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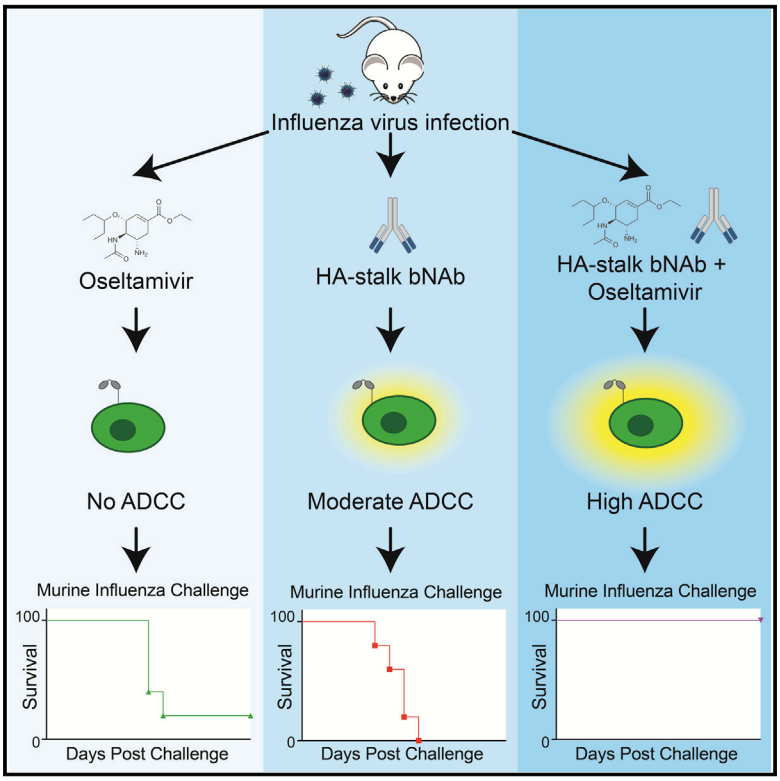
Hemagglutinin stalk-binding antibodies enhance effectiveness of neuraminidase inhibitors against influenza via Fc-dependent effector functions

Graphical abstract



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In brief

Broadly neutralizing antibodies (bNAbs) that bind to the hemagglutinin stalk domain are an attractive therapeutic that protect against multiple strains of influenza virus. Here, Zhang et al. demonstrate that neuraminidase inhibitors cooperate with both monoclonal and polyclonal bNAbs to protect against influenza by potentiating Fc-dependent effector cell functions.

Highlights

- NA inhibitors potentiate Fc-mediated activation of immune effector cells by bNAbs
- bNab-NA inhibiter combination therapy is superior to either monotherapy
- bNAb-NA inhibitor combination therapy is effective therapeutically and prophylactically
- Pre-existing bNAb titers may predict effectiveness of NA inhibitor treatment

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Hemagglutinin stalk-binding antibodies enhance effectiveness of neuraminidase inhibitors against influenza via Fc-dependent effector functions

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SUMMARY

The conserved hemagglutinin stalk domain is an attractive target for broadly effective antibody-based therapeutics and next-generation universal influenza vaccines. Protection provided by hemagglutinin stalk-binding antibodies is principally mediated through activation of immune effector cells. Titers of stalk-binding antibodies are highly variable on an individual level and tend to increase with age as a result of increasing exposures to influenza virus. In our study, we show that stalk-binding antibodies cooperate with neuraminidase inhibitors to protect against influenza virus infection in an Fc-dependent manner. These data suggest that the effectiveness of neuraminidase inhibitors is likely influenced by an individual's titers of stalk-binding antibodies and that neuraminidase inhibitors may enhance the effectiveness of future stalk-binding monoclonal antibody-based treatments.

INTRODUCTION

Influenza causes 3–5 million serious illnesses and 290,000–645,000 deaths every year worldwide.[1] Seasonal influenza vaccination induces production of mostly neutralizing antibodies directed against the hemagglutinin (HA) head domain and is the most effective way to prevent infection. The HA head domain undergoes continuous antigenic drift, which necessitates annual reformulation of seasonal vaccines to narrow strain-specific protection. Vaccine strategies designed to elicit immune responses against more conserved protein domains, such as the HA stalk domain by broadly neutralizing antibodies (bNAbs), may achieve more broad and durable protection against influenza.

The HA stalk domain is more conserved between influenza virus strains compared with the HA head domain due to functional constraints and minimal immune pressure.[2,3] Protection against influenza by HA stalk-binding antibodies is mediated primarily by Fc-dependent activation of immune effector cells.[4–6] Optimal Fc activation by these antibodies requires two points of contact: the antibody Fab-HA stalk interaction, and the sialic acid-HA head interaction[7] (Figure 1A). Neuraminidase (NA), the other major influenza virus surface glycoprotein, cleaves sialic acids from the HA head domain during viral budding. Antibody-dependent cellular cytotoxicity (ADCC) is a process through which infected cells are eliminated by Fc receptor (FcR)-bearing immune cells after binding of antibodies to surface-bound antigen. Antibodies that bind to NA can induce modest levels of ADCC on their own and can also cooperate with stalk-binding antibodies to enhance ADCC induction.[6] HA stalk-binding antibodies can, in turn, partially inhibit NA activity, contributing to their potency of virus neutralization.[8] Since cleavage of the sialic acid-HA head interaction by NA may destabilize one of the two points of contact required for optimal stalk antibody-mediated activation of immune effector cells, we reasoned that chemical inhibition of NA activity may also potentiate ADCC induction by HA stalk-binding antibodies.

Here, we show that chemical inhibition of NA increases stalk antibody Fc-mediated activation of immune effector cells in a dose-dependent manner. HA stalk antibodies potentiated the efficacy of the NA inhibitor oseltamivir in both prophylactic and therapeutic contexts. This effect is preserved in the contexts of both monoclonal stalk antibody and polyclonal sera. Cooperativity between NA inhibition and HA stalk antibodies was dependent on Fc-FcR interaction. Together, these data suggest that the efficacy of oseltamivir treatment may be dependent on HA stalk antibody titers and that monoclonal HA stalk antibodies and NA inhibitors may represent an effective combination therapy in the future.

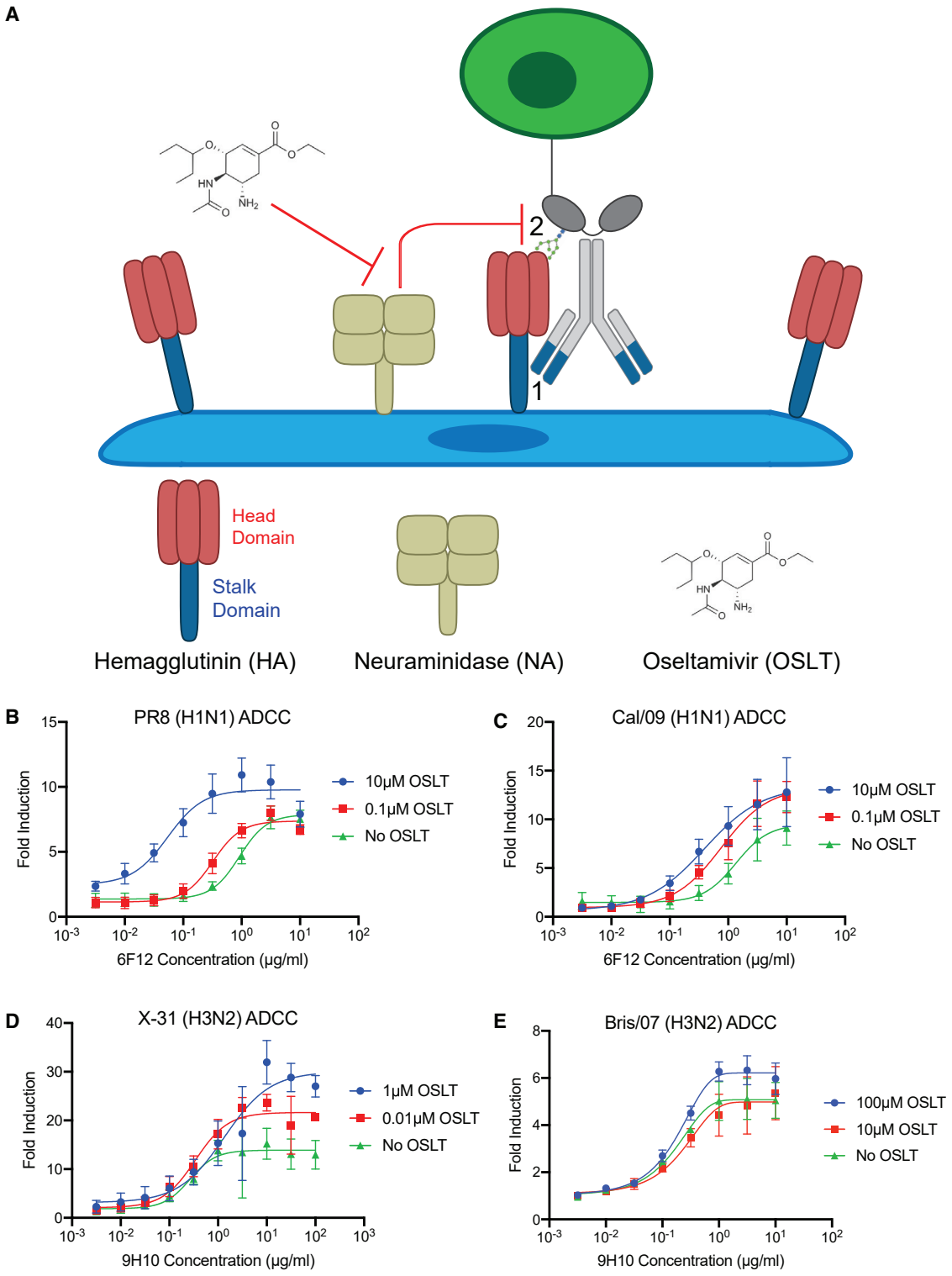

Figure 1. Oseltamivir potentiates ADCC induction by monoclonal stalk-binding broadly neutralizing antibodies in a dose-dependent manner

(A) Diagram of broadly neutralizing antibodies (bNAbs) facilitating the interaction between immune effector cell and infected cell via two points of contact. The immune effector cell with an Fc receptor is depicted in green. The stalk-binding antibody is shown in gray and blue. The stalk-binding antibody interacts with the HA stalk domain via its Fab portion (1) and binds to the Fc receptor of the effector via its Fc portion. The HA head domain interacts with sialic acid residues on the

(legend continued on next page)

RESULTS

Oseltamivir cooperates with stalk-binding antibodies to enhance antibody-dependent cell cytotoxicity of cells infected with group 1 and group 2 influenza A viruses

We first set out to determine the role of NA on HA stalk-binding antibody-mediated activation of immune effector cells. We first quantified the NA activity of three strains of influenza virus, A/Puerto Rico/8/1934 H1N1 (PR8), A/California/07/2009 H1N1 (Cal/09), and X-31 H3N2 (Figures S1A–S1C).[9] We then tested the potency of oseltamivir in inhibiting the NA activity of these strains (Figures S1D–S1F) and in reducing viral replication (Figures S1G–S1I). Having established these dose-response relationships with oseltamivir, we performed ADCC assays using modified Jurkat effector cells expressing murine FcγRIV. This is an extremely sensitive, highly quantitative assay that we have used extensively in the past and has been validated to correlate closely with the activation of primary natural killer (NK) cells.[6,10] A549 target cells were infected with PR8, Cal/09, X-31, or A/Brisbane/10/2007 H3N2 (Bris/07) at a multiplicity of infection (MOI) of 5. The infected cells were then incubated with oseltamivir and murine monoclonal stalk-binding bNAbs 6F12 for cells infected with PR8 or Cal/09 (which express group 1 HA) and 9H10 for cells infected with X-31 or Bris/07 (which express group 2 HA). Addition of oseltamivir resulted in a dose-dependent enhancement of ADCC induction in PR8-, Cal/09-, X-31-, and Bris/07-infected cells (Figures 1B–1E). Oseltamivir by itself or with mouse immunoglobulin G (IgG) isotype control did not induce activation of the effector cells (Figure S2B). We next used 6F12 with the D265A mutation in our ADCC assays. D265A is a well-characterized mutation in the CH2 domain that completely abrogates Fc-FcR interactions.[11] Enhancement of bNAb-mediated ADCC by oseltamivir requires Fc-FcR interactions, since 6F12 D265A failed to induce ADCC of PR8-infected cells, even at the highest oseltamivir concentration used (Figure S2A). Furthermore, the D265A antibody retained binding properties of the wild-type (WT) 6F12 antibody to the HA stalk domain, as reported previously (Figure S2D).[5] Lastly, enhancement of bnAb-mediated ADCC induction by NA inhibition was specific, as addition of baloxavir marboxil, an inhibitor of influenza cap-snatching endonuclease, had no effect on ADCC induction (Figure S2C). Together, these results demonstrate that NA inhibition by oseltamivir enhances ADCC induction by bNAbs that bind to the HA stalk domain, demonstrating cooperative activation.

The efficacy of oseltamivir in preventing and treating influenza virus is potentiated by bNAbs

To determine whether bNAbs could enhance protection mediated by oseltamivir *in vivo*, we conducted influenza challenge experiments in a murine model system. To test efficacy in a prophylaxis setting, six groups of 6- to 8-week-old BALB/c mice (n = 5 mice/group) were administered 1 mg/kg 6F12, 6F12 D265A, or IgG isotype control intraperitoneally and 1 mg/kg oseltamivir or PBS by oral gavage. The mice were then infected with 5 × LD_{50} of PR8 (500 plaque-forming units [PFUs]) intranasally 2 h later. Mice continued to receive either oseltamivir or PBS twice daily by oral gavage for 5 days and were monitored for 14 days, with the humane endpoint defined as 80% of initial body weight (Figures 2A–2C). Mice that received negative control treatments all reached endpoint by day 7 post-infection. Of the mice that did not receive oseltamivir, treatment with 1 mg/kg 6F12 resulted in similar disease progression compared with the IgG isotype control. Similarly, mice that received oseltamivir alone experienced significant weight loss. In contrast, mice that received both oseltamivir and 6F12 lost minimal weight throughout the experimental period, with none reaching endpoint. Mice that received both oseltamivir and 6F12 D265A displayed similar disease progression compared with mice that received oseltamivir alone, demonstrating that Fc-FcR interactions are crucial for the enhanced protection offered by co-administration of 6F12 and oseltamivir.

To determine if bNAbs could also enhance the activity of oseltamivir when treating an established influenza infection, we infected four groups of 6- to 8-week-old BALB/c mice (n = 5 mice/group) with 5 × LD_{50} of PR8 (500 PFUs) and treated them with 10 mg/kg 6F12 or 10 mg/kg oseltamivir starting 2 days post-infection (Figures 2D and 2E). Antibody or oseltamivir alone were insufficient to protect mice from severe disease, whereas the combination of 6F12 and oseltamivir yielded complete protection. These results demonstrate a substantial improvement in protection against influenza morbidity and mortality when oseltamivir and stalk-binding antibodies are administered together in the context of both prophylaxis and treatment. The cooperativity of oseltamivir and stalk-binding antibodies was not due to their independent activities (i.e., direct NA inhibition and virus neutralization) but was instead dependent upon Fc-FcR interactions mediated by the stalk-binding antibodies.

Titers of bNAbs in human serum predict effectiveness of oseltamivir treatment

The previous results demonstrate that oseltamivir cooperates with monoclonal stalk-binding antibodies to protect against clinical signs of influenza virus infections by enhancing antibody Fc-mediated immune effector cell activation. We next set out to determine if titers of bNAbs in human serum could predict the effectiveness of oseltamivir treatment. First, we conducted ADCC assays using serum from four adult donors who had received the seasonal influenza vaccine 14 days prior to serum collection. Titers of bNAbs in these samples were first quantified

effector cell (2). NA enzymatically cleaves sialic acids from the HA head domain, abrogating the second point of contact. NA inhibitors, such as oseltamivir, restore the second point of contact by preventing the enzymatic cleavage of HA and sialic acid.

(B–E) *In vitro* ADCC assays were completed using A549 cells infected with PR8 (H1N1), Cal/09 (H1N1), X-31 (H3N2), or Bris/07 (H3N2) at an MOI of 5. Fold induction depicts activation above background (infected cells without antibody). Concentrations of oseltamivir carboxylate (OSLT) are denoted in the legend. 6F12 (Pan H1 stalk-binding antibody) was used to target PR8- and Cal/09-infected cells, while 9H10 (group 2 HA stalk-binding antibody) was used to target X-31- and Bris/07-infected cells. Fold induction data are shown as mean ± SD with biological triplicates.

See also Figures S1 and S2.

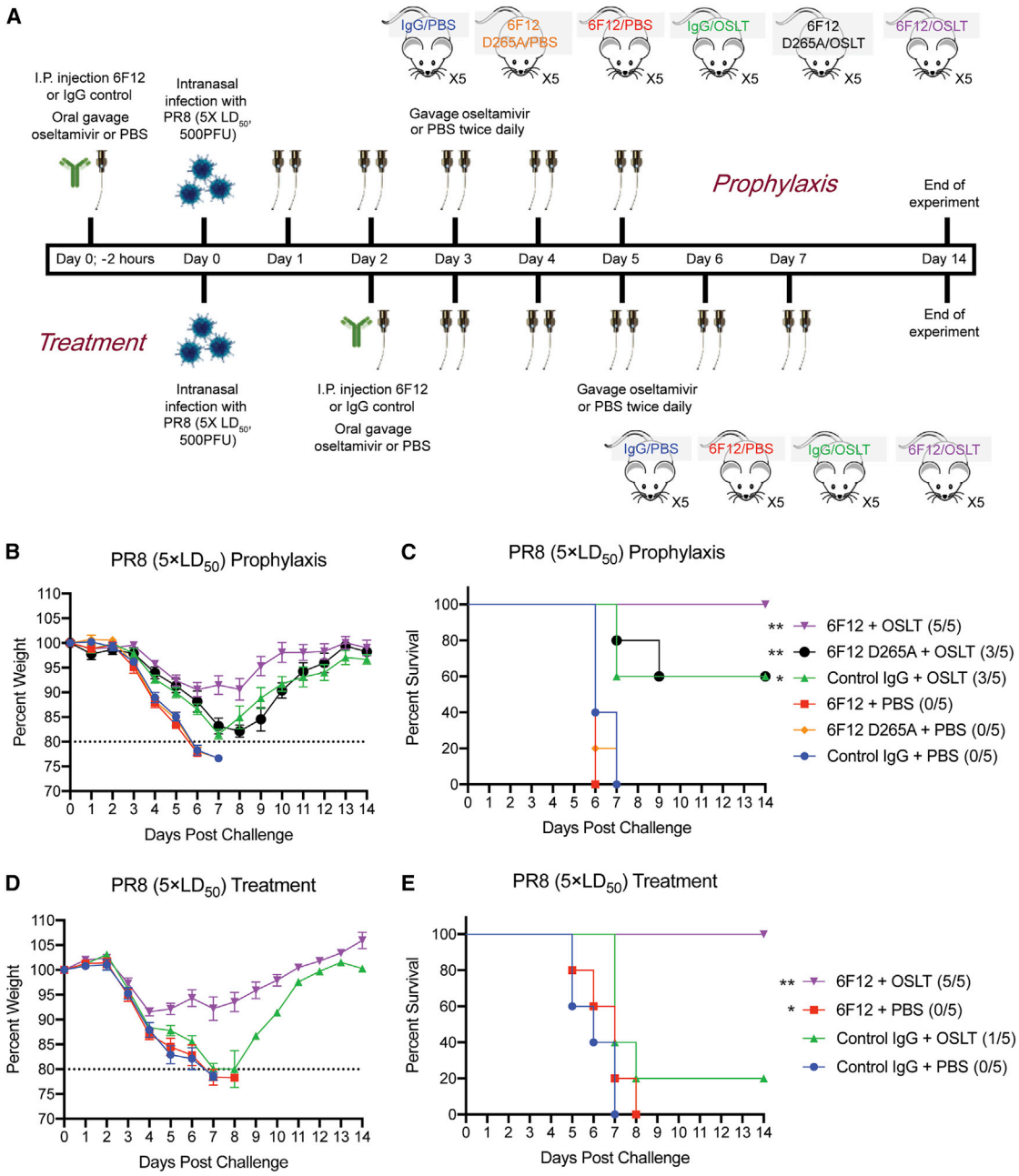

Figure 2. Oseltamivir administered in combination with bNAbs is superior at preventing and treating influenza clinical signs compared with either therapeutic alone

(A) Six- to eight-week-old female BALB/c mice were infected intranasally with 500 PFUs of PR8 (5 × LD_{50}). The mice were also administered intraperitoneally with 6F12 or PBS and oral gavage of oseltamivir or PBS. A dose of either 1 mg/kg 6F12 and/or 1 mg/kg oseltamivir (prophylaxis) or 10 mg/kg 6F12 and/or 10 mg/kg oseltamivir (treatment) were used. The first round of therapeutics was given either 2 h before infection (prophylaxis) or 48 h after infection (treatment). Mice were then given oseltamivir or PBS by oral gavage twice daily for 5 additional days following the first round of therapeutics. Weight change was monitored daily, and the animals were sacrificed when they reached 80% of initial weight.

(B and C) Weight loss and Kaplan-Meier survival curves of the mice treated prior to infection (prophylaxis group). Weight loss is shown as percentage of initial weight with mean ± SEM, n = 5/group. Statistical comparisons are shown against the control IgG + PBS group; *$p < 0.05$, **$p < 0.01$. Numbers in brackets denote number of surviving mice and total number of mice per group.

(D and E) Weight loss and Kaplan-Meier survival curve of the mice treated after infection (treatment group). Weight loss is shown as percentage of initial weight with mean ± SEM, n = 5/group. Statistical comparisons are against the control IgG + PBS group; *$p < 0.05$, **$p < 0.01$. Numbers in brackets denote number of surviving mice and total number of mice per group.

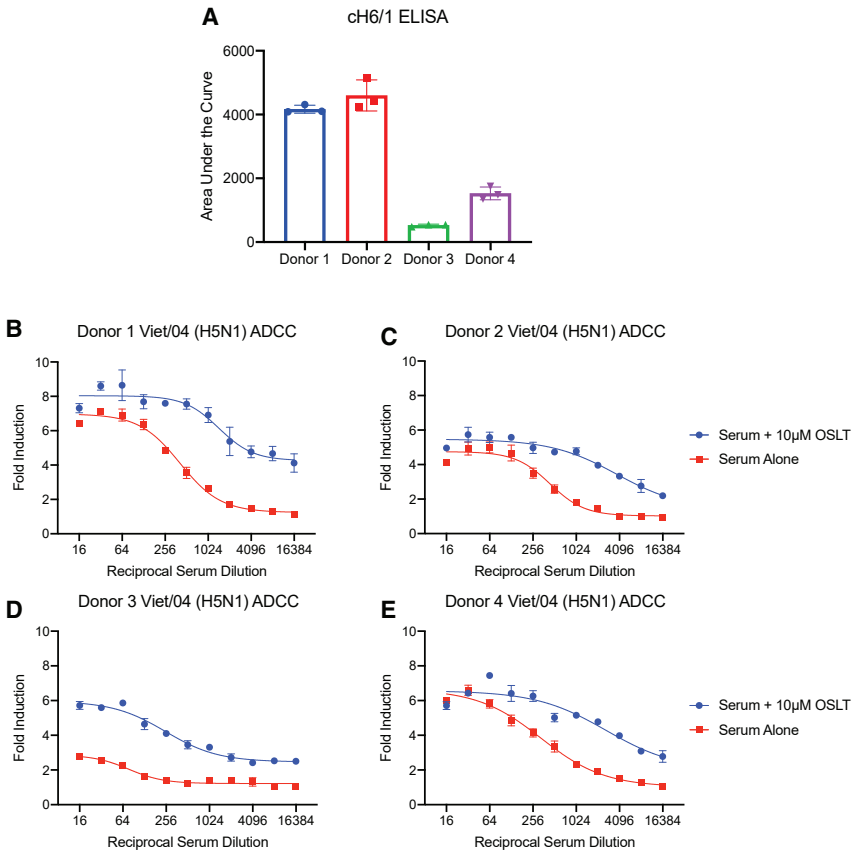

Figure 3. Oseltamivir increases the potency of ADCC induction by polyclonal stalk-binding antibodies

(A) ELISAs were performed using recombinant cH6/1 HA to measure the titer of H1 stalk-binding antibodies in the serum samples. The area under the curve is shown as mean ± SEM. Experiments were performed in technical triplicate.

(B–E) *In vitro* ADCC assays were completed using A549 cells infected with Viet/04 (H5N1) at an MOI of 5. Fold induction denotes activation above infected cells without antibody. Serum was obtained from 4 healthy donors who had been previously vaccinated with seasonal influenza virus vaccine. Fold induction data are shown as mean ± SD. Experiments were performed in technical triplicate.

by ELISA using a chimeric cH6/1 HA protein, which contains the head domain from A/Mallard/Sweden/81/02 H6N1 and the stalk domain from A/Puerto Rico/8/1934 H1N1 (Figure 3A). The cH6/1 chimera has been used extensively in the past to quantify antibodies that specifically bind to the group 1 stalk domain, as humans are rarely exposed to H6 influenza viruses, and we have previously shown that human sera lacks hemagglutination inhibition (HAI) activity against the H6 head domain.[12–15] Consistent with the observations made using monoclonal bNAbs, ADCC induction by polyclonal bNAbs from human donors was enhanced by oseltamivir (Figures 3B–3E).

We next set out to assess the effectiveness of human sera in combination with oseltamivir in protecting against a "pandemic-like" virus. We then chose to use A/Vietnam/1203/2004 H5N1 HAlo (Viet/04), which is an H5N1 virus that is highly pathogenic in mice but has had the polybasic cleavage site of the H5 protein removed to enhance safety.[16] ELISAs were first performed on the serum from two additional healthy adult donors using the chimeric cH6/1 HA protein to quantify bNAb titers (Figures S3A). To ensure that the serum samples used in these assays did not contain HAI+ antibodies to the head domain of Viet/04 H5, we conducted an HAI assay (Figure S3B). No HA inhibition was observed, indicating that both donors were naive to this virus. In ADCC assays, the donor with higher titers of HA stalk-binding antibodies based on ELISA data (Figure S3A) also elicited higher activation of reporter cells, consistent with expectations (Figure S3C).

Next, six groups of 6- to 8-week-old BALB/c mice were either administered 150 μL serum with low titers of stalk-binding antibodies (low serum), 150 μL serum with high titers of stalk-binding antibodies (high serum), or 150 μL PBS (vehicle). The mice were also given either 0.1 mg/kg oseltamivir or PBS by oral gavage. The mice were infected with 5 × LD_{50} of Viet/04 H1N1 (200 PFUs) intranasally 2 h after passive transfer of serum (Figure 4A). The data are displayed as two sets of graphs for clarity, with shared negative control groups (Figures 4B–4E). Mice that received either serum or oseltamivir alone experienced significant morbidity, with most animals in those groups reaching endpoint. When oseltamivir was administered to mice that had received serum containing low titers of bNAbs (low serum), weight loss was similar to that of mice treated with serum or oseltamivir alone; however, mortality in this group improved substantially, with only one out of five mice reaching endpoint. In contrast, when oseltamivir was administered to mice that received serum containing high titers of bNAbs (high serum), minimal weight loss was observed, and full protection against mortality was achieved (Figures 4B–4E). Taken together, these results demonstrate that titers of bNAbs found in human serum correlate with effectiveness of oseltamivir treatment.

DISCUSSION

NA inhibitors are a major class of antivirals used for treatment and prevention of influenza virus. While NA inhibitors tend to be highly effective in preventing influenza when administered prophylactically, effectiveness is much more modest in most therapeutic contexts.[17] Maximum benefit is achieved therapeutically when NA inhibitors are administered early after symptom onset.[18–20] However, there is considerable heterogeneity in the effectiveness of these drugs at the individual level, and the

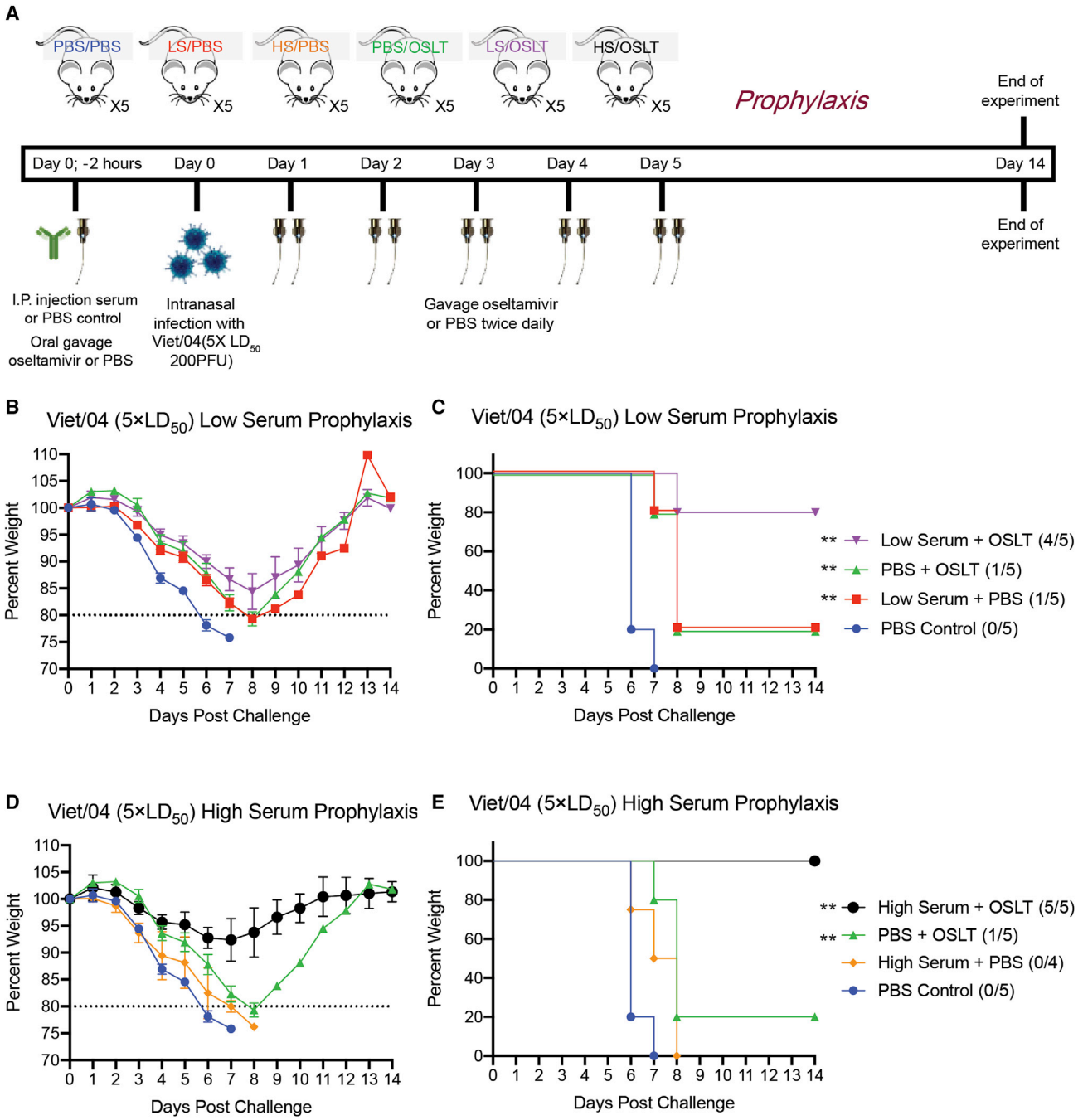

Figure 4. Oseltamivir is more effective at preventing influenza clinical signs in animals with higher HA stalk-binding antibody titers

(A) Six- to eight-week-old female BALB/c mice were administered 150 μL human serum or PBS intraperitoneally (i.p.) and oral gavage of 0.1 mg/kg oseltamivir or PBS. The serum contains either high titers of polyclonal stalk-binding antibodies (high serum [HS]) or low titers of polyclonal stalk-binding antibodies (low serum [LS]). The mice were then infected intranasally with 200 PFUs of Viet/04 (5 × LD_{50}) 2 h later. Mice were given oseltamivir or PBS by oral gavage twice daily for 5 days. Weight change was monitored daily, and the animals were sacrificed when they reached 80% of initial weight.

(B and C) Weight loss and Kaplan-Meier survival curve of the LS group. Weight loss is shown as percentage of initial weight with mean ± SEM, n = 5/group. Statistical comparisons are against the PBS control group; *$p < 0.05$, **$p < 0.01$. PBS + OSLT and PBS control groups are shared between graphs depicting HS and LS experiments for clarity. Numbers in brackets denote number of surviving mice and total number of mice per group.

(D and E) Weight loss and Kaplan-Meier survival curve of the HS group. Weight-loss graph is shown as percentage of initial weight as mean ± SEM, n = 5/group unless otherwise indicated. Statistical comparisons are against the PBS control group; *$p < 0.05$, **$p < 0.01$. PBS + OSLT and PBS control groups are shared between graphs depicting HS and LS experiments for clarity. Numbers in brackets denote number of surviving mice and total number of mice per group.

See also Figure S3.

host factors that determine outcome remain elusive.[21] Furthermore, NA inhibitor-resistant viruses circulate naturally, and therefore strategies that mitigate the selection of these resistant variants are critical to prolonging the utility of this class of drug.[22]

The HA stalk domain is an attractive target for antibody-based therapeutics and universal influenza vaccines due to its high degree of conservation among diverse influenza viruses. The main mechanism by which these antibodies protect against influenza is through Fc-dependent effector cell functions, such as ADCC, NETosis, phagocytosis, and secretion of cytokines.[4,5,23,24] Elegant studies by the Yewdell laboratory previously demonstrated that NA inhibition by anti-HA stalk antibodies contributes to viral neutralization and induction of FcγR-mediated activation of innate immune cells.[8] We have also previously shown that antibodies with NAI activity can cooperate with bNAbs that bind to the HA stalk to potentiate ADCC.[6] NA inhibition potentiates bNAb-mediated effector cell function by stabilizing the interaction between HA and sialic acids on the effector cell.[7] The additional point of contact results in increased levels of downstream signal transduction and activation of Fc-dependent effector functions. Our current study demonstrates that the efficacy of NA inhibitors is influenced by pre-existing titers of bNAbs and that protection from influenza *in vivo* is mediated by potentiation of Fc-dependent effector functions of immune cells mediated by NA inhibition.

We used the well-characterized monoclonal murine antibodies 6F12 and 9H10, which bind to H1 and group 2 HA, respectively, in our ADCC assays.[25,26] Our ADCC assay uses engineered Jurkat effector cells, which express murine FcγRIV, and firefly luciferase driven by the nuclear factor of activated T cells (NFAT) response element. ADCC induction in these assays correlate well with classical CD107a NK cell degranulation assays.[10] We found that oseltamivir potentiated ADCC induced by bNAbs in a dose-dependent manner. Interestingly, the magnitude and pattern of potentiation of ADCC induction varied by strain of virus and antibodies that we used. Among the two H1N1 viruses, the increase in ADCC potency (decreased half maximal effective concentration [EC_{50}]) was more pronounced with PR8 compared with Cal/09, even though both viruses were similarly susceptible to NA inhibition by oseltamivir. The pattern of potentiation was also different when comparing the H1N1 viruses with the H3N2 viruses. Whereas oseltamivir increased both the potency and efficacy (maximum induction) of bNAb-mediated ADCC in H1N1 viruses, only the efficacy was increased by oseltamivir in experiments with H3N2 viruses. The differences in the precise pattern of ADCC activation mediated by NA inhibitors is likely mediated by a variety of factors including differences in density of HA on infected cell, NA activity, and affinities and specific epitopes bound by the antibodies.

Intact Fc-FcR interactions were absolutely necessary for this potentiation, as introduction of a D265A mutation in the CH2 domain of 6F12, which abolishes the Fc-FcR interaction, negated effector cell induction.[11,27,28] The addition of oseltamivir had similar effects in improving ADCC mediated by serum from healthy donors. Effector cell activation is strikingly enhanced even in samples with low titers of bNAbs. Antibodies targeting the less immunogenic NA, M2, and NP have also been shown to induce effector cell activation.[4,29–31] Our experiments here show that chemical inhibition of NA can further enhance induction of ADCC even in a complex, polyclonal context.

Consistent with previous studies, we found that co-administration of oseltamivir with a sub-protective dose of monoclonal HA stalk-binding antibodies improves protection against lethal influenza challenge in mouse models.[32,33] However, our work clearly demonstrates that this enhancement is dependent upon Fc-FcR interactions and is not due to the combined, independent activities of oseltamivir and bNAbs. To test if titers of bNAbs in human serum impact the effectiveness of oseltamivir treatment, we passively transferred mice with human sera containing either "low" or "high" titers of bNAbs. The effectiveness of oseltamivir treatment was proportional to the titers of bNAbs transferred to the mice. Together, these data expand on our reductionist experiments with monoclonal antibodies to demonstrate that the phenomenon is maintained in a polyclonal context and suggest that an individual's pre-existing titers of bNAbs may correlate with the effectiveness of oseltamivir treatment.

Our data provide insights that help to explain the heterogeneity in effectiveness of NA inhibitors at the individual level. In the future, clinical trials that directly assess whether pre-existing titers of bNAbs predict the effectiveness of NA inhibitor treatment could have important clinical implications that inform selection of the most appropriate antiviral treatments for individuals with influenza—especially in light of the recent approval of baloxavir marboxil, which targets the cap-snatching endonuclease activity of the viral polymerase complex.[34] These drugs have similar clinical effectiveness, and thus an evidence-based framework to guide which drug may be most effective for individual patients is sorely needed. While rapid screening of bNAb titers may be possible in the future, it is also known that titers of these antibodies tend to increase with age.[35,36] Our study also suggests that the effectiveness of future bNAb-based monoclonal antibody therapies could be improved by co-administration with NA inhibitors. This class of drugs may be especially important for providing first-line defense in the event of a future influenza virus pandemic, since prediction of which strains are likely to cause pandemics remains a considerable challenge to strain-specific pandemic vaccine design.[37]

Limitations of the study

The *in vitro* ADCC assays in our study use reporter cell lines that express only one specific activating FcR (murine FcγRIV or human FcγRIIIa) and therefore may not recapitulate all features of endogenous FcR signaling in NK cells. However, these specific assays have been previously shown to correlate well with conventional CD107a NK cell degranulation assays.[10] Finally, although our study suggests that individuals with higher levels of HA stalk-binding bNAbs may be better protected by oseltamivir treatment, that work was based on a murine model system and should be further evaluated in a clinical study of human participants.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100718.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.Z. and M.S.M.; methodology, A.Z. and M.S.M.; formal analysis, A.Z., H.C., Y.A., J.C.A., and M.S.M.; investigation, A.Z., H.C., Y.A., M.R.D., J.C.A., and Y.T.; writing, A.Z. and M.S.M.; supervision, M.S.M.

DECLARATION OF INTERESTS

M.S.M. is a scientific advisory board member for Seqirus and has received honoraria from Sanofi for "COVOICES" educational programming.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
6F12 antibody	[25]	N/A
6F12 D265A antibody	This paper	N/A
9H10 antibody	[38]	N/A
Mouse IgG Isotype Control	Invitrogen	Cat#31903,
Goat anti-Human IgG (H+L) Secondary Antibody, HRP	Invitrogen	Cat#31410, RRID#AB_228269
Bacterial and virus strains		
A/Puerto Rico/8/1934 H1N1 (PR8)	Gift from Dr. Peter Palese, Icahn School of Medicine at Mount Sinai, NY	NCBI taxon ID#211044
A/California/07/2009 H1N1 (Cal/09)	Gift from Dr. Peter Palese, Icahn School of Medicine at Mount Sinai, NY	NCBI taxon ID#641501
A/X-31 H3N2 (X-31)	Gift from Dr. Peter Palese, Icahn School of Medicine at Mount Sinai, NY	NCBI taxon ID#132504
A/Brisbane/10/2007 H3N2 (Bris/07)	Gift from Dr. Brian Ward, McGill University, QC	NCBI taxon ID#476294
A/Vietnam/1203/2004 H5N1 HAlo (Viet/04)	Gift from Dr. Florian Krammer, Icahn School of Medicine at Mount Sinai, NY	N/A
MAX Efficiency™ DH10Bac Competent Cells	Gibco/Thermo Fisher	Cat#10361012
Biological samples		
Participant sera	This study	N/A
Chicken erythrocytes in Alsever's solution	Canadian Food Inspection Agency (CFIA)	N/A
Chemicals, peptides, and recombinant proteins		
cH6/1 hemagglutinin (HA) construct	Engineered using plasmids encoding A/Mallard/Sweden/81/02 H6 head domain and a A/Puerto Rico/8/1934 (PR8) group 1 stalk	N/A
Cal/09 HA construct	Gift from Dr. Florian Krammer, Icahn School of Medicine at Mount Sinai, NY	N/A
DMEM	Gibco	Cat#11965092
RPMI	Gibco	Cat#11875093
MEM	Sigma-Aldrich	Cat#M0275
Hybridoma SFM	Gibco	Cat#12045076
HyClone SFX insect cell culture media	Cytiva	Cat#SH3027802
TNM-FH insect cell media	Sigma-Aldrich	Cat#T1032
Expi293 Expression Medium	Gibco	Cat#A1435101
Heat Inactivated FBS	Gibco	Cat#12484028
BSA	Sigma-Aldrich	Cat#A8412
Sodium Bicarbonate Solution	Sigma-Aldrich	Cat#S8761
MEM Vitamin Solution	Gibco	Cat#11120052
MEM Amino Acids Solution	Gibco	Cat#11130051
Low IgG FBS	Gibco	Cat#16250078
Trypsin-EDTA	Gibco	Cat#15400054
Penicillin-Streptomycin	Gibco	Cat#15140122
Glutamax Supplement	Gibco	Cat#35050061
Trypsin treated with N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)	Sigma-Aldrich	Cat#4370285
Oseltamivir Carboxylate	Toronto Research Chemicals	Cat#O700980

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oseltamivir Phosphate	Toronto Research Chemicals	Cat#O701000
Baloxavir Marboxil	Selleck Chemicals	Cat#S5952
Critical commercial assays		
NA-STAR Influenza Neuraminidase Inhibitor Resistance Detection Kit	Invitrogen	Cat#4374422
SigmaFast OPD Peroxidase Substrate	Sigma-Aldrich	Cat#P9187
pFastBac Dual Expression Vector	Gibco	Cat#10712024
ADCC Reporter Bioassay, V Variant (Human)	Promega	Cat#G7102
Mouse FcγRIV ADCC Bioassay	Promega	Cat#M1212
Bio-Glo Luciferase Assay System	Promega	Cat#G7940
Expi293 expression system	Gibco	Cat#A14635
Experimental models: Cell lines		
A549	American Type Culture Collection (ATCC, VA, USA)	Cat#CCL-185
MDCK.2	American Type Culture Collection (ATCC, VA, USA)	Cat#CRL-2936
Expi293F	Gibco	Cat#A14527
Trichoplusia ni High Five (BTI-TN5B1-4) insect cells	American Type Culture Collection (ATCC, VA, USA)	Cat#CRL-10859
Spodoptera frugiperda Sf9 insect cells	American Type Culture Collection (ATCC, VA, USA)	Cat#CRL-1711
Experimental models: Organisms/strains		
BALB/c Mice	Charles River	Strain Code: 028
Specific-pathogen-free (SPF) chicken eggs	Canadian Food Inspection Agency (CFIA)	N/A
Recombinant DNA		
pFUSE2ss-CHIg-hG1 D265A 6F12	This Paper	N/A
pFUSE2ss-CLIg-hK 6F12	This Paper	N/A
Software and algorithms		
Graphpad Prism v.9.0.2	https://www.graphpad.com/scientific-software/prism/	SCR_002798
BioTek Synergy Gen5 Software	https://www.biotek.com/products/software-robotics-software/gen5-microplate-reader-and-imager-software/	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Matthew S. Miller (mmiller@mcmaster.ca).

Materials availability

All unique reagents generated in this study are available from the lead contact, Matthew S. Miller (mmiller@mcmaster.ca) with a completed Materials Transfer Agreement.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyse the data reported in this work is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Murine influenza infections

6–8 week old female BALB/c mice (Charles River) were anesthetized with isoflurane and intranasally infected with 40 μL of PR8 (500 PFU, 5 × LD_{50}) or Viet/04 (200 PFU, 5 × LD_{50}) diluted in PBS. The mice were administered 1 mg/kg or 10 mg/kg 6F12, 150μL of undiluted serum, or 1 mg/kg or 10 mg/kg Mouse IgG Isotype Control (ThermoFisher) intraperitoneally either 2 hours before infection, or 48 hours post infection as described in the main text. The mice also received 1 mg/kg or 10 mg/kg oseltamivir phosphate (Toronto

Research Chemicals) by oral gavage as described in the main text. Mice were monitored daily and were sacrificed if they reached endpoint, defined as 20% reduction in initial body weight. Mice were monitored continuously by experienced animal technicians. Mice were acclimatised at the animal facility for 1 week before experiments commenced. Five mice were housed per cage with unrestricted access to food and water. All animal experiments in this study were approved by the Animal Research Ethics Board at McMaster University.

Cell lines

Human adenocarcinoma alveolar basal epithelial (A549) cells (ATCC, VA, USA) and Madin-Darby Canine Kidney (MDCK) cells (ATCC, VA, USA) were maintained at 37°C at 5% CO_2 in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100U/mL penicillin-streptomycin (Gibco), 2mM GlutaMAX supplement (ThermoFisher). Expi293 cells (Gibco) were grown in Expi293 Expression Medium (ThermoFisher) at 37°C at 8% CO_2 with regular agitation (150RPM). *Trichoplusia ni* High Five (ATCC) insect cells were maintained in HyClone SFX insect cell culture media (Cytiva/Fisher Scientific) at 28°C with regular agitation (70RPM). *Spodoptera frugiperda* Sf9 insect cells (ATCC) were maintained as adherent cultures in Roux flasks in full TNM-FH insect cell media (Sigma) supplemented with 10% FBS, 1% penicillin-streptomycin (Gibco) and 0.1% Pluronic F68 solution (Gibco) at 27°C.

Viruses

PR8 H1N1, X-31 H3N2, Bris/07 H3N2, and Viet/04 H5N1 HAlo viruses were kindly gifted by Dr. Peter Palese (Icahn School of Medicine at Mount Sinai, NY) and propagated in embryonated chicken eggs (Canadian Food Inspection Agency).[9,16] Cal/09 H1N1 was propagated in MDCK cells.

Chicken eggs

All viruses used in this study, except Cal/09 H1N1 were propagated in specific pathogen free embryonated chicken eggs (Canadian Food Inspection Agency).[9,16] Eggs were incubated at 37°C in a dry incubator for 10–12 days. Viruses were inoculated by injection of virus stock into the allantoic cavity followed by two additional days of incubation at 37°C. Eggs were then chilled at 4°C for at least 24 hours before allantoic fluid containing virus was harvested.

Recombinant viral antigens

All viral antigens were made using the baculovirus expression system and constructs kindly gifted by Dr. Peter Palese and Dr. Florian Krammer (Icahn School of Medicine at Mount Sinai, NY). The cH6/1 HA construct was composed of a A/Mallard/Sweden/81/02 H6 head domain and a A/Puerto Rico/8/1934 (PR8) group 1 stalk. Briefly, construct sequences were cloned into pFastBac Dual Expression Vector (Gibco/ThermoFisher) plasmids containing a C-terminal trimerization domain and hexahistidine tag, followed by transformation into DH10Bac bacteria (Gibco/ThermoFisher). The resulting bacmids were transfected into Sf9 cells to generate recombinant baculovirus, which was subsequently used to infect High Five insect cells. Viral supernatants were harvested via centrifugation at 5500g for 20 min at 4°C, and proteins purified by Ni-resin affinity chromatography.

METHOD DETAILS

Human samples

Blood from four male participants born between 1981 and 1994 were used in this study. Informed consent was obtained from all study participants. All study procedures were approved by the McMaster University Research Ethics Review Board.

Serum collection and processing

Blood (6 mL) was collected into ethylenediaminetetraacetic acid (EDTA) coated tubes (BD) by venipuncture. 3 mL of Histopaque 1119 and 3mL of Histopaque 1077 was added to a 15 mL conical centrifuge tube before adding 6mL of participant blood. The mixture was centrifuged at 931 × g for 3 minutes. The top layer of plasma was aspirated and inactivated by heating at 56°C for 30 min prior to experimentation.

NA-Star neuraminidase assays

Neuraminidase inhibition assays were completed using the NA-Star kit (Invitrogen). Viruses were diluted in NA-Star Assay Buffer in white 96 well opaque flat-bottom plates to a final volume of 25 μL per well. An additional 25 μL per well of oseltamivir carboxylate (Toronto Research Chemicals) diluted in NA-Star Assay Buffer was added in half-log dilutions to each well. Plates were then incubated for 20 minutes at 37°C after brief shaking. After the incubation, 10 μL of NA-Star Substrate was added to each well and was then incubated for 30 minutes at room temperature after a brief shaking. After the incubation, 60 μL of NA-Star Accelerator was added to each well, and luminescence was quantified using a SpectraMax i3 plate reader (Molecular Devices).

Virus quantification by plaque assay

MDCK cells were seeded at a density of 1×10^6 cells per well in 6 well plates. The next day, cells were infected with serial dilutions of virus diluted in 1× minimum essential medium (MEM, Sigma) supplemented with 0.6% BSA (Sigma). After the 1-hour infection at 37°C, the media was replaced with 1x MEM, 0.6% BSA, 1 μg/mL N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin, 0.01% DEAE-dextran, and 0.5% agar. The infected cells were then incubated at 37°C for 2 days. After the incubation, the cells were fixed with 10% buffered formalin for 30 minutes at room temperature. The agar layer was then removed, and the cells were stained with crystal violet to visualize plaques.

ADCC reporter assay

A549 cells were seeded at a density of 2×10^4 cells per well in white 96 well opaque flat-bottom plates. 24 hours after seeding, cells were infected with PR8, Cal/09, X-31, Bris/07 or Viet/04 at a multiplicity of infection (MOI) of 5. 16 hours after infection, the media was replaced with 50 μL of assay buffer (RPMI 1640 supplemented with 4% (vol/vol) low IgG FBS(Gibco)) containing oseltamivir carboxylate (Toronto Research Chemicals) or baloxavir marboxil (Selleck Chemicals) and serial dilutions of antibody or serum. After a 30-minute incubation at 37°C, 25 μL of 7.5×10^4 Jurkat effector cells expressing either human FcγRIIIa or murine FcγRIV (Promega) resuspended in assay buffer were added to each well. The cells were incubated for an additional 6 hours at 37°C before 75μL of Bio-Glo Luciferase Assay Reagent (Promega) was added to each well. After a 5-minute incubation at room temperature, luminescence in relative light units (RLU) was quantified using a SpectraMax i3 plate reader (Molecular Devices). Fold induction was obtained by dividing the RLU of the wells of interest by the mean of control wells containing infected target cells and Jurkat effector cells with no monoclonal antibodies/serum.

Antibody purification

Murine 6F12 and 9H10 were obtained from hybridoma cultures kindly gifted by Dr. Peter Palese (Icahn School of Medicine at Mount Sinai, NY) as previously described.[25,26] Briefly, hybridomas were thawed and expanded in ClonaCell-HY Growth Medium E (Stem Cell Technologies) up to a volume of 300mL. The media was then changed to Hybridoma-SFM (Gibco). The hybridoma cultures were harvested by centrifugation at 3000 × g for 20 minutes when cultures reached maximal cell density. The supernatant was then nutated overnight at 4°C with 1 mL of Protein G Sepharose 4 Fast Flow slurry (Invitrogen) per 25mL of supernatant. After incubation, the supernatant/Sepharose bead slurry was passed through a 5 mL polypropylene gravity flow column (Qiagen). The column was washed with 1 column volume of PBS before being eluted with 9 mL of Elution Buffer (0.1M Glycine/HCl buffer, pH 2.2) into 1 mL of Neutralization Buffer (2M Tris-HCl, pH 10). The eluate was then concentrated and buffer exchanged to PBS using 30 kDa Amicon Ultra-15 Centrifugal Filter Units (Millipore).

The variable heavy and light chain sequences of 6F12 were cloned into the EcoRI/NheI sites of pFUSEss-CHIg-mG2a (D265A, Invivogen) and the EcoRI/BstAPI sites of pFUSE2ss-CLIg-mk (Invivogen) respectively. The Expi293 Expression System was used to produce the 6F12 D265A antibody (ThermoFisher). A 2:1 molar ratio of light to heavy chain plasmids was used to transfect Expi293F cells. The culture supernatant was harvested by centrifugation at 3000 × g for 20 minutes when cell viability reached 60%. Antibodies were purified from cell culture supernatant as described above.

ELISA

Purified soluble Cal/09 HA and cH6/1 comprised of the HA head domain of A/Mallard/Sweden/81/02 H6N1 and the stalk domain of PR8 with a C-terminal T4 trimerization domain and a 6-His tag was generated using a baculovirus expression system as previously described.[14] Clear flat-bottom 96-well Immulon 4 HBX plates (ThermoFisher) were coated with 50 μL of 2 μg/mL of Cal/09 HA or cH6/1 diluted in ELISA coating buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$, pH 9.4) overnight at 4°C. After the incubation, the plates were blocked for 1 hour with 100 μL of blocking buffer (5% milk powder in PBS with 5% Tween-20 (PBS-T)). Serum serially diluted in blocking buffer was then added to the wells and incubated for 2 hours at room temperature. After the incubation, the plate was washed 3 times with PBS-T. Anti-human IgG (Fab specific) – peroxidase-conjugated antibody (Sigma) diluted 1:5000 in blocking buffer was added to the wells and incubated for 1 hour at room temperature. After 3 additional washes with PBS-T, 50μL of reconstituted SIGMAFAST OPD (Sigma) was added to each well. The reaction was stopped 10 minutes later by adding 50 μL of 3M HCl to each well, and the absorbance at 490nm was read using a SpectraMax i3 plate reader (Molecular Devices).

Hemagglutinin inhibition assay

Chicken red blood cells diluted 1:1 in Alsever's solution (Canadian Food Inspection Agency) were diluted 1:10 in PBS and centrifuged at 300 × g for 5 minutes. The supernatant was removed, and 125 μL of the red blood cell (RBC) pellet was added to 25 mL of PBS to make 0.5% chicken RBC. Serum samples were inactivated by trypsin-heat-periodate treatment by first adding 10 μL of 8 mg/mL trypsin to 20 μL of serum before heating at 56°C for 30 minutes. After cooling the serum to room temperature, 60 μL of 0.011M KIO_4 was added followed by a 15-minute incubation at room temperature. The periodate treatment was inactivated by adding 60 μL of 1% glycerol (v/v) in PBS followed by a 15-minute incubation at room temperature. Lastly, 50 μL of 0.85% NaCl (w/v) in distilled water was added to each sample to make a final 1:10 dilution of serum. The inactivated serum was then diluted 1:2 across

clear V-bottom plates (Sigma) to a final volume of 25 μL. Then, 4 HA units of virus diluted PBS to a final volume of 25 μL was added to each well. Lastly, 50 μL of 0.5% chicken RBC was added to each well. The plates were read after a 1-hour incubation at 4°C.

Statistical analyses

Statistical analyses were performed using GraphPad Prism v.9.0.2. Log-rank (Mantel-Cox) tests were used to assess differences in survival between different groups of mice (n = 5 unless otherwise indicated) in Figures 2C, 2E, 4C, and 4E. Differences between groups were considered significant at $p < 0.05$.