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Characterization of non-neutralizing human monoclonal antibodies that target the M1 and NP of influenza A viruses

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ABSTRACT Improved broad-spectrum influenza virus vaccines are desperately needed to provide protection against both drifted seasonal and emerging pandemic influenza A viruses (IAVs). Antibody-based protection from influenza A virus-induced morbidity and mortality is traditionally associated with neutralizing antibodies. As such, vaccine efforts have solely focused on the hemagglutinin (HA) as a vaccine target; however, the HA is mutation prone resulting in the need for annual vaccine reformulation. Broad-spectrum vaccines could be achieved through non-neutralizing antibodies that target conserved influenza virus antigens. Here, we describe six human monoclonal antibodies (mAbs) isolated from two H3N2-infected donors that showed robust binding against the conserved internal nucleoprotein (NP) or matrix protein 1 (M1) of IAV strains. Despite the capacity for potent antigen binding, substantial morbidity was observed in mice prophylactically treated with these mAbs and then challenged with A/Netherlands/602/2009 (H1N1) or A/Switzerland/9715293/2013 (H3N2) viruses. While our findings need to be confirmed with a larger number of mAbs and with polyclonal serum, these findings suggest that human NP and M1 antibodies that are elicited following IAV infection/vaccination do not protect from substantial weight loss in the mouse model and imply that protection afforded targeting these antigens following vaccination/infection is most likely the result of cellular-based immunity.

IMPORTANCE Currently, many groups are focusing on isolating both neutralizing and non-neutralizing antibodies to the mutation-prone hemagglutinin as a tool to treat or prevent influenza virus infection. Less is known about the level of protection induced by non-neutralizing antibodies that target conserved internal influenza virus proteins. Such non-neutralizing antibodies could provide an alternative pathway to induce broad cross-reactive protection against multiple influenza virus serotypes and subtypes by partially overcoming influenza virus escape mediated by antigenic drift and shift. Accordingly, more information about the level of protection and potential mechanism(s) of action of non-neutralizing antibodies targeting internal influenza virus proteins could be useful for the design of broadly protective and universal influenza virus vaccines.

KEYWORDS influenza, antibodies, nucleoprotein, matrix protein

Influenza virus infections result in mild to severe respiratory illness in humans and are a major health burden worldwide, with considerable annual morbidity and mortality (1–4). Before the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, seasonal influenza virus infections resulted in around 3–5 million cases of severe disease and 290,000–650,000 global deaths annually (5). Moreover, influenza pandemics happen at unpredictable and irregular intervals (2). Vaccination is the cornerstone in preventing seasonal influenza virus infections, although vaccines are only effective when vaccine strains match circulating strains (6–8). Vaccines elicit a robust humoral response toward

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The Icahn School of Medicine at Mount Sinai has filed patent applications regarding an influenza virus vaccine. F.K. is named as coinventor on these applications.

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the immuno-dominant globular head domain of the major surface glycoprotein, the hemagglutinin (HA) (3, 9). Anti-HA antibodies can prevent influenza virus infection by blocking virus binding to its sialic acid receptor and subsequent viral entry and are, therefore, called neutralizing antibodies. Neutralizing antibody titers are a correlate of vaccine-elicited protection (10–12). These antibodies, including monoclonal antibodies (mAbs) with the same activity, can also protect mice from influenza virus infection and/or mortality via passive transfer in a prophylactic (13–22) and/or therapeutic setting (16–24). In spite of the effectiveness of neutralizing anti-HA head antibodies, their applicability as a vaccine target is restricted as these types of vaccines induce narrow, strain- or clade-specific immune responses (10, 11, 25). Specifically, the plastic globular head domain of HA undergoes constant antigenic drift, especially within epitopes recognized by neutralizing antibodies (antigenic sites), circumventing the established immunity induced by the previous year's vaccine, and thereby forcing annual vaccine reformulation and re-administration (9, 26–28). Several neutralizing antibodies that target more conserved regions of the HA head domain have also been described, including mAbs that are directed to the receptor-binding site (RBS) (22, 29–36), to sites outside of the RBS (37), to the trimer interface (38–40), and to the vestigial esterase domain (16, 41). Nonetheless, the neutralization breadth of these HA head-directed antibodies is still mostly restricted to specific influenza virus subtypes, clearly highlighting the need for novel broader protective or universal vaccines that offer protection against different influenza virus strains.

Unlike neutralizing antibodies, non-neutralizing antibodies (noneAbs) cannot prevent viral infection *in vitro* (42). These antibodies are, therefore, not readily detectable in classical *in vitro* neutralization or hemagglutination inhibition assays. Hence, it was presumed that noneAbs could not contribute to protective immunity against influenza viruses. This has been challenged by increasing evidence that suggests that HA-directed noneAbs can provide some protection *in vitro* (10) and *in vivo* (8, 27, 43–47) against both seasonal and pandemic influenza viruses. The protective effects employed by noneAbs are thought to encompass mechanisms involving the fragment crystallizable (Fc) of antibodies, such as antibody-dependent cellular cytotoxicity (ADCC) (28, 42, 48–54), antibody-dependent complement activation (55–57), and antibody-dependent cellular phagocytosis (ADCP) (27, 58–60). Interestingly, Fc-dependent noneAbs target either conserved regions on the HA, primarily in the stalk region (10, 27, 42, 43, 52, 61–66), or the neuraminidase (NA) (42, 67–71). In addition, such noneAbs can also target internal influenza virus antigens, such as the matrix protein (M1), matrix protein 2 (M2, which has small ectodomain), and nucleoprotein (NP) (8, 42, 45, 71). These latter proteins are relatively well conserved and could, therefore, represent compelling candidates for the induction of broad protection (1, 9, 72). Indeed, noneAbs targeting internal influenza virus proteins induced by prior seasonal influenza virus infection have been reported to offer a level of protection against subsequent pandemic influenza virus infection *in vivo* (44). In agreement, broadly protective, heterosubtypic humoral immune responses directed toward the M2 protein were observed after vaccination in mice [(73) and reviewed in detail in reference (74)]. Similarly, in a mouse model, antibodies directed against NP provided protection, although weak, against influenza virus challenge (45, 46). These studies provide evidence that noneAbs might provide an alternative pathway to induce broad cross-reactive protection.

Given the broad cross-protection of some highly efficacious noneAbs that target conserved, sometimes internal, influenza virus proteins, efforts to create a universal vaccine should not ignore the contribution of these long-underappreciated antibodies in anti-influenza virus immune responses. Whereas protective noneAbs to HA and NA have been relatively well defined, less is known about the level of protection induced by noneAbs that target the internal proteins M1 or NP. Therefore, in this study, we analyzed the breadth and potential mechanism of protection of a panel of six human anti-M1 and anti-NP noneAbs that were cloned from plasmablasts isolated from two H3N2-infected individuals. First, a characterization of the binding profile of the mAbs was performed via

a cell-based enzyme-linked immunosorbent assay (ELISA) followed by ADCC and ADCP *in vitro* bioreporter assays with influenza A virus (IAV)-infected cells. To translate the potential of *in vitro* noneAb-mediated Fc-effector mechanisms toward *in vivo* protection, prophylactic passive transfer challenge experiments in mice were subsequently performed. Information about the mechanisms of partial protection induced by these noneAbs could give important insights in the development of future broad-spectrum vaccines against both drifted seasonal and emerging pandemic influenza viruses.

RESULTS

Isolation of human mAbs from two H3N2-infected individuals

Plasmablasts (defined as $CD19^{+}IgD^{-}CD38^{+}CD20^{-}CD71^{hi}$) were single sorted from peripheral blood mononuclear cells (PBMCs) collected from two individuals with confirmed H3N2 influenza virus infection on day 4 and day 5 after symptom onset during the 2017–2018 H3N2-predominant influenza virus season. Corresponding immunoglobulin heavy and light variable (IGHV and IGLV) chain genes were cloned into expression vectors and expressed as described previously (75). A total of 55 antibodies were isolated from these individuals. Most antibodies bound to HA; several also bound to NA. A total of six clonally distinct mAbs reactive to seasonal influenza virus vaccine but non-reactive to recombinant HA or NA were produced; three of the six antibodies were derived from subject 1718002 (1H01, 1F11, and 1B06), and three were from subject 1718003 (1F03, 1D11, and 1H06). Sequence analysis of the generated mAbs showed that they predominantly belonged to the IGHV3 family, which is compatible with the occurrence of this family in human repertoires; IGHV4 and 5 families included the remnant of the mAb population (Fig. S1A). The light chains were distributed among IGKV1-3 families, with the IGKV1 family representing 50%. The IGHV gene complementarity-determining region 3 (CDR3) lengths ranged from 13 to 20 amino acid residues (Fig. S1B). As expected, the CDR3 lengths of the IGLV gene were relatively similar and ranged from 11 to 13 residues (Fig. S1C). The variety of IGHV and IGLV families, in addition to IGHV CDR3 lengths, suggest distinct modes of interaction with the influenza virus proteins.

Human mAbs bind to the internal M1 and NP proteins of IAVs

The mAbs were first screened for their breadth of binding to IAV- and influenza B virus (IBV)-infected cells via cell-based ELISAs. Three different viruses, A/Netherlands/602/2009 (H1N1), which represents a pandemic strain; A/Hong Kong/4801/2014 (H3N2), which is seemingly closely related to the strain that caused the infection; and B/Colorado/06/2017, which belongs to the B/Victoria/2/1987-like lineage, were chosen for this assay and for downstream *in vitro* characterization. mAbs CR9114, a cross-reactive influenza virus HA stem binding mAb (41), and 1D04, an anti-chikungunya virus (CHIKV) E1 glycoprotein mAb, were used as positive and negative controls, respectively. Five out of the six mAbs showed robust binding to the H1N1- and H3N2-infected cells, while none of the mAbs recognized the IBV-infected cells (Fig. 1).

To determine which antigens these mAbs bind, we assessed binding to HA, NA, M1, and NP in an ELISA. Protein-based ELISAs revealed that none of these mAbs recognized recombinant H3 HA or N2 NA proteins from the A/Singapore/INFIMH-16-0019/2016 (H3N2) strain in contrast to the positive controls CR9114 (a pan anti-HA mAb) and 1G01 (a pan anti-NA mAb) (3) (Fig. 2A and B). Instead, two mAbs—1F11 and 1H01—displayed binding to recombinant M1 proteins derived from strains A/Puerto Rico/8/1934 (H1N1) and A/Michigan/45/2015 (H1N1) (Fig. 2C and D). The four remaining mAbs—1B06, 1D11, 1F03, and 1H06—bound the influenza virus NP of A/Singapore/INFIMH-16–0019/2016 (H3N2) (Fig. 2F). Three of those mAbs—1B06, 1F03, and 1H06—also bound to recombinant NP protein of A/Michigan/45/2015 (H1N1) (Fig. 2E). In agreement with the cell-based ELISAs, none of the NP-binding mAbs recognized the recombinant B/Colorado/06/2017 NP protein (Fig. 2G), indicating that these mAbs only recognize proteins of IAVs. Similarly, none of the HA, NA, M1, and NP recombinant proteins were

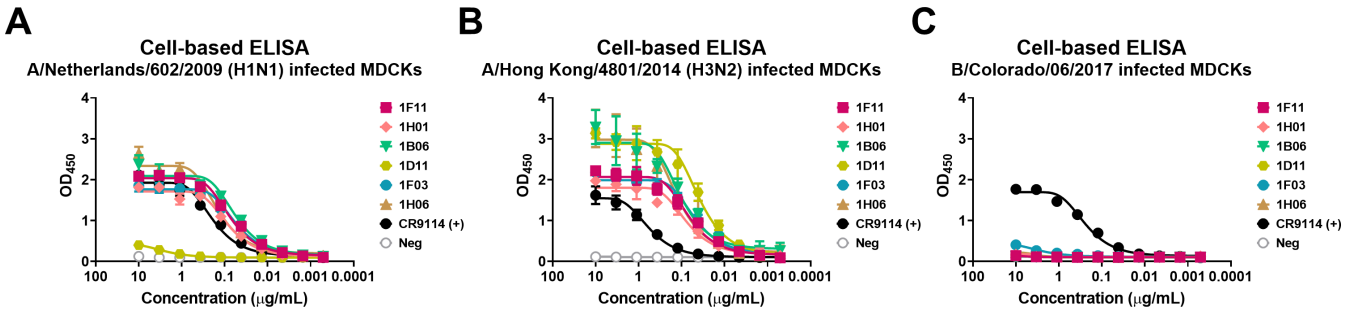

FIG 1 mAb reactivity to different influenza virus strains in a cell-based ELISA. Binding profiles of six human mAbs against A/Netherlands/602/2009 (H1N1) (A), A/Hong Kong/4801/2014 (H3N2) (B), and B/Colorado/06/2017 (C) viruses measured by ELISA. CR9114, a cross-reactive influenza virus HA stem binding mAb, was used as a positive control. 1D04, an anti-CHIKV E1 glycoprotein mAb, was used as a negative control. For all panels, OD_{450} values are shown. The non-linear regression for each group is indicated. Data are representative of one experiment with duplicate measurements.

recognized by the negative control mAb, 1D04 (Fig. 2). Taken together, these results indicate that the H3N2 infections in these two individuals induced robust plasmablast responses with cross-reactive mAbs specific for the internal M1 and NP proteins of IAVs.

Anti-M1 and anti-NP antibodies are non-neutralizing *in vitro*

Next, we characterized the mAbs in an *in vitro* neutralization assay to evaluate their ability to neutralize diverse influenza viruses. Typically, mAbs directed against internal M1 and NP proteins are not able to prevent infection, since they cannot compete with the influenza virus for binding to the cell surface receptor and, consequently, do not show any activity in *in vitro* neutralization assays (8, 42, 76, 77). In order to assess possible antiviral effects downstream of entry, the mAbs remained in the overlay at all times during the incubation period. mAbs CR9114 (a pan anti-HA mAb) and 2E01 (an IBV NA mAb) (78) served as positive controls, whereas the mAb 1D04 functioned as the negative control. As expected, although all anti-M1 and anti-NP mAbs bound to IAV-infected cells (Fig. 1), none of them showed neutralizing activity and never reached a 50% inhibition endpoint, against any of the tested viruses (A/Netherlands/602/2009 (H1N1), A/Hong Kong/4801/2014 (H3N2), and B/Colorado/06/2017) (Fig. 3). Accordingly, the isolated broadly reactive anti-M1 and anti-NP mAbs were non-neutralizing.

Anti-NP, but not anti-M1, noneAbs exhibit ADCC and ADCP activity *in vitro*

FcR-mediated effector functions are a pivotal mechanism for broadly protective noneAbs (45, 48, 49, 52, 79). Although internal proteins, patches of typically intracellular NP antigens are transiently expressed on the surface of influenza virus-infected cells, and the M1 has been reported to become readily accessible in cells that die following influenza virus infection (57, 77, 80). This makes NP and M1 proteins reachable targets for antibody-mediated Fc-receptor functions. Therefore, to examine potential mechanisms of the six isolated mAbs, *in vitro* ADCC bioreporter assays were performed and functioned as a surrogate for *in vivo* Fc-FcR based effector functions. Here, Madin Darby canine kidney (MDCK) cells were infected with A/Netherlands/602/2009 (H1N1), A/Hong Kong/4801/2014 (H3N2), or B/Colorado/06/2007 strains, and ADCC induction of mAbs in varying concentrations was evaluated. CR9114 and 1D04 functioned as a positive and negative control mAb, respectively. Indeed, all anti-NP mAbs induced a significant ADCC activity toward IAVs when compared to the negative control antibody (Fig. 4A and B). More precisely, two anti-NP mAbs—1B06 and 1F03—facilitated ADCC induction in response to the two influenza A virus strains, while the other two anti-NP mAbs—1D11 and 1H06—only mediated ADCC activity against A/Hong Kong/4801/2014 (H3N2). In agreement with the ELISA data, none of the anti-NP mAbs mediated ADCC activity against the B/Colorado/06/2017 strain (Fig. 4C). Notably, none of the anti-M1 mAbs displayed ADCC activity to any of the influenza viruses (Fig. 4A through C), which is

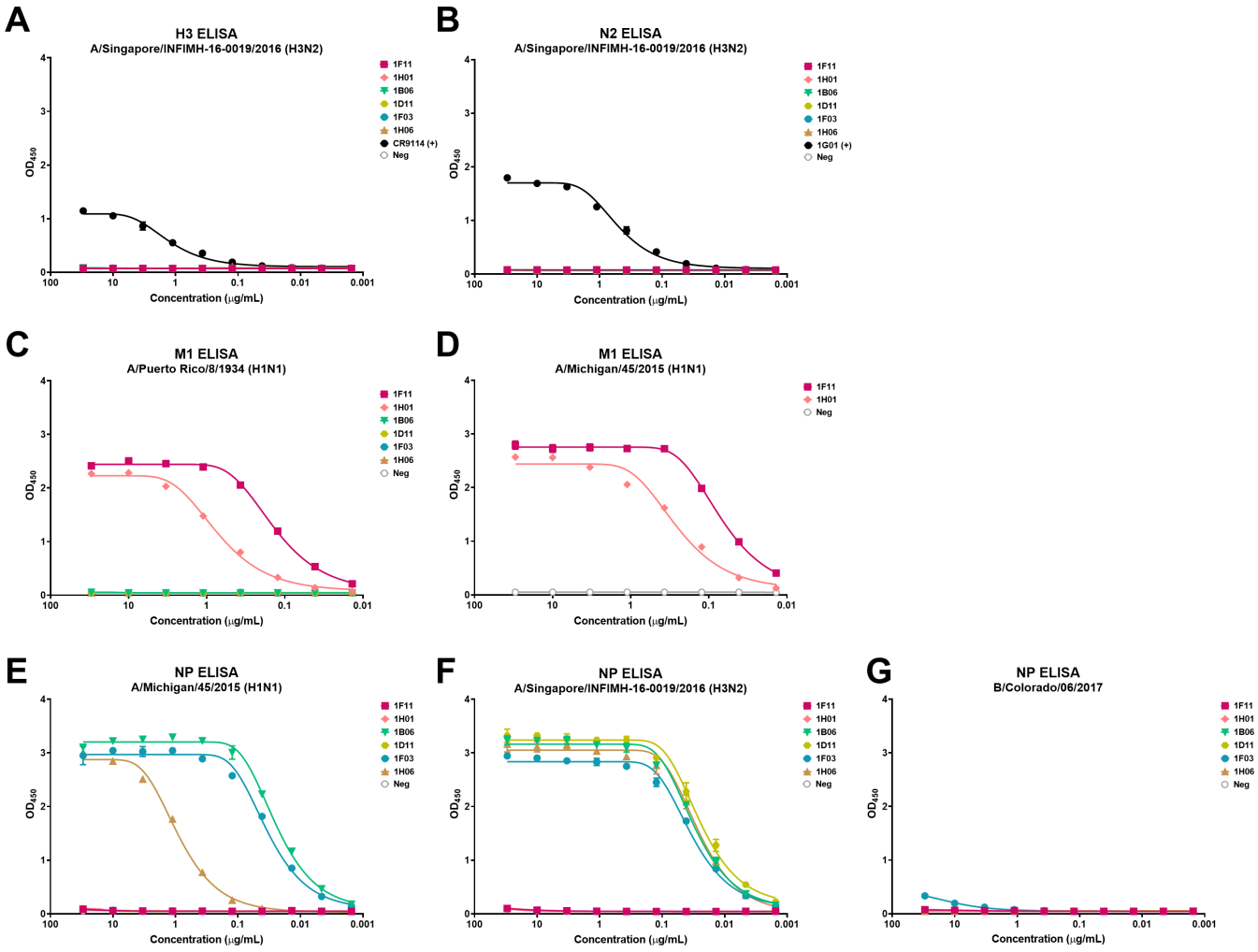

FIG 2 Binding profile of mAbs isolated from two H3N2-infected individuals to influenza virus proteins. Binding of the human mAbs against A/Singapore/INFIMH-16-0019/2016 H3 (A), A/Singapore/INFIMH-16-0019/2016 N2 (B), A/Puerto Rico/8/1934 M1 (C), A/Michigan/45/2015 M1 (D), A/Michigan/45/2015 NP (E), A/Singapore/INFIMH-16-0019/2016 NP (F), and B/Colorado/06/2017 NP (G). 1D04, an anti-CHIKV E1 glycoprotein mAb, functioned as a negative control. The OD_{450} values are displayed in each panel. The non-linear regression for each group is displayed. Data are representative of one experiment with duplicate measurements.

consistent with the absence of studies reporting M1's ADCC potency. The ADCC assays were repeated, and the data were shown in Fig. S3A through C.

Although less intensively investigated in the setting of influenza viruses, another antibody-mediated Fc-effector function is ADCP. Recent studies reported that antibody-mediated phagocytosis significantly contributed to the elimination of influenza virus-infected cells and, hence, protection against influenza virus infection (58, 60, 64, 81). In addition, this Fc-mediated mechanism has been shown to contribute to protection conferred by anti-influenza virus noneAbs (27, 42, 59). Therefore, we subsequently assessed the ability of anti-M1 and anti-NP noneAbs to induce ADCP activity in an *in vitro* ADCP bioreporter assay. The positive and negative control mAbs included CR9114 and 1D04, respectively. Interestingly, similar results were found to the ADCC bioreporter assays (Fig. 4A through C). Specifically, anti-NP mAbs induced a significant level of ADCP in response to influenza A, but not B, virus-infected cells when compared to the negative control antibody (Fig. 4D through F). In agreement with the binding profiles, mAb 1D11 only conferred ADCP activities against the A/Hong Kong/4801/2014 (H3N2) strain (Fig. 4E). In contrast, anti-NP mAbs 1B06, 1F03, and 1H06 showed heterosubtypic activity

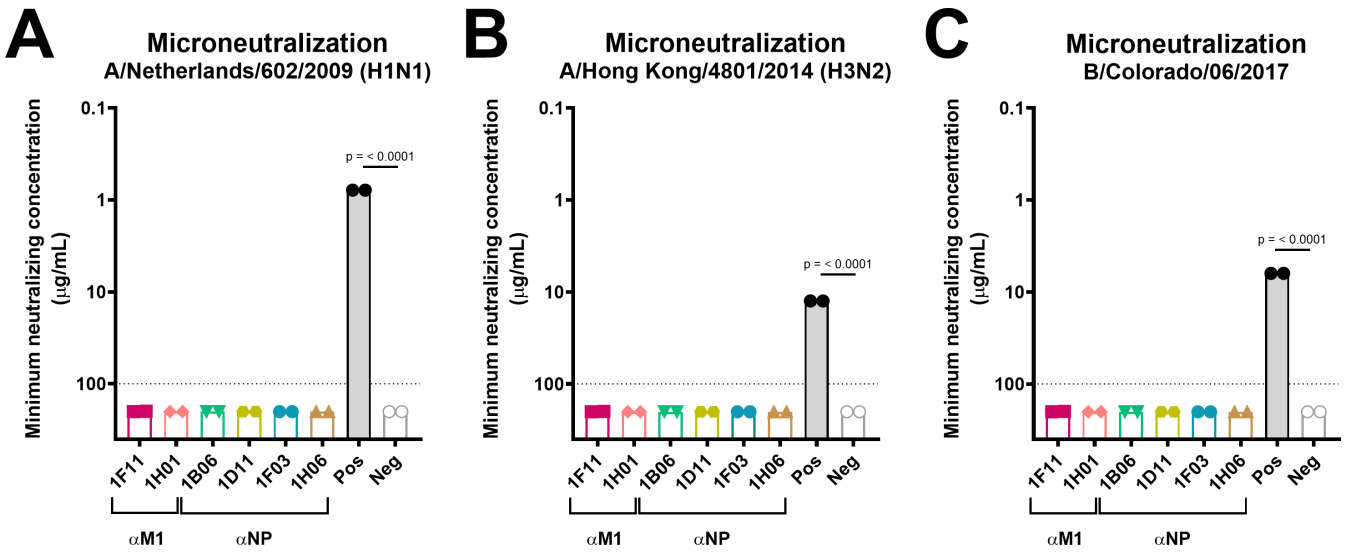

FIG 3 Neutralization activity assessment of IAVs and an IBV for anti-M1 and anti-NP mAbs. Neutralization of A/Netherlands/602/2009 (H1N1) (A), A/Hong Kong/4801/2014 (H3N2) (B), and B/Colorado/06/2017 (C) strains measured in microneutralization assays. The minimum neutralization concentration in micrograms per milliliter of the respective mAb is displayed and was established via a hemagglutination-based readout. The minimum neutralizing concentration represents the lowest antibody concentration at which no hemagglutination was detected. The data are presented as individual technical replicates of a single experiment, and the linear regression for each mAb group is represented. The dashed vertical gray line represents the assay detection limit (100 μg/mL). mAbs that did not reach this limit were assigned a value of 200 μg/mL. CR9114, which has known neutralizing activity against H1N1 and H3N2 viruses, or 2E01, which has known neutralizing activity against influenza B viruses, and a CHIKV E1 virus mAb, 1D04, were used as positive and negative controls for all experiments, respectively. *P* values indicate the statistical significance of the difference between the groups and the irrelevant antibody control assessed by one-way analysis of variance (ANOVA) followed by a multiple-comparison test. Only *P* values lower than 0.05 are presented.

against both tested IAV strains (Fig. 4D and E). However, ADCP activity could not be observed for anti-M1 mAbs (Fig. 4D through F), which could indicate that these mAbs potentially function via alternative Fc-dependent mechanisms and/or Fc receptors.

Anti-M1 and anti-NP mAbs show low protectiveness *in vivo*

To evaluate whether *in vitro* ADCC and/or ADCP activity of the anti-NP mAbs is sufficient to provide protection against influenza A viral challenge *in vivo*, a prophylactic passive transfer challenge experiment in mice was performed. The anti-M1 mAbs were also included to investigate their protective capacity *in vivo*, as they displayed reactivity to diverse IAV-infected cells (Fig. 1). Mice were intraperitoneally injected with 10 mg/kg of mAb 3 h prior to intranasal A/Netherlands/602/2009 (H1N1) or mouse-adapted A/Switzerland/9715293/2013 (H3N2) challenge, and for a period of 2 weeks, weight loss and survival were monitored daily. The anti-NA mAb, 1G01, and anti-SARS-CoV-2 mAb, 1C11, served as positive and negative controls, respectively. All mice displayed high mAb titers, which is indicative of successful transfer, and hence, no mice were excluded from the experiments (Fig. S2A and B). Following challenge with the A/Switzerland/9715293/2013 (H3N2) virus, we found that all mice administered anti-M1 and the negative control, 1C11, mAbs crossed the 25% weight loss humane endpoint and were euthanized and scored dead (Fig. 5A). Of the anti-NP mAbs, all mice administered 1D11 and 1F03 did not survive. However, mice administered 1B06 or 1H06 showed 40% or 20% survival, respectively. Similar results were obtained in a biological replicate, in which 40% or 80% of the mice survived the A/Switzerland/9715293/2013 challenge when administered the anti-NP mAb 1B06 or 1H06, respectively (Fig. S4A and B). For the A/Netherlands/602/2009 (H1N1) challenge, we observed that mice administered the anti-M1 mAbs, 1F11 and 1H01; the anti-NP mAbs, 1B06, 1D11, or 1F03; or the negative control mAb, 1C11, lost a substantial amount of weight (Fig. 5B). It should be noted that the A/Netherlands/602/2009 (H1N1) challenge was performed at a lower lethal dose

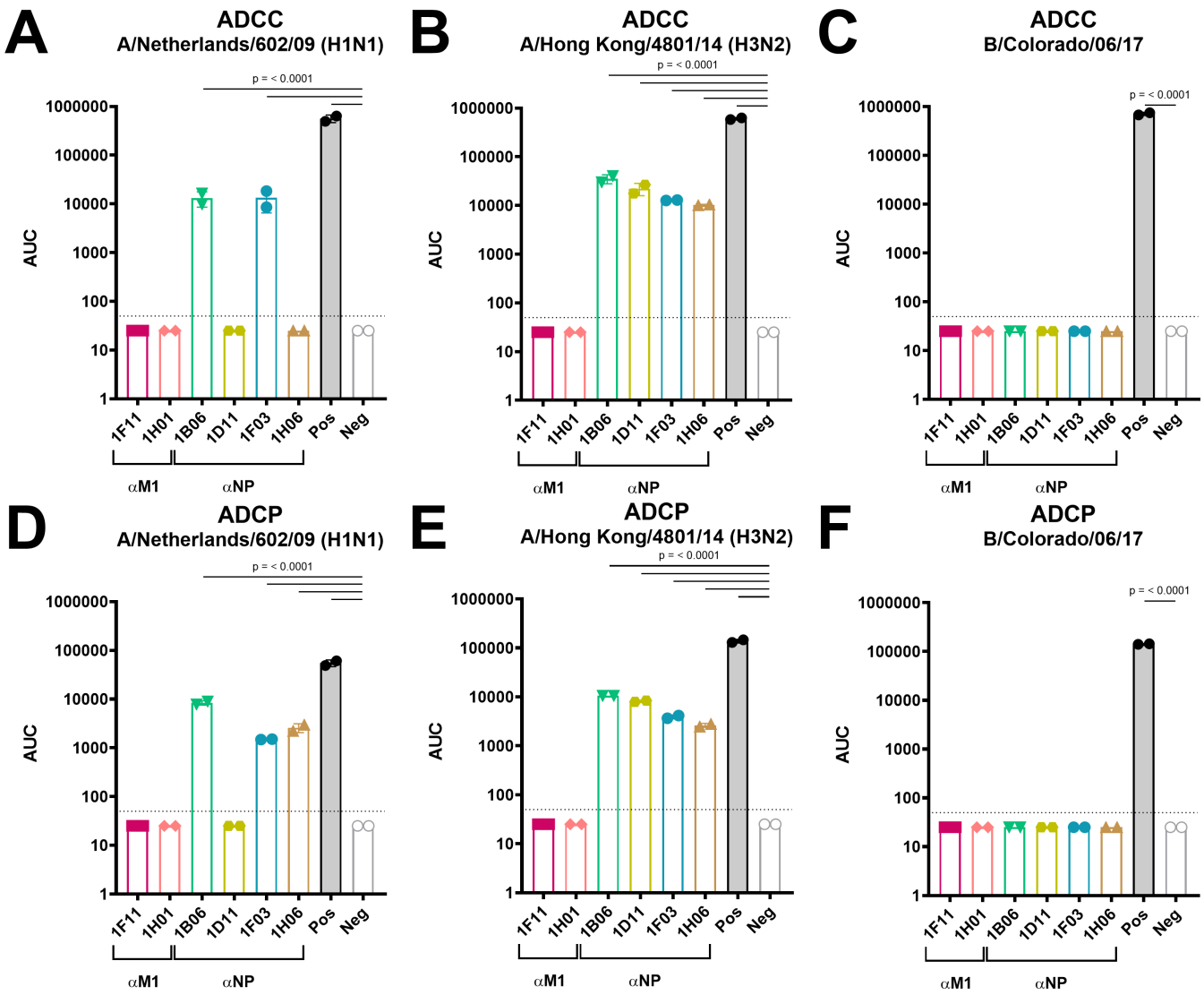

FIG 4 Functional *in vitro* characterization of ADCC and ADCP Fc-effector mechanisms mediated by anti-M1 and anti-NP mAbs. *In vitro* ADCC activity of anti-M1 and anti-NP mAbs against A/Netherlands/602/2009 (H1N1) (A), A/Hong Kong/4801/2014 (H3N2) (B), and B/Colorado/06/2017 (C). *In vitro* ADCP activity of anti-M1 and anti-NP mAbs against A/Netherlands/602/2009 (H1N1) (D), A/Hong Kong/4801/2014 (H3N2) (E), and B/Colorado/06/2017 (F). CR9114 functioned as a positive control, whereas the anti-CHIKV E1 mAb (1D04) served as a negative control. Data are presented as areas under the curve (AUCs) calculated with a lower detection limit of the average background plus three standard deviations (SDs). Every symbol represents the AUC of a single mAb and bars denote mean ± SD of one experiment conducted with two technical replicates. For statistical significance calculations, values were first log(y) transformed, and subsequently, a one-way ANOVA corrected for multiple comparison test was performed. Only *P* values lower than 0.05 are shown.

than anticipated, as 40% of the negative control mice survived the challenge. As the anti-NP mAbs 1B06 and 1H06 were partially protective in both challenge experiments, the A/Netherlands/602/2009 challenge was repeated with these mAbs (Fig. S4C and D). Upon repeating the challenge at 5 murine 50% lethal doses (mLD_{50}), all mice administered the anti-NP mAbs, 1B06 and 1H06, and the negative control mAb lost weight rapidly and were euthanized by day 7 post-infection. Collectively, the data indicate that our noneAbs that target either internal M1 or NP proteins do not robustly protect mice from influenza virus-induced morbidity and mortality.

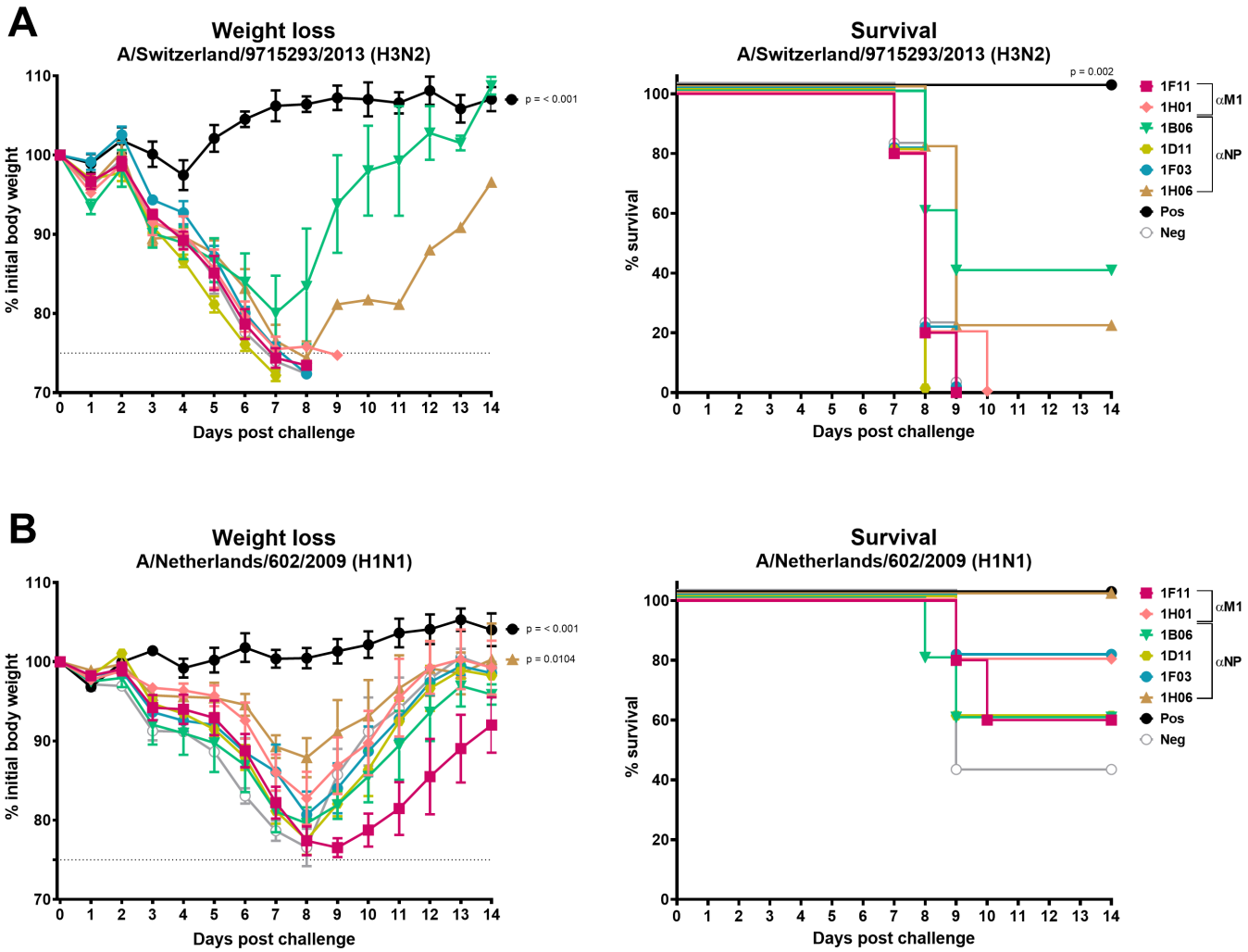

FIG 5 Assessment of anti-M1 and anti-NP mAb protection in a prophylactic setting *in vivo*. Morbidity in A/Switzerland/9715293/2013 (H3N2) (A) and A/Netherlands/602/2009 (H1N1) (B) infected mice following intraperitoneal administration of anti-M1 (1F11 and 1H01) or anti-NP (1B06, 1D11, 1F03, and 1H06) mAbs. Five animals per experimental group were used. Weight loss and survival were monitored for 14 days post challenge. The percentage (%) of weight loss and survival is shown. Error bars indicate the standard error of the mean (SEM). 1G01, a pan NA mAb, functioned as a positive control, whereas the SARS-CoV-2 mAb, 1C11, served as a negative control. The dashed vertical gray line represents a 75% cutoff of the initial weight, which was defined as the humane endpoint. *P* values indicate the statistical significance of the difference between the groups and the irrelevant antibody control assessed by one-way ANOVA followed by a multiple-comparison test for weight loss or a log-rank (Mantel-Cox) test for survival. For weight loss comparisons, the maximum weight loss in percentage for each mouse was used. Mice that reached the human endpoint were assigned 75%. Only *P* values lower than 0.05 are displayed.

DISCUSSION

Classical antibody-based protection from influenza A virus-induced morbidity and mortality correlates with neutralizing antibodies that prevent virus binding to sialic acid receptors and subsequent viral entry (82). Hence, studies of antibody-induced immunity to influenza viruses historically focused on the highly variable HA as a target (83, 84). However, recently, this has been challenged by the discovery of the importance of another subset of antibodies, namely, noneAbs (42, 48, 62, 76). Whereas the protective role of noneAbs against HA and NA is relatively well defined (11, 27, 42, 61, 85), the relevance and mechanism(s) by which noneAbs against internal proteins, like M1 and NP, exactly operate in the setting of influenza virus infection remained largely elusive. Therefore, in this study, we characterized a panel of six novel human anti-M1 and anti-NP mAbs that showed robust binding to IAVs. Both the internal M1 and NP influenza virus proteins represent highly conserved targets for noneAbs (8, 42). While

none of the mAbs had neutralizing activity in an *in vitro* neutralization assay, all anti-NP mAbs were able to mediate ADCC and/or ADCP activity in response to diverse IAV infections in reporter assays. In an attempt to translate the potential of *in vitro* noneAb-mediated Fc-effector mechanisms toward *in vivo* protection against IAVs, a prophylactic passive transfer challenge experiment in mice was performed. Notably, anti-IAV M1 mAbs did not robustly protect mice from influenza virus-associated morbidity and mortality. In contrast, the anti-IAV NP noneAbs, 1B06 and 1H06, partially protected against A/Switzerland/9715293/2013 (H3N2) challenge. Hence, our study suggests that noneAbs that bind internal IAV antigens, particularly the M1 protein, only provide limited protection *in vivo*. These findings, although based on a relatively small panel of mAbs, can provide important information for the design of broadly protective and universal influenza virus vaccines. An important caveat here is that human antibodies were used in the mouse model and crosstalk between human Fc and murine FcRs may not have been optimal. However, low-affinity non-neutralizing human anti-H7 mAbs showed protection in the mouse model despite this caveat (27). We are, therefore, confident that this system would detect a robust Fc-mediated protective effect.

Our work found that anti-M1 and anti-NP mAbs had robust binding to the two tested IAV strains, A/Netherlands/602/2009 (H1N1; pandemic) and A/Hong Kong/4801/2014 (H3N2), while no measurable reactivity was found toward the B/Colorado/06/2017 virus strain. Internal M1 and NP proteins exhibit over 90% amino acid sequence identity across a range of IAV subtypes and strains sampled from both humans and other host species (28, 72, 76, 86–90). In contrast, comparative analysis of the influenza A virus NP amino acid sequence with that of the influenza B virus NP revealed only a low 37% direct homology in the aligned regions (91). Similarly, a 25.4% direct homology was found between influenza A and B virus M1 amino acid sequences (92). Thus, the influenza virus internal proteins are only highly conserved within one influenza virus type, and it is, consequently, not surprising that the mAbs assessed in this study and others (77) do not provide cross-reactivity between diverse types.

Upon further assessment of the anti-M1 and anti-NP mAbs, we identified that the anti-NP mAbs induced significant levels of ADCC and ADCP activities when measured with IAV-infected cells in reporter assays compared to the negative control antibody, while the anti-M1 mAbs did not mediate any of these activities. These data might implicate that the anti-M1 mAbs conferred partial protection against the low dose A/Netherlands/602/2009 (H1N1) viral challenge via a different mechanism of action that has not been tested in this study, such as antibody-mediated complement-dependent cytotoxicity (CDC). Indeed, the Fc fragment of anti-M2e noneAbs has been demonstrated to bind to complement and thereby trigger the complement cascade leading to CDC (55, 56). Similarly, stalk (93) and NP (57) reactive mAbs promote complement-mediated virolysis upon binding to IAV-infected cells. In contrast, another anti-NP mAb was unable to mediate CDC following the recognition of IAV-infected cells (77). This discrepancy may be due to differences in the experimental conditions used or the inherent biological effect of the divergent anti-NP mAbs. Alternatively, the results might also be skewed by the M1 epitope accessibility on only dead, and not living, influenza virus-infected cells (2, 57, 77, 80). Accordingly, it might be conceivable that the *in vitro* ADCC and ADCP bioreporter assays were performed in the absence of accessible M1 proteins. Therefore, future experiments with a longer infection period and/or with M1 transfected cells might clarify which Fc-effector function(s) is mediated by mAbs targeting the internal M1 protein. In addition, it should be noted that the ADCC assays used in these studies are based on human FcRs, but mice—as mentioned above—express murine FcRs, which in our experiments perhaps would reduce the interaction between human mAbs and mouse FcRs that are required for protection in the mouse model. Besides, to verify the binding of mAbs 1F11 and 1H01 to influenza A virus internal M1 proteins, fluorescence-activated cell sorting analysis with living cells could be performed. Collectively, these findings firmly indicate that the antigen specificity was responsible for the different Fc-effector profiles obtained for anti-M1 and anti-NP mAbs, in which

future studies are required to elucidate on which Fc-effector mechanism or mechanisms anti-M1 mAbs depend for providing *in vivo* partial protection against the low dose A/Netherlands/602/2009 (H1N1) viral challenge.

Upon assessment of the anti-M1 and anti-NP mAbs in a prophylactic model, we found that all mice passively immunized with anti-M1 noneAbs succumbed to A/Switzerland/9715293/2013 (H3N2) infection. In contrast, these anti-M1, as well as the four anti-NP, reduced morbidity following challenge with A/Netherlands/602/2009 (H1N1). Although it should be highlighted that the A/Netherlands/602/2009 challenge was performed at a lower lethal dose than anticipated, as 40% of the negative control mice survived challenge. When the H1N1 study was repeated with an actual dose of 5× mLD_{50} for the anti-NP noneAbs, 1B06 and 1H06, as well as a negative control mAb, all mice showed severe weight loss and reached the 25% weight loss humane endpoint around day 7. This indicates that anti-NP noneAbs are unable to protect mice from A/Netherlands/602/2009 (H1N1) virus-induced mortality when a lethal dose of 5× mLD_{50} is used. For the A/Switzerland/9715293/2013 (H3N2) challenge, however, anti-NP mAbs 1B06 and 1H06 were able to partially protect mice against morbidity and mortality, and this may be because the mAbs were isolated from an influenza A H3N2-infected patient. This is reminiscent of the mouse mAbs 1H5 (a broadly reactive H7 HA head mAb) (85), 07-5E01 (a cross-reactive H7 HA stalk mAb) (27), 41-5D06 (a heterosubtypic H7 HA head binding mAb) (27), and 2D1 (a H7 HA stem reactive mAb) (11), which do not neutralize avian influenza A (H7N9) viruses but do significantly protect *in vivo* via, at least in part, Fc-dependent effector functions. Importantly, the observed protection in our H3N2 study was not as robust as seen for these and some other noneAbs that target HA (11, 27, 62–64, 85) or NA (3, 69) proteins. It, therefore, seems that noneAbs that target external, rather than internal, proteins on the IAV surface confer better protection *in vivo*. These differing protective profiles might be due to differential protein expression levels and/or protein accessibility on living and/or dead influenza virus-infected cells. Indeed, when comparing the affinity of our anti-M1 and anti-NP mAbs with other non-neutralizing HA stalk antibodies that better protect *in vivo* (27, 62), it becomes clear that our antibodies reveal lower minimum binding concentrations excluding the possibility that differences in antibody affinity were responsible for the divergent protective capacities conferred *in vivo* by our anti-M1 and anti-NP and the reported anti-HA noneAbs. Another possible explanation for the differing protective profiles is related to a limitation of our study. DBA2/j mice have a CD94 deficiency and, as a result, dysregulated NK cells, which may be why the noneAbs were not as protective following A/Switzerland/9715293/2013 (H3N2) challenge compared to the low dose A/Netherlands/602/2009 (H1N1) challenge (94). Another limitation with our animal studies is the challenge dose. We challenged with a virus dose that induces significant weight loss in challenged mice, and perhaps, the protective effects of these mAbs would be better assessed with a lower challenge dose. As mentioned above, one of our anti-NP mAbs—1H06—was able to completely protect *in vivo* when a low H1N1 challenge dose was used (an average maximum weight loss of 14% was observed). The protection afforded seemed to be contingent on *in vitro* ADCP activity as this mAb did not mediate ADCC activity against A/Netherlands/602/2009 (H1N1) virus-infected cells. As such, further studies that investigate different and especially lower virus doses for prophylactic passive transfer studies in mice would be critical to gain a better understanding of the potential cross-protective profile of the described anti-M1 and anti-NP mAbs.

Anti-M1 and anti-NP immune responses are primarily associated with the induction of broadly reactive cellular but not humoral immune responses. Along these lines, we and others (8, 45, 77, 95) have demonstrated that anti-M1 and anti-NP mAbs do not neutralize influenza viruses, which is seen as the main correlate of mAb-mediated protection. In contrast, broad-spectrum T-cells that primarily recognize conserved internal influenza virus antigens can provide protection by curbing influenza viral spread and limiting transmission (96–99). T-cell responses targeting these epitopes have been demonstrated to be highly protective in mice (100–102), and both $CD4^+$ and $CD8^+$ T-cells

have been described as correlates of protection in humans (99, 103–106). In fact, two studies performed in mice (9) and ferrets (1) illustrated that a combined prime-boost vaccination regimen of sequential exposure to chimeric HAs [cHAs; contain a highly variable head, but a conserved stalk domain (107–110)] with a replication-deficient chimpanzee adenovirus-vectored vaccine expressing both NP and M1 proteins, called ChAdOx1 NP + M1 (111), protected against H3N2 infection through the induction of a robust anti-HA stalk humoral in combination with a long-lived cellular immune response. Of note, in the former study, the combination of both sets of vaccination strategies offered augmented protection against influenza virus challenge in comparison to vaccination with the monovalent (cHA or M1 + NP) viral vectors (9). These and corresponding (112, 113) data implicate a potential synergy between vaccine-induced humoral and cellular immune responses. Hence, efforts focusing on developing a universal influenza virus vaccine should consider the development of vaccines that are able to harness both types of adaptive immunity. More precisely, if such a vaccination platform would be able to elicit a humoral immune response that consists of a cocktail of broadly neutralizing and non-neutralizing antibodies that target non-overlapping but protective epitopes, influenza virus immune escape could be avoided as a consequence of complementary or alternative mechanisms induced by these two different types of antibodies [detailed in reference (6)]. Accordingly, it would be interesting and worthwhile to examine the interplay between neutralizing and non-neutralizing mAbs, in addition to distinct noneAbs that mediate different Fc-effector mechanisms, to gain a better understanding on which antibody patterns would be able to induce synergistical protection and would be suitable candidates for the design of future universal anti-influenza virus vaccines.

In conclusion, we demonstrate that on a monoclonal basis in the mouse model, broadly reactive non-neutralizing anti-IAV M1 antibodies do not significantly protect against an influenza virus challenge. Anti-IAV NP antibodies, on the other hand, were more protective than anti-IAV M1 antibodies and had increased levels of Fc-effector functions. These results need to be confirmed with a larger panel of mAbs, as well as with polyclonal serum, and ideally in a mouse model with humanized FcRs. Our results, nonetheless, suggest that efforts focusing on developing a universal influenza virus vaccine that incorporate M1 and NP antigens should perhaps focus on the cellular immune responses induced following vaccination with these antigens since protection from mAbs against NP and M1 is not robust.

MATERIALS AND METHODS

Patients

Human PBMCs were collected from two individuals enrolled in a prospective observational cohort study at the Barnes Jewish Hospital Emergency Department (EDFLU study) (114). The Institutional Review Board of Washington University in Saint Louis (Missouri, USA) approved the EDFLU study (approval #2017-10-220). Subject 1718002 was 35 years old, female, and had a history of asthma. She was infected with sequencing-confirmed H3N2 IAV and presented on the fourth day of symptomatic illness when the PBMC and serum samples were obtained. She had not received the 2017–2018 seasonal influenza vaccine prior to disease onset but reported that she received other seasonal influenza vaccines in the past 5 years. Subject 1718003 was 34 years old, female, and had a history of diabetes, asthma, and congestive heart failure. She was infected with sequencing-confirmed H3N2 IAV and presented on the fifth day of symptomatic illness when PBMC and serum samples were obtained. She received the 2017–2018 seasonal influenza vaccine prior to disease onset and reported receiving other seasonal influenza vaccines in the past 5 years. Both subjects required admission to hospital for their illness but were successfully treated and discharged within a week of arrival in the emergency department. A total of 55 antibodies were isolated from these two individuals (22 mAbs from subject 1718002 and 33 mAbs from subject 1718003). Most antibodies bound to

HA; several also bound to NA. The remaining antibodies were screened for binding to NP and M1.

Cells

MDCK (CCL-34, American Type Culture Collection, Manassas, USA) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Waltham, USA) accompanied with penicillin-streptomycin (Pen/Strep) antibiotics solution (100 U/mL of Pen and 100 µg/mL Strep; Gibco), 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Gibco) and fetal calf serum (10%; Corning, Corning, USA) resulting in complete DMEM at 37°C in a humidified incubator with 5% carbon dioxide (CO_2). Expi293F cells (A14527, Thermo Fisher Scientific, Waltham, USA) were grown in Expi293 Expression Medium (Gibco) in 1-L Erlenmeyer shaker flasks (Corning) at 37°C and 125 revolutions per minute (rpm) in a humidified incubator with 8% CO_2. Single-use aliquots of ADCC bioassay effector Fcgamma(γ)RIIIa (CD16a) and ADCP bioassay effector FcγRIIa-H (CD32a) cells (Promega, Madison, USA) were thawed directly prior to use.

Viruses

The IAV strains, A/Netherlands/602/2009 (H1N1), A/Hong Kong/4801/2014 (H3N2), and mouse-adapted A/Switzerland/9715293/2013 (H3N2), were propagated in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (Charles River Laboratories, Massachusetts, USA) for 2 days at 37°C. The IBV strain, B/Colorado/06/2017, was grown in 10-day-old SPF embryonated chicken eggs for 3 days at 33°C. Eggs were subsequently inactivated over night at 4°C. Following inactivation, allantoic fluid was harvested and centrifuged (Centrifuge 5810 R, 15 amp version, Eppendorf, Hamburg, Germany) at 4,000 × *g* for 10 min at 4°C to pellet cellular debris, and eventually, allantoic fluid was aliquoted and stored at −80°C prior to performing plaque assays to determine virus titers, as described previously (1, 115).

Antibodies

Antibodies were expressed by transient co-transfection of 17 µg of heavy and 33 µg of light chain plasmids in Expi293F (70×10^6 cells/50 mL) cells using the ExpiFectamine 293 Transfection kit (Thermo Fisher Scientific) as per the manufacturer's recommendations and as has been previously described (74). Seven days post transfection, cell cultures were harvested by low-speed centrifugation at 4,000 × *g* for 30 min and filtered through a 0.22 µM sterile filtration unit. Next, mAbs were purified using protein A chromatography (GoldBio, Missouri, USA) and were eluted into a 50-mL Falcon tube (Denville Scientific, Inc., New Jersey, USA) each with 0.1 M glycine-hydrogen chloride (HCl; pH 2.7) and were directly neutralized with 1 M Tris-HCl (pH 9.0). mAbs were then concentrated and buffer exchanged with phosphate-buffered saline (PBS) using Amicon Ultra filter units (EMD Millipore) with 30-kDa cutoffs, and finally, mAb concentrations were determined at 280-nm absorbance with a Nanodrop spectrophotometer (Thermo Fisher Scientific).

Recombinant proteins

Recombinant H3 from A/Singapore/INFIMH-16-0019/2016 (H3N2), N2 from A/Singapore/INFIMH-16-0019/2016 (H3N2), and M1 from A/Michigan/45/2015 (H1N1) or A/Puerto Rico/8/1934 (H1N1) proteins were expressed and purified using the well-established baculovirus expression system as described previously (63, 116, 117). Recombinant NP proteins from A/Michigan/45/2015 (H1N1), A/Singapore/INFIMH-16-0019/2016 (H3N2), and B/Colorado/06/2017 were expressed via transient transfection of Expi293F cells according to the manufacturer's instructions (Thermo Fisher Scientific).

Recombinant protein supernatants were harvested after 3 days, centrifuged at 4,000 × *g* for 20 min at 4°C, and passed through a 0.22-µm filter. Supernatants were subsequently mixed and incubated with nickel nitrilotriacetic acid (Qiagen, Hilden,

Germany) agarose beads on a shaker (~125 rpm) for 2 h at room temperature (RT). Proteins were then purified via gravity flow columns (Qiagen), eluted as described previously (117), concentrated, and buffer exchanged using Amicon Ultra centrifugal units via three-time centrifugation at 4,000 × *g* for 30 min at 4°C and finally re-suspended in PBS. Purified proteins were quantified using the Quick Start Bradford 1× Dye Reagent (Bio-Rad; California, USA) and based on a standard curve. Recombinant proteins were analyzed via reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis to check for protein purity and integrity.

Cell-based ELISA

Ninety-six-well cell culture plates (Corning) were seeded with 100 µL/well of MDCK cells at a density of 2×10^5 cells/mL and incubated over night at 37°C with 5% CO_2. The following day, cells were washed with PBS and infected with 100-µL virus inoculum at a multiplicity of infection (MOI) of 5 [corresponding to about 2×10^5 plaque-forming units (PFU) of virus; diluted in 1× minimal essential medium (MEM, Gibco)] for 16–20 h at 37°C with 5% CO_2 for IAV or at 33°C with 5% CO_2 for IBV strains. Cells were then fixed with 3.7% paraformaldehyde (Thermo Fisher Scientific; 200 µL/well) and stored at 4°C for 24 h. The subsequent day, plates were washed with PBS, and the cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Missouri, USA) diluted in PBS and incubated for 15 min at RT. Afterwards, plates were washed once and incubated for 1 h at RT with 200 µL/well blocking solution containing 3% milk in PBS. The mAbs were then diluted to a starting concentration of 10 µg/mL, serially diluted threefold across plates, and incubated for 2 h at RT. Plates were subsequently washed three times with PBS before adding the secondary anti-human IgG (Fab specific) horseradish peroxidase (HRP) (produced in goat, Sigma-Aldrich) antibody at a concentration of 1:3,000 in blocking solution for 1 h at RT. After incubation, plates were washed three times to remove residual secondary antibody and were subsequently developed by adding 3,3′-5,5′-tetramethylbenzidine substrate (Sigma-Aldrich; 100 µL/well). The reaction was stopped after 10 min with 4 M sulfuric acid (Thermo Fisher Scientific; 50 µL/well). The optical density (OD) of each plate was measured at 450 nm on a Synergy H1 microplate reader (BioTek, Vermont, USA), which represented the readout for this assay. Results were analyzed in Microsoft Excel and GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, California, USA). To visualize the binding curves, a non-linear regression model was used based on one individual experiment with technical duplicates.

The ELISA to measure mAb titers in the pre-challenge sera from mice was performed similarly. Here, MDCK cells were infected with the A/Hong Kong/4801/2014 (H3N2) virus strain at a MOI of 5. The plates were treated and blocked as described above. Pre-diluted sera (1:10) were added to the first well to obtain a final concentration of 1:100 in blocking solution. The sera were then serially diluted threefold and incubated for 2 h at RT. Next, the secondary antibody, an anti-human IgG (Fab specific) HRP (produced in goat, Sigma-Aldrich) diluted at a concentration of 1:3,000 in blocking solution, was added. Subsequently, the ELISA was further processed as described above. Data are presented as the area under the curve (AUC) calculated with a cutoff value of the average of the OD values of the background plus three standard deviations (SDs).

Protein-based ELISA

Protein-based ELISAs were performed as described previously (3). In brief, 96-well nonsterile, flat-bottom, microtiter plates (Immunolon 4HBX; Thermo Fisher Scientific) were coated with a final concentration of 2 µg/mL of recombinant protein (50 µL/well) in PBS overnight at 4°C. The next day, plates were washed three times with PBS containing 0.1% Tween 20 (PBS-T; Fisher BioReagents, Pennsylvania, USA). Subsequently, plates were blocked for 1 h at RT with 220-µL blocking solution consisting of 3% goat serum (Life Technologies, Carlsbad, USA) and 0.5% milk in PBS-T. After blocking, mAbs were diluted to a starting concentration of 30 µg/mL in blocking solution and were serially diluted threefold in a final volume of 100 µL/well and incubated for 2 h at RT. The rest of

the assay was conducted as described in the Cell-based ELISA section with the only alteration that the final washing step included four washes with PBS-T with intermitted shaking in between each washing step.

Microneutralization assay

MDCK cells were seeded in 96-well cell culture plates (Corning) at a density of 2 × 10^4 cells/well with a total volume of 100 µL in each well and were incubated over night at 37°C in a humidified incubator under 5% CO_2. The following day, mAbs were diluted to a 100 µg/mL concentration and then twofold serially diluted across a 96-well plate in infection medium [1× MEM (Gibco), 2 mM L-glutamine (Gibco), 0.1% sodium bicarbonate (wt/vol, $NaHCO_3$; Gibco), 0.01 M HEPES buffer (Gibco), Pen/Strep (100 U/mL penicillin, 100 µg/mL streptomycin; Gibco), 0.2% bovine serum albumin (MP Biomedicals, California, USA), and 1 µg/mL tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich)]. Next, 60 µL of 100× tissue culture infectious dose 50 of virus in infection medium and 60 µL of serially diluted mAb were incubated on a shaker for 1 h at RT. Prior to the end of the incubation time, MDCK cells were washed once with 200-µL PBS, and then, 100 µL of the incubated mAb-virus mixture was added for 1 h at 37°C with 5% CO_2 for IAV or 33°C with 5% CO_2 for IBV strains, respectively. Afterwards, the virus inoculum was aspirated, and MDCK cells were again washed with 200-µL PBS and incubated with 100 µL of mAb (at the same concentrations) for 48 h at 37°C under 5% CO_2 for the IAV plates or for 72 h at 33°C under 5% CO_2 for the IBV plates. To visualize the neutralizing potency of the mAbs, a classical hemagglutination assay was conducted. Results were analyzed in Microsoft Excel and GraphPad Prism 9. Data are displayed as the minimum neutralizing concentration, and this value represents the lowest antibody concentration at which no hemagglutination could be detected. The detection limit included 100 µg/mL, as this was the highest antibody concentration used for this assay, and mAbs that did not reach neutralizing activity at this limit were assigned a value of 200 µg/mL for graphing purposes. Each experiment was performed in duplicate. CR9114, a broadly cross-reactive influenza A and B virus HA stalk binding mAb with known neutralizing activity (41), and 2E01, an IBV NA mAb (78), were used as positive controls, and 1D04, an anti-CHIKV E1 virus mAb, was used as a negative control.

ADCC bioreporter assay

ADCC activity of the mAbs was tested by performing *in vitro* ADCC bioreporter assays as per the manufacturer's protocol (Promega). In short, 2 × 10^5 MDCK cells/well (100 µL/well) were cultured over night at 37°C under 5% CO_2 in white, flat-bottom, 96-well cell culture plates (Corning). The following day, the cells were washed with 200-µL PBS and infected with a virus inoculum at an MOI of 5 (equivalent to 200,000 PFU, diluted in 1× MEM: 100 µL/well). Plates were incubated for 16 h at 37°C with 5% CO_2 for IAVs or at 33°C with 5% CO_2 for IBVs. Next, mAbs were diluted to 30 µg/mL and twofold or threefold serially diluted in assay buffer consisting of Roswell Park Memorial Institute Medium 1640 (Gibco). An appropriate amount of human ADCC bioassay effector cells (Jurkat cells engineered to express FcγRIIIa, high affinity version H131, Promega) were then thawed in a 37°C water bath. Media were aspirated from the cell plates; plates were once washed with 100 µL/well PBS; and afterwards, 25 µL/well of assay buffer, 25 µL/well of serially diluted mAbs, and 25 µL/well of ADCC effector cells in a final concentration of 7.5 × 10^4 cells/25 µL were added. For the blank wells, 50 µL/well of assay buffer and 25 µL/well of bioeffector cells were added. Plates were incubated for 6 h at 37°C with 5% CO_2 in a humidified incubator, followed by temperature equilibration for 15 min at RT and addition of 75 µL/well Bio-Glo Luciferase assay reagent (Promega, #G7940, G7941) for 10 min in the dark at RT. After incubation, the luciferase-induced luminescence was assessed using a Synergy H1 microplate reader (BioTek). Data were analyzed in Microsoft Excel and GraphPad Prism 9. Data are presented as the AUC calculated with a cutoff value of the average of the OD values of the background plus five SDs.

ADCP bioreporter assay

A commercial ADCP reporter assay kit (Promega) was used to investigate the ADCP activity of the mAbs. The protocol is similar to the ADCC bioreporter assay described above with the only difference being that human bioeffector FcγRIIa cells were used instead of the human FcγRIIIa cells.

Prophylactic passive transfer studies in mice

Animal experiments were performed following the Icahn School of Medicine Institutional Animal Care and Use Committee guidelines. Animals were given *ad libitum* access to food and water and were maintained in a 12-h light-dark cycle. Mouse passive transfer experiments to test the prophylactic efficacy of the mAbs were conducted as described earlier (3, 78). In brief, naïve six-week-old female BALB/c (for H1N1 studies) or DBA2/j (for H3N2 studies) mice (Jackson Laboratories) were intraperitoneally injected with 10 mg/kg of mAb (100 µL; 1F11, 1H01, 1B06, 1D11, 1F03, and 1H06; $n = 5$ mice/mAb tested). Positive control mice received 1G01, a pan NA mAb which has been documented to protect against lethal challenges with both influenza A and B viruses (3), and negative control mice received 1C11, an irrelevant human anti-SARS-CoV-2 mAb, or 8H9, an irrelevant mouse anti-H6 mAb, at an equivalent dose and volume. Three hours post mAb transfer, mice were bled to verify successful mAb transfer by ELISA as described above and were afterwards intranasally challenged with 1,600 PFU/50 µL of A/Netherlands/602/2009 (H1N1) (or 1,778.3 PFU of retitered virus/50 µL in repeated studies) or 1,600 PFU/50 µL mouse-adapted A/Switzerland/9715293/2013 (H3N2) virus strains under anesthesia (0.15 mg/kg/ketamine and 0.03 mg/kg/xylazine per mouse). Weight loss and survival were monitored daily for a period of 12–14 days, and data were analyzed in Microsoft Excel and GraphPad Prism 9. Mice that lost 25% or more of their day 0 body weight were euthanized, according to institutional guidelines.

Statistics and reproducibility

For line graphs, data are expressed as means. For bar graphs, data are expressed as individual values, and the average, presented as the mean. Error bars represent SD or SEM. Significance between antibody responses or weight loss data was analyzed by one-way analysis of variance (ANOVA) followed by a multiple-comparison test. Groups were compared to the irrelevant antibody control group. For the antibody response ADCC and ADCP data, the one-way ANOVA analysis was performed on log(y)-transformed AUC values. For the weight loss comparisons, the maximum weight loss in percentage for each mouse was used. Mice that reached the humane endpoint were assigned 75%. Survival curve comparisons were analyzed using a log-rank (Mantel-Cox) test. Data were considered statistically significant at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 9.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (JVI01646-22-S0001.pdf). Fig. S1 to Fig. S4.

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