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A non-neutralizing antibody broadly protects against influenza virus infection by engaging effector cells

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A non-neutralizing antibody broadly protects against influenza virus infection by engaging effector cells

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Abstract

Hemagglutinin (HA) is the immunodominant protein of the influenza virus. We previously showed that mice injected with a monoglycosylated influenza A HA (HA_{mg}) produced cross-strain-reactive antibodies and were better protected than mice injected with a fully glycosylated HA (HA_{fg}) during lethal dose challenge. We employed a single B-cell screening platform to isolate the cross-protective monoclonal antibody (mAb) 651 from mice immunized with the HA_{mg} of A/ Brisbane/59/2007 (H1N1) influenza virus (Bris/07). The mAb 651 recognized the head domain of a broad spectrum of HAs from groups 1 and 2 influenza A viruses and offered prophylactic and therapeutic efficacy against A/California/07/2009 (H1N1) (Cal/09) and Bris/07 infections in mice. The antibody did not possess neutralizing activity; however, antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis mediated by nature killer cells and alveolar macrophages were important in the protective efficacy of mAb 651. Together, this study highlighted the significance of effector functions for non-neutralizing antibodies to exhibit protection against influenza virus infection.

Author Summary

The protective efficacy of antibodies is generally related to their neutralization potency. Here, we isolated a monoclonal antibody from mice injected with monoglycosylated hemagglutinin

protein-based universal flu vaccine, and demonstrated a head-domain recognizing, but non-neutralizing, monoclonal antibody carried prophylactic and therapeutic efficacy against a broad spectrum of influenza virus infections *in vivo* via effector functions.

Introduction

Influenza viral infections cause a contagious respiratory illness of the upper airways and lungs. Approximately half a million deaths worldwide are due to seasonal flu each year [1]. Influenza viruses belong to the *Orthomyxoviridae* family, whose genomes encode 11 proteins, including the major surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) [2]. Influenza viruses are classified into three subtypes: A, B, and C. Among which, the A and B subtypes are responsible for the seasonal flu in humans [2]. Although the influenza vaccine is currently in use for controlling the spread of seasonal flu each year, the trivalent inactivated influenza vaccine only confers protection in around two thirds of those vaccinated [3]. More severe complications resulting from influenza viral infection and a loss of influenza vaccine efficacy have been found in older adults [4, 5]. Thus, there remains a need to develop new strategies to overcome the moderate responses and narrow coverage range of the current flu vaccine.

Advances in antibody engineering technologies have enabled the isolation of several neutralizing antibodies against influenza viruses from either infected patients or donors with extensive vaccinations [6-10]. These anti-flu neutralizing monoclonal antibodies (mAbs) target HA, which comprises a variable immunodominant globular head domain or a more conserved immune-subdominant stem domain [11, 12]. Most of the broadly neutralizing antibodies target the stem region of HA and have less potent direct neutralization activity [11,

12]. Other neutralizing antibodies bind to the head region of HA and generally possess a potent ability to directly inhibit virus entry [12]. Recently, non-neutralizing mAbs recognizing the HA globular head domain trimer interface have been reported that limit the spread of influenza viruses and protect against infection by various flu strains in mice [10, 13]. In addition to the abovementioned modes of action, antibodies can eliminate infected cells through effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) [14], in which nature killer (NK) cells with Fc receptors (FcRs) are the primary effector cells [15]. It has been demonstrated that mAbs targeting the stem region of HA can mediate ADCC [16]. Additionally, antibody-dependent cellular phagocytosis (ADCP), which involves the ingestion of antibody-opsonized influenza virus particles by alveolar macrophages, has also been shown to be involved in the clearance of influenza viruses [17].

Changing the glycan abundance of HA can affect its immunogenicity [18, 19]. We developed a monoglycosylated HA (HA_{mg}) protein-based universal flu vaccine by treating HA with endoglycosidase H. HA_{mg} served as an effective vaccine providing broader protection against infection by various influenza virus strains than the fully glycosylated HA (HA_{fg}) in animal models [20, 21]. One mAb, 651, isolated from HA_{mg}-immunized mice, was subjected to functional characterization in this study. Although it lacks neutralizing activities *in vitro*, 651 is able to recognize the head region of a broad range of HA proteins and protect against infection by several H1N1 viruses through effector functions *in vivo*. Thus, we demonstrated a

unique mode of action for a non-neutralizing mAb that can offer cross-strain protection against influenza virus infection through FcR-mediated effector functions. The results highlight the potential significance of non-neutralizing antibodies in host responses against viral infections.

Results and Discussion

Generation of mAb 651 from Bris/07 HA_{mg}-immunized mice

We previously demonstrated that the HA_{mg} vaccine elicits antibody responses that recognize a broader spectrum of influenza viruses than the HA_{fg} vaccine [21]. We aimed to isolate broadly neutralizing mAbs from mice immunized with HA_{mg} proteins prepared from the A/Brisbane/59/2007 (H1N1) (Bris/07) influenza virus. By using a single B-cell antibody screening technique, we isolated and expressed the HA_{mg}-specific chimeric mAbs (Fig 1A). We identified on a specific mAb, 651, which recognized the broad recognition of HA proteins from different strains of influenza virus, including group 1 and group 2 influenza A virus subtypes (Fig 1B). Quantitative measurement using an antibody affinity assay showed that 651 possessed a higher affinity to HA_{mg} than HA_{fg} from both A/Brisbane/59/2007 (H1N1) (Bris/07) and A/California/7/09 (H1N1) (Cal/09) (Fig 1C).

The structure and conformation of HA are important for its recognition by 651, as decreased binding was observed after HA was treated with 2-ME, which disrupted its protein

conformation (Fig 1D). ELISA results showed that 651 bound to the intact HA, but not to the stem region, of Bris/07 (Fig 1E), implying that 651 mainly recognizes the globular head domain of HA. Supporting this notion, the results of a hydrogen-deuterium exchange-mass spectrometry (HDX-MS) assay showed that there was one region recognized by 651. The mAb 651 interacts with the globular head region near a glycosylation site of HA from Bris/07 (S1A Fig). Sequence alignment of the identified Bris/07 HA peptide with the HA proteins from several influenza viruses revealed that this 651-binding region in the head globular domain is relatively conserved in Bris/07 and Cal/09, and is close to the N-glycosylation site (S1B Fig). Typically, anti-influenza virus antibodies that recognize the globular head domain possess hemagglutination inhibition (HI) and neutralizing activity but show less cross-reactivity compared with antibodies against the stem region [12, 22]. Although 651 recognizes the HA of a panel of influenza viruses, it does not possess neutralizing activity against Bris/07 (H1), Cal/09 (H1), NIBRG-14 (H5), or Vic/11 (H3) influenza viruses (Fig 1F). Furthermore, 651 did not show HI activity against the Cal/09 virus (S1C Fig). Therefore, we have isolated a mAb 651 derived from Bris/07 HA_{mg}-immunized mice that targets the globular head region of various HA proteins but has no neutralizing activity.

mAb 651 confers preventive and therapeutic efficacy through FcγR

Because 651 binds to various strains of influenza virus, we next sought to understand its biological functions *in vivo*. Mice were intraperitoneally injected with two different 651 doses 2 h before viral infection (Fig 2A). To our surprise, pretreatment with 651 provided a dose-dependent protection against Bris/07 infection (Fig 2B) and a complete protection against Cal/09 infection (Fig 2C). The better prophylactic efficacy against Cal/09 correlated with the stronger binding affinity of 651 to the HA of the Cal/09 strain than to that of Bris/07 (Fig 1C).

We then examined the therapeutic efficacy of 651. Mice were administered the antibodies 1 day after infection with influenza virus (Fig 2D). Again, the survival rates were significantly improved in the 651-treated groups after Cal/09 infection. Improved, although not statistically significant, survival rates were observed in the high-dose 651-treated mice after Bris/07 infection (Fig 2E). Remarkably, mice provided a high dose of 651 1 day after Cal/09 infection were completely protected from a lethal dose Cal/09 challenge on day 14 (Fig 2F). Weight loss following viral infection was also prevented in the mice treated with 651 before or after infection (S2A–S2D Fig). F10 was used as a positive control antibody, as it has prophylactic and therapeutic efficacy against a broad spectrum of group 1 human influenza A viruses [23]. Our results showed 651 and F10 had comparable preventive and therapeutic efficacies (Fig 2A–2F and S2A–S2D Fig). Thus, 651 provided notable protective efficacy against influenza viral infection *in vivo*, despite being a non-neutralizing antibody.

In addition to being able to directly neutralize pathogens, antibodies can eliminate infection through FcR-mediated reactions, such as ADCC or ADCP [12]. Because 651 was unable to neutralize the influenza virus, we next examined whether the protective efficacy of 651 was due to FcR-mediated effector functions. We generated an Fc-region mutant with leucine 234 and 235 to alanine substitutions, called 651-LALA, which mitigates antibody effector function by abolishing antibody binding to the FcR [24]. We found that mice given 651-LALA had similar mortality rates as those pretreated with PBS (Fig 2G), demonstrating the significance of the effector function of 651. The γ chain of the Fc receptor is a critical component of the high-affinity receptor for IgG [15]. Mice lacking the FcR γ chain displayed impaired NK-cell-mediated ADCC and macrophage-mediated ADCP [25]. Significantly reduced survival rates were also found in 651-pretreated *Fcer1g*-knockout mice compared with 651-pretreated wildtype mice after Cal/09 infection (Fig 2H). However, a lack of *Fcer1g* did not abolish the protective effect of F10, indicating that neutralizing activity predominantly contributed to the function of F10 (Fig 2H). Following challenge with a lethal dose of Cal/09 virus, mice pretreated with 651-LALA or *Fcer1g*-knockout mice consistently showed similar dramatic bodyweight loss as the PBS treated group (S2E and S2F Fig). Together these results indicate that the protective effects mediated by 651 rely on FcR-mediated effector functions.

651-pretreatment alleviates virus replication and ameliorates lung inflammation

155 Upon influenza viral infection, both virus-induced virulence and immunopathology contribute
156 to inflammation and tissue injury in the respiratory tract [26]. We next examined whether 651
157 leads to viral clearance and alleviates lung inflammation. Virus replication was abolished in
158 mice pretreated with F10 2 h before Cal09 infection by 3 days post infection, which is likely
159 to be due to the potent influenza-virus-neutralizing ability of F10 (Fig 3A). Notably, a
160 significant improvement in viral clearance was seen on day 5, but not day 3, in Cal09-infected
161 mice pretreated with 651 (Fig 3A). Hematoxylin and eosin (H&E) staining further showed the
162 reduced pulmonary edema and immune cell infiltration in mice pretreated with F10 or 651.
163 However, mice treated with 651-LALA displayed lung damage and immune cell infiltration as
164 severe as that seen in PBS-treated mice on day 3 after Cal09 infection (Fig 3B).

165 The viral nucleic acids released upon influenza virus infection can be sensed by various
166 pattern-recognition receptors, which trigger robust downstream signaling [27, 28]. Several
167 cytokines in the lungs, including the type I IFN, MCP-1, IL-6, and TNFα produced by various
168 cells after influenza infection, lead to cytokine storms—one of the major causes of severe
169 flu-associated complications [27]. We next assessed whether the production of cytokines in
170 the lungs is influenced by 651 administration. We found that the production of cytokines,
171 including IFNα, IFNβ, MCP-1, IL-6, and TNFα, was elevated on days 3 and 5 after Cal/09
172 infection (Fig 3D and 3E). Pretreatment with F10 or 651 significantly reduced the release of
173 all cytokines on day 3 (Fig 3D). At 5 days post infection, the production of IFNβ, MCP-1, and

IL-6 remained significantly reduced by pretreatment with F10 or 651 (Fig 3E). Type III IFN, IFNλ, has been shown to have therapeutic efficacy against the influenza virus, but it does not induce proinflammatory side effects [29]. It is noteworthy that the IFNλ level was elevated in mice pretreated with F10 or 651 on day 3 post infection (Fig 3D). The 651-mediated reduction of cytokine production was reverted when Leu234/Leu235 sites were mutated (Fig 3D and 3E). Therefore, similarly to pretreatment with F10, pretreatment with 651 alleviated lung inflammation after influenza virus challenge.

ADCC and ADCP mediated by 651

The HA of influenza virus was reported to be the antigenic determinant responsible for the generation of antibodies with ADCC [30]. Several immune cell types, including NK cells and neutrophils, target antibody-labeled cells after infection and release cytotoxic granules and cytokines to kill the virus-infected cells [31]. Following Cal/09 challenge, the percentage of NK cells, defined as $CD49b^{+}CD3e^{-}$, in the lungs increased on day 3 (Fig 4A) but then declined to a level similar to that in uninfected mice on day 5 (Fig 4B). We found an increased NK cell population in the lungs on day 5 post infection in F10- and 651-, but not in 651-LALA-, pretreated mice (Fig 4A and 4B), suggesting the potential role of NK cells in mice provided with protective antibody treatment. Furthermore, we used HEK293T cells to express HA proteins from various strains of influenza virus, including Bri/09 H1N1, Cal/09

193 H1N1, H3N2, and H7N9, to examine whether 651 provided ADCC *in vitro*. Compared with
194 the effects in the control IgG-treated cells, both 651 and FI6 showed greater dose-dependent
195 cytotoxicity towards cells expressing Bris/07, Cal/09, and H3 (Fig 4C), but 651 did not
196 provide ADCC against H7-expressing cells (Fig 4C). This is consistent with our data showing
197 the lack of binding to H7 by 651 (Fig 1B). Since ADCC relies on the Fc-FcR interaction,
198 651-LALA could not trigger effective ADCC against Bris/07 and Cal/09 HA (Fig 4D).
199 Besides ADCC, ADCP also contributes to protection against influenza virus infection.
200 Non-neutralizing antibodies have been shown to offer protective effects through alveolar
201 macrophages but not NK cells [32]. We found that on days 3 and 5 after Cal/09 challenge, the
202 frequency of alveolar macrophages significantly declined (Fig 4E and 4F), which is consistent
203 with a previous report showing that alveolar macrophages dramatically diminished in
204 BLAB/c mice after influenza virus infection [33]. Pretreatment with F10 or 651 significantly
205 rescued the decline of alveolar macrophages caused by influenza virus infection on days 3 and
206 5 post infection (Fig 4E and 4F). We subsequently used THP-1 as effector cells to confirm
207 whether 651 utilizes ADCP for influenza virus clearance *in vitro*. The antibody opsonized
208 Cal/09 virus was incubated with sialidase-treated THP-1 cells, and intracellular viral N
209 protein (NP) was detected. We found that the uptake of viruses by THP-1 cells was
210 accelerated by opsonized 651 (Fig 4G) compared with uptake by the IgG- and
211 651-LALA-opsonized group. Moreover, a dose-dependent ADCP by 651 was observed (Fig

4H). ADCP activity was not found for various doses of 651-LALA opsonized with Cal/09 (Fig 4G and 4H). Therefore, we demonstrated that 651 possesses ADCC and ADCP activity *in vitro*.

NK cells and alveolar macrophages coordinate the antiviral responses mediated by 651

Having demonstrated the ADCC and ADCP mediated by 651 *in vitro*, we next examined the involvement of NK cells and alveolar macrophages in 651-mediated protection during virus challenge *in vivo*. Mice were administered an anti-ASGM1 antibody to deplete NK cells (S3A Fig) and intranasally administered clodronate liposomes to deplete alveolar macrophages (S3A Fig). As expected, both F10 and 651 still possessed prophylactic activity in Cal/09-infected mice injected with the control antibody or liposomes, as shown by the significantly improved survival (Fig 5A). Remarkably, the prophylactic effects of 651 diminished when NK cells and alveolar macrophages were co-depleted (Fig 5B), while 651 still protected mice lacking either NK cells or alveolar macrophages. In contrast, F10-administered mice remained resistant to influenza virus challenge, even when NK cells and alveolar macrophages were co-depleted (Fig 5B), implying the predominant neutralizing activity of F10 [23]. The body weight loss results demonstrated that the co-depletion of NK cells and alveolar macrophages was required to eradicate the protective effect of 651 (S3B and S3C Fig). Consistently, the production of IFN-β and proinflammatory cytokines, such as

231 IL-6 and MCP-1, in the lungs was comparable between the 651- and PBS-pretreated mice
232 co-depleted with NK cells and alveolar macrophages on day 3 (Fig 5C) and day 5 (Fig 5D)
233 post infection. The viral titers on day 5 post infection were also similar between the PBS- and
234 651-pretreated mice co-depleted with NK cells and alveolar macrophages (Fig 5E). These
235 results suggest that the protective efficacy of 651 *in vivo* could be attributed to both NK cells
236 and alveolar macrophages.

237 From our study, we concluded that 651 is a non-neutralizing mAb with a broad spectrum
238 of recognition range that is able to alleviate the inflammatory responses and ameliorate the
239 mortality associated with influenza viral infection. This broad-spectrum mAb was isolated
240 from HA_{mg}-immunized mice. It remains to be ascertained if a natural influenza viral infection
241 or influenza vaccination in humans can elicit a significant abundance of such
242 broad-recognition and non-neutralizing anti-HA antibodies that confer protection through
243 effector functions. One recent study demonstrated the antibody-dependent enhancement of
244 influenza virus infection involved the recognition of the head domain and the promotion of
245 viral fusion [34]. Studying the structure of HA, 651, and the FcR tertiary complex will further
246 reveal the allosteric preferences of engaging target cells and effector cells for effective ADCC.
247 Nevertheless, the Fab of 651 recognizes the globular head region, near the glycan
248 modification sites of HA, whose sequences are conserved in Bris/07 and Cal/09. We consider
249 that removal or truncation of the glycan may allow the exposure of hidden epitopes of HA

250 that are relatively conserved across influenza viruses.

251 Accumulating efforts have improved the potency of antibody-mediated effector functions
252 by generating Fc variants or modulating the glycan compositions of Fc [35-37]. Further
253 modifications of 651 may optimize its protection efficacy *in vivo*. We found that, in addition
254 to NK-cell-mediated ADCC, alveolar macrophage-mediated ADCP is also critical to the
255 protective effects of 651. In response to viral infections, alveolar macrophages are activated,
256 becoming phagocytic and producing large quantities of inflammatory cytokines, including
257 type I IFNs [38]. Some mAbs have been demonstrated to confer protection against influenza
258 viral infection through the action of antibody-induced inflammation and ADCP mediated by
259 alveolar macrophages [32]. However, neither F10 nor 651 increased the production of
260 inflammatory cytokines in the lungs during influenza virus infection, possibly because of the
261 differential cytokine kinetics resulting from the different doses and types of virus used.
262 Although awaiting confirmation in future studies, it is also plausible that the antibody affinity
263 in this study selectively modulated certain subsets of the heterogeneous alveolar macrophages,
264 which are, at minimum, composed of resident macrophages that populate the lungs during
265 embryogenesis and circulating blood monocytes recruited to the lungs following infection
266 [39].

267 In conclusion, the study described herein revealed the protective effects of a
268 broad-recognition and non-neutralizing mAb against influenza infection, which may prove

valuable in future assessments of vaccine efficacy and therapeutic antibody development.

Materials and Methods

Influenza viruses, HA expression plasmids and HA proteins

The vaccine strains of influenza viruses A/H1N1/California/07/2009 (Cal/09), A/H1N1/Brisbane/59/2007 (Bris/07), A/H5N1/Vietnam/1194/2004/NIBRG14 (NIBRG14) and A/H3N2/Victoria/361/2011 (Vic/11) were from the reference collection of the National Institute for Biological Standards and Control. All viruses were inoculated into the allantoic cavities of 10-d-old specific pathogen-free embryonated chicken eggs for 2 days at 35°C. The 50% tissue culture infective dose ($TCID_{50}$) of viruses in Madin-Darby canine kidney (MDCK) (CCL-34; American Type Culture Collection) cells and LD_{50} of virus in BALB/c mice were determined before experiments. Methods for expression and purification of the ectodomains of HA from Cal/09, Bris/07, NIBRG14, Vic/11, A/H1N1/WSN/1933 (WSN), A/H1N1/Puerto Rico/8/1934 (PR8), A/H7N9/Shanghai/2/2013, and B/Victoria/2/87 were described previously [20]. Methods for generation and purification of stem region of HA from Bris/07 as well as HA_{mg} and HA_{fg} from Cal/09 and Bris/07 were described previously [21, 40].

Flow cytometry

To determine the immune cell subsets in lungs of infected mice, whole lung tissues were

removed, followed by isolation of single cell suspensions using the lung dissociation kit (Miltenyi Biotec). Cells were harvested and suspended in FACS buffer (2% FBS in PBS) at a density of 10^6/ml. The antibodies used in this study are anti-mouse B220 antibody (BD, clone RA3-6B2), anti-mouse IgG1 antibody (BD, clone X56), anti-mouse CD38 antibody (Biolegend, clone 90), anti-mouse CD45 antibody (BD, clone 30-F11), anti-mouse CD49b antibody (Biolegend, clone DX5), anti-mouse CD3e antibody (BD, clone 145-2c11), anti-mouse Siglec-F antibody (BD, clone E50-2440), anti-mouse CD64 antibody (BD, clone X54-5/7.1), anti-mouse CD11b antibody (BD, clone M1/70), and anti-mouse CD11c antibody (Biolegend, clone N418). Cellular fluorescence intensity was analyzed by FACSCanto (BD Biosciences) and FCS Express 3.0 software.

Cloning and expression of HA-specific mAbs

Ig genes from a single B cell were isolated primarily following the protocols reported previously [21, 41, 42]. Briefly, single cell was collected from the spleens of BALB/c mice immunized i.m. two times at 2 weeks apart with 20 μg Bris/07 HA_{fg} or HA_{mg}. The $HA_{fg}^+B220^+IgG1^+CD38^+$ splenic B cells were isolated on day 29 by a cell sorter (FACSAria II), and the single B cell was sorted into 96-well PCR plates (Thermal Scientific). RT-PCR reactions were performed as described previously [21, 41, 42]. Aliquots of nested PCR products were sequenced and analyzed using IMGT/V-Quest (http://www.imgt.org) to

identify the highest homology gene loci of germ-line V, D, and J genes. Those candidate Ig heavy- and light-chain cDNA segments were further subcloned to a chimeric Ig expression vector modified from the tandem chimeric antibody expression (TCAE) vector and the pIgG1(κ) vector (provided by Dr. T. W. Chang, Genomics Research Center, Academia Sinica, Taipei, Taiwan). The generation of the mutations at Fc region of mAb 651, Leu234Ala/Leu235Ala (LALA), was performed by substituting two leucine (L) residues with alanine (A) at a.a. 234 and 235 through site-directed mutagenesis of cDNA. The positive control of anti-HA antibodies FI6 [6] and F10 [23] were expressed and purified following previously described procedures [43].

Purification and binding efficacy of recombinant mAbs

Ig expression vector was transfected into HEK293F cells by using Expi293™ Expression System Kit (Thermo Fisher Scientific). Three days later, supernatant was collected for antibody purification by using Protein A Sepharose (GE) chromatography. Antibody was dissolved in PBS. Enzyme-linked immunosorbent assay (ELISA) was used to determine the binding of recombinant mAbs with HA. Briefly, purified HA of Cal/09, Bris/07, WSN/33, PR8/34, H3, H5, H7 and Flu B viruses was coated on the 96-well plates (0.1 μg/well in 100 μL) for 2 h. The HA-coated plates were then incubated with the 2-fold serial dilutions of recombinant mAbs starting from the highest concentration at 0.05 μg/mL for 2 h. The

captured recombinant antibodies were detected by HRP-conjugated anti-human antibodies and peroxidase substrate solution substrate (BD Biosciences). The OD was read by the SpectraMax M2 Microplate Reader (Molecular Devices). The bio-layer interferometry (BLI) was performed on Octet RED 96 instrument (FortéBio, Inc.). Antibody was immobilized onto anti-human AHC biosensors (FortéBio, Inc.) and incubated with HAs at 0.013–3.17 μM for 90 seconds for association and then incubated in 20 mM Tris, pH 8.0, 150 mM NaCl, 0.005% Tween 20 for dissociation for 90 seconds. The signals for each binding event were measured with a 1:1 Langmuir binding model for k_{on}, k_{off} and K_d value determination.

Microneutralization assay

Microneutralization assay was performed as previously described [37]. The freshly prepared virus was quantified with the median TCID50. The 10-fold TCID50 of virus was mixed in equal volume with 2-fold serial dilutions of mAbs in 96-well plates and incubated for 1 h at 37 °C. The mixture was added onto the MDCK cells (1.5×10^4 cells per well) in the plates followed by incubation at 37 °C for 16-20 h. The cells were washed with PBS, fixed in acetone/methanol solution (1:1 vol/vol), and blocked with 5% (wt/vol) skim milk in PBS. Quantification of virus was detected by ELISA with a polyclonal antibody against influenza A nucleoprotein (NP) protein. The anti-NP primary antibody was added and incubated for 1 h at 37 °C. After washing with PBST (PBS + 0.01% Tween 20), the secondary antibody (rabbit

345 anti-goat IgG HRP conjugated) was added and incubated for 1 h at 37 °C. Peroxidase
346 substrate solution was then added and incubated for 15 min at room temperature in the dark,
347 followed by adding stop solution. The absorbance (OD) of the wells was read at 450/620 nm.
348
349 **Hemagglutination inhibition assay (HI) assay**
350 The hemagglutination units of Cal/09 virus were determined following a previous report [21].
351 Briefly, the 2-fold serial dilutions of Cal/09 virus were added into the 96-well round (U)
352 bottom plates, and 0.2% turkey red blood cells (Jianrong Farm, Taiwan) were added into each
353 well and mixed well. After 30 min, the hemagglutination unit (HAU) was identified. For
354 antibody mediated hemagglutination inhibition detection, 1024 HAU of Cal/09 virus was
355 mixed with 2-fold serial dilutions of mAbs and incubated for 30 min. After incubation, 0.2%
356 turkey red blood cells were added into each well and mixed well. After 30 min, the
357 hemagglutination inhibition was determined.
358
359 **Virus challenge in mice**
360 Female BALB/c mice, purchased from National Laboratory Animal Center, Taiwan, at 8 wk
361 old were injected i.p. with 300 μg (15 mg/kg) or 150 μg (7.5 mg/kg) purified mAb 2 h before
362 or 24 h after intranasal challenge with H1N1 Bris/07 or Cal/09 virus with a lethal dose (100
363 LD50). In some experiments, FcγR knockout mice (purchased from The Jackson Laboratory)

364 at 8 wk old were used. Mouse body weight and survival data were measured and recorded
365 every day afterwards. In some experiments, NK cells were depleted by anti-ASGM1 (50
366 μL/mouse, WAKO) antibody or PBS (as the control) by ip injection 2 d before mAb injection.
367 Macrophages were depleted by clodronate liposomes (100 μL/mouse, ClodronateLiposomes)
368 2 and 4 d before mAb injection. All animal experiments were evaluated and approved by the
369 Institutional Animal Care and Use Committee of Academia Sinica.
370
371 **ADCC assay**
372 ADCC was performed as previously reported [37]. HEK293T cells transfected with
373 HA-expression vectors (Bris/07, Cal/09, H3, and H7) for 48 h and human peripheral blood
374 mononuclear cells (PBMCs) were used as target cells and effector cells, respectively. Human
375 PBMCs from healthy donors were obtained from Taipei Blood Center with the consent
376 procedures approved by the Academia Sinica Research Ethics Committee. PBMCs were
377 isolated by density gradient centrifugation with Ficoll-Paque at 400×*g* for 30 min without
378 brake at 22°C. mAbs at 2-fold serial dilutions were added to the co-culture composed of
379 5×10^3 293T cells transiently expressing HA proteins from indicated strains of influenza
380 viruses and 2.5×10^5 effector PBMCs (E:T ratio= 50), and incubated for 5 h at 37°C. The
381 supernatant of the co-culture was collected and analyzed by CytoTox 96 Non-Radioactive
382 Cytotoxicity Assay Kit (Promega).

383

384 **ADCP assay**

385 ADCP was performed essentially as a previous report [44]. Briefly, Cal/09 virus was
386 incubated with indicated antibodies at 37 °C for 1 h and added to the sialidase (0.5 unit/mL,
387 Sigma) pre-treated THP-1 cells (from ATCC). After 1 h incubation, THP-1 cells were washed
388 three times with RPMI and incubated at 37 °C. After 6 h incubation, THP-1 cells were fixed
389 in 4% paraformaldehyde, permeabilized and stained with anti-influenza NP antibody (Abcam,
390 Ab20921, 1:100 dilution). The levels of phagocytosis were monitored by flow cytometry.

391

392 **Histology analysis, cytokine levels and viral titers in lung analysis**

393 The perfused mouse lungs isolated at day 3 and day 5 after infection were fixed in 4%
394 formaldehyde, embedded in paraffin and cut into 3 μm-thick sections for Hematoxylin &
395 Eosin (H&E) staining. To determine the viral titers in the infected lungs, infected mice were
396 sacrificed on day 3 and day 5 post-infection. The whole lungs were collected and
397 homogenized in PBS (2.5 ml/g lung). The viral titers from the homogenates were determined
398 by immunoplaque assay on MDCK cells following previously established protocols [45].
399 Briefly, MDCK cells were seeded into 24-well plates. One day later, the MDCK monolayers
400 were washed once with PBS. Ten-fold serial dilutions of lung homogenates, made in in
401 serum-free RPMI containing 0.5 μg/mL TPCK-trypsin, were added to the MDCK cells. After

1 h incubation, the cells were washed once with PBS and overlaid with DMEM containing 0.5 μg/mL TPCK-trypsin and 0.5% agarose. After overnight incubation, MDCK cells were fixed with 10% formaldehyde and the agarose were removed. The cells were fixed, permeabilized and stained with anti-influenza NP antibody and the focus forming units were determined. The lung homogenates were used for determining the levels of cytokines by using IFN-β and IFN-α ELISA kits from PBL Assay Science, MCP-1, IL-6, and TNF-α ELISA kits from eBioscience, and IFN-λ2/3 ELISA kit from R&D Systems.

Hydrogen-Deuterium Exchange-Mass Spectrometry (HDX-MS) Assay

HDX-MS was performed essentially as previously reported [46]. Briefly, the Fab regions of the antibodies were prepared by using Pierce™ Fab Preparation Kit (Thermo). HA protein (from Bris/07) and Fab region of antibody were co-incubated for 30 min at 37 °C, digested by PNGase F (NEB) for 2 h at 37 °C. Hydrogen was exchanged to deuterium by adding D_2O to the reaction for 10 and 20 min at room temperature and stopped the exchange by increasing urea concentration to 2 M and low temperature (4°C). After pepsin and protease type XIII digestion, the sample was ready for MALDI TOF analysis with ESI mass spectrometry (Velos Pro LTQ, Thermo Scientific, mass spectrometric core facility, Genomics Research Center, Academia Sinica) to determine the interacting region between HA and Fab.

Statistical analysis

Data are shown as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 8 software. Analysis of differences between two groups was performed by an unpaired two-tailed Student's t test. Comparisons between multiple groups were performed using a one-way ANOVA, followed by Dunnetts' honestly significant difference post hoc test. Survival rate differences between groups were analyzed by using log-rank (Mantel-Cox) test.

Online supplemental material

S1 Fig shows the *in vitro* characterizations of mAb 651-binding to HA.

S2 Fig shows the weight changes of mice pretreated or post-treated with mAbs, followed by influenza virus infection.

S3 Fig shows the depletion efficiency of NK cells and alveolar macrophages (AMs), and the weight changes of mice depleted with AMs and/or NK cells after mAb pretreatment and Cal/09 virus challenge.

Author contributions

K.-I Lin conceived the study. Y.-A. Ko, Y.-H. Yu, Y.-F. Wu, Y.-C. Tseng, C.-L. Chen, K.-S. G., H.-Y. Liao, and T.-H. Chen performed the experiments and data analysis. T.-J. R. Cheng, A.-S. Yang, C.-H. Wong, and C. Ma provided critical reagents and analytic tools. Y.-A. Ko, Y.-H.

Yu and K.-I Lin contributed to writing and revising the manuscript.

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622

Figure Legends

Figure 1. Generation of 651 from Bris/07 HA_{mg}-immunized mice

(A) Bris/07 (H1N1) HA proteins with two types of glycan modifications, HA_{fg} (HA carrying the typical complex type N-glycans) and HA_{mg} (HA carrying GlcNAc at N-glycosylation sites only), were used to immunize mice, followed by isolation of mAbs with a single B cell screening platform. (B) The resulting recombinant mAb 651 showed cross-recognition of several strains of group A influenza virus. (C) BLI assay showing 651 possessed a higher affinity for HA_{mg} from Cal/09. (D) Immunoblot of reduced (5% 2-ME in sample buffer) and non-reduced Bris/07 HA proteins showing 651 recognized the structural epitope of HA. (E) ELISA showing 651 was unable to recognize the stem region of HA from Bris/07. (F) Effects of 651 on the microneutralization of various strains of influenza viral infections. FI6 and F10 were used as the positive controls.

Figure 2. Prophylactic and therapeutic efficacy of 651 in mice challenged with lethal dose of H1N1

(A-C) Cumulative survival rate of mice treated (i.p.) with indicated mAb 2 h before challenge with 100 LD50 of H1N1 Bris/07 virus (B) or Cal/09 virus (C). (D-F) Cumulative survival rate of mice injected (i.p.) with 651 24 h after challenge with 100 LD50 of H1N1 Bris/07 virus (E) or Cal/09 virus (F). (G) Cumulative survival rate of mice treated with F10, 651, or a mutation

642 of 651 (651-LALA) after challenge with Cal/09 at 100 LD50. (H) Cumulative survival rate of
643 FcγR knockout (KO) mice and control WT mice after F10 or 651 pretreatment and Cal/09
644 virus challenge. Data were analyzed by log-rank (Mantel-Cox) test. The statistical
645 significance was calculated for the differences between PBS control and mAb treated groups
646 (in B, C, E, F, and G), or between WT and FcγR knockout (KO) mice receiving the same kind
647 of mAb (in H). Six-8 mice were used per group in each experiment. *p < 0.05; **p < 0.01;
648 ***p < 0.001. N.S. = no significant difference.
649
650 **Figure 3. Pretreatment with 651 reduced viral replication and alleviated lung**
651 **inflammation after respiratory challenge with Cal/09 virus**
652 (A and B) Fluorescent focus assay showing the viral titers in lungs of various indicated mAb-
653 or PBS-pretreated mice on day 3 (A) and day 5 (B) after Cal/09 infection. (C) H&E staining
654 showing the histopathology of lungs in mAb-pretreated mice on day 3 after Cal/09 infection.
655 Results from two individual mice from each group are shown. Scale bar = 100 μm. (D and E)
656 Pulmonary IFNα, IFNβ, MCP-1, IL-6, TNFα, and IFNλ2/3 protein levels on day 3 (D) and
657 day 5 (E) after Cal/09 infection were measured by ELISA using samples from mice receiving
658 indicated pretreatments. NC represents mice without infection. Results are mean ± SEM. (n =
659 4-10). *p < 0.05; **p < 0.01; ***p < 0.001.
660

Figure 4. 651 possessed ADCC or ADCP against influenza viruses *in vitro*

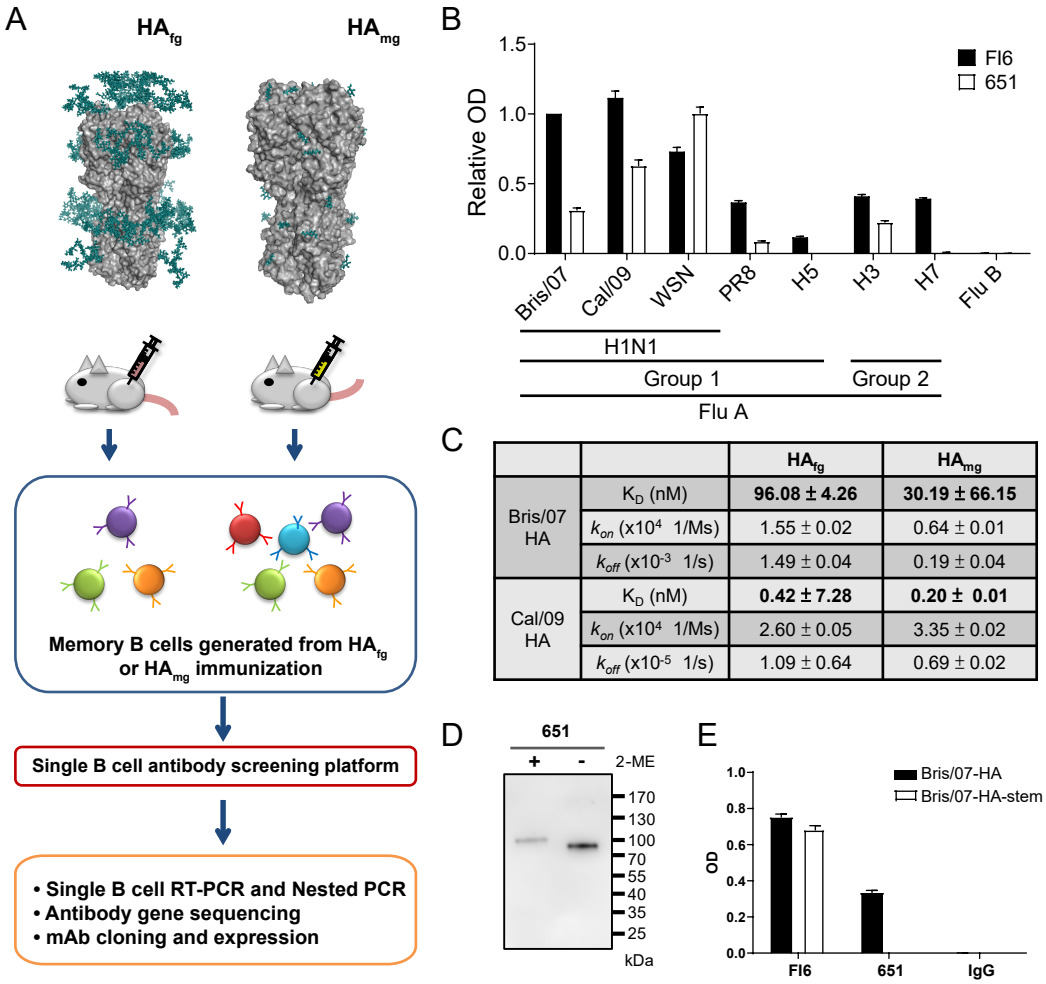
(A and B) FACS analysis showing the percentage of pulmonary NK cells ($CD49b^+$) in $CD45^+CD3e^-$-gated populations in indicated mAb-pretreated mice on day 3 (A) and day 5 (B) post challenge of Cal09 virus. (C) 651 showed ADCC against H1 (Bris/07 and Cal/09) and H3 but not H7-expressing 293T cells. (D) 651-LALA mutation abolished ADCC mediated by 651 to kill cells expressing H1 from Bris/07 or Cal/09 viruses. IgG was used as the negative control in C and D. (E and F) FACS analysis showing the percentage of alveolar macrophages (AMs) ($SiglecF^+CD64^+$) in $CD45^+CD11b^-CD11c^+$ gate in indicated mAb- or PBS-pretreated mice on day 3 (E) and day 5 (F) post-challenge with Cal/09 virus. (G) FACS showing the NP positive signals in sialidase- (0.5 unit/mL) treated THP-1 cells incubated with 651-, 651-LALA-, or IgG- (all at 100 ng/ml) opsonized Cal/09 virus. Percentage of NP positive cells is indicated. (H) FACS results showing the percentage of NP positive signals in sialidase treated THP-1 cells incubated with serial doses of 651 or 651-LALA mAbs and Cal/09 virus. Results are mean ± SEM. (n = 3-9). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure 5. 651-mediated protection in mice requires both alveolar macrophages and NK cells

(A and B) Cumulative survival rate of mice receiving control reagents, including liposome alone, control antibody, or both (A), or clodronate liposomes or/and anti-ASGM1 antibody (B)

prior to i.p. injection of mAbs and 100 LD50 Cal/09 intranasal challenge. (C and D) Comparison of pulmonary IFNβ, MCP-1, and IL-6 protein levels measured by ELISA in alveolar macrophages (AM)-NK co-depleted mice treated with either PBS or 651 on day 3 (C) and day 5 (D) post-Cal/09 infection. (E) Fluorescent focus assay showing the comparable influenza virus titers in AM-NK co-depleted mice treated with either PBS or 651 on day 5 after Cal/09 infection. Data in A and B were analyzed by log-rank (Mantel-Cox) test. Data in C-E are mean ± SEM. (n = 4-12). *p < 0.05; **p < 0.01; ***p < 0.001. N.S. = no significant difference.

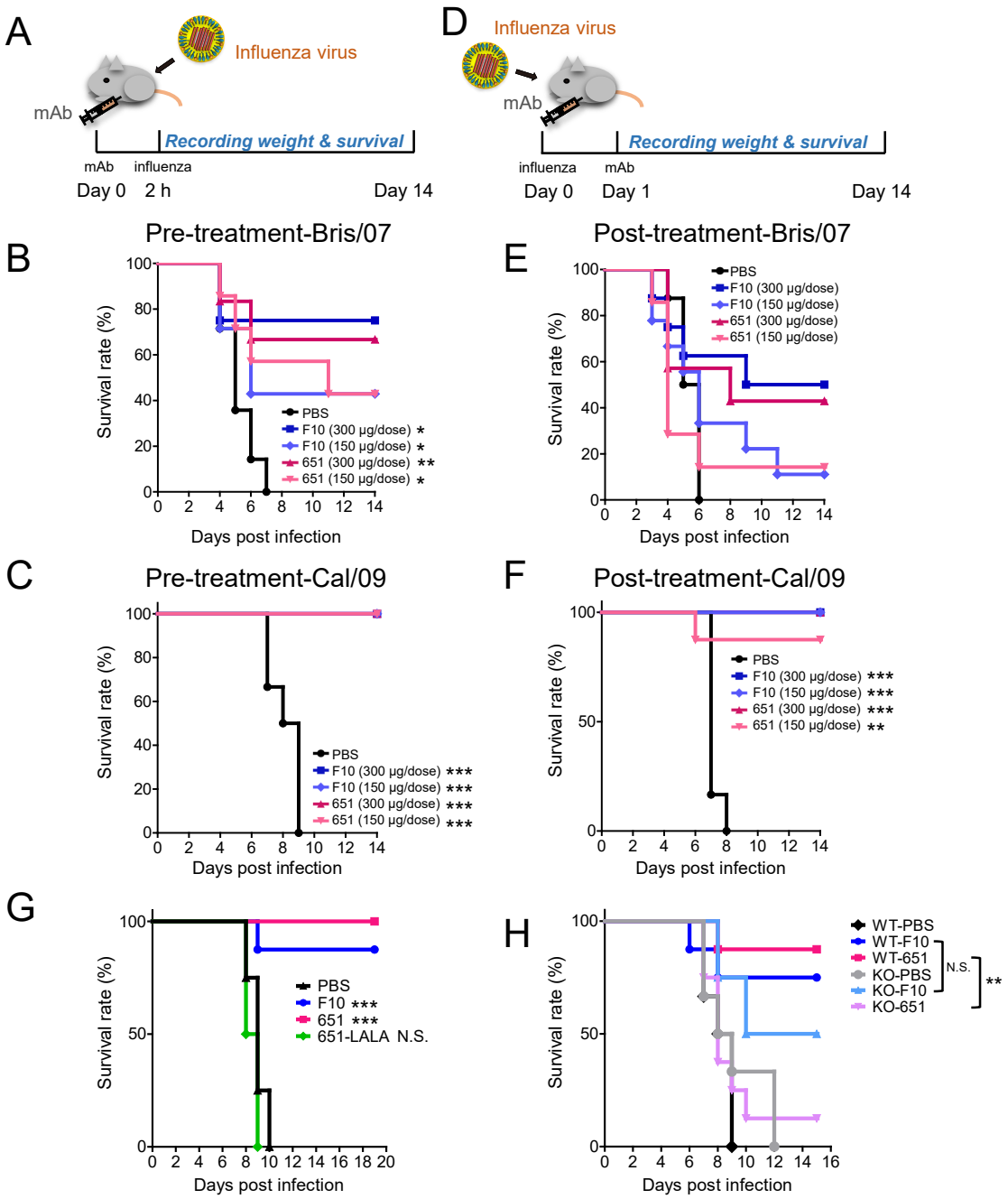
Ko et al. Figure 1



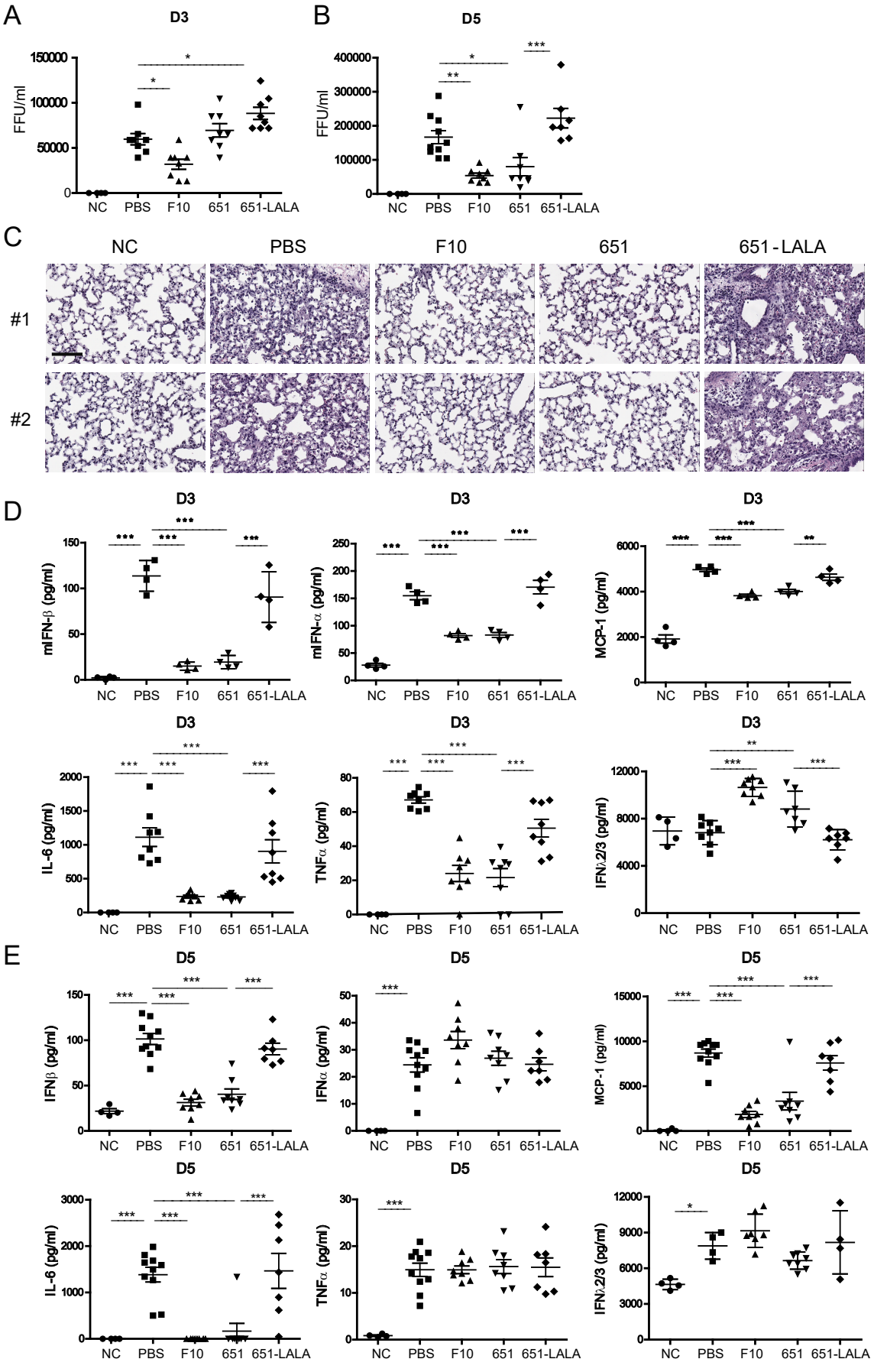
C

		HA_{fg}	HA_{mg}
Bris/07 HA	K_D (nM)	**96.08 ± 4.26**	**30.19 ± 66.15**
	k_{on} (x10^4 1/Ms)	1.55 ± 0.02	0.64 ± 0.01
	k_{off} (x10^{-3} 1/s)	1.49 ± 0.04	0.19 ± 0.04
Cal/09 HA	K_D (nM)	**0.42 ± 7.28**	**0.20 ± 0.01**
	k_{on} (x10^4 1/Ms)	2.60 ± 0.05	3.35 ± 0.02
	k_{off} (x10^{-5} 1/s)	1.09 ± 0.64	0.69 ± 0.02

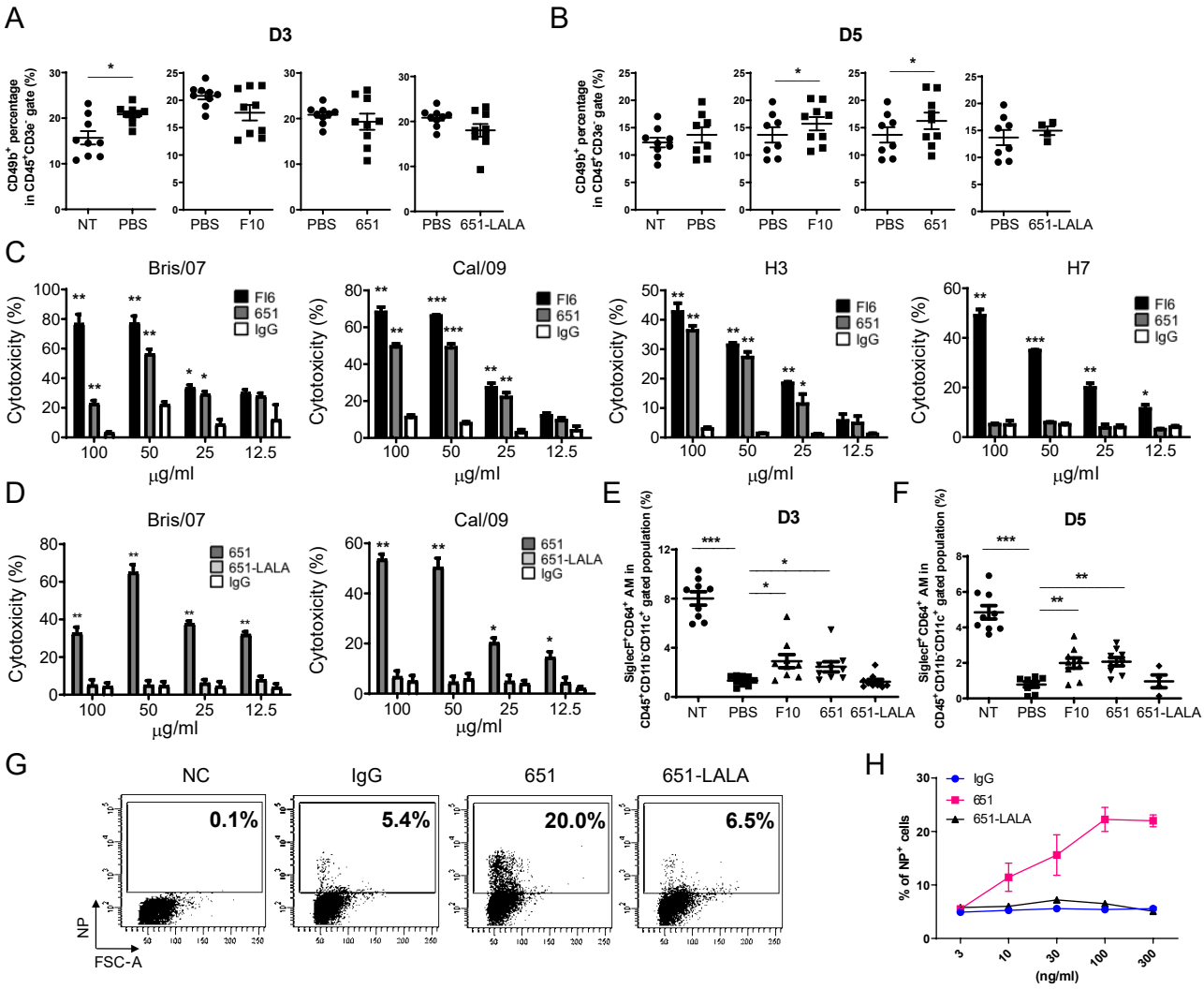
Ko et al. Figure 2



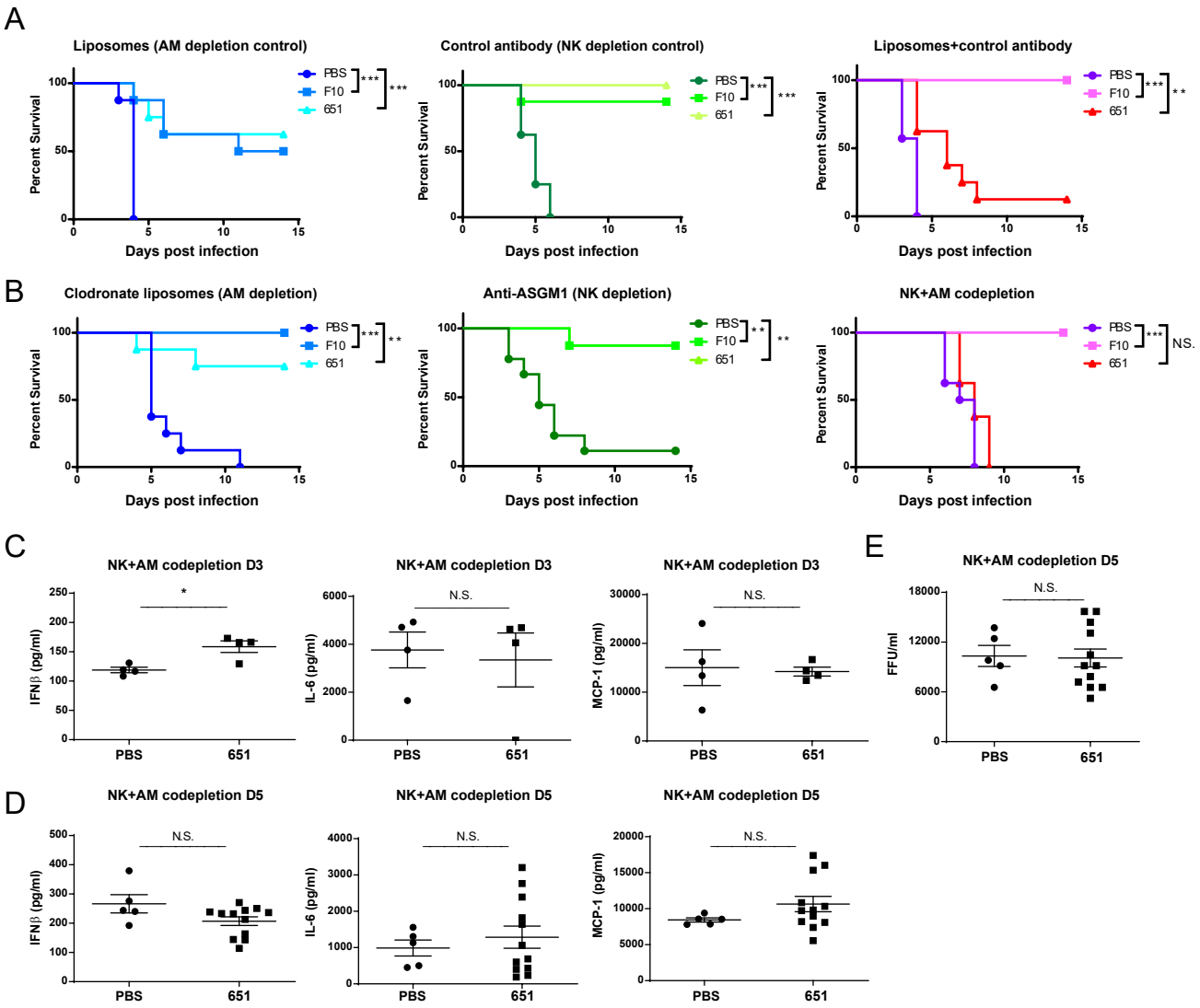
Ko et al. Figure 3



Ko et al. Figure 4



Ko et al. Figure 5



Supplementary figures