

BRIEF DEFINITIVE REPORT

Identification of a seasonal influenza vaccine-induced broadly protective neuraminidase antibody

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Seasonal influenza viruses cause significant global illness and death annually, and the potential spillover of avian H5N1 poses a serious pandemic threat. Traditional influenza vaccines target the variable hemagglutinin (HA) protein, necessitating annual vaccine updates, while the slower-evolving neuraminidase (NA) presents a promising target for broader protection. We investigated the breadth of anti-NA B cell responses to seasonal influenza vaccination in humans. We screened plasmablast-derived monoclonal antibodies (mAbs) from three donors, identifying 11 clonally distinct NA mAbs from 268 vaccine-specific mAbs. Among these, mAb-297 showed exceptionally broad NA inhibition, effectively protecting mice against lethal doses of influenza A and B viruses, including H5N1. We show that mAb-297 targets a common binding motif in the conserved NA active site. Our findings show that while B cell responses against NA following conventional, egg-derived influenza vaccines are rare, inducing broadly protective NA antibodies through such vaccination remains feasible, highlighting the importance of improving NA immunogens to develop a more broadly protective influenza vaccine.

Introduction

Influenza viruses pose a persistent threat due to their high mutation rates and the significant morbidity and mortality they cause globally, with an estimated 290,000–650,000 deaths annually (Iuliano et al., 2018). The two major influenza virus surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) play crucial roles in the viral replication cycle. HA facilitates viral entry into host cells through binding to sialic acids on the surface of the host cell, and NA aids in the release of progeny viruses by cleaving sialic acid residues. Despite the importance of both glycoproteins, currently licensed seasonal influenza vaccines primarily target the immunodominant head domain of HA, which undergoes frequent antigenic drift. This high mutation rate limits the long-term effectiveness of seasonal influenza vaccines, necessitating biannual updates to vaccine formulations to match circulating strains. However, NA exhibits slower

antigenic drift and evolves independently of HA (Kilbourne et al., 1990). This suggests that NA-based immunogens could help broaden protection against drifted strains and emerging zoonotic strains with novel HAs, including avian and bovine H5N1, avian H7N2 and H9N2, and, to a lesser extent, avian strains with heterosubtypic NAs, such as H7N9. NA-antibody responses have been shown to correlate with protection and reduction of viral shedding in animal models and humans independent of HA responses (Couch et al., 2013; McMahon et al., 2019, 2023; Memoli et al., 2016; Monto et al., 2015; Tan et al., 2022).

Characterization of broadly protective monoclonal antibodies (mAbs) targeting NA demonstrates that NA is a promising target for next-generation influenza vaccines (Chen et al., 2018; Gilchuk et al., 2019; Hansen et al., 2023; Jiang et al., 2020;

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Lederhofer et al., 2024; Lei et al., 2023; Madsen et al., 2020; Momont et al., 2023; Stadlbauer et al., 2019; Yasuhara et al., 2022; Zhu et al., 2019). These NA-mAbs exhibit broad protective capabilities by either inhibiting the enzymatic activity of NA through direct binding to the active site or by sterically blocking access. Additionally, they can engage fragment crystallizable-dependent effector functions such as antibody-dependent cell-mediated cytotoxicity (Wohlbold et al., 2017). Notably, the human mAbs 1G01 (Stadlbauer et al., 2019) (isolated from H3N2 infection), DA03E17 (Yasuhara et al., 2022) (isolated from H1N1 infection), and FNI9 (Momont et al., 2023) (isolated from baseline screenings) have demonstrated broad protection in mice across influenza A and B virus strains (pan-NA mAbs) by direct binding to the NA active site, highlighting the potential use of NA as a universal influenza vaccine immunogen.

Most previously characterized NA-mAbs have been isolated after infection, and no vaccine-induced pan-NA mAbs have been reported so far. This disparity may be attributed to the generally weak NA response induced by licensed influenza vaccines, which are optimized to induce a robust HA response without a standardized amount of NA required for licensure. Notably, a previous study isolating NA-mAbs after influenza virus infection or vaccination found that NA-specific plasmablasts accounted for a quarter of the total plasmablast response to H3N2 infection compared with only 1–2% of the plasmablast response to vaccination with subunit or split virus inactivated influenza vaccine (IIV) (Chen et al., 2018). Additionally, the conformational stability of the NA tetramer, which is important for NA immunogenicity and NA active site antibody binding (McMahon et al., 2020), is poorly retained in IIVs compared with its native transmembrane form during infection (Ellis et al., 2022).

This study describes a vaccine-induced pan-NA mAb that targets the NA active site following vaccination with a standard dose (0.5 ml intramuscularly) of Fluarix Quadrivalent 2022–2023 Northern Hemisphere seasonal inactivated influenza vaccine. This was a subunit vaccine purchased from GlaxoSmithKline Biologicals containing 15 µg of HA and an unquantified amount of NA from each of the following viruses: A/Wisconsin/588/2019 (H1N1) pdm09-like virus, A/Darwin/9/2021 (H3N2)-like virus, B/Austria/1359417/2021 (B/Victoria/2/87-like lineage)-like virus, and B/Phuket/3073/2013 (B/Yamagata/16/88-like lineage)-like virus. This mAb demonstrated broad binding across Group 1 and 2 NAs from human and avian influenza A viruses and influenza B virus NAs, spanning 80 years of antigenic drift. Additionally, it demonstrated potent inhibition of NA activity in vitro and conferred protection against both influenza A and B virus strains in vivo. Our findings confirm the feasibility of inducing highly functional, broadly protective NA mAbs through vaccination, demonstrating the potential of targeting NA for next-generation influenza vaccine development. Furthermore, we identified key binding motifs shared among both infection-induced and vaccine-induced mAbs targeting the NA active site, suggesting a common mechanism of NA recognition and inhibition. These findings offer insights into the potential benefits of utilizing these conserved NA interaction sites for universal influenza vaccine efforts.

Results and discussion

Seasonal influenza vaccination induces rare NA-specific plasmablasts in humans

Influenza viruses display two major glycoproteins on their surface: the HA and the NA. The development of influenza vaccines has historically focused on targeting the HA antigen due to its central role in inducing protective antibody responses that block viral attachment to host cells. However, NA's relatively conserved antigenic properties suggest its potential use as a target for broader protection. To investigate NA-directed B cell responses following seasonal influenza vaccination, we analyzed peripheral blood mononuclear cells (PBMCs) and draining axillary lymph node fine needle aspiration (FNA) samples from three healthy adults, referred to as Donor A, B, and C (Fig. 1 A). These individuals were part of a larger study characterizing the HA-specific germinal center (GC) response to influenza vaccination (Matz et al., 2024, Preprint). A total of 1,396 clonally distinct mAbs had previously been expressed from the plasmablast (PB), GC B cell, and lymph node plasma cell (LNPC) compartments of these individuals ($n = 491$ from Donor A; $n = 412$ from Donor B; $n = 493$ from Donor C). By screening all 1,396 expressed mAbs against recombinant N1 (A/Auckland/1996) and N2 (A/Hong Kong/1073/1999) proteins, we detected 11 clonally distinct NA-mAbs ($n = 4$ mAbs from Donor A; $n = 7$ from Donor B; $n = 0$ from Donor C) (Fig. 1, B and C). We next overlaid the NA-binding data on the B cell subtypes transcriptionally identified by single-cell RNA sequencing (scRNA-seq) of blood and FNA samples from Donors A and B (Fig. 1 D). The scRNA-seq analysis yielded a total of 29,912 heavy and light chain pairs for Donor A and 35,872 pairs for Donor B. The 4 NA-mAbs from donor A, along with a total of 38 HA-mAbs isolated from this donor, accounted for 8.6% of the 491 successfully expressed, clonally distinct mAbs screened from this subject, representing 656 clonally related B cells from the scRNA-seq dataset ($n = 29$ NA-binding and $n = 627$ HA-binding). From Donor B, the 7 NA-mAbs and 165 HA-mAbs accounted for 41.7% of the expressed mAbs, representing 1,992 B cells ($n = 66$ NA-binding and $n = 1926$ HA-binding).

Notably, mAb-297 from Donor B, which originated from the V_H1-69 germline, exhibited the largest clonal expansion among the NA-specific clones, comprising 30 cells (fivefold higher than the median NA-specific clone size) and accounting for 45% (30/66) of the NA-binding B cells detected in this individual (Fig. 1 E). The clone represented by mAb-297 was exclusively found in the week-1 PB compartment, with no clonal overlap of mAb-297 detected in the donor's pre- or 26-wk post-vaccination memory B cell (MBC) compartments (Table S1). The alignment of the heavy chain sequences of mAb-297 revealed 11/288 (3.8%) nucleotide differences from the V_H1-69 germline gene (Fig. 1 F). We further characterized the binding specificities of these 11 NA-mAbs against a panel of 26 different NA proteins, including 11 influenza virus NAs with avian origin (Fig. 1 G). The majority (6/11 mAbs) bound broadly within the N2 subtype, including avian-origin N2s from A/shorebird/Delaware/127/1997 (H6N2), A/turkey/Wisconsin/1/1966 (H9N2), and A/Hong Kong/1073/99 (H9N2). The mAbs 117 and 297 displayed particularly broad binding, cross-binding influenza A and B virus NAs. The mAb-

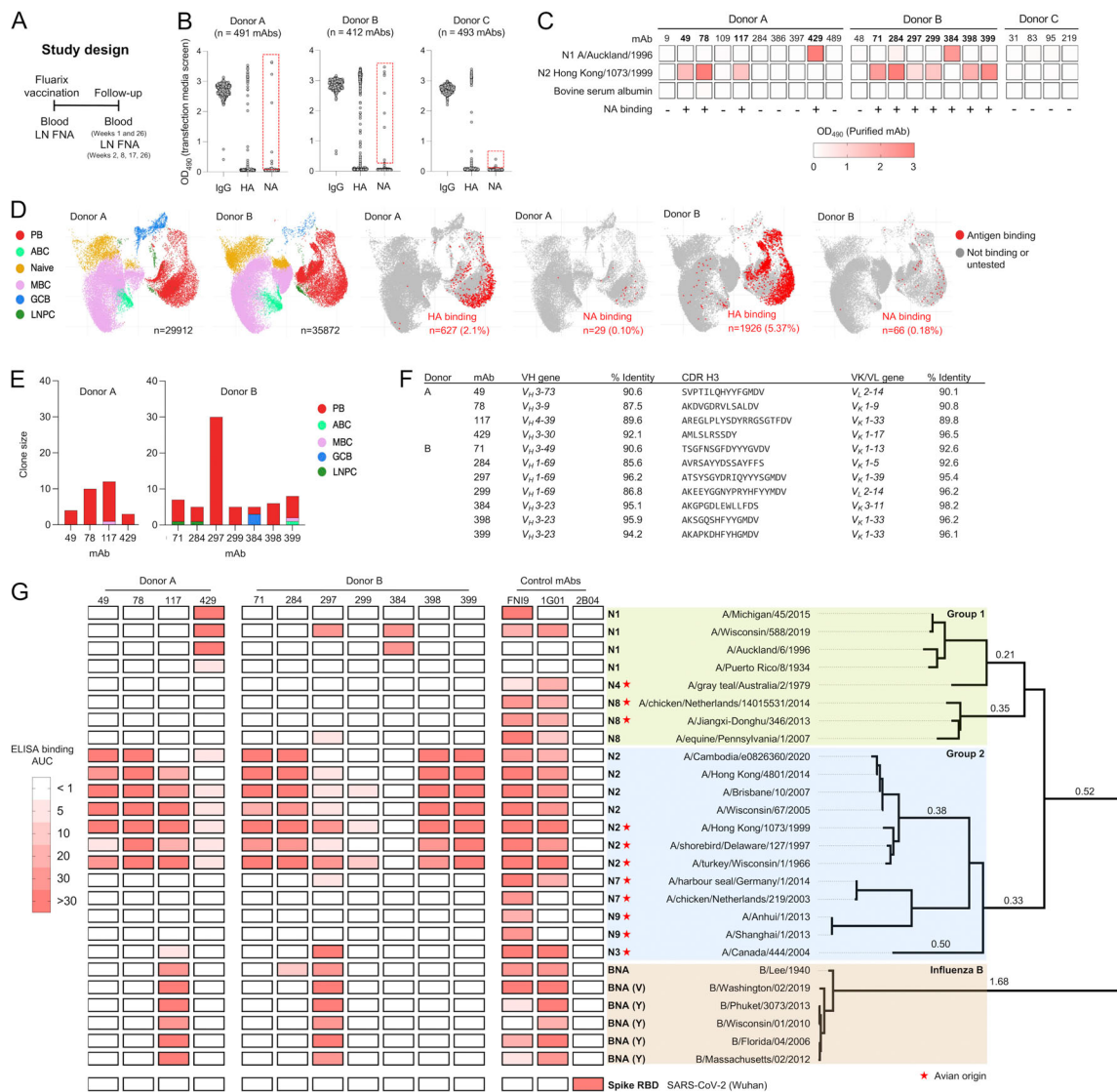


Figure 1. Identification and characterization of vaccine-induced NA-antibodies. (A) Study design. Three healthy adults (Donors A–C) aged 24–36 received the inactivated influenza vaccine Fluarix intramuscularly. Blood was collected before vaccination and at 1, 2, 8, 17, and 26 wk after vaccination. FNAs of ipsilateral axillary lymph nodes were collected before vaccination and at 2, 8, 17, and 26 wk after vaccination. (B) A total of 1,396 clonally distinct mAbs were expressed and tested for antigen specificity by screening the transfection media by ELISA against pooled HA proteins from A/Wisconsin/588/2019 (H1), A/Darwin/6/2021 (H3), B/Phuket/3073/2013, and B/Austria/1359417/2021, and against pooled NA proteins from A/Auckland/6/1996 (N1) and A/Hong Kong/1073/1999 (N2). A donkey anti-human IgG control was used to test for successful mAb expression. Red boxes indicate transfection media with detectable NA binding that was selected for purification. (C) The purified mAbs with detectable NA binding were screened against each NA protein to verify binding, resulting in $n = 11$ NA positive mAbs ($n = 4$ Donor A; $n = 7$ Donor B; $n = 0$ Donor C) indicated in bold. Bovine serum albumin was used as a negative control. (D) UMAP plots from donors A and B showing transcriptional clusters of B cells identified in blood and FNAs (all time points combined). Each dot represents a single cell. ABC, activated B cells; Naive, naïve B cells; GCB, germinal center B cells. Total number of cells is shown at the bottom right. The four rightmost plots indicate HA and NA positive cells in red, with the number and proportion (%) of total cells shown at the bottom right. (E) Bar graphs showing all distinct B cell clones represented by all NA-mAbs identified in Donors A and B. B cell clones were identified via the same V_H and V_K/V_L gene usage and similar CDR H3 sequences. The y-axis indicates the total number of paired heavy- and light-chain sequences analyzed, with colors matching B cell subsets from panel C. (F) Immunoglobulin gene usage, percentage identity to germline, and CDR H3 sequences of identified NA-mAbs. (G) Heat map showing binding of NA-mAbs to recombinant NA proteins. The area under the curve (AUC) from ELISA is shown. Phylogenetic tree of NA proteins with branch length indicated. Proteins from virus strains with avian origin are indicated with red stars. Control mAbs 1G01 and FNI-9 are known pan-NA binders (Momont et al., 2023; Stadlbauer et al., 2019); 2B04 binds to SARS-CoV2 RBD and serves as a negative control mAb (Alsoussi et al., 2020). (V), B/Victoria/2/87-like; (Y), B/Yamagata/16/88-like.

297 was the most broadly crossbinding mAb, binding NAs from all three phylogenetic groups (Group 1, Group 2, and influenza B virus NA), including animal-origin N2, N3, N7, and N8 proteins and the ancestral influenza virus B/Lee/1940 NA.

While these findings highlight the broad crossreactivity of the 11 NA clones identified, it is important to acknowledge the potential bias introduced by our screening method. We used two heterologous NA antigens (A/Auckland/6/1996 N1 and A/Hong Kong/1073/1999 N2) to test for NA binding, which may have

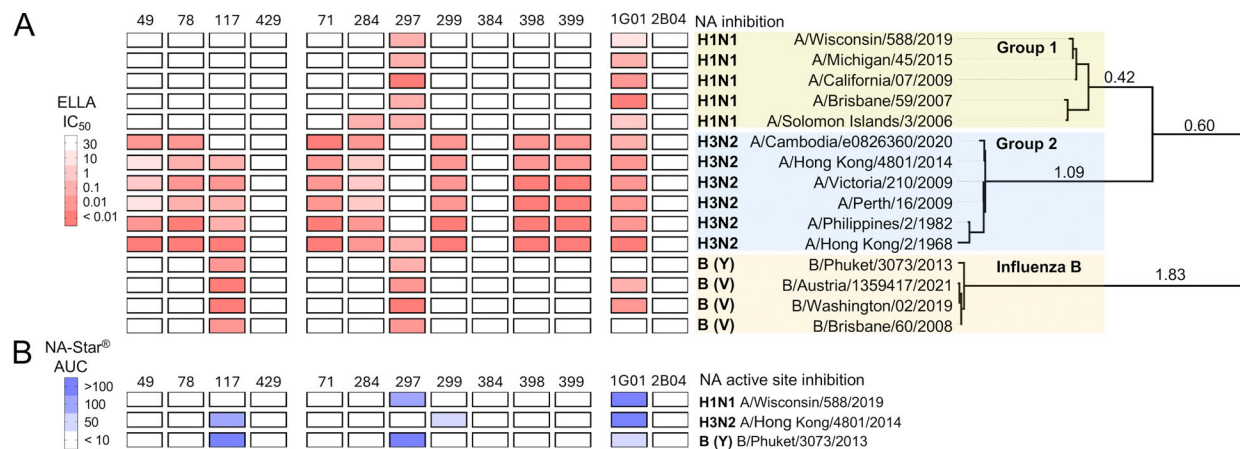


Figure 2. **Functional characterization of NA-mAbs in vitro.** (A) Heat map showing the NA-inhibition of mAbs to various influenza virus strains, IC₅₀ measured by ELLA. The phylogenetic tree categorizes the virus strains by NA protein, with branch lengths indicated. Mabs 1G01 (pan-NA mAb) (Stadlbauer et al., 2019) and 2B04 (SARS-CoV-2 RBD-mAb) (Alsoussi et al., 2020) serve as controls. (B) Heat map displaying NA active site inhibition by NA-specific mAbs, assessed using the NA-Star assay. AUC values are shown. B (V), B/Victoria/2/87-like; B (Y), B/Yamagata/16/88-like.

missed influenza A virus vaccine strain-specific clones and influenza B virus NA-specific clones that do not crossbind with these antigens. As a result, our study could not directly compare the quantity of HA-activated and NA-activated plasmablasts after vaccination. Nevertheless, despite these limitations, the observed NA-to-HA B cell ratios of 1:22 (Donor A) and 1:30 (Donor B) were higher than the 1:87 ratio reported in an unbiased screening (Chen et al., 2018), suggesting that our screening method remained effective in identifying NA clones.

Vaccine-induced NA mAbs exhibit broad NA inhibition in vitro

We next assessed the functional activities of NA-specific mAbs derived from the vaccinated donors against a panel of influenza A and B virus strains (Fig. 2). The enzyme-linked lectin assay (ELLA), which measures the cleavage of sialic acid from the large sialylated glycoprotein fetuin, revealed that 9 out of 11 mAbs effectively inhibited NA enzymatic activity (Fig. 2 A). While most mAbs showed inhibition within their respective phylogenetic NA subtypes, mAbs 117 and 297 exhibited broader activity toward both influenza A and B viruses. Specifically, mAb-117 inhibited NAs from crosslineage influenza B virus and influenza A virus Group 2 NA strains, while mAb-297 demonstrated inhibition across influenza A virus Group 1 NAs and the ancestral N2 strain, A/Hong Kong/2/1968 (X-31), as well as cross-lineage influenza B viruses. The inhibition assay by ELLA appeared to be more sensitive to some NAs than the binding ELISA (such as for A/Michigan/45/2015 NA and A/Hong Kong/4801/2014 NA), likely due to the more stable conformation of NA in its native form in the ELLA compared with the recombinant proteins used in ELISA.

The mechanisms of inhibition detected by ELLA include mAbs either binding directly to the enzymatic site, thereby blocking its catalytic function, or binding outside the catalytic site and preventing access to fetuin through steric hindrance. In contrast, the NA-Star assay uses a much smaller substrate, thereby detecting mAbs that bind directly or close to the catalytic site. The mAbs 117 and 297 showed significant inhibition in the NA-Star assay, likely due to direct binding to the enzymatic site. The mAb-117

maintained inhibition of H3N2 and influenza B virus, while mAb-297 continued to inhibit H1N1 and influenza B virus (Fig. 2 B).

Vaccine-induced mAb-297 protects against human influenza A and B and avian H5N1 virus challenge in mice

The ability of a vaccine to induce crossprotective B cell responses is essential not only for combating seasonal antigenic drift but also for protecting against novel influenza virus strains that may emerge from zoonotic reservoirs. This includes the highly pathogenic avian H5N1 virus, the causative agent of the ongoing avian influenza virus outbreak in U.S. cattle (Caserta et al., 2024). Given mAb-297's broad crossreactivity identified in vitro, we assessed its in vivo protective efficacy against human seasonal influenza viruses and the highly pathogenic avian H5N1 strain. To evaluate this, a murine challenge study was conducted in BALB/c mice infected with 5 × 50% lethal dose (LD₅₀) of influenza virus. In a prophylactic setting, mAb-297 was administered at doses of 10, 3, 1, 0.3, and 0.1 mg/kg (*n* = 5 mice per dose tested) 2 h before infection, with weight loss and survival monitored over 14 days after challenge. The mAb-297 demonstrated the most potent protection against the influenza B/Victoria/2/87-like virus strain (B/Malaysia/2506/2004), achieving 100% survival and <10% weight loss even at a mAb dose of 0.3 mg/kg (Fig. 3, A and B). In addition, mAb-297 protected robustly against both human (A/Singapore/GP1908/2015; IVR-180 H1N1) and avian (A/bald eagle/FL/W22-134-OP/2022 PR8 H5N1) N1 strains, with 100% survival rates at a dose of 3 mg/kg (Fig. 3, C–F). However, mAb-297 was less effective against an N2 strain (A/Philippines/2/1982; X-79 H3N2), only achieving a 40% survival rate at the highest dose of 10 mg/kg (Fig. 3, G and H). Overall, the protective efficacy of mAb-297 agreed with the NA inhibition observed against the same challenge strains by ELLA (Fig. S1).

To our knowledge, these results represent the first demonstration of an NA-mAb induced by seasonal vaccination to protect against avian influenza virus in vivo. Our previous observation that 91% (10/11) of the NA-mAbs we isolated exhibited crossbinding to one or more avian influenza virus strains

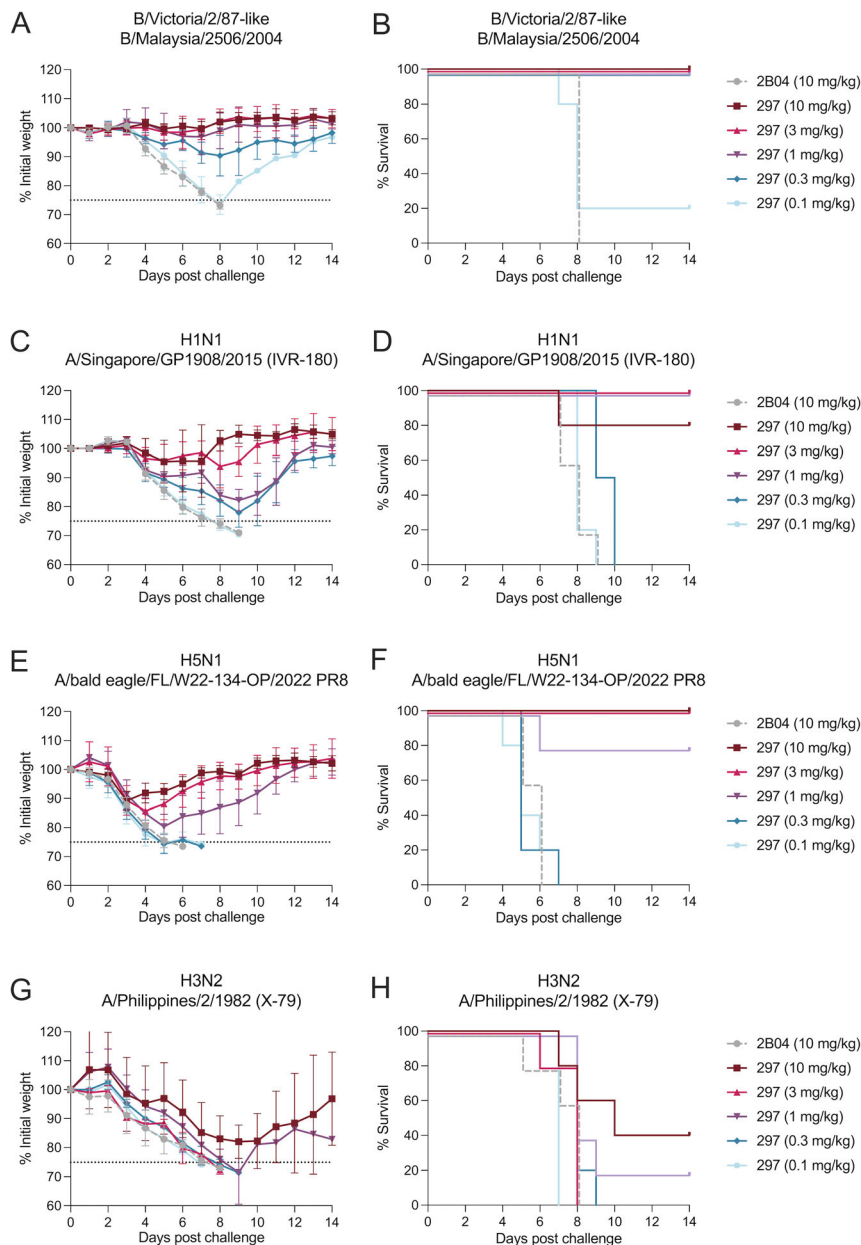


Figure 3. mAb-297 exhibits broad in vivo protection against human and avian influenza. (A–H) 6-wk old female BALB/c mice ($n = 5$ /group) were challenged with $5 \times LD_{50}$ of B/Malaysia/2506/2004 (B/Victoria/2/87-like) (A and B), A/Singapore/GP1908/2015 (H1N1, IVR-180) (C and D), A/bald eagle/FL/W22-134-OP/2022 (H5N1, A/PR/8/1934 reassortant with the polybasic cleavage site removed) (E and F), or A/Philippines/2/1982 (H3N2, X-79) (G and H) 2 h after prophylactic administration of mAb-297 ($n = 30$ mice per challenge virus). For each challenge virus, the mAb was administered in serial dilutions (10, 3, 1, 0.3, and 0.1 mg/kg) with $n = 5$ mice per dilution. A SARS-CoV-2 RBD-mAb (2B04) (Alsoussi et al., 2020) was used as a negative control (grey dotted line). **(A, C, E, and G)** Colored lines show the average change in body weight from the initial weight on day 0. The dotted horizontal line indicates the humane endpoint. Error bars represent SD. **(B, D, F, and H)** Kaplan–Meier survival curves show rates.

indicates that boosting NA responses could confer some immunity against emerging avian influenza virus strains. Cross-protection to avian NAs was previously demonstrated after humans' H1N1 and H3N2 infection (Chen et al., 2018; Hansen et al., 2023; Stadlbauer et al., 2019). Additionally, targeted immunization with monovalent H7N9, H5N1, and H10N8 has yielded NA-mAbs with in vivo protection against avian influenza virus strains with a homologous NA subtype in mice (Job et al., 2018; Shoji et al., 2011; Wilson et al., 2016; Wohlbold et al., 2015a; Xiong et al., 2020) and humans (Gilchuk et al., 2019).

Structural analysis of mAb-297 reveals a convergent binding motif in the complementarity-determining region (CDR) H3

Our understanding of antibody responses to NA at the molecular level continues to grow with the increasing number of structurally characterized NA-mAbs. Recently, several key epitopes

outside the enzymatic active site have been identified. These include the vaccine-induced N2-specific mAbs 3C08, 1F04, and 3A10; the infection-induced N2-specific mAbs NDS.3 and NDS.1; and the N1-specific mAbs 2H08, 3H03, and CD6 (Hansen et al., 2023; Lederhofer et al., 2024; Lei et al., 2023; Wan et al., 2015). These antibodies bind to the lateral surface and underside of the NA globular head, providing broad protection within the NA subtype, including protection against avian virus strains. However, the highly conserved NA active site remains the primary target for pan-NA mAbs (Momont et al., 2023; Stadlbauer et al., 2019).

To understand the molecular basis of mAb-297 binding to NA, we determined the cryogenic electron microscopy (cryo-EM) structure of the fragment antigen-binding (Fab) of mAb-297 in complex with recently circulating H1N1 A/Victoria/4897/2022 virus NA at a resolution of 2.65 Å (Table S4). The Fab binds to

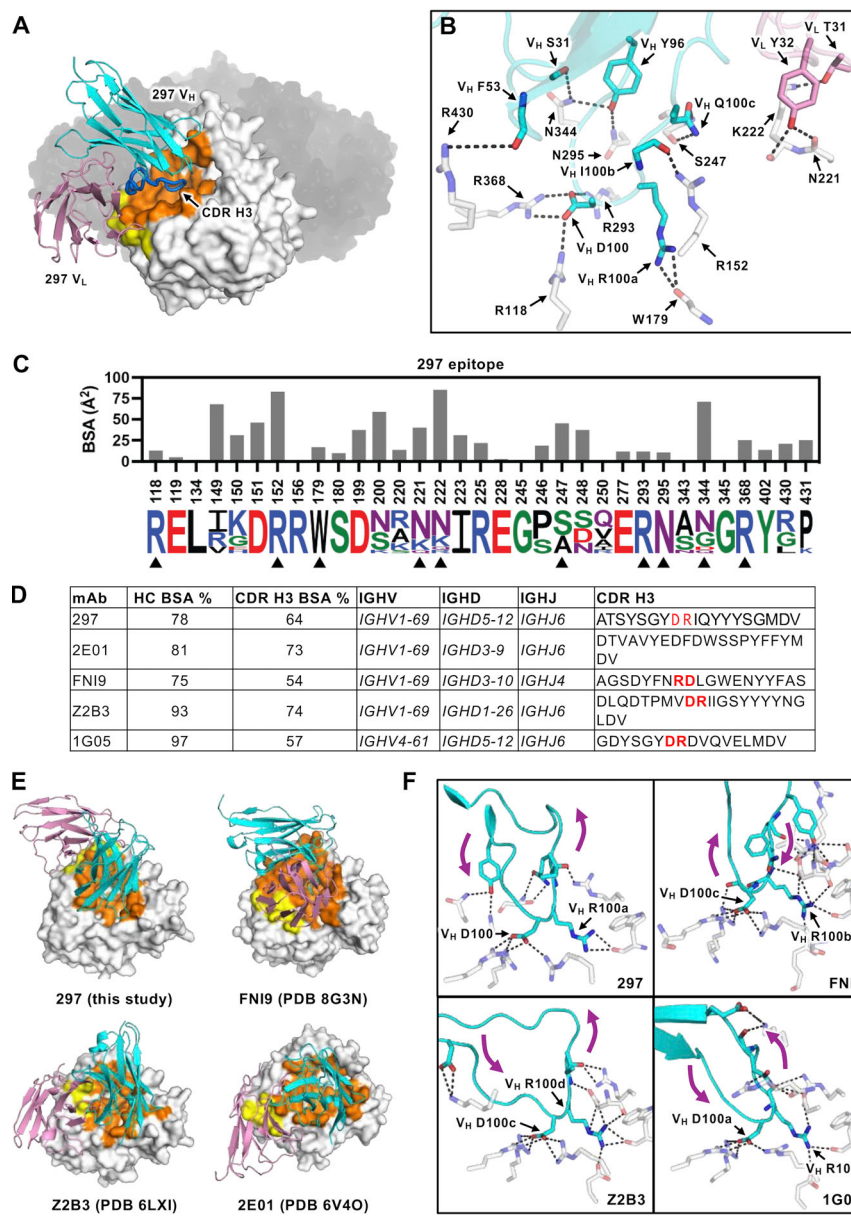


Figure 4. Structural characterization of mAb-297 in complex with influenza virus NA. (A) The structure of NA from H1N1 A/Victoria/4897/2022 virus is shown as surface representation, with one protomer in white and the remaining three protomers in black. Epitope residues interacting with the heavy chain are shown in orange, whereas those interacting with the light chain are shown in yellow. The variable domain of Fab-297 is shown as a cartoon representation (heavy chain: cyan, light chain: pink), with the CDRH3 highlighted in blue. **(B)** Hydrogen bonds and salt bridges between Fab-297 (heavy chain: cyan, light chain: pink) and H1N1 A/Victoria/4897/2022 NA (white) are shown as black dashes. Fab-297 residues are labeled based on the Kabat system. **(C)** The bar chart shows the BSA of each residue in the Fab-297 epitope upon binding. The sequence logo represents sequence diversity from 10 NAs that Fab-297 inhibited their functional activity based on the ELLA (Fig. 2 A), together with H1N1 A/Victoria/4897/2022. Residues forming H-bonds or salt bridges with Fab-297 are indicated by black triangles under the sequence logo. **(D)** Previously published antibodies that bind the NA active site and share the same Ig heavy chain variable region (IGHV), diversity region (IGHD), or joining region IGHJ gene(s) as mAb-297 are listed. These antibodies are 2E01 (Madsen et al., 2020), FN19 (Momont et al., 2023), Z2B3 (Jiang et al., 2020), and 1G05 (Madsen et al., 2020). The BSA contribution of the heavy chain (HC) and CDRH3 in the percentage of the total BSA contribution of paratope is listed for each Fab. CDRH3 sequences (Kabat numbering scheme) of these antibodies are also shown. The key binding motifs shown in panel F are highlighted in red. **(E)** NA (surface representation) in complex with active-site-binding antibodies derived from the V_H1-69 germline gene (cartoon representation), namely mAb-297 (this study), FN19 (PDB 8G3N) (Momont et al., 2023), Z2B3 (PDB 6LXI) (Jiang et al., 2020), and 2E01 (PDB 6V40) (Madsen et al., 2020) are shown (colored as in panel A). **(F)** CDRH3 of antibodies mAb-297, FN19, Z2B3, and 1G05 are compared using similar representations as in B. Curved purple arrows indicate the direction of descent and ascent (N-terminal to C-terminal) of the CDRH3.

the active site of NA, primarily utilizing its heavy chain, which accounts for 78% of the buried surface area (BSA; heavy chain: 749 Å², light chain: 202 Å²). Specifically, Fab-297 inserts its CDR H3 into the NA active site and forms hydrogen bonds with four highly conserved arginine residues (R118, R152, R293, and R368; N1 numbering) that directly interact with sialic acids (Zhu et al., 2012) (Fig. 4, A and B). Additionally, CDRs H3 and L1 engage with other conserved residues adjacent to the active site (Fig. 4, B and C). The highly conserved nature of the mAb-297 epitope explains its binding breadth (Fig. 1 G and Fig. 4 C).

Besides mAb-297, several V_H1-69 antibodies targeting the NA active site have previously been reported, including influenza B virus-specific mAb 2E01 (Madsen et al., 2020), pan-NA mAb FN19 (Momont et al., 2023), and mAb Z2B3, which has been shown to crossreact with N1 and N9 NAs (Jiang et al., 2020; Rijal et al., 2020) (Fig. 4 D). These V_H1-69 antibodies approach the NA

active site at different angles (Fig. 4 E). Specifically, while mAb-297 and FN19 bind to the NA active site with a similar angle, the orientations of their heavy and light chains are opposite. Besides, the binding angle of mAb-297 and FN19 is nearly perpendicular to that of mAbs Z2B3 and 2E01 (Fig. 4 E). Furthermore, mAb-297 and Z2B3 share a convergent usage of an aspartic acid-arginine (DR) motif in the CDR H3 to insert into the NA active site, which mimics the interaction with sialic acid (Zhu et al., 2012) (Fig. 4, D and F). This motif is also present in mAb 1G05, encoded by V_H4-61, and targets the active site of influenza B virus NA. Interestingly, the CDR H3 of mAb FN19 ascends into the NA active site in the opposite direction (from N- to C-terminus) compared with mAb-297, Z2B3, and 1G05 but still achieves similar interactions with the active site using its RD motif in CDR H3 (Fig. 4 F). These findings illustrate that V_H1-69 antibodies targeting the NA active site have diverse binding

angles and orientations yet exhibit strong convergence in utilizing the DR/RD motif in CDR H3 for binding. The consistency of this binding motif across previously characterized mAbs from the V_H1-69 germline highlights a common mechanism of NA recognition. This offers valuable insights for the design of broadly protective influenza vaccines targeting these conserved interaction points within the NA enzyme.

A key somatic hypermutation in CDR H2 broadens mAb-297 crossreactivity

The paratope of mAb-297 contains three somatic hypermutations (SHMs), namely S30F in CDR L1 as well as I53F and F54L in CDR H2 (Fig. S2 A and Fig. S3 A). To assess their importance for binding, we individually reverted these SHMs to their corresponding germline amino-acid variants (i.e., V_L F30S, V_H F53I, and V_H L54F). We tested their binding to a recombinant H1N1 NA (A/Wisconsin/588/2019), a recombinant H3N2 NA (A/turkey/Wisconsin/1/1966), and two recombinant influenza B virus NAs (B/Washington/02/2019 and B/Phuket/3073/2013). All germline-reverted mutants had a minimal effect on binding to influenza B virus NAs (Fig. S3, B and C). By contrast, germline-reverted mutant V_H F53I increased binding to N1 and N2 compared with the wild type, indicating that the SHM V_H I53F is deleterious for binding to N1 and N2 but not to influenza B virus NA. This observation suggests that influenza B virus NA may have initially elicited mAb-297 and that SHM V_H I53F might have optimized binding to influenza B NA at the expense of crossbinding to N1 and N2.

Interestingly, the germline-reverted mutant V_H L54F substantially disrupted the binding of mAb-297 to both N1 and N2. In our cryo-EM structure, V_H L54 contributes to binding with N1 via van der Waals interactions (Fig. S2 B). Structural modeling indicated that V_H L54F would sterically clash with the CDR H3 unless accompanied by a change in the backbone conformation of either CDR H2, CDR H3, or both (Fig. S2 B). Altering the conformation of CDR H2 would potentially disrupt the hydrogen bond between V_H F53 and NA R430 (Fig. 4 B). Consistently, R430 is present in both N1 NA (A/Wisconsin/588/2019) and N2 NA (A/turkey/Wisconsin/1/1966) (Fig. S2 C), which bound poorly to germline-reverted mutant V_H L54F. By contrast, influenza B virus NA has a Gly at position 430 (Fig. S2 C), which may explain why the binding of mAb-297 to influenza B virus NAs (B/Washington/02/2019 and B/Phuket/3073/2013) was not affected by V_H L54F (Fig. S3). The germline-reverted mutant V_H L54F could also alter the conformation of the adjacent CDR H3, thereby affecting its interaction with the core arginine cluster in the N1/N2 active site (Fig. S2 B). Overall, these results suggest that V_H F54L is a key SHM for mAb-297 to evolve cross-reactivity to N1 and N2 NAs.

Our results highlight the potential of NA-based immunogens to generate broadly protective antibody responses across human and avian influenza virus subtypes, suggesting that further exploration of NA-targeting strategies could significantly enhance vaccine efficacy to antigenically drifted strains. Despite being exceedingly rare, we report that currently licensed egg-derived seasonal influenza vaccines can elicit broadly crossreactive NA antibodies, with mAb-297 being the first vaccine-induced pan-

NA human antibody that targets the NA active site to be described. Several vaccine trials use novel vaccine platforms to enhance NA responses, including adjuvanted recombinant NA-based vaccines with established good manufacturing practice production processes (Hoxie et al., 2024; McMahon et al., 2023; Strohmeier et al., 2021, 2022; Wohlbold et al., 2015b) and, more recently, mRNA vaccines encoding NA as a component (Freyn et al., 2020, 2021; McMahon et al., 2022; Pardi et al., 2022), including a phase 1/2 clinical trial registered as NCT05333289 at [ClinicalTrials.gov](https://clinicaltrials.gov). Our discovery of the vaccine-induced broadly protective mAb-297 points to the possibility of improving the breadth of seasonal influenza vaccine-induced antibody responses by optimizing NA immunogens included in next-generation vaccines. The observed breadth in V_H1-69 encoded antibodies, including mAb-297, highlights the potential of germline targeting vaccines. Such strategies, by priming V_H1-69 B cells to target conserved NA and HA epitopes, could enhance the breadth of protection against diverse influenza strains. Focusing on conserved NA interaction sites could produce vaccines that offer long-lasting protection against various influenza virus strains, significantly enhancing global efforts toward influenza prevention and preparedness.

Materials and methods

Human subjects

Human PBMCs and FNAs from axillary lymph nodes were obtained from three donors (Donor A, B, and C) enrolled in the prospective observational cohort study WU397 (Matz et al., 2024, Preprint) at Washington University in St. Louis. The study was reviewed and approved by the Washington University in Saint Louis Institutional Review Board (approval #2208058), and written informed consent was obtained from all participants. Participants were aged 23–51 years old. None of the participants had received influenza vaccine within the last year. Prior records of influenza vaccinations or infections were unavailable. Blood samples were collected at week 0, 1, and 26 after immunization in ethylenediaminetetraacetic acid (EDTA) evacuated tubes (BD), and PBMCs were enriched by density gradient centrifugation over Lymphopure (BioLegend). The residual red blood cells were lysed with ammonium chloride lysis buffer (BioLegend), and PBMCs were washed with PBS supplemented with 2% FBS and 2 mM EDTA (P2) and immediately used or cryopreserved in 10% dimethylsulfoxide (DMSO) in FBS. FNAs were sampled by a qualified radiologist, who made six passes using 25-gauge needles that were subsequently flushed with 3 ml of Roswell Park Memorial Institute 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin, followed by three 1-ml rinses. Samples were then treated with ammonium chloride lysis buffer (BioLegend) for red blood cell lysis and washed twice with P2 before immediate use or cryopreserved in 10% DMSO in FBS.

Vaccine

All participants received one dose of Fluarix-quadrivalent inactivated influenza virus vaccine (Northern Hemisphere, 2022–2023 season) purchased from GlaxoSmithKline

Biologicals. It contained 15 µg of HA and an unquantified amount of NA from the following viruses: A/Wisconsin/588/2019 (H1N1) pdm09-like virus, A/Darwin/9/2021 (H3N2)-like virus, B/Austria/1359417/2021 (B/Victoria/2/87-like lineage)-like virus, and B/Phuket/3073/2013 (B/Yamagata/16/88-like lineage)-like virus.

Cell lines

Expi293F cells were cultured in Expi293 Expression Medium (#A1435102; Gibco). Madin-Darby Canine Kidney cells were grown in minimal essential media (MEM; Gibco) supplemented with 5% FBS, 0.2% sodium bicarbonate (Gibco), 1x MEM amino acids, 1x MEM vitamins, 2 mM L-glutamine, and 1% penicillin-streptomycin (Gibco).

Recombinant proteins and viruses

Recombinant proteins were either provided by Moderna, Inc. or Florian Krammer's laboratory, or purchased from Sino Biological and Biodefense and Emerging Infections Research Resources Repository (BEI Resources) as detailed in Table S2. Influenza viruses listed in Table S3 were either provided by Dr. Richard Webby (St. Jude Children's Research Hospital, Memphis, TN, USA) or purchased from International Reagent Resource. For the production of recombinant NA protein used in cryo-EM study, the ectodomain of H1N1 A/Victoria/4897/2022 NA, which contained residues 83–469 (N1 numbering), and the N-terminal repeat tetramerization domain from human vasodilator-stimulated phosphoprotein was cloned into the baculovirus transfer vector, and the protein was expressed in insect Sf9 cells as described previously (Xu et al., 2008). Soluble recombinant NA was purified from the supernatant using Ni Sepharose excel resin (Cytiva) and then via size exclusion chromatography using a HiLoad 16/100 Superdex 200 prep grade column (Cytiva) in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, and 10 mM CaCl₂. The tetramerization domain on the NA was not cleaved after purification.

Flow cytometry and cell sorting

Cryopreserved PBMCs were thawed and resuspended in P2 for staining. The cells were incubated for 30 min on ice with purified CD16 (3G8, 1:100), CD32 (FUN-2, 1:100), and CD64 (10.1, 1:100) then stained for 30 min on ice with the following antibodies: CD20-Pacific Blue (2H7, 1:400), IgD-PerCP-Cy5.5 (IA6-2, 1:200), CD19-PE (HIB19, 1:200), CD38-BV605 (HIT2, 1:100), CD3-FITC (HIT3a, 1:200), and Zombie NIR (all BioLegend) diluted in P2. After staining, the cells were washed twice. PBs from PBMCs collected at day 7 (live singlet CD3⁺CD19⁺IgD^{lo}CD20^{lo}CD38⁺), and MBCs collected at day 0 and 180 (identified as live singlet CD3⁺CD19⁺IgD^{lo}) were sorted using a Bigfoot Spectral Cell Sorter (Thermo Fisher Scientific) into PBS supplemented with 0.05% bovine serum albumin and immediately processed for scRNA-seq.

Samples for scRNA-seq

Sorted PBs and MBCs were processed using the following 10x Genomics kits: Chromium Next GEM Single Cell 5' Kit v2 (PN-1000263); Chromium Next GEM Chip K Single Cell Kit (PN-

1000286); BCR Amplification Kit (PN-1000253); and Dual Index Kit TT Set A (PN-1000215). Chromium Single Cell 5' Gene Expression Dual Index libraries and Chromium Single Cell V(D)J Dual Index libraries were prepared according to the manufacturer's instructions. Both gene expression and V(D)J libraries were sequenced on a NovaSeq 6000 (Illumina), targeting a median sequencing depth of 50,000 and 5,000 read pairs per cell, respectively.

Processing of 10x Genomics single-cell BCR reads

Demultiplexed paired-end FASTQ reads were preprocessed using Cell Ranger v.6.0.1. Initial germline V(D)J gene annotation was performed using IgBLAST (Ye et al., 2013) v.1.18.0 with the deduplicated version of IMGT/V-QUEST reference directory release 202150-3 (Brochet et al., 2008). Isotype annotation was extracted from the "c_call" column in the "filtered_contig_annotations.csv" files generated by Cell Ranger. Additional quality controls were performed as previously described (Kim et al., 2022). Cross-sample contamination was checked by identifying overlaps between samples for BCRs with identical unique molecular identifiers (UMI) and V(D)J nucleotide sequences. For each group of cells with identical UMI and V(D)J sequences, only one cell with transcriptomic data was kept; if multiple cells had transcriptomic data, none were retained. Individualized genotypes were inferred using TiGER (Gadala-Maria et al., 2015) v.1.0.0 to finalize V(D)J annotations. Non-productively rearranged sequences annotated by IgBLAST were excluded from further analysis.

Clonal lineage inference and BCR analysis

B cell clonal lineages were inferred for each donor individually using productively rearranged sequences as described previously (Kim et al., 2022). Paired heavy and light chains were first grouped based on common V and J gene annotations and CDR3 lengths. Within each group, pairs with heavy chain CDR3 nucleotide sequences within a 0.15 normalized Hamming distance were clustered as clones. After clonal inference, full-length clonal consensus germline sequences were reconstructed using Change-O v.1.2.0 (Gupta et al., 2015). A B cell clone was classified as NA-specific if it included any sequence matching a recombinant mAb synthesized from single-cell BCRs that tested positive for binding in an ELISA.

Processing of 10x Genomics single-cell 5' gene expression data

Demultiplexed paired-end FASTQ reads were first preprocessed on a per-sample basis. Samples from participants A–C, together with the other participants of the WU397 study (Matz et al., 2024, Preprint) were subsampled to the same effective sequencing depth and aggregated using Cell Ranger v.6.0.1, as previously described (Kim et al., 2022). Quality control was conducted on the aggregated gene expression data using SCANPY v.1.8.2. Briefly, cells with mitochondrial content exceeding 30% of all transcripts were removed to exclude likely lysed cells. To eliminate potential doublets, cells with over 8,000 features or 80,000 total UMIs were excluded. Cells with no detectable expression of any of a list of 34 housekeeping genes (Kim et al., 2022) were also removed. The feature matrix was

filtered to include only protein-coding, immunoglobulin, and T cell receptor genes that were expressed in at least 0.05% of the cells in any sample. Additionally, cells with detectable expression of <200 genes were removed. The same quality control criteria were applied when samples were analyzed on a per-participant basis for selecting B cells for monoclonal antibody expression.

Single-cell gene expression analysis

Transcriptomic data was analyzed using SCANPY v1.8.2 (Wolf et al., 2018) as previously described (Matz et al., 2024, Preprint). Briefly, overall clusters were first identified using Leiden graph-clustering with a resolution of 0.12. Uniform Manifold Approximation and Projection (UMAPs) were faceted by participants and inspected for convergence to assess whether there was a need for integration. Cluster identities were assigned by examining the expression of a set of marker genes (Andrews et al., 2019) for different cell types. To remove potential contamination by platelets, cells with a log-normalized expression value of >2.5 for *PPBP* were removed. Cells from the overall B cell cluster were further clustered to identify B cell subsets using Leiden graph-clustering with a resolution of 0.95 (Fig. 1 D). B cell cluster identities were assigned by examining the expression of a set of marker genes (Andrews et al., 2019) for different B cell subsets along with the availability of BCRs. Cells found in the PB/LNPC clusters that came from blood samples were labeled PB, while those that came from FNA samples were labeled LNPC. Cells found in the GC B cell clusters but which came from blood samples and which had a PB-like expression profile were labeled PB. Two clusters were excluded from the final B cell clustering: one expressing relatively high levels of CD2 and CD3E, and another showing no marked expression level of any marker gene. Heavy chain SHM frequency and isotype usage of the B cell subsets were inspected for consistency with expected values to further confirm their assigned identities.

Selection of single-cell BCRs for expression

For B cell clones identified in the PB compartment but not found in the GC B cell or LNPC compartments, we selected a cell from the PB compartment from each clone with a size of at least four cells. Conversely, for clones found within the GC B cell or LNPC compartments but not in the PB compartment, a cell was chosen from the GC B cell or LNPC compartment for each clone with a size of at least three cells. When clones were present in both the PB and either GC B cell or LNPC compartments, the selection process differed based on donor origin. From Donor A and C, a cell from the PB, GC B, or LNPC compartments was chosen from each clone, regardless of clone size. From Donor B, a cell from the PB, GC B cell, or LNPC compartments was selected from clones with a size of at least three cells. In cases where multiple compartments or time points provided selection options, we initially chose a compartment or time point randomly. We then selected the cell with the highest heavy chain UMI count from the matched compartment or time point. In the event of a tie, the cell with the higher IGHV SHM frequency was selected. The selected BCRs were curated as previously described (Kim et al., 2022) prior to synthesis.

Production of recombinant mAbs

Heavy and light chain BCR sequences for the selected clones were synthesized by GenScript and sequentially cloned into IgG1 and Igκ/λ expression vectors. The resulting plasmids were co-transfected into Expi293F cells (Thermo Fisher Scientific) to produce recombinant mAbs, which were subsequently purified using protein A agarose resin (GoldBio). To produce recombinant Fab-297, the Fab heavy and light chain sequences were synthesized by IDT and cloned into pCMV3 expression vector, which were transfected into Expi293F cells. Fab-297 was purified with CaptureSelect CH1-XL beads (Thermo Fisher Scientific) and subsequently with size exclusion chromatography using a HiLoad 16/100 Superdex 200 column (Cytiva) in 20 mM Tris-HCl pH 8.0, 100 mM NaCl.

Passive transfer experiments in mice

All animal procedures in this study were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines and have been approved by the Icahn School of Medicine at Mount Sinai IACUC. Female BALB/c mice (6–8 wk old) sourced from Jackson Laboratories were injected intraperitoneally with 100 μl of each mAb at concentrations ranging from 10 to 0.1 mg/kg ($n = 5$ mice per concentration tested). A negative control group received a mAb specific for the receptor-binding domain (RBD) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), mAb 2B04 (Alsoussi et al., 2020) (10 mg/kg, $n = 5$ mice). 2 h after mAb transfer, the mice were challenged intranasally with $5 \times \text{LD}_{50}$ of the challenge virus while under deep anesthesia induced by a ketamine/xylazine mixture. Survival and weight loss were monitored daily over a 14-day period, with euthanasia performed for mice that lost 25% or more of their initial body weight.

ELISA

To identify HA- and NA-specific mAbs, all mAbs were screened against recombinant HA and NA proteins (A/Wisconsin/588/2019 H1, A/Darwin/6/2021 H3, B/Phuket/3073/2013 HA, B/Austria/1359417/2021 HA, A/Auckland/6/1996 N1, and A/Hong Kong/1073/1999 N2). 96-well microtiter plates (Thermo Fisher Scientific) were coated with 1 μg/ml recombinant protein in PBS and incubated overnight at 4°C. The next day, the wells were blocked with 200 μl of PBS containing 0.05% Tween-20 (T-PBS) and 10% FBS, followed by a 1.5-h incubation at room temperature (RT). After removing the blocking solution, 1:30 dilutions of mAb transfection culture supernatant or at 10 μg/ml for purified mAbs were added to the wells. mAbs identified as NA-specific were additionally titrated against a panel of NA proteins from 26 different influenza strains (Table S2) with threefold serial dilutions of purified mAbs starting at 10 μg/ml. The plates were then incubated for 1 h at RT and washed three times with T-PBS. Next, horseradish peroxidase (HRP)-conjugated anti-human IgG (Jackson ImmunoResearch) was diluted 1:2,500 in blocking solution and added to each well (100 μl/well). After a 1-h incubation at RT, the plates were washed three times with T-PBS and three times with PBS. Then, 100 μl of substrate solution (phosphate-citrate buffer with 0.1% H₂O₂ and 0.4 mg/ml o-phenylenediamine

dihydrochloride [OPD, Sigma-Aldrich]) was added to each well and incubated for 5 min. The reaction was stopped by adding 100 μ l of 1 M hydrochloric acid (HCl) to each well. The absorbance was measured at 490 nm using a Bio-Tek microtiter plate reader. All mAbs were initially screened against the four homologous HAs from the vaccine strains A/Wisconsin/588/2019 H1, A/Darwin/6/2021 H3, B/Phuket/3073/2013 (B/Yamagata/16/88-like) HA, and B/Austria/1359417/2021 (B/Victoria/2/87-like) HA, and two influenza A virus NAs: A/Auckland/6/1996 N1 and A/Hong Kong/1073/1999 N2. HA- and NA-positive mAbs were identified as mAbs binding with an OD four times higher to NA or HA than bovine serum albumin.

ELLA

ELLA was performed to measure the inhibition of NA activity by mAbs. In brief, 96-well flat-bottom microtiter plates (Thermo Fisher Scientific) were coated with 100 μ l of fetuin (Sigma-Aldrich) at a concentration of 25 μ g/ml in 1 $\times$ coating buffer (KPL coating solution; SeraCare) and incubated overnight at 4°C. The following day, the plates were washed three times with T-PBS. mAbs were serially diluted twofold in sample diluent (PBS containing 1% bovine serum albumin [Sigma-Aldrich] and 0.5% Tween-20 [Sigma-Aldrich]) starting from a concentration of 30 μ g/ml. 50 μ l of each mAb dilution was then transferred to the fetuin-coated plates in duplicate wells. Subsequently, 50 μ l of virus at 90% maximal effective concentration (EC_{90}) was added to the plates and incubated for 18 h at 37°C. The next day, the plates were washed six times with PBS-T, and then 100 μ l of peanut agglutinin-HRP (Sigma-Aldrich) at a concentration of 1 μ g/ml in PBS with 1% bovine serum albumin was added to each well. After a 2-h incubation at room temperature, the plates were developed using 100 μ l of SigmaFast OPD. The reaction was stopped after 10 min by adding 100 μ l of 1 M HCl (Thermo Fisher Scientific), and the absorbance was measured at 490 nm using a Bio-Tek microtiter plate reader. The 50% inhibition concentration (IC_{50}) was determined as the mAb concentration required to inhibit 50% of the NA activity compared with the negative control (virus without mAb).

Neuraminidase inhibition by NA-Star assay

The inhibition of NA activity by mAbs was also assessed using the NA-Star Influenza NA Inhibitor Resistance Detection Kit (Applied Biosystems). This kit measures the inhibition of NA's ability to cleave a 1,2-dioxetane chemiluminescent substrate, indicating the binding of mAbs to the enzymatic site of NA. The procedure followed the manufacturer's instructions. In summary, mAbs were first diluted to 30 μ g/ml in NA-Star Assay Buffer and then serially diluted in NA-Star Assay Buffer in a 1:3 ratio. From each dilution, 25 μ l were placed into a white, flat-bottom 96-well plate. To these wells, 25 μ l of virus at $2 \times EC_{50}$ was added and incubated for 20 min at 37°C. After incubation, 10 μ l of the NA-Star Substrate was added to each well and the plates were left at room temperature for 30 min. Following this, 60 μ l of the NA-Star accelerator solution was added before reading the plates. The resulting chemiluminescent signal was detected using a Bio-Tek microtiter plate reader.

Influenza strains phylogenetic tree

The NA sequences for generating phylogenetic trees were downloaded from the Global Initiative on Sharing Avian Influenza Data (<https://www.gisaid.org>) and The Influenza Virus Resource at the National Center for Biotechnology Information (Bao et al., 2008). The amino acid sequences were then submitted to <https://ngphylogeny.fr/workflows/oneclick/> for phylogenetic reconstruction using NgPhylogeny.fr (Lemoine et al., 2019). The phylogenetic tree was visualized using FigTree version 1.4.4. Sequence information for three strains (A/shorebird/Delaware/127/1997, A/Canada/444/2004, A/equine/Pennsylvania/1/2007) was not available. In these cases, the following strains from the same NA subtype were used instead: A/ruddy turnstone/Delaware/203/1996, A/chicken/Canada/CN006/2004, and A/equine/New York/146066/2007.

Cryo-EM sample preparation and data collection

The ectodomain of NA from H1N1 A/Victoria/4897/2022 was mixed with the Fab from mAb-297 in a 1:4 M ratio and incubated overnight at 4°C before size exclusion chromatography on the Superose 6 Increased 10/300 column (Cytiva). The complex peak was concentrated to 1 mg/ml and mixed with n-octyl- β -D-glucoside (Anagrade) to 0.1% wt/vol of the final concentration of the detergent. Cryo-EM grids were prepared using a Vitrobot Mark IV machine. An aliquot of 3 μ l sample was applied to a 300-mesh Quantifoil R1.2/1.3 Cu grid pretreated with glow-discharge. High-resolution cryo-EM movies were collected on an FEI Titan Krios at 300 kV with a Gatan K3 detector. A total of 4,076 movies of 40 frames per movie were collected with a total accumulated dose of 57.35 electrons per $\text{\AA}^2$ at a pixel size of 0.529 $\text{\AA}$.

Cryo-EM image processing and model building

Data processing was performed with CryoSPARC (Punjani et al., 2017) (version 4.5). Movies were subjected to motion correction and contrast transfer function (CTF) estimation, and particles were picked with CryoSPARC blob picker followed by 2D classification. Best classes from the blob picker were used as templates for CryoSPARC template pickers, and the resulting particles were cleaned up by multiple rounds of 2D classification before ab initio reconstruction. Due to substoichiometric quantities of bound Fab-297, the data was processed in C1 symmetry to preserve the overall quality of the one complete copy of the Fab visible in the reconstruction. The best class from ab initio reconstruction was subjected to homogenous refinement, reference-based motion correction, another round of homogenous refinement, local and global CTF estimation, and non-uniform refinement, which yields a final map with an overall resolution of 2.65 $\text{\AA}$. The map was sharpened with DeepEMhancer (Sanchez-Garcia et al., 2021), and the initial atomic model was built using ModelAngelo (Jamali et al., 2024), supplied with a sequence of full-length H1N1 A/Victoria/4897/2022 NA and Fab-297. Structure refinement was performed using real-space refinement in Phenix (Afonine et al., 2018) (version 1.21) and iterations of refinement using Coot (Casañal et al., 2020) (version 0.9.8). The model was validated in Molprobit and Phenix (Afonine et al., 2018; Williams et al., 2018).

Online supplemental material

Fig. S1 provides details on the NA-inhibition of mAb-297 to the mouse challenge strains. **Fig. S2** describes somatic hypermutations of mAb-297. **Fig. S3** shows the binding of mAb-297 after single amino acid germline reversions. Table S1 list the number of NA-binding B cell clones. Table S2 details the list of the recombinant protein used. Table S3 details the list of the virus strains used. Table S4 provides the cryo-EM data collection, refinement and validation statistics.

Data availability

Map generated from electron microscopy data was deposited in the Electron Microscopy Data Bank with accession number EMD-47102, and the refined model in the Protein Data Bank with accession number 9DPC. 10x Genomics sequencing data has been deposited at Sequence Read Archive under accessions SRR27203025–SRR27203056, SRR27211114–SRR27211141, and SRR27205963–SRR27205992. All data are available from ImmPort under the following identifier: SDY2837. Any additional information required to reanalyze the data reported in this paper is available upon request from the corresponding authors.

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Disclosures: J.S. Turner reported "other" from Abbvie outside the submitted work. F. Krammer reported that The Icahn School of Medicine at Mount Sinai has filed patent applications relating to SARS-CoV-2 serological assays, NDV-based SARS-CoV-2 vaccines influenza virus vaccines and influenza virus therapeutics that list F. Krammer as co-inventor. F. Krammer has received royalty payments from some of these patents. Mount Sinai has spun out a company, Kantaro, to market serological tests for SARS-CoV-2 and another company, Castlevax, to develop SARS-CoV-2 vaccines. F. Krammer is the cofounder and scientific advisory board member of Castlevax. F. Krammer has consulted for Merck, GSK, Sanofi, Curevac, Seqirus, and Pfizer, and is currently consulting for Third Rock Ventures, Gritstone, and Avimex. The Krammer laboratory is also collaborating with Dynavax on influenza vaccine development and with VIR on influenza virus therapeutics. N.C. Wu reported personal fees from Helixon outside the submitted work. A.H. Ellebedy reported grants from Moderna during the conduct of the study; and the Ellebedy laboratory received funding from Emergent BioSolutions, and AbbVie, which is unrelated to the data presented in the current study. A.H. Ellebedy has received consulting and speaking fees from InBios International, Fimbrion Therapeutics, RGAX, Mubadala Investment Company, Moderna, Pfizer, GSK, Danaher, Third Rock Ventures, Goldman Sachs, and Morgan Stanley and is the founder of ImmuneBio Consulting. No other disclosures were reported.

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Supplemental material

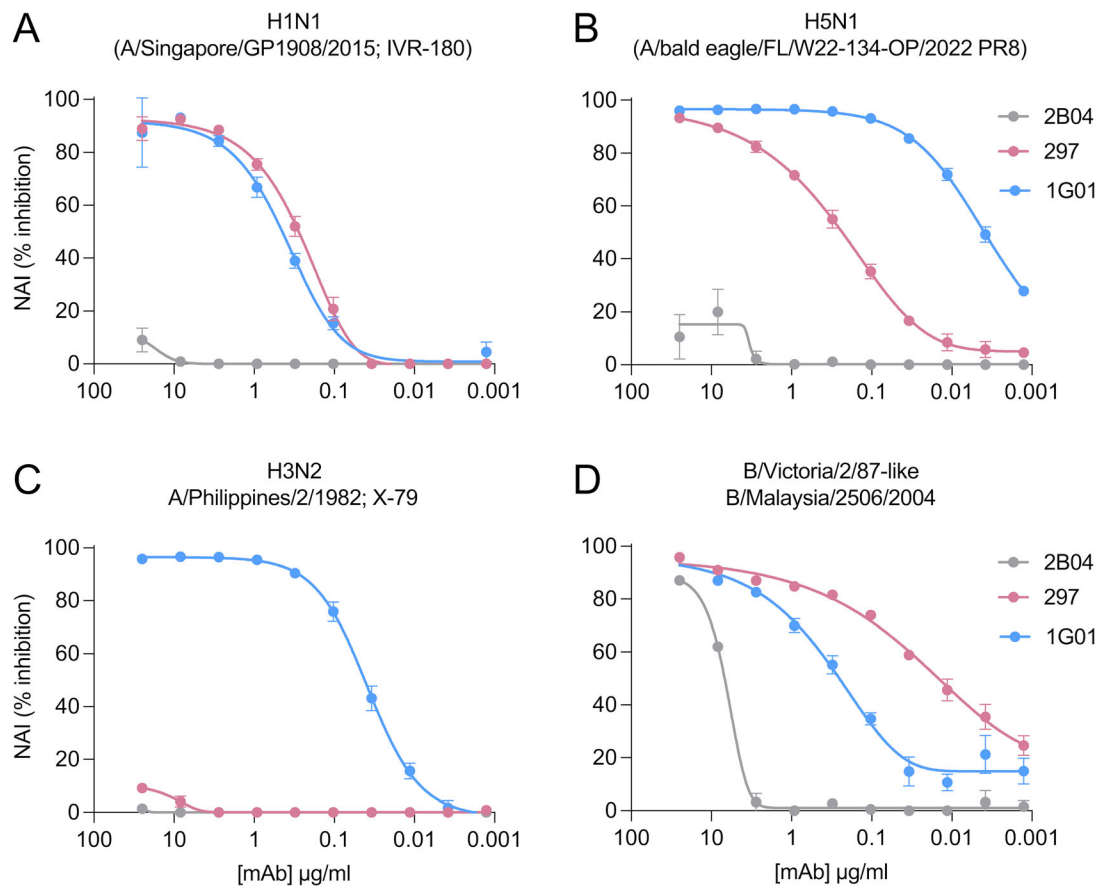


Figure S1. **In vitro NA inhibition of mouse challenge strains by mAb-297.** (A–D) The NA-inhibition (NAI) of mAb-297 to the mouse challenge strains was measured by ELLA. Lines indicate percent inhibition across various concentrations of mAb-297 (pink line). Negative control mAb 2B04 (Alsoussi et al., 2020) is shown as grey line, and the pan-NA mAb 1G01 (Stadlbauer et al., 2019) is shown as a blue line. Symbols represent the mean of three replicates with the SD indicated by error bars.

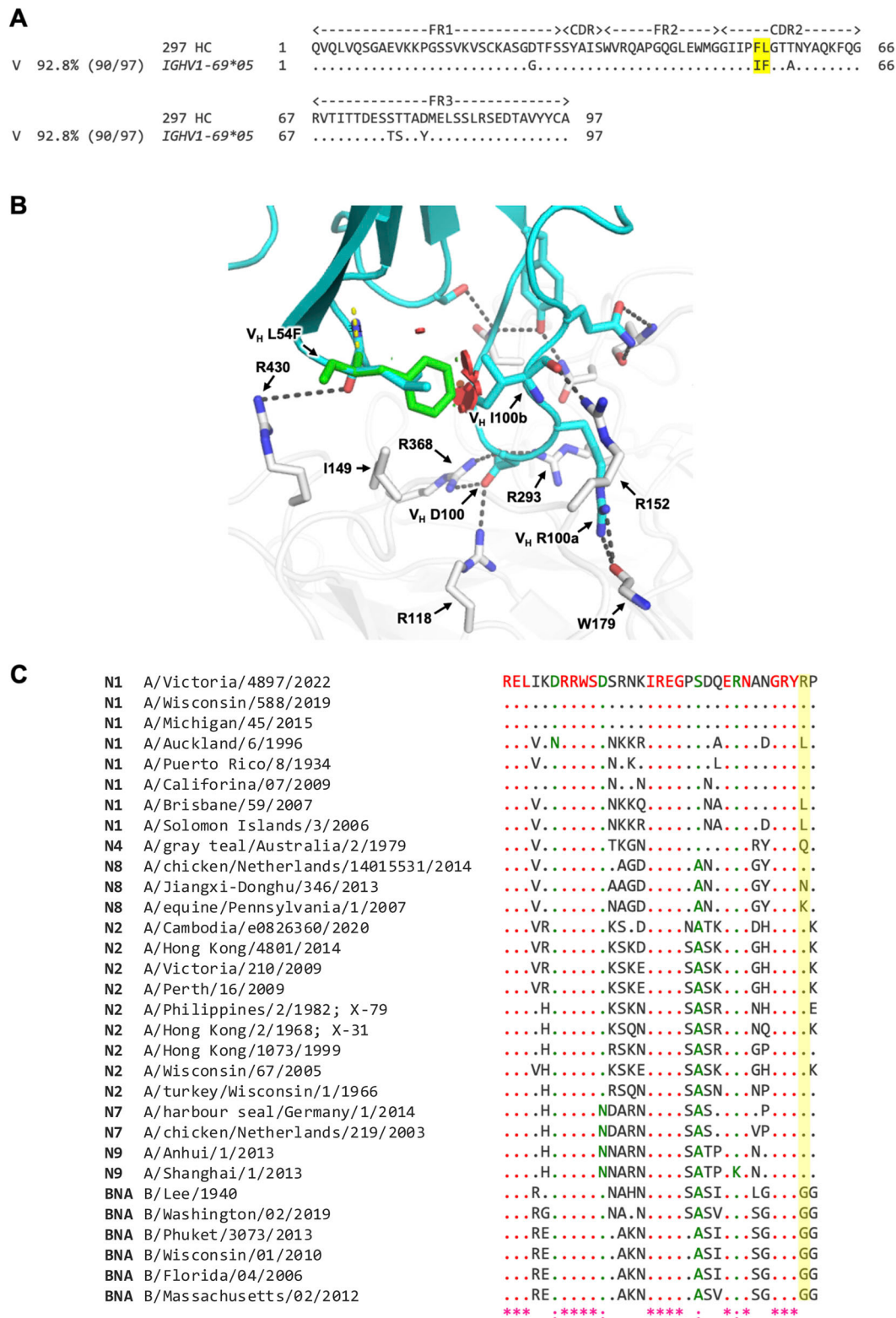


Figure S2. **Somatic hypermutations in mAb-297.** (A) Sequence alignment between the V_H domain of mAb-297 and the V_H1-69 germline gene using IgBlast (Ye et al., 2013). The V_H I53F and F54L are highlighted in yellow. (B) The germline-reverted mutation V_H L54F is modeled computationally into the structure of mAb-297 in complex with H1N1 A/Victoria/4897/2022 NA using PyMOL Molecular Graphics System, Version 3.0 Schrödinger, LLC. The NA and mAb-297 are in white and cyan, respectively, with some key side chains shown as sticks. The side chain of the modeled V_H F54 is in green. Black dashes represent hydrogen bonds or salt bridges. Clashes with the modeled V_H F54 are shown as red blobs. (C) Sequences of NAs used in experiments in Fig. 1 and Fig. 2 are aligned to the sequence of H1N1 A/Victoria/4897/2022 NA. Only the epitope residues are shown and aligned (as in Fig. 4 C). The alignment of NA residues in the same position as R430 of H1N1 A/Victoria/4897/2022 NA is highlighted in yellow. Asterisks (*) denotes fully conserved residues, while a colon (:) indicates positions with strongly similar properties among the residues.

A

Heavy chain

```

<-----FR1-----><---CDR1---><-----FR2-----><---CDR2---><-----FR3-----><---CDR3--->
mAb-297 VH_WT      QVQLVQSGAEVKKPGSSVKVCKASGDTFSSYAISWVRQAPGQGLEWMGGIIPFLGTTNYAQKFQGRVTITDESSTADMESSLRSEDVAVYYCATSYSGYDRIQYYSMDV
mAb-297 VH_F53I    QVQLVQSGAEVKKPGSSVKVCKASGDTFSSYAISWVRQAPGQGLEWMGGIIPFLGTTNYAQKFQGRVTITDESSTADMESSLRSEDVAVYYCATSYSGYDRIQYYSMDV
mAb-297 VH_L54F    QVQLVQSGAEVKKPGSSVKVCKASGDTFSSYAISWVRQAPGQGLEWMGGIIPFLGTTNYAQKFQGRVTITDESSTADMESSLRSEDVAVYYCATSYSGYDRIQYYSMDV
VH 1-69*05 germline .....G.....A.....ISS.....Y.....S.....S.....L.....Q.....P.....E.....F.....A.....T.....S.....Y.....R.....

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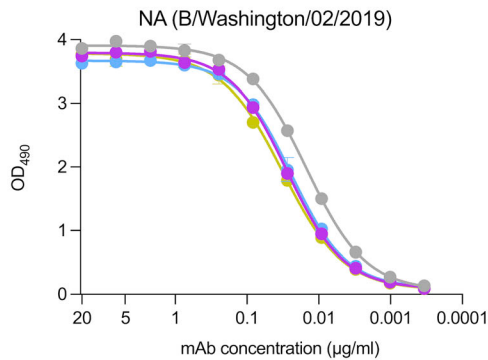
Light chain

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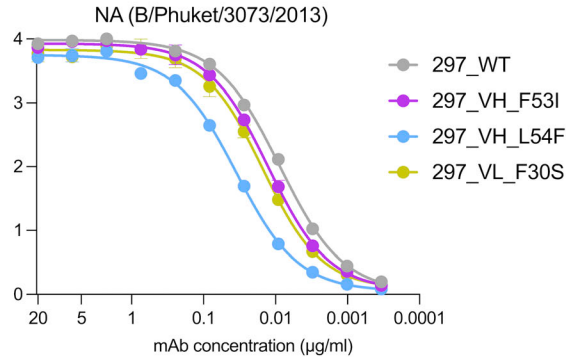
<-----FR1-----><---CDR1---><-----FR2-----><---CDR2---><-----FR3-----><---CDR3--->
mAb-297 VL_WT      DIQMTQSPSSLSASVDRVTITCRSSQSFTYLNWYQKPKGAPKLLISAASTLQSGVPSRFSGSGSGTDFTLTINSLQPEDFATYYCQQSFSTPLT
mAb-297 VL_F30S    DIQMTQSPSSLSASVDRVTITCRSSQSFTYLNWYQKPKGAPKLLISAASTLQSGVPSRFSGSGSGTDFTLTINSLQPEDFATYYCQQSFSTPLT
VL 1-39*01 germline .....G.....A.....ISS.....Y.....S.....S.....L.....Q.....P.....E.....F.....A.....T.....S.....Y.....R.....

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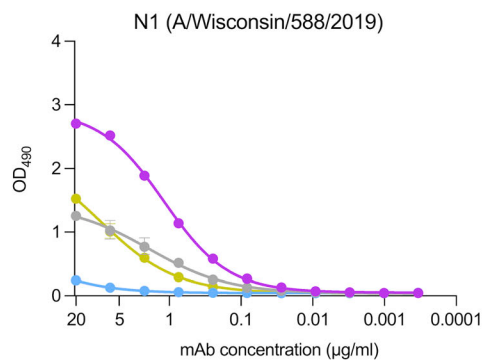
B



C



D



E

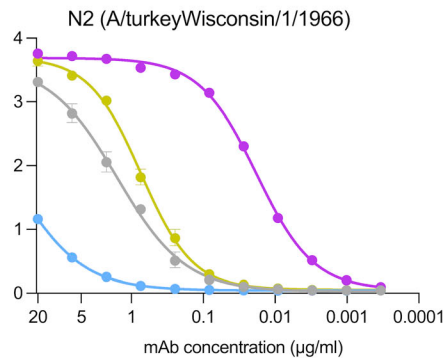


Figure S3. NA binding of mAb-297 with single amino acid germline reversions. (A) Sequence alignment between mAb-297 mAb and its V_H and V_L germline, along with three mutant mAbs with single germline revisions (V_H F53I, V_H L54F, and V_L F30S) indicated in red. (B–E) Binding curves from ELISA of the wild type mAb-297 (grey line) along with three mutant mAbs V_H F53I (purple line), V_H L54F (blue line), and V_L F30S (green line). Symbols represent the mean of two replicates, with the SD indicated by error bars.

Provided online are Table S1, Table S2, Table S3, and Table S4. Table S1 list the number of NA-binding B cell clones. Table S2 details the list of the recombinant protein used. Table S3 details the list of the virus strains used. Table S4 provides the cryo-EM data collection, refinement and validation statistics.