.3.9	CQQSHSAPYTF
AF9C	C; 23	1-69*01, or 1-69D*01	6*02	4-17*01	91.7	16	8.8.19	CARDLAPYGDRFYFHYGMDVW	κ	1-9*01	5*01	94.6	9	6.3.9	CQQLNNYPFTF
Z2B3	Z; 6	1-69*01, or 1-69D*01	6*02	5-18*01	96.5	8	8.8.25	CARDLQDTPMVDRIIGSYYYYNGLDVW	λ	2-14*01	2*01, or 3*01	96.9	8	9.3.10	CSSYTRSSSVVF
Z2C2	Z; 6	3-66*01, or *04	6*02	2-21*02	93.3	13	8.7.27	CASWSFCGGDCYPDRMQEKFHYSYGMDVW	κ	1D-12*01	4*01	95.7	9	6.3.9	CQQAYSFPLTF
Z1A11	Z; 6	1-46*01, *02 or *03	6*02	3-22*01	92.7	17	8.8.19	CARNSYYYDTDRPYYNGMDVW	κ	2-28*01, or 2D-28*01	5*01	96.9	5	11.3.9	CMQAVQTPRTF
W1C7	W; 7	3-9*01	3*02	4-17*01	99.7	0	8.8.13	CAKDVGGDYHAFDIW	κ	3-15*01	4*01	99.6	1	6.3.10	CQQYNNWPPLTF
P17C	K; 39	3-23*04	5*02	2-15*01	99.3	1	8.8.14	CAKDGRWLLGNWFDPW	λ	2-14*01	1*01	99.3	2	9.3.10	CSSYTSSSTFVF
F4C	K; 39	4-59*01	4*02	4-17*01	99.7	1	8.7.10	CARGYYGDYDYW	λ	1-40*01	2*01, or 3*01	100.0	0	9.3.11	CQSYDSSLSGVVF

Table 3. List of sequences of secreted NA proteins

Virus Identifier	**Neuraminidase sequence [Signal sequence - 6x His tag - Tetramerisation domain - Thrombin cleavage site - ectodomain (69 - 469, N1 numbering)]**
A/Vietnam/1203/2004 N1 HM006761.1	MKANLLVLLCALAAADAADPHHHHHHSSSDYSDLQRVKQELLEEVKKELQKVKEEIIEA FVQELRKRGSLVPRGSPSRSISNTNFLTEKAVASVKLAGNSSLCPINGWAVYSKDNSIRI GSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGEAP SPYNSRFESVAWSASACHDGTSWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQ ESECACVNGSCFTVMTDGPSNGQASHKIFKMEKGKVVKSVELDAPNYHYEECSCYPN AGEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGVFGDNPRPNDGTGSCGPVSSN GAYGVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGWTETDSSFSVKQDIVAITD WSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWTSGSSISFCGVNSDTVGWS WPDGAELPFTIDK*
A/Indonesia/05/2005 N1 EU146623.1	MKANLLVLLCALAAADAADPHHHHHHSSSDYSDLQRVKQELLEEVKKELQKVKEEIIEA FVQELRKRGSLVPRGSPSRSISNTNPLTEKAVASVTLAGNSSLCPIRGWAVHSKDNNIRI GSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGEAP SPYNSRFESVAWSASACHDGTSWLTIGISGPDNEAVAVLKYNGIITDTIKSWRNDILRTQ ESECACVNGSCFTVMTDGPSNGQASYKIFKMEKGKVVKSVELDAPNYHYEECSCYPD AGEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGVFGDNPRPNDGTGSCGPMSP NGAYGVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGWTGTDSSFSVKQDIVAITD WSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWTSGSSISFCGVNSDTVSWS WPDGAELPFTIDK*
A/Egypt/2321-NAMRU3/2007 N1 EF535822.1	MKANLLVLLCALAAADAADPHHHHHHSSSDYSDLQRVKQELLEEVKKELQKVKEEIIEA FVQELRKRGSLVPRGSPSRSISNTKFLTEKAVASVTLAGNSSLCPISGWAVYSKDNSIRI GSRGDVFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGEAP SPYNSRFESVAWSASACHDGTSWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQ ESECACVNGSCFTVMTDGPSSGQASYKIFKMEKGKVVKSVELDAPNYHYEECSCYPD AGEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGVFGDNPRPNDGTGSCGPVFPN GAYGVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGWTGTDSSFSVKQDIVAITD WSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWTSGSSISFCGVNSDTVSWS WPDGAELPFTIDK*
A/Guizhou/1/2013 N1 EPI420387	MKANLLVLLCALAAADAADPHHHHHHSSSDYSDLQRVKQELLEEVKKELQKVKEEIIEA FVQELRKRGSLVPRGSPSRSIRNTNFLTENAVASVTLAGNSSLCPIRGWAVHSKDNSIRI GSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGEAP SPYNSRFESVAWSASACHDGTSWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQ ESECACVNGSCFTVMTDGPSDGQASYKIFKMEKGKVVKSVELNAPNYHYEECSCYPD AGEIICVCRDNWHGSNRPWVSFNQNLEYQIGYICSGVFGDNPRPNDGTGSCGPVSPN GAYGIKGFSFKYGNGVWIGRTKGTNSRSGFEMIWDPNGWTGTDSDFSVKQDIVATTD WSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWTSGSSISFCGVNSDTVSWS WPDGAELPFTIDK*
A/Vietnam/14012902/2014 N1 EPI624924	MKANLLVLLCALAAADAADPHHHHHHSSSDYSDLQRVKQELLEEVKKELQKVKEEIIEA FVQELRKRGSLVPRGSPSRSISNTNFHTEKAVVSAKLAGNSSLCPINGWAVYSKDNSIR IGSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTAKDRSPHRTLMSCPVGEA PSPYNSRFESVAWSASACHDGTSWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRT QESECACVNGYCFTVMTDGPSNGQASHKIFKMEKGKVVKSVELDAPNYHYEECSCYP DAGEITCVCRDNWHGSNRPWISFNQNLEYQIGYICSGVFGDNPRPNDGKGSCGPVSS NGAYGVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGWTETDSSFSVKQDIVAITD WSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKEGTIWTSGSSISFCGVSGDTVGWS WPDGAELPFTIDK*

A/Egypt/682/2015 N1 EPI642538	MKANLLVLLCALAAADAADPHHHHHHSSSDYSDLQRVKQELLEEVKKELQKVKEEIIEA FVQELRKRGSLVPRGSPSRSISNTKFLAEKAVASVTLAGNSSLCPVSGWAVYSKDNSIR IGSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPHRTLMSCPVGEA PSPYNSRFESVAWSASACHDGTSWLTIGISGPDSGAVAVLKYNGIITDTIKSWRNNIMR TQESECACVNGSCFTIMTDGPSSGQASYKIFKMEKGKVIKSVELDAPNYHYEECSCYP DAGEITCVCRDNWHGSNRPWISFNQNLEYQIGYICSGVFGDNPRPNDGTGSCGPVFP NGAYGVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGWTGTDSSFSVKQDIVAITE WSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWTSGSSISFCGVNGDTVSWS WPDGAELPFTIDK*
A/Indonesia/NIHRD/ 15023/2015 N1 EPI643070	MKANLLVLLCALAAADAADPHHHHHHSSSDYSDLQRVKQELLEEVKKELQKVKEEIIEA FVQELRKRGSLVPRGSPSRSTSNNNPLTEKTVASVTLAGNSSLCHTRGWAVHSKDNNI RIGSKGDVFVIREPFISCSHLECRTFFLTHGALLNDKHSNGTVKDRSPHRTLMSCPLGE APSPYNSRFESVAWSASACHDGTSWLTIGISGPDNEAVAVLKYNGIITDTIKSWRNNIM RTQESECVCVNGSCFVVVTDGPSNGQASYKIFKMKKGKVVKSVELDAPNYHYEECSC YPDAGEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGVFGDNPRPNDGTGSCGP MSSNGAYGVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGWTETDSSFSVKQDIV AITDWSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWTSGSSISFCGVNSDTV SWSWPDGAELPFIIDK*
A/Cal/07/2009 N1 FJ981613.1	MKANLLVLLCALAAADAADPHHHHHHSSSDYSDLQRVKQELLEEVKKELQKVKEEIIEA FVQELRKRGSLVPRGSPSRSISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSV RIGSKGDVFVIREPFISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEV PSPYNSRFESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRT QESECACVNGSCFTVMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPD SSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGDNPRPNDKTGSCGPVSSN GANGVKGFSFKYGNGVWIGRTKSISSRNGFEMIWDPNGWTGTDNNFSIKQDIVGINEW SGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKENTIWTSGSSISFCGVNSDTVGWSW PDGAELPFTIDK*
A/Brevig Mission/1/2018 N1 AF250356.2	MKANLLVLLCALAAADAADPHHHHHHSSSDYSDLQRVKQELLEEVKKELQKVKEEIIEA FVQELRKRGSLVPRGSPSRSISNTNVVAGQDATSVILTGNSSLCPISGWAIYSKDNGIRI GSKGDVFVIREPFISCSHLECRTFFLTQGALLNDKHSNGTVKDRSPYRTLMSCPVGEAP SPYNSRFESVAWSASACHDGMGWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRT QESECACVNGSCFTIMTDGPSNGQASYKILKIEKGKVTKSIELNAPNYHYEECSCYPDT GKVMCVCRDNWHGSNRPWVSFDQNLDYQIGYICSGVFGDNPRPNDGTGSCGPVSSN GANGIKGFSFRYDNGVWIGRTKSTSSRSGFEMIWDPNGWTETDSSFSVRQDIVAITDW SGYSGSFVQHPELTGLDCMRPCFWVELIRGQPKENTIWTSGSSISFCGVNSDTVGWS WPDGAELPFSIDK*
A/Anhui/1/2013 N9	The protein was kindly provided by Donald Benton (Benton et al., 2017)