Broadly protective murine monoclonal antibodies against influenza B virus target highly conserved neuraminidase epitopes

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A substantial proportion of influenza-related childhood deaths are due to infection with influenza B viruses, which co-circulate in the human population as two antigenically distinct lineages defined by the immunodominant receptor binding protein, haemagglutinin. While broadly cross-reactive, protective monoclonal antibodies against the haemagglutinin of influenza B viruses have been described, none targeting the neuraminidase, the second most abundant viral glycoprotein, have been reported. Here, we analyse a panel of five murine anti-neuraminidase monoclonal antibodies that demonstrate broad binding, neuraminidase inhibition, in vitro antibody-dependent cell-mediated cytotoxicity and in vivo protection against influenza B viruses belonging to both haemagglutinin lineages and spanning over 70 years of antigenic drift. Electron microscopic analysis of two neuraminidase–antibody complexes shows that the conserved neuraminidase epitopes are located on the head of the molecule and that they are distinct from the enzymatic active site. In the mouse model, one therapeutic dose of antibody 1F2 was more protective than the current standard of treatment, oseltamivir, given twice daily for six days.

Influenza B viruses (IBVs) co-circulate in humans as two lineages based on the genetic and antigenic differences of the haemagglutinin (HA) glycoprotein. The two lineages—Yamagata (named after the B/Yamagata/16/88 strain) and Victoria (named after the B/Victoria/1/87 strain)—are thought to have diverged from a common ancestor strain in the 1970s1–3. Although IBVs are responsible for 20–30% of influenza cases per year on average, IBV is the predominant cause of influenza disease in some years4–6. Current studies challenge the notion that influenza B cases are clinically milder than those of influenza A, with the finding of no difference between influenza B and influenza A in terms of the length of hospital stay, intensive care unit admission frequency, or rate of death among hospitalized influenza patients7. Additionally, epidemiological data suggest that IBVs disproportionately afflict children. During the 2010–2011 influenza season in the USA, IBVs accounted for 25% of all influenza infections but caused 38% of influenza-related paediatric deaths, and nearly half of these children had no pre-existing health conditions8.

Neuraminidase (NA) inhibitors are the only antivirals officially recommended by the Advisory Committee on Immunization Practices (ACIP) for the treatment of influenza virus infection9. This is particularly problematic for IBV infections, because oseltamivir has been shown to be less effective when treating influenza B than when treating influenza A in both paediatric and adult outpatient populations10–12. Furthermore, zanamivir (an alternative NA inhibitor) is not approved for children under the age of seven13. Given the substantial disease burden attributable to IBV despite the availability of vaccines and antivirals, the development of therapeutics, such as the monoclonal antibodies (mAbs) presented here, is crucial.

There have been reports of murine and human mAbs against the IBV HA13–15, but no broadly cross-reactive, protective mAbs binding the IBV NA have been reported thus far. The potential of the IBV NA globular head domain to harbour highly conserved epitopes has been recognized for some time16. mAbs against the IBV NA were previously isolated, yet the antibodies were not assessed for in vivo protection, and the structures of the antibody bound to NA were not solved16–18. Although the importance of anti-NA immunity in protection from viral infection has been extensively demonstrated19–28, far less is known about NA epitopes compared to HA epitopes. Although NA does not serve as the receptor binding protein, it is critically responsible for freeing nascent virus from host cells and virus in the airway from mucins29–31, so antibodies that bind to the NA and interfere with its activity may confer protection through several mechanisms. Here, for the first time, we extensively characterize a panel of broadly cross-reactive mouse mAbs against the IBV NA in vitro and in vivo. We also present structures of recombinant IBV NA in complex with the fragment antigen-binding (Fab) portion of the antibody, allowing for a direct comparison of structural binding footprints with critical binding residues (mapped using escape mutagenesis). Furthermore, such studies highlight the potential benefits of targeting the conserved regions of the NA when designing innovative influenza virus vaccines.

Results

Using hybridoma technology, we identified a panel of five broadly cross-reactive IBV NA-binding mAbs from the spleen of a single female BALB/c mouse that was serially immunized with IBVs from both the Victoria (V) and Yamagata (Y) lineages. All IgG-producing
hybridoma clones were initially screened for binding to the ancestral B/Lee/40 strain (purified, whole virus), binding to recombinant, baculovirus-expressed NA (rNA) from B/Yamagata/16/88 and inhibiting neuraminidase activity of B/Wisconsin/1/2010 (Y) virus. Five hybridomas—1F2 (IgG2a), 1F4 (IgG2a), 3G1 (IgG2a), 4B2 (IgG2a) and 4F11 (IgG2b)—were selected based on robust reactivity and were further tested for binding and neuraminidase inhibition (NI) activity to a wide array of IBVs covering both lineages and spanning 73 years of antigenic drift. The five mAbs were encoded by different light chain/heavy chain combinations.

![Graphs showing binding and inhibition activity of the selected mAbs.](image)

**Fig. 1** | In vitro binding of IBV anti-NA mAbs. a, Bar graphs represent the minimal binding concentrations of anti-NA mAbs to either rNA (top, coated at 2 µg ml⁻¹) or purified whole virus (bottom, coated at 5 µg ml⁻¹), measured by ELISA. rHA from B/Yamagata/16/88 was used as a negative control substrate. Binding at concentrations higher than 10 µg/ml was detected for some mAb/NA combinations but is not displayed. b, mAbs were tested via ELLA to assess NI activity. Bar graphs represent IC₅₀ values. Technical duplicates were performed in a and b, with the mean displayed graphically. Victoria lineage strains (with lineage referring to the HA) are shown in red, Yamagata lineage strains in blue, and the ancestral B/Lee strain in green. mAb 8H9 (anti-H6, murine IgG) was used as a negative control. c, Phylogenetic tree based on the amino acid sequence of the B NAs of 280 randomly subsampled IBV strains spanning all years since IBV was first isolated (1940–present). Strains used to assess mAb binding are labelled using the same colour scheme as in a and b. The scale bar represents a 1% difference in amino acid sequence.
with various degrees of mutations as compared to the germline sequences (Supplementary Data Set 1). All five mAbs displayed broad cross-reactivity to purified whole virus and rNA in enzyme-linked immunosorbent assays (ELISAs) and functionally inhibited NA enzymatic activity in enzyme-linked lectin assays (ELLAs) (Fig. 1a,b). Most mAbs displayed minimal binding concentrations to rNA in the sub-micromolar range, with binding to certain rNA substrates reaching minimal binding concentrations in the nanomolar range (1.5–150 nM). These findings were corroborated by affinity measurements via biolayer interferometry where all but one mAb (4B2) reached sub-nanomolar binding affinities (Supplementary Fig. 1). However, for some mAb/NA combinations binding was only observed at higher antibody concentrations (>10 μg/ml). Because whole virus was coated based on amount of total protein in the preparation, the minimal binding concentrations in these ELISAs tended to be greater, on average, than those in which rNA was used as a substrate; other differences in binding between whole virus and rNA may be attributed to variation in protein folding and epitope availability. As expected, if a mAb did not bind the NA from a particular strain at a concentration of 10 μg/ml, it did not display NI activity against that strain. Binding and NI activity against the ancestral B/Lee/40 strain was weak, but still present, compared to the negative control. By displaying a subsample of IBV NA amino-acid sequences as a phylogenetic tree, we observe that the strains used in this study are representative of the amino-acid sequence diversity of IBV NAs, strengthening the conclusion that these anti-NA mAbs are broadly cross-reactive (Fig. 1c).

Protective epitopes on the IBV NA have yet to be described. To map the relevant epitopes on the surface of the NA protein, we carried out electron microscopic analysis of the complex between rNA and the Fab portions of antibodies 1F2 and 4F11. We focused our analysis on 4F11 (because it displayed the widest binding breadth and greatest binding affinities overall) and 1F2 (because it displayed the strongest binding affinity to the ancestral B/Lee/40 strain) Fab fragments. The resulting density maps (resolution of ~25 Å) were interpreted (Fig. 2a,b) using coordinates from the X-ray structures of NA from B/Brisbane/60/2008 (PDB ID: 4CPL) and a Fab that binds an IBV HA (PDB ID: 4FQL). Oblique and top views of the complex (Fig. 2a,b) enable visualization of the locations of the bound antibodies at the periphery of the NA tetramer, indicating that 1F2 has an orientation that is tilted relative to the plane of the NA tetramer, while 4F11 binds in an orientation in which the Fab is in the same plane as that of the tetramer. In Fig. 2c, the top view of antibody binding is shown with respect to the NA enzymatic active site. Detailed inspection of the footprints of the bound Fab molecules shows that the structural footprints of 4F11 and 1F2 are adjacent to each other, but separate (Fig. 2d). The amino-acid residues of the binding footprints are highly conserved across all IBVs, consistent with the broad binding profiles of mAbs 4F11 and 1F2 (Fig. 2e). Interestingly, the binding sites of both Fabs appear not to directly overlap the enzymatic active site of NA (Fig. 2e). Our reconstructions thus show that antibodies that inhibit NA activity need not contact the catalytic site directly and instead may function by binding and sterically hindering access of the NA to the substrate.

Residues critical for antibody binding were identified through the generation of escape mutant IBVs. Escape mutants generated against four of the five mAbs showed drastic loss of binding to mAbs via immunofluorescence staining of infected Madin–Darby canine kidney (MDCK) cells (Supplementary Fig. 2a,b). Interestingly, the 4B2 escape mutant retained robust binding to the antibody, but is nevertheless a true escape mutant, as demonstrated by its ability to grow to high titres compared to wild-type (wt) virus in the presence of antibody (Supplementary Fig. 2c). In this instance, mutations in other gene segments may have contributed to the ability of the mutant virus to escape antibody pressure. The critical residues identified in 1F2 and 4F11 escape mutants (E338K and G385R, respectively) are located either within or at the periphery of the binding footprints determined by electron microscopy (Supplementary Fig. 2d). The critical residue identified in the 3G1 escape mutant (G346R) maps to a location close to the active site, consistent with the finding that this was the only one of the analysed mAbs that could inhibit NA enzymatic activity to levels comparable to oseltamivir when using a small-molecule substrate (Supplementary Fig. 3f). Interestingly, mAb 1F2, which has a footprint adjacent to the 3G1 escape mutation, competed with mAb 3G1 in an ELISA assay (Supplementary Fig. 4). This was an asymmetric interaction because mAb 3G1 was unable to block 1F2 binding. The critical residue identified in the 1F4 escape mutant (Q453R) mapped close to the monomer–monomer interface of the NA tetramer. Of relevance, a quaternary, protective epitope spanning two monomers of the NA from pandemic H1N1 (A/California/07/2009) has been previously reported as the target of a human mAb. It is conceivable that 1F4 binds to IBV NA in a similar manner. mAb 1F4 asymmetrically competed with mAb 4F11,
suggesting that it binds closer to the 4F11 footprint than to the 1F2 footprint (Supplementary Figs. 2 and 4). Finally, deep sequencing of the 4B2 escape mutant did indeed reveal a non-synonymous mutation in NA (G344E), although the mutation does not strongly alter the binding of mAb 4B2 as assessed by immunofluorescence (Supplementary Fig. 2d). The mutation found in the 4B2 escape mutant is located right above both the 1F2 and the 4F11 footprint. All generated escape mutants lost sensitivity to the respective mAbs in an NI assay with the exception of the 4B2 escape mutant, which was still inhibited by mAb 4B2 (Supplementary Fig. 6). However, this virus does not seem to escape by abolishing antibody binding to the NA, as discussed above (Supplementary Fig. 2c).

As previous reports have demonstrated the potential of influenza A virus NA-directed antibodies to protect in vivo,[25,27] we decided to test the in vivo efficacy of our panel of mAbs in a well-established influenza virus challenge model, using female BALB/c mice. For all five mAbs, mice were fully protected from morbidity (Supplementary Fig. 5a,d) and mortality (Fig. 3a,d) when administered antibody prophylactically at the highest tested dose (5 mg kg⁻¹ ) and challenged with five murine lethal doses (mLD₅₀) of either B/Malaysia/2506/04 (Victoria lineage) or B/Florida/04/06 (Yamagata lineage) virus strains. At lower prophylactic doses of 1 mg kg⁻¹ and 0.5 mg kg⁻¹, the mAbs did not prevent morbidity (Supplementary Fig. 5b,c), but nevertheless prevented mortality against B/Malaysia/2506/04 virus, with 1F2 demonstrating 100% protection at both of the lower doses tested (Fig. 3b,c). All five mAbs were 100% protective when administered to mice 24 h post infection (h.p.i.) with 5 mLD₅₀ B/Malaysia/2506/04 virus, and three of five were 100% protective when administered 48 h.p.i. (Fig. 3e,f and Supplementary Fig. 5e,f).

To investigate the in vivo protective breadth of the mAb panel, mice were prophylactically administered with antibodies as in Fig. 3, but were killed on days 3 or 6 for lung titre analysis. When mice were challenged with B/Malaysia/2506/04 virus, lung titres were significantly reduced on day 6, but not day 3, post infection relative to the negative control group (P ≤ 0.0001), suggesting enhanced viral clearance as a possible mechanism of protection (Fig. 4a). A similar pattern was also seen when mice were challenged with B/Florida/04/06 virus (Fig. 4b).

**Fig. 3** | In vivo efficacy of iBV anti-NA mAbs. a–d, To test prophylactic efficacy, female BALB/c mice (five per group) were administered either 5, 1 or 0.5 mg kg⁻¹ of mAb i.p. 2 h before a 5 mLD₅₀ challenge with B/Malaysia/2506/04 virus (a–c) or administered 5 mg kg⁻¹ of mAb i.p. 2 h before a 5 mLD₅₀ challenge with B/Florida/04/06 virus (d). e,f, To test the therapeutic efficacy, mice were administered 5 mg kg⁻¹ of each antibody either 24 h (e) or 48 h (f) after challenge with 5 mLD₅₀ B/Malaysia/2506/04 virus. Murine mAb 8H9 was used as a negative control in all experiments. Differences in survival were analysed using a Mantel–Cox log-rank test. Statistical significance is indicated, where tested, as follows: NS, P > 0.05; *P ≤ 0.05; **P ≤ 0.01.
Fig. 4 | Non-neutralizing iBV anti-NA mAbs reduce viral lung titres in mice, activate ADCC, inhibit activity of a drug-resistant iBV and demonstrate superior effectiveness to oseltamivir. a, Female BALB/c mice (three per group) were administered 5 mg kg$^{-1}$ antibody prophylactically, challenged with B/Malaysia/2506/04 virus in identical fashion to Fig. 3a, and killed on day 3 or 6 p.i. for lung titre analysis. Lung titres in groups treated with anti-NA mAbs are significantly reduced on day 6 p.i. compared to negative-control mAb 8H9. b. c. When added to both the infectious inoculum and the solid agar overlay in a PRNA, iBV anti-NA mAbs did not reduce plaque number (b), but reduced the plaque size (c), of B/Malaysia/2504/06 virus in a titratable fashion compared to negative control mAb 8H9. The exception was 3G1, which in addition to reducing plaque size, was also able to reduce plaque number up to ~50%. A neutralizing murine mAb against the iBV HA was used as a positive control. d, Anti-NA mAbs incubated with MDCK cells infected with B/Malaysia/2504/06 virus (MOI = 3) were able to engage Fc receptors and activate ADCC reporter activity in vitro. Fold induction is defined as relative light units (RLU) induced by antibody/RLU (no antibody control background). Murine mAb 2G12 (anti-Ebolavirus Gp) is used as negative control. e, NI assay against wild-type (W) and oseltamivir-resistant (R) B/Perth/211/2001 virus. Bar graphs represent IC$_{50}$ values. Technical duplicates were performed in b, c, e, d, and technical triplicates were performed in d. f, Female BALB/c mice (five per group) were administered either 5 mg kg$^{-1}$ of mAb 1F2 i.p., 5 mg kg$^{-1}$ negative-control mAb 8H9 i.p., or placed on a twice daily, 20 mg kg$^{-1}$ regimen of oseltamivir delivered via oral gavage and initiated at 72 h.p.i. Per cent survival is shown. Lung virus titres were compared using a one-way ANOVA corrected for multiple comparisons. Differences in survival were analysed using a Mantel–Cox log rank test. Statistical significance is indicated, where tested, as follows: NS, $P > 0.05$; *$P \leq 0.05$; ***$P \leq 0.001$. Error bars in b–d represent standard deviation.
B/Yamagata/16/88, B/Victoria/2/87 and B/Lee/40 viruses, respectively (Supplementary Fig. 3a–c).

NA antibodies, which are typically considered non-neutralizing, have been shown to decrease plaque size, but not plaque number, in plaque reduction neutralization assays (PRNA)s, an in vitro phenomenon that stems from their ability to inhibit viral egress. Upon viral entry, the Fc receptor (FcγR) is activated by NA antibodies. The critical importance of Fc effector functions such as ADCP and ADCC to the mechanism of protection of non-neutralizing antibodies against the influenza virus is becoming increasingly appreciated. It has been shown using Fc receptor knockout mice and mAbs deficient in Fc receptor binding (DA265-mutant Fc), for instance, that the protection mediated by broadly reactive HA-stalk antibodies in vivo is dependent on Fc-Fc receptor interactions. Furthermore, Leon et al. demonstrated that the Fc activation mediated by HA-directed mAbs is epitope-dependent. Whether or not the NA mAbs described in this study depend on Fc receptor activation to mediate protection or whether or not the ADCC activation by NA-binding mAbs is epitope-dependent remains unknown, but may be elucidated using similar approaches. Of note, a human NA antibody activated ADCC to a lesser extent than an HA-stalk antibody in A549 cells infected with H3N2 influenza virus. However, when titrated into the assay, the NA-binding mAb was able to increase the level of ADCC achieved by the stalk-binding mAb in an additive fashion. This finding implies that NA antibodies may be able to cooperatively enhance the levels of effector functions achieved by HA antibodies during natural infection, most probably by providing increased contact points for Fc-Fc receptor engagement on the surface of virus or infected cells.

Finally, we demonstrated that mAb 1F2 exhibited superior efficacy to the standard of care, oseltamivir treatment, when administered 72 h.p.i. in a mouse challenge model. Conceivably, the combined ability of an anti-NA antibody to interfere with NA enzymatic activity while engaging in effector functions that allow for increased, immune-mediated viral clearance may explain why mAb treatment is able to outperform treatment with NA inhibitor in vivo. The mAbs in this study possess the potential to be developed into therapeutics for high-risk patient populations or against drug-resistant strains of IBV, highlighting the benefits of targeting broadly protective NA epitopes in innovative influenza virus vaccine formulations. As the HA and NA drift independently, vaccines that elicit robust NA titres may prove especially useful in influenza seasons in which an HA drift event occurs.

Methods

Cells, viruses and proteins. As described previously, MDCK cells (originated from MDCK (NBL-2), ATCC CCL-34) were grown in complete Dulbecco’s modified Eagle medium (DMEM, Life Technologies) supplemented with antibiotics (100 U ml−1 penicillin, 100 μg ml−1 streptomycin [Pen-Strep], Gibco), 10% fetal bovine serum (FBS, HyClone) and 1 ml of 1 M HEPES (Life Technologies). Sf9 insect cells (originated from ATCC CRL-1711) were grown in TNM-FH insect medium (Gemini Bioproducts) supplemented with antibiotics (Pen-Strep) and 10% FBS and High Five cells (BTI-TN-5B1-4 subclone, Vienna Institute of Biotechnology) were grown in serum-free SFX-insect cell medium
ELISA. ELISAs were performed as described previously. An endpoint titre was defined as the final concentration at which the antibody signal remained greater than 50% of the maximum signal observed. To create purified virus preparations, allantoic fluid containing virus was collected and subjected to low-speed centrifugation (at 3,000 g for 2 h) to remove cellular debris. Viruses were pelleted through a 30% sucrose cushion (30% sucrose in NTE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA), pH 7.4) by ultracentrifugation (Beckman L-765 ultracentrifuge with SW-28 rotor at 25,000 r.p.m. for 2 h). Once all of the supernatant was aspirated, virus pellets were resuspended in phosphate-buffered saline (PBS). B/Perth/211/1991, B/Perth/211/2011 and B/Perth/211/2011 were provided by A. Hurt and E. Govorkova and are part of the ‘Panel of Influenza A and B Viruses for Assessment of Neuraminidase Inhibitor Susceptibility’ provided by the International Society for Influenza and other Respiratory Virus Diseases (ISIRV - https://isirv.org/site/images/stories/avg_documents/Resistance%20v%20Neuf%20Nov12.pdf). The recombiant proteins used (B/Yamagata/16/88, B/Malaysia/2506/04, B/Florida/04/06, B/Perth/211/2001 198D, B/Perth/211/2001 198E, B/Memphis/1B/2003, B/Malaysia/2506/04, B/Wisconsin/1/10, B/New Jersey/1/12, B/Massachusetts/2/12, B/Texas/2/13 and escape mutant viruses were grown in 8- to 10-day-old embryonated chicken eggs, and titres were determined on MDCK cells in triplicate 106. The HA (viral fusion) and NA (viral neuraminidase) genes were PCR amplified, sequenced and the obtained sequences were deposited into the IMGT/V-QUEST database tool (http://www.imgt.org/IMGT_vquest/ share/textes/) to determine the complete variable-region sequence as well as unique germline genes. In cases where the quality of the sequencing read was not sufficient, the PCR product was first cloned into Stratagene vectors (Agilent Technologies), which were then amplified in Stratagene competent cells, purified using a QIAprep Spin Miniprep Kit (Qiagen) and then sequenced before entering them into the IMGT/V-Quest.

**Antibody/antigen biotinylation.** Antibodies and rNA were biotinylated using an EZ-Link NHS-PEG, Biotin kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

**Kd determination using biolayer interferometry.** Antbody dissociation constants (Kd values) were determined by biolayer interferometry using an Octet Red96 instrument (Fortebio), as described previously. Biosensors were loaded with rNA from B/Malaysia/2506/04.

**Phylogenetic tree generation.** A subsample of 280 IBV NA amino acid sequences were chosen to form the phylogenetic tree. For years in which 10 or fewer sequences were available, all sequences were used, and for years with more than 10 sequences, 10 sequences were randomly chosen to minimize the bias of more recent isolates. Sequences were aligned using MUSCLE and manually edited using the MEGA 6.0 software when applicable. A phylogenetic tree was assembled using the Clustal Omega web server with a neighbour-joining clustering method and default settings. The tree was cleaned and edited using FigTree.

**Negative‐stain electron microscopy.** Recombinant NA and FabS were diluted with buffer (5 mM HEPES, 150 mM NaCl pH 7.3) to −0.02 mg ml−1 and 0.04 mg ml−1, respectively. To prepare Fab bound samples, equal volumes of NA and Fabs 1F2 or 4F11 were mixed and incubated for 5–10 min. The samples were adsorbed to plasma‐cleaned (Solarus Model 950 cleaner, Gatan) electron microscope grids coated with continuous carbon film, which were subsequently washed with buffer and air dried. Images were collected using EPU software (FEI) on a Tecnai T12 electron microscope (FEI) fitted with a 4K charge‐coupled device camera (Gatan) at an effective pixel size of 0.18 nm in the specimen plane. The software package RELION 1.4 (ref. 50) was used to obtain three-dimensional reconstructions. The maps for unbound NA and for the complexes with 1F2, and 4F11 were reconstructed using 47,592, 4,326 and 13,665 particles, respectively, and visualized using UCSF Chimera software.

**Modelling Fab binding footprints.** NA and Fab (Influenza B HA Fab CR8033) X-ray coordinates (PDB IDs: 4CPL and 4FQL, respectively) were fitted to density maps using UCSF Chimera software. To highlight the binding footprints of the Fabs, the regions that NA more closely interacted with each Fab were identified by manual inspection.

**Multiple sequence alignment.** Sequences were obtained from the Global Initiative on Sharing All Influenza Data (GISAID, http://platform.gisaid.org/), with any laboratory-associated strains or truncated sequences removed from analysis. There were a total of 2,409 sequences in the final file used for alignments. Sequence alignments were performed using MEGA 6.0 software (MUSCLE alignment).

**Per cent conservation calculation.** Escape residues were isolated from the whole NA protein sequence alignment using sequence editing tools in MEGA 6.06. A subsample of 944 sequences was used for calculations. For years in which 50 or fewer sequences were available, more than 50 sequences, a random selection of 50 sequences was chosen to minimize the bias of more recent isolates. To obtain the percentage of sequences that contained a specific amino acid at each escape residue location, the amino-acid phenotypes at the site were sorted and then divided by the total number of sequences.

**Generation of IBV anti-NA mAb escape mutant viruses.** mAb escape mutant variants of B/Malaysia/2506/04 virus were generated based on the methods described in ref. 29. mAbs (250 μg) and virus (105 p.f.u.) were combined (total...
volume, 800 μl, incubated for 1 h at room temperature (RT), split evenly into thirds and injected into three 8-day-old embryonated chicken eggs. After incubating for 72 h at 33 °C, allantoic fluid was collected and plated in the presence of mAb (100 μg/ml* in both inoculum and overlay). After incubating for 72 h at 33 °C, plaque assays were inspected for escape variants (evidenced by large plaque size). Large plaques were picked and inoculated into 10-day-old embryonated chicken eggs for amplification. All escape mutant variants, excluding that of mAb 4B2, were generated in this way. mAb 4B2 did not produce a viral escape mutant when using this technique, therefore a second technique, which is more difficult target epitopes, was applied as follows. For generating escape mutants of 4B2, B/Malaysia/2506/04 virus was serially passaged on an MDCK cell monolayer in the presence of increasing amounts of mAb, with a starting concentration of 0.25 × 10^6 (as calculated from the NI assay against B/Malaysia/2506/04 virus). Initially, MDCK cells in one well of a six-well plate were infected with B/Malaysia/2506/04 virus at an MOI of 0.1 in the presence of 0.5 × 10^6 of mAb. After incubating for 72 h at 33 °C, 10 μl of supernatant was collected and used to directly inoculate a fresh monolayer of MDCK cells in the presence of increased mAb concentration. This process was repeated for 15 passages, until the final antibody concentration was ~1 mg ml⁻¹. Throughout serial passaging, successful infection was confirmed by the presence of the cytopathic effect (CPE) or, if CPE was not clear, positive staining with polyclonal anti-IBV mouse serum (the detailed immunostaining procedure is described in the section ‘Evaluation of prophylactic and therapeutic efficacy in mice’). Both CPE and positive immunostaining were present in the last passage. Virus was also passaged in the presence of an irrelevant mouse mAbs (SC12, 12B10) and previously in ref 7 throughout all experiments to control for mutational variants obtained from passaging alone. Viruses were plaque purified once serial passaging was completed to create monoclonal stocks for deep sequencing and growth curve analysis.

Deep sequencing of escape mutant variants. RNA from the escape mutant variants was obtained using a Direct-zol RNA kit (Zymo Research). Samples were processed using the Illumina TruSeq RNA Sample Preparation Kit according to the manufacturer’s instructions and sequenced using a MiSeq Illumina instrument. Reads were consolidated and aligned to B/Malaysia/2506/04 using Bowtie2. The assembled genomes and minority variants were visualized with the Integrative Genomic Viewer (IGV) (Broad Institute).

Immunofluorescence. To screen for escape variants, MDCK cells were plated in 96-well, sterile, flat-bottom tissue culture plates (Sigma) and subsequently infected with either wt B/Malaysia/2506/04 or mutant viruses at an MOI of 10. After incubation for 18 h at 33 °C in MEM lacking TPCK-trypsin (to limit viral growth to one infectious cycle), medium was removed and cells were fixed with 3.7% formaldehyde for at least 1 h at 4 °C. The formaldehyde was discarded and the cell monolayer was blocked with 3% milk in PBS for at least 1 h. For the primary antibody step, plates were incubated with either the respective IBV anti-NA mAb (30 μg/ml⁻¹), a positive infection control (a polyclonal cocktail of purified mouse IgGs against the IBV HA (1:1,000 dilution), or irrelevant negative control mouse IgGs (1:1,000 dilution). The plates were washed three times with PBS and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody in PBS, 1% milk (100 μl per well) for 1 h at RT, while shaking. Plates were washed three times with PBS and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody in PBS, 1% milk (100 μl per well) for 1 h at RT in the dark. Finally, after washing three additional times, cells were visualized via fluorescent microscopy.

Growth curve analysis. To compare viral fitness in the presence of mAbs, growth curves were performed in MDCK cells. Cells were plated as a confluent monolayer in 12-well tissue culture plates (SigmA) and infected with virus at an MOI of 0.01 (final volume of 1 ml per well). The experiment was performed in triplicate for each time point and each antibody condition. mAb was added to the infection medium at a concentration of 10 μg/ml⁻¹. Cells were incubated at 33 °C and supernatant was collected at 12, 24, 48, and 72 h p.i. The collected supernatant was clarified by centrifugation (at a relative centrifugal force of 3,000 g for 10 min at 4 °C) and immediately stored at −80 °C. For simplicity, only titres at 72 h p.i. are reported here. Viral titres were assessed via haemagglutination assays, as described previously.

Three-dimensional mapping of escape mutations. Escape mutations were represented on a three-dimensional structure of the NA of B/Brisbane/60/2008 (3D model). The model was visualized using a Jmol add-on (Jmol). For producing escape mutants, RNA PRNAs were performed according to the protocols described by Tan et al. and Wohlbild et al., with some modifications. In duplicate, fivefold dilutions of mAbs (highest concentration, 100 μg/ml⁻¹; lowest concentration, 3.2 × 10⁻⁴ μg/ml⁻¹) were prepared in serum-free 1 × MEM, and each dilution was incubated with 100 p.f.u. of virus for 1 h at 30 °C on a shaker. The inocula were then plated on MDCK cell monolayers in either 12-well (B/Victoria/2/87, B/Yamagata/16/88, B/Victoria/2/87 viruses) or 6-well (B/Malaysia/2506/04 virus) plates, similar to the protocol used to plaque lungs titres (described earlier). After 3–4 days of incubation at 33 °C, the cells were fixed with 3.7% formaldehyde for at least 1 h at 4 °C and blocked with 3% milk in PBS for at least 1 h. For the primary antibody step, plates were then incubated with a cocktail of broadly reactive, anti-IBV HA mouse mAbs (1:5,000 dilution in PBS, 1% milk) for 1 h at RT, while shaking. Plates were washed with PBS and incubated with an anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma) for 30 min at 37 °C. Finally, plates were washed and stained with 5-bromo-4-chloro-3-indolyl phosphate (BrdIP) and 3 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl phosphate. The plaques were visualized and counted. The plaques were counted in each mAb dilution and the per cent inhibition for each mAb at each dilution was calculated based on a no-antibody control. An irrelevant murine IgG (81H9) was used as negative control. The data were analysed using Prism software (GraphPad). We also assessed the decrease in plaque size upon incubation with antibody. To analyse average plaque diameter, 10 plaques were randomly selected in each well using a technique described previously.

Mouse ADCC reporter assays. Assessment of the ability of mAbs to trigger ADCC was performed using a commercial ADCC reporter kit (Promega) and according to the manufacturer’s instructions. Briefly, MDCK cells were seeded into white flat-bottom, 96-well cell culture plates (Costar) at a density of 3.0 × 10⁵ cells per well and incubated overnight at 37 °C and 5% CO₂. The following day, the cells were infected with B/Yamagata/16/88, B/Malaysia/2506/04 or B/Florida/04/06 virus at an MOI of 3 and incubated at 33 °C, 5% CO₂. Sixteen hours later, the cell medium was exchanged for threefold serial dilutions of antibody in assay buffer, starting at 30 μg/ml⁻¹. Effector cells were added and, after another 6 h of incubation (37 °C, 5% CO₂), Bio-Glo luminescence reagent and substrate (Promega) were added and luminescence was measured on a Synergy H1 microplate reader (BioTek). Data were analysed using Prism 6 software (GraphPad).

NA-Star assay. The NA-STAR influenza neuraminidase inhibitor resistance detection kit (Applied Biosystems) was used to assess mAb (or Fab) inhibition of the ability of NA to cleave a small, soluble, chemiluminescent substrate (sodium (2-chloro-5-[4-methoxy]phenyl-1,2-dioxetane-3,2'-dioxetano) (Star Assay NA-STAR assay buffer (starting concentration, 100 μg/ml⁻¹; final volume per well, 25 μl) in white, flat-bottom, 96-well cell culture plates. A 25 μl volume of virus at the determined 3 × EC₅₀ (half-maximum effective concentration) was added to each cell well, and the plates were incubated. For competition inhibitor binding, the remaining of the assay was performed in an identical manner to the method described above, beginning with the addition of NA-Star substrate. Data points were expressed as per cent inhibition of maximal NA enzymatic activity, which was...
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**Author contributions**

T.J.W., K.A.P., S.S. and F.K. designed experiments and wrote the manuscript. T.J.W., K.A.P., V.C. and P.M. performed experiments. J.T. and F.A. assisted with experiments. F.A. and G.S.T. generated reagents. T.J.W., K.A.P., V.C., V.F., J.T., E.K., B.R.t., P.P., S.S. and F.K. analysed and interpreted data.

**Competing interests**

The Icahn School of Medicine at Mount Sinai has filed patents regarding use of the described mAbs as therapeutics (application no. 62/483,262). T.J.W., P.P. and F.K. are named as inventors on the application.

**Additional information**

Supplementary information is available for this paper at doi:10.1038/s41564-017-0011-8.

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1. **Experimental design**

   1. **Sample size**
      
      Describe how sample size was determined.

      No initial power analysis was done to select the number of mice used. An n of 3 mice/group was chosen for lung titer analysis and an n of 5 mice/group for challenge studies based on available resources and according to the general practices in the influenza field.

   2. **Data exclusions**
      
      Describe any data exclusions.

      No data were excluded.

   3. **Replication**
      
      Describe whether the experimental findings were reliably reproduced.

      All experiments contained at least technical duplicates. Experimental findings were reliably reproduced. Specifically, the described mAbs are now being further developed as potential therapeutics and in vivo and in vitro results could be reproduced by independent operators.

   4. **Randomization**
      
      Describe how samples/organisms/participants were allocated into experimental groups.

      Mice were randomly assigned to infection and treatment groups without a specific algorithm.

   5. **Blinding**
      
      Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

      The researchers were not blinded to group allocation.

   6. **Statistical parameters**
      
      For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

      - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
      - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
      - A statement indicating how many times each experiment was replicated
      - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
      - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
      - The test results (e.g. p values) given as exact values whenever possible and with confidence intervals noted
      - A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
      - Clearly defined error bars

      See the web collection on statistics for biologists for further resources and guidance.
Software

7. Software

Describe the software used to analyze the data in this study.

Prism Version 6

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The Nature Methods guidance for providing algorithms and software for publication may be useful for any submission.

Materials and reagents

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

Not applicable, there are no restrictions on the materials used in this study.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

This study describes the initial characterization of monoclonal antibodies produced in mice. In addition, the following proprietary antibodies were used as secondary antibodies (respective catalogue numbers are provided):
(1) Anti-mouse IgG, horseradish peroxidase linked whole antibody (from sheep)- #NXA931
(2) Alexa Fluor 488 donkey anti-mouse IgG- #A21202

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

MDCK - originally obtained from ATCC, maintained and passaged in the lab
SP2/0 mouse myeloma cell line - originally obtained from ATCC, maintained and passaged in the lab

b. Describe the method of cell line authentication used.

None of the cell lines used in the study have been specifically authenticated.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines tested negative for mycoplasma.

d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used in this study.

Animals and human research participants

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

6-8 week old, female BALB/c mice (Jackson labs) were used for all experiments.

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.