A potent broad-spectrum protective human monoclonal antibody crosslinking two haemagglutinin monomers of influenza A virus

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Effective annual influenza vaccination requires frequent changes in vaccine composition due to both antigenic shift for different subtype hemagglutinins (HAs) and antigenic drift in a particular HA. Here we present a broadly neutralizing human monoclonal antibody with an unusual binding modality. The antibody, designated CT149, was isolated from convalescent patients infected with pandemic H1N1 in 2009. CT149 is found to neutralize all tested group 2 and some group 1 influenza A viruses by inhibiting low pH-induced, HA-mediated membrane fusion. It promotes killing of infected cells by Fc-mediated antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. X-ray crystallographic data reveal that CT149 binds primarily to the fusion domain in HA2, and the light chain is also largely involved in binding. The epitope recognized by this antibody comprises amino-acid residues from two adjacent protomers of HA. This binding characteristic of CT149 will provide more information to support the design of more potent influenza vaccines.

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Influenza epidemics of variable severity recur every year with winter season peaks in temperate regions of the world. In addition, four influenza pandemics have been recorded since the beginning of the last century: the 1918 Spanish flu, the 1957 Asian flu, the 1968 Hong Kong flu and the 2009 swine flu, along with the reemergence of H1N1 virus in 1977 (ref. 3). The impact of these pandemics ranged from an estimated 300,000 to 50 million deaths worldwide for pandemics4-5. Vaccination is the most effective intervention available to mitigate seasonal and pandemic influenza morbidity and mortality. The current strategy to rapidly immunize the human population against an emerging pandemic relies, for the most part, on quickly adapting to the antigen composition of seasonal influenza vaccines and scaling-up manufacturing as fast as possible. Because massive pandemic vaccination cannot achieve herd immunity until sufficient quantities of vaccine have been produced, pandemic viruses, such as A(H1N1)pdm09, are free to spread for several months. For this reason, pandemic preparedness plans also rely on antiviral medications to mitigate the impact of the pandemic, especially in the very early stages of the response. Neuraminidase inhibitors are currently the most widely recommended class of antiviral drugs as they are available in stockpiles for immediate use. Unfortunately, some viruses have shown the capacity to develop resistance to these drugs without loss of transmissibility6. Therefore, other antiviral small molecules and therapeutic monoclonal antibodies (mAbs) are being developed as alternatives for the treatment of influenza infections7.

Passive immunization with plasma-derived antibody products has been suggested and used to treat influenza patients with encouraging results8-12, although production of hyperimmune sera to influenza viruses is not scalable for wide use in a pandemic response. mAbs produced by immortalized cells in industrial bioreactors would offer an unlimited supply of homogeneous antibody for therapeutic or prophylactic use, yet the highly variable nature of most neutralization epitopes on the influenza hemagglutinin (HA) molecule proposes a problem. This has motivated the search for influenza-neutralizing mAbs that recognize highly conserved neutralizing epitopes on HA. Okuno, et al.13,14 reported a mouse mAb, C179, that showed broad cross-subtype neutralizing activity in vitro and protection in mice that were passively immunized. Recent reports indicate that human mAbs targeting a conserved region on the stem of the HA spikes are protective in mouse models of infection15-20. These mAbs could be derived from VH and VL (variable region sequences of the heavy-chain (VH) and light-chain (VL)) loci complementary DNA (cDNA) phage display libraries prepared from human immunoglobulin M (IgM)-positive memory cells of influenza-vaccinated or non-vaccinated donors15-17. Similar mAbs were also identified by screening phage libraries from non-immune human B cells18 or human plasma cells from influenza-vaccinated or infected donors19,20. Here we present the isolation of a broadly neutralizing antibody, CT149, from convalescent patients infected with A(H1N1)pdm09. We reveal that CT149 exhibits potent neutralization in cell-based tests for divergent HA subtypes and good protection from H1N1, H3N2 and H5N1 subtype viruses and, especially, from the recently emerged human-infecting H7N9 virus21-23 in the mouse model. By further structural analysis, we show that the epitope recognized by CT149 is present in the stem region spanning across two adjacent protomers.

Results

Isolation and characterization of CT149 mAb. In this study, we used the ISAAC (immunospot array assay on a chip) method24 using peripheral blood mononuclear cells (PBMCs) from convalescent patients infected with A(H1N1)pdm09 to screen mAbs that might protect against heterosubtypic influenza A virus infection. The mAbs generated directly from single human B cells were screened by enzyme-linked immunosorbent assay (ELISA) against groups 1 and 2 HAs (A/California/04/2009 (H1N1)pdm09 (CA/09) and A/Brisbane/10/2007 (H3N2) (BR/07), respectively). Three of the mAbs (CT149, CT164 and CT166) neutralized representative subtype H5N1 and H3N2 viruses, but failed to neutralize representative H1N1 and H2N2 viruses in an in vitro microneutralization (MN) assay (Supplementary Table 1 and Fig. 1). Although the mAbs neutralized various influenza viruses, they failed to inhibit the haemagglutination of turkey erythrocytes by BR/07 virus at concentrations of up to 20 µg ml^-1 (Supplementary Table 1). These results imply that the antibodies bind in the HA stem region and/or the adjacent region of the globular head, away from the receptor binding site. CT149, which showed the greatest neutralization potency among the three mAbs (Fig. 1), revealed cross-neutralizing activity against virus subtypes with groups 1 and 2 viruses.

Database searches with the VH and VL nucleotide sequences from mAb CT149 (Supplementary Fig. 1) indicated 88.07 and 91.49% of identity to IgHV1-18*01 and IgKV3-20*01, respectively. Several broadly neutralizing anti-HA antibodies have been reported previously such as CR6261, CR8020, F10, F16 and CR9114 (refs 17-19,25). Most of them are originated from the IgHV1-69 germline locus and bind to the stem region of HA only with their heavy chain, mainly using its hydrophobic CDR2. However, F16 and antibody 3.1, which are derived from IgHV3-30, use both heavy and light chains to bind HA19,26. Notably, both CT149 and CR8020 derive from the VH1-18 germ line, but CR8020 is specific for group 2 HAs, whereas CT149 can neutralize from groups 1 and 2 HA subtypes. Thus, CT149 probably binds differently to HA, compared with CR8020.

CT149 neutralizes divergent HA subtypes. To investigate the breadth of the broadly neutralizing antibody CT149 against divergent H3 HAs, 10 H3N2 viruses isolated between 1968 and 2007 were tested in MN assays. CT149 neutralized infectivity by all tested H3N2 isolates, although the neutralization potency varied by nearly two orders of magnitude (0.156-5 µg ml^-1; Table 1). CT149 also showed neutralizing activity against some tested viruses of group 1 HA subtypes (one H1N1pdm09 virus among the six H1N1 tested, the two H5N1 viruses and two H9N2

![Figure 1](https://example.com/figure1.png)
strains) and all tested viruses of group 2 (Table 1). Of note, CT149 neutralized two representative subtype H7N9 viruses, A/Anhui/1/2013 (AH1) and A/Shanghai/2/2013 (SH2), which have caused hundreds of severe and fatal human infections since March 2013 in China.12–15

The binding affinity of CT149 for different HA subtypes was determined by surface plasmon resonance using soluble HAs. CT149 binding to H1 was between 30.6 and 345 nanomolar (Kd = 3.45 × 10⁻⁹ to 3.06 × 10⁻⁸ M), whereas CT149 bound with higher affinity to H3 HAs, with apparent Kd values ranging from 1.81 × 10⁻⁹ to 4.56 × 10⁻¹¹ M. CT149 showed similar binding affinities for H5 and H7 recombinant HAs (rHAs) tested with Kd values of 2.94 × 10⁻⁹ and 1.83 × 10⁻¹⁰ M, respectively (Table 2 and Supplementary Fig. 2).

Recombinant CHO (Chinese Hamster Ovary) stable cell lines constitutively expressing a full-length HA of CA/09, A/Japan/305/1957 (H2N2), BR/07 and A/Vietnam/1203/2004 (H5N1) (VN/04) were developed to evaluate CT149 in a low pH-induced cell–cell fusion–inhibition (multinucleated syncytium) assay. CT149 were developed to evaluate CT149 in a low pH-induced cell–cell fusion of recombinant cells expressing H1, H3 and H5 HAs, but not H2 HA (Supplementary Fig. 3), whereas an isotype-matched negative control mAb (CT-P6) had no effect on cell–cell fusion by any of the four HA subtypes analysed. These observations correlated with the observed neutralization activities of CT149.

Structures of CT149/H3 and CT149/H7 complexes. The epitope recognized by CT149 was likely to be located in the HA stem region, based on the inhibition of syncytia formation (a surrogate for virion–endosomal membrane fusion; Supplementary Fig. 3), but not haemagglutination (measures receptor function interference by antibody binding to the HA globular head; Supplementary Table 1). CT149 Fab fragments were prepared and co-crystallized with H3–HA (A/Hong Kong/1/1968) and H7–HA (A/Anhui/1/2013). Final statistics for data collection and structure refinements indicated a resolution of 2.9 and 3.6 Å, respectively (Table 3). Crystal structures of CT149/H3 and CT149/H7 complexes revealed that CT149 indeed recognized residues in the stem region of HA (Fig. 2). In both structures, each HA trimmer was decorated by three CT149 Fab molecules (Fig. 2a,b). The heavy chain of CT149 binds into a shallow groove in the fusion subdomain of a single HA protomer, whereas the light chain binds to two separate protomers, one

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**Table 1 | Neutralization activity of CT149 against viruses from diverse HA subtypes and antigenic drift variants.**

| Group | Subtype | Virus | MN endpoint (µg ml⁻¹) |
|-------|---------|-------|----------------------|
| 1     | H1N1    | A/Ohio/83 | >10                   |
|       |         | A/Solomon Islands/2006 | >10                   |
|       |         | A/Ohio/07/2009 | 5                     |
|       |         | A/Texas/05/2009-RG15 | >10                   |
|       |         | A/Texas/18/2009-RG18 | >10                   |
|       |         | A/California/04/2009 | >10                   |
| H2N2  |         | A/Ann Arbor/6/60 ca | >10                   |
| H5N1  |         | A/Vietnam/1203/04 | 2.5                   |
|       | (VN/H5N1-PR8/CDC-RG) | 0.625              |
|       | A/Anhui/01/2005(H5N1)-PR8-IBCDC-RG6 | 0.625 |
| H9N2  |         | A/c/k/HK/G9/97(H9N2)-PR8-IBCDC-2 | 0.312               |
|       | A/Green-winged teal/209/TX/2009 | 0.156               |
| 2     | H3N2    | A/Hong Kong/68 | 2.5                   |
|       |         | A/Philippines/2/1982 | 0.625               |
|       |         | A/Beijing/353/89-X109-H3N2 PR8 | 0.156               |
|       |         | A/Beijing/32/92-R-H3N2 PR8 reassortant | 0.078               |
|       |         | A/Johannesburg/33/94 R-H3N2 PR8 reassortant | 0.625               |
|       |         | A/Nanchang/933/95 | 0.625               |
|       |         | A/Sydney/5/97 | 0.625               |
|       |         | A/Panama/2007/99 | 0.312               |
|       |         | A/Wyoming/3/03 rg | 5                     |
|       |         | A/Brisbane/10/07 | 0.625               |
| H7N2  |         | A/turkey/Virginia/2002(H7N2)/PR8-IBCDC-5 | 10                   |
| H7N9  |         | A/Anhui/1/2013 | 0.904               |
|       | A/Shanghai/2/2013 | 1.17               |

**HA:** hemagglutinin; **MN:** microneutralization; **RG:** reverse genetics.

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**Table 2 | SPR analysis of CT149 with recombinant HAs.**

| Subtype | HA donor virus | Kd, M |
|---------|----------------|-------|
| H1N1    | A/California/04/2009* | 3.06E-08  |
|         | A/Texas/05/2009* | 3.38E-08  |
|         | A/Solomon Island/03/2006 | 3.45E-07  |
|         | A/Ohio/07/2009† | 5.13E-08  |
| H3N2    | A/Philippines/2/1982* | 4.56E-11  |
|         | A/Brisbane/10/2007† | 1.81E-09  |
| H5N1    | A/Vietnam/1203/2004* | 2.94E-09  |
| H7N9    | A/Anhui/1/2013 | 1.83E-10  |

**HA:** hemagglutinin; **SPR:** surface plasmon resonance.  
*Immune Technology  
†Sino Biological

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**Table 3 | Data collection and refinement statistics (molecular replacement).**

| CT149/H7 | CT149/H3 |
|----------|----------|
| **Data collection** |
| Space group | R₃₂ | P₃₁ |
| Cell dimensions |
| a, b, c (Å) | 126.9, 126.9, 409.6 | 128.7, 128.7, 428.3 |
| a, b, c (Å) | 90.00, 90.00, 120.00 | 90.00, 90.00, 120.00 |
| Resolution (Å) | 50.0–2.8 (2.90–2.80)* | 50.3–3.5 (3.63–3.50) |
| RSym or Rmerge |
| L/α |
| Completeness (%) | 99.5 (99.9) | 99.5 (100) |
| Redundancy | 7.8 (7.7) | 3.7 (3.9) |
| **Refinement** |
| Resolution (Å) | 48.4–2.8 (2.90–2.80) | 48.3–3.5 (3.50–3.59) |
| No. of reflections | 31,755 | 94,551 |
| Rwref/Rwork | 26.4/31.1 | 23.9/27.8 |
| No. of atoms |
| Protein | 5,650 | 42,616 |
| Ligand/ion | 0 | 636 |
| Water | 25 | 0 |
| B-factors |
| Protein | 75,313 | 120.4 |
| Ligand/ion | 0 | 138.4 |
| Water | 58,999 | 0 |
| R.m.s.d. |
| Bond lengths (Å) | 0.004 | 0.019 |
| Bond angles (°) | 0.780 | 1.58 |

*Indicates that the values in the parentheses are for the highest-resolution shell.
and the light chain is coloured in yellow. The residues contacting the HA are coloured in pink for the heavy chain and coloured in orange for the light chain. In addition, the HCDR2 loop was observed to cross-helix A, at an angle of 45 degrees, enabling residues V101, V105 and V107 to create hydrogen bonds and polar interactions may also be formed in the contacting site.

For the interaction between the light chain of CT149 and HA, the LCDR1, LCDR2, LCDR3 and FR3 loops were observed to form fewer contacts with the residues in the HA, as compared with the heavy chain (Supplementary Table 2a,b). Other than hydrophobic interactions, potential hydrogen bonds and polar interactions may also be formed in the contacting site.

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Doses of 30, 15 or 7.5 mg kg\(^{-1}\) weight were administered by intranasal injection. The LC/DR2 and FR3 loops interact with I278 on the HA1 and heavy chain, respectively, at 24 or 48 h after infection. Mice treated with CT149 antibody (30 mg kg\(^{-1}\) body weight, intraperitoneally) 24 h after maCA/09 virus challenge showed full protection (Fig. 5a).

High levels of protection (80% remained healthy) were also evident in mice that were treated with lower doses of the antibody (15 mg kg\(^{-1}\) body weight). However, when mice were treated with 7.5 mg kg\(^{-1}\) at 24 h after virus challenge, only 50% remained healthy. Furthermore, 60, 40 and 20% of mice treated with 30, 15 and 7.5 mg kg\(^{-1}\) of CT149 at 48 h post infection remained healthy, respectively (Fig. 5a). Full protection against highly pathogenic VN/04 was observed when mice (n = 10 per group) were treated with various doses of CT-P149 (30, 15 or 7.5 mg kg\(^{-1}\)) at 24 h after inoculation (Fig. 5b).

Mice (n = 5 per group) treated with CT149 (20 or 10 mg kg\(^{-1}\)) 24 h before or after virus challenge were fully protected (100% survival) from challenge with maHK/68 (Fig. 5c). Significant levels of protection, 100% and 80%, were also observed in mice (n = 5 per group) given 20 and 10 mg kg\(^{-1}\) of CT149, respectively, at 48 h after inoculation with the maHK/68 virus (Fig. 5c). The in vivo protective effect of CT149 against AH1 viral infection was tested in mice (n = 10 per group) at doses of 30, 15 or 7.5 mg kg\(^{-1}\) of CT149 given 24 h after intranasal virus challenge.
challenge. A single treatment of 30 mg kg\(^{-1}\) CT149 in mice provided 70% protection against lethal challenge from H7N9 virus. A lower dose of 7.5 mg kg\(^{-1}\) was observed to be partially protective; 40% of mice treated with CT149 survived (Fig. 5d).

Taken together, these results indicated that passive protection by CT149 in mice is dependent on challenged virus, the timing of treatment relative to inoculation and the mAb dose used.

**Fc-dependent activity of CT149.** The in vivo protective efficacy of CT149 in the mouse model contrasted with its minimal neutralization activity against some of the group 1 influenza viruses tested in the in vitro MN assay (Table 1 and Fig. 5a). Immunoglobulin Fc region-mediated effector pathways have been implicated in the protective activity of antibodies in vivo by engaging host effector cells in killing virus-infected cells rather than preventing cell infection\(^{5,36}\). To identify potential host-dependent antiviral functions of CT149, we analysed its activity in antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays with CHO cells expressing HA or virus-infected Raji cells. Low concentrations (~40–200 ng ml\(^{-1}\)) of CT149 resulted in ~40% cytotoxicity in the ADCC assay using CHO cells expressing CA/09 HA as targets and PBMCs as effectors, whereas the isotype-matched negative control antibody, CT-P6, achieved only 10% cytotoxicity (Supplementary Fig. 5a). The ADCC effect was also confirmed with virus-infected cells (Supplementary Fig. 5c,d).

The CDC assay with the same target cells revealed that CT149 (~3 μg ml\(^{-1}\)) mediated ~50% cytotoxicity in the presence of human complement (Supplementary Fig. 5b), in contrast to the low cytotoxicity (<10%) levels recorded for isotype-matched mAb, CT-P6.

To evaluate the contribution of Fc-mediated mechanisms to protection in the mouse model of infection, we generated human/mouse chimeric versions of CT149 by exchanging the heavy-chain constant region of CT149 with mouse IgG1
(CT149mIgG1) or mouse IgG2a (CT149mIgG2a). Passive immunization with the chimeric CT149mIgG2a, CT149mIgG1 and the original CT149 revealed protection, albeit at different levels, in animals (n = 5 per group) that received a low dose (3 mg kg\(^{-1}\)) of mAb at 24 h post inoculation with an otherwise lethal dose of maCA/09 (Supplementary Fig. 6). The observed trend towards improved protection with CT149mIgG2a is consistent with a postulated Fc-mediated effector function resulting from the higher-affinity Fc-Fc/γR for IgG2a relative to IgG1 (refs 37,38). Taken together, these data suggest that CT149 also depends on Fc-mediated effectors for in vivo protection as reported for other stem-binding human mAbs38,39.

Discussion

The identification of broadly neutralizing human mAbs against multiple influenza virus subtypes and the emergence of new subtypes of zoonotic influenza viruses from domestic animals, for example, H7N9, have sparked new interest in the use of antibody therapy in the treatment of severe influenza39. The broad and heterogeneous reactivity of the antibodies is clearly beneficial and suggests that these antibodies could be used as novel antivirals against current and future circulating viruses. In addition, the information obtained from structural analysis of antibody–HA complexes may be used as guides for the rational design of therapeutic molecules and, especially, vaccines39.

In this study, we obtained PBMCs from six patients recovered from infection with A(H1N1)pdm09 virus and isolated several mAbs from the single B cells. We focused our attention on CT149 because of its unique profile of reactivity with viruses from both groups 1 and 2. In addition, CT149 was selected particularly owing to its potent efficacy against H3N2 viruses isolated during the past 40 years, and against newly emerging avian influenza A (H7N9) viruses with high propensity for human infections. The neutralization of widely divergent virus subtypes by CT149 indicates that donors infected with A(H1N1)pdm09 can harbour broadly protective mAbs to influenza viruses with groups 1 and 2 HAs as described previously19.

The crystal structures of the CT149/HA complexes have confirmed that the mAb recognizes epitopes within highly conserved HA1/HA2 interfaces in the stem. In stark contrast with previously reported broad neutralization epitopes in the stem region, the light chain of CT149 makes extensive contacts with two protomers on the HA16–19,34, crosslinking the two monomers. These structural features may help explain the poor in vitro neutralization of H1N1 subtype viruses by CT149, even though it was derived from convalescent patients. These viruses feature a highly conserved glycosylation motif for N289 in the HA1 region, adjacent to the extended loop between helix A and B of HA2 (Fig. 4). According to the analysis of H1/CT149 complex model (Fig. 4), we postulate that the N289 glycan may cause steric hindrance by colliding with the light chain of CT149. However, we have not obtained the crystal structure of CT149 in complex with a group 1 HA (for example, H5 or H1), which is a shortcoming to comprehensively elucidate the molecular basis of CT149 neutralization. We cannot rule out the possibility that the N289 glycan may not sterically hinder the binding of CT149 to H1 HA. This should be pursued in the near future.

As the in vitro neutralization studies would suggest, mice challenged with maHK/68 (H3N2), VN/04 (H5N1) and AH1 (H7N9) were protected from an otherwise lethal inoculation. In contrast to predictions based on in vitro results, CT149 protected mice challenged with maCA/09, VN/04. These observations may be reconciled by considering the Fc-dependent effector functions of CT149 observed in ADCC and CDC assays (Supplementary Fig. 5), as was demonstrated also for Fl6 and other mAbs38,39. Recently, CR9114 demonstrated passive protection of mice challenged with influenza type A and B viruses, although this mAb was unable to neutralize type B viruses in vitro38. These findings in model systems are thought to be medically relevant since human sera from either convalescent patients or vaccinated individuals can induce ADCC of influenza virus-infected cells, presumably mediated by antibodies to HA and NA35. Killing of influenza virus-infected cells by CDC with human mAbs was also observed by Terajian et al.36. Overall, we found a good correlation between CT149 binding to various HA subtypes, function in vitro assays such as neutralization, fusion–inhibition, host factor-mediated killing and in vivo protection, but exceptions were noted. For example, the binding affinities to HA epitopes tended to correlate with neutralization potency with some exceptions as previously reported40. These differences can be attributed to either intrinsic functional difference between divergent Has, for example, fusion pH optima or host cell specificity, or idiosyncrasies imposed by binding and neutralization assay systems. For example, HA density on virions and particle morphology could modulate the relevant interaction of IgG with HA stem epitopes on neighbouring trimers and affect neutralization potency as measured in vitro34. Similarly, mAb binding assay results could be influenced by undetected structural changes differentially affecting the target HA32,43.

Similarly, the overall concordance between CT149 neutralization and low pH-induced polykaryon (syncytia) formation in cells expressing a cognate HA included a notable exception in the case of CA/09 expressing cells. CT149 failed to neutralize the wild-type virus but blocked fusion in this assay, suggesting that the cell-based assay is less stringent than neutralization. Alternatively, differences in HA density, membrane curvature and lipid composition between CHO cell plasma membranes and virions could explain the outcomes of these assays44.

To date, a few ‘headless’ HA immunogens and chimeric HAs have been designed as potential ‘universal’ vaccines that re-direct antibody specificity towards the more conserved regions of the fusion subdomain of the HA stem region45. In this study, an antibody that uses both its heavy and light chains to contact the HA could potentially neutralize viruses from divergent groups 1 and 2 HA subtypes, and the breadth of neutralization would be dependent on the interaction between the light chain of antibody and the HA, as the heavy chain usually generates a conserved binding site in the shallow pocket on the fusion subdomain of HA17–20,25. This finding may assist in the vaccine design to elicit a more potent crossreactive neutralizing antibody response.

Methods

Recombinant HA. The following commercially available monomeric recombinant HAs expressed from both baculovirus and mammalian expression systems were utilized for the studies: A/California/04/2009 (H1N1)pdm09 (Sino Biological, Beijing, China, cat. no. 1055-V08H), A/California/07/2009 (H1N1)pdm09 (Immune Technology, NY, USA, cat. no. IT-003-SW12ATAmp), A/Texas/05/2009 (H1N1)pdm09 (Immune Technology, cat. no. IT-003-SW16ATAmp), A/Solomon Island/03/2006(H1N1) (Immune Technology, cat. no. IT-003-011ATAmp), A/Oslo/07/2009 (H1N1)pdm09 (Sino Biological, cat. no. 40007-V08H), A/Brisbane/10/2007 (H3N2) (Sino Biological, cat. no. 11056-V08H), A/Philippines/2/1982 (H3N2) (Immune Technology, cat. no. IT-003-0041ATAmp), A/Wisconsin/67/1961(514N2) (Immune Technology, cat. no. IT-003-041ATAmp), A/Texas/05/2004 (H5N1) (Immune Technology, cat. no. IT-003-0515p) and A/Anhui/Anhui/1/2013 (H7N9) (Sino Biological, cat. no. 40103-V08H). In addition, trimeric recombinant HAs that have folded trimerization sequences were obtained from the Influenza Reagent Resource (http://www.influenzareagentresource.org): A/California/04/2009 (cat. no. FR-180), A/Brisbane/10/2007 (cat. no. FR-61) and A/Vietnam/1203/2004 (cat. no. FR-39).

Viruses and cells. Viruses used in this study comprised wild-type isolates and re-assortants, containing internal genes from A/Puerto Rico/8/1934 or A/Ann Arbor/1960, which were developed as candidate vaccine viruses for vaccine
manufacturing (Table 1). Viruses were propagated in Madin–Darby canine kidney (MDCK) cells or in embryonated eggs. All infectious wild-type H5N1 viruses were handled in Biosafety Level 3 (BSL-3) facilities including enhancements required by the US Department of Agriculture and the Select Agent Program36.

For passive protection studies in mice, wild-type A/Vietnam/1203/2004 (H5N1), mouse-adapted A/California/04/2009 (H1N1) and A/Anhui/1/2013 (H7N9) (AN/13) were amplified in embryonated eggs, and mouse-adapted A/Hong Kong/1968 (H2N2) was propagated in MDCK cultures. Virus titres were determined by plaque assay in MDCK cells (plaque-forming unit per ml) or by end point dilution in MDCK cells (TCID50) or eggs (EID50 per ml). MDCK (CCL-34) and CHO (CHO-K1 and CCL-61) cells were obtained from the American Type Culture collection (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, cat. no. 11965) with or without 10% fetal bovine serum (FBS).

Isolation of PBMCs from patients recovered from influenza. Following laboratory confirmation of influenza A (A/California/04/2009) infection, blood was obtained from patients within 2–4 weeks after the onset of symptoms and was processed immediately. All donors gave written informed consent for research use of blood samples following protocols approved (approval number: 4-2009-0461) by the Institutional Review Board at Severance Hospital, Yonsei University, Seoul, Korea. PBMCs were isolated from the collected blood using lymphoprep (Axis-Shield, Norway, 1114545). The isolated PBMCs were suspended at 2 × 10^6 cells per ml in KM banker II freezing medium (Cosmobio, Japan, cat. no. KOJ-16092010) and stored in a liquid nitrogen tank for later use.

Identification and cloning of VH and VL sequences. B cells secreting antigen-specific antibodies were screened using the ISACA method as previously described24. Briefly, the PBMCs were added to each well of the prepared microcarriers at a density of one cell per well. Antibody secretion from single cells was confirmed by binding to pre-coated anti-human IgG antibody. HA-specific antibody-secreting cells were selected with fluorescein isothiocyanate-labelled A/California/04/09 rHA (ref. 45.7). The VH and VL of antibodies from each individual antibody-secreting cell were obtained by single-cell 5’-rapid amplification of cDNA ends (5’-RACE), consisting of amplification of an amplicon generated by a reverse transcription–PCR using degenerate primer sets flanking the variable region loci of the heavy and light immunoglobulin chains. Heavy-chain and light-chain DNA amplicons were cloned into pcDNA 3.1 (+) expression vectors (Invitrogen, CA, USA, cat. no. V790-20) for fusion with the constant gamma heavy and kappa light-chain coding sequences to prepare expression vectors producing each of the specific immunoglobulins for further characterization. Two chimeric antibodies, CT149mIgG1 and CT149mIgG2a, were also produced via subcloning of the Fc region (G02 and H03) of the human IgG1 by those of mouse IgG1 and IgG2a, respectively, to evaluate the role of Fc-mediated effector functions of CT149 in a mouse model.

Production of mAbs in mammalian cells. The VH and VL chain genes of selected mAbs were recloned from the pcDNA 3.1-based vectors into the MarEx-based expression vector, pCT107 (patent US 8772021 B2 (2006)), by replacing the heavy and light chains of pCT107 with those from the pcDNA vectors. mAbs for functional evaluation in MN and haemagglutinin inhibition (HI) assays were produced by transient expression. To this end, plasmids encoding mAbs of interest were transfected into human host cell line, F2N78 (ref. 48), and mAbs were harvested by serum-free medium. The complete coding regions of HA from A/California/04/2009 (H1N1), A/Japan/305/1957 (H2N2), A/Brisbane/10/2007 (H3N2) and A/California/04/2009 (H1N1)pdm09 (Sino Biological, cat. no. FR-61)) (50 μl, 250 ng ml^{-1} 2 carbonic anhydrase coating buffer) and blocked with 1% bovine serum albumin in PBS. Antibodies (threefold dilutions starting from 1 μg ml^{-1} 2) were added and incubated at room temperature for 1 h, and was followed by incubation with horseradish peroxidase-conjugated goat anti-human IgG (Zymed, cat. no. 62.8420). After washing with 0.05% Tween-20, 0.1 M sodium phosphate buffer, 0.15 M NaCl (pH 7.2), the plate was incubated with tetramethylbenzidine (Sigma-Aldrich, DL, USA, cat. no. T0440), and the incubation was stopped by adding 1 N HCl. The absorbance at 450/570 nm was recorded by a plate reader (Spectramax M5 Microplate reader (488 nm excitation/530 nm emission/515 nm cutoff). The maximal ADCC response of CT149 was evaluated as specific % cell lysis by detecting calcine-AM release. Calculation of the percentage of specific lysis from triplicate experiments was performed using the equation of % Cell lysis = (E-S)/(M-S) × 100’, where E represents the AFU of test sample, S represents the AFU of supernatant with identical calcine-AM inclusion, and M represents the AFU of maximal calcine lysis by target cells on lysis by detergent (2% Triton X-100). For ADCC assay with virus-infected cells, Raji cells were infected with influenza viruses (A/California/04/09 at 0.03 multiplicity of infection (MOI)), A/Penn/16/09 at 0.1 MOI) in OptiPro SFM (Gibco) for 24 h. The infected cells were stained with calcine-AM (Invitrogen) and washed twice. The stained cells (1 × 10^6 cells) were added into 96-well plates pre-treated with serially diluted antibodies (CT149 and CT-P6) and incubated for 30 min. The interleukin-2-
treated PBMC (the frozen ePBMC, Cellular Technology Ltd, cat. no. CTL-UP1) effectors were added into the 96-well assay plates at a 25:1 ratio of effector to target cells in triplicate and incubated for 4 h. The fluorescent signals from samples and controls in triplicate were measured and the cytotoxicity effect of ADCC (%) was analysed as following: \( \text{cytotoxicity} = \frac{\text{mean sample release-mean spontaneous release}}{\text{mean maximum release-mean spontaneous release}} \times 100 \).

In the case of CD8+ T-CHO-K1 cell lines constitutively expressing HA from A/California/04/2009 (H1N1) (PBMC) were used. Target cells were depleted at a cell density of 3.0 \( \times \) 10^5 cells per well in 96-well plates in duplicates and incubated for 16–20 h at 37 °C in a humidified 5% CO2 incubator. Following incubation, cells were washed once with 200 µl per well of assay media (20 mM HEPES and 0.1% bovine serum albumin in DMEM/F12 (1:1 medium) and filled with 100 µl per well of assay media. An amount of 50 µl of serially diluted CT149 and CT-P6 (from 50,000 to 82 ng ml \(^{-1} \)) were then added to each well. Thereafter, 50 µl of diluted normal human serum complement (Quidel, San Diego, USA, cat. no. A113) with assay media was added and incubated for 2 h. Finally, 10 µl of cell-counting kit (CCK-8, Dojindo, Kumamoto, Japan, cat. no. CK04) reagent was added to each well and incubated >8 h at 37 °C in a humidified 5% CO2 incubator. Absorbance was read on a SpectraMax M5 microplate multireader (450–650 nm) to estimate cell viability from the dye-reducing activity levels. The CDC activity of antibodies was reported as cytotoxicity (loss of cell viability) according to the following formula: cytotoxicity (loss of cell viability) = (baseline reaction – OD of experimental reaction)/(OD of baseline reaction – OD of maximal reaction) \times 100.

Mice. Virus challenge with mouse-adapted A/Hong Kong/1968 (H3N2) was performed using 5-week-old female BALB/c mice purchased from Orient Bio (Korea). These mice were housed at the pathogen-free facility located at the International Vaccine Institute, Seoul, Korea.

For all tests at a dose of 30 mg kg \(^{-1} \) of CT-P6 was used as the negative control.

Recombinant HA for crystallization. For production of recombinant H3, residues comprising the HA ectodomain from A/Hong Kong/1/1968 (H3N2) were codon optimized, synthesized, subcloned into the pXGP67-B baculovirus shuttle vector (Bac-to-Bac) and expressed. TriCoplus m (High 5) cells (Invitrogen) were infected with recombinant baculovirus at an MOI of 5–10 at 28 °C for 72 h. The secreted H3 protein was purified from the tissue culture supernatant by metal affinity chromatography and subsequent size-exclusion gel filtration chromatography (Superdex 200 16/60 column, GE Healthcare). For crystallization, the trimeric terminus was removed from the H3 protein by thrombin treatment using 3 U enzyme per mg H3 overnight at 4 °C. Then 7.5, 15 or 30 mg kg \(^{-1} \) of CT-P6 was used as the negative control.

For challenge with mouse-adapted A/Hong Kong/1968 (H3N2), groups of five mice were anesthetized and inoculated with 5 LD50 of virus diluted with 50 µl of PBS. Following abdominal skin disinfection with 70% ethanol, mice were intraperitoneally injected with CT149, CT149mGlG1 or CT149mGlG2a at a dose of 3 mg kg \(^{-1} \) of body weight. For therapeutic efficacy, groups of 10 mice were injected with only CT149 (H3) to achieve 100% body doses (7.5 mg kg \(^{-1} \) at 4 h). The mice were monitored for 4 days before conducting the experiment. Housing and care of the animals were performed in the Animal BSL-3–4 facilities located within Biolab.

Studies using mouse-adapted A/California/04/2009 (H1N1) with CT149 and chimeric CT149 were performed utilizing 9-week-old female BALB/c mice (Vital River Laboratories, Beijing, China) were used. Mice were housed in the Animal BSL-3–4 facility located in the Beijing Institute of Microbiology and Epidemiology.

IgG Fabs for crystallization. CT149 was digested using papain (Roche ref. no.10180014001) protease at an antibody to papain ratio of 100:1 at 37 °C for 1 h. After desalting the column, the material was loaded into a Mabslect Sure column (GE Healthcare, cat. no. 17-3438-03) by applying the flowthrough mode to eliminate the Fc region and undigested antibody. Flowthrough Fab material was concentrated and purified to homogeneity by size-exclusion gel filtration chromatography (Superdex 200 10/500 GL, GE Healthcare, cat. no.17-5175-01) with PBS buffer.

For the challenge study with A/Vietnam/1203/04 (H5N1), groups of 10 mice were inoculated with 50 µl of cell-counting kit (CCK-8, Dojindo, Kumamoto, Japan, cat. no. CK04) reagent was added to each well and incubated >8 h at 37 °C in a humidified 5% CO2 incubator. Absorbance was read on a SpectraMax M5 microplate multireader (450–650 nm) to estimate cell viability from the dye-reducing activity levels. The CDC activity of antibodies was reported as cytotoxicity (loss of cell viability) according to the following formula: cytotoxicity (loss of cell viability) = (baseline reaction–OD of experimental reaction)/(OD of baseline reaction–OD of maximal reaction) \times 100.

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Formation and purification of Fab/HA complexes. CT149 Fab was mixed with purified, His-tag-depleted, recombinant H3 and H7 HA trimers at a molar ratio of 1:5. For each one part HA, 4 parts Fab were added. For H7, HA trimers were codon adapted and expressed from M7V7, which was used to express CT149 Fab-H3 HA (CT149/H3) and CT149 Fab-H7 HA (CT149/H7) complexes were purified from unbound substrates by size-exclusion gel filtration chromatography (Superdex 200 10/300 column, GE Healthcare) in a buffer comprising 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl, and collected protein fractions were concentrated to 10 mg ml \(^{-1} \). For challenge with mouse-adapted A/Hong Kong/1968 (H3N2), groups of five mice were anesthetized and inoculated with 5 LD50 of virus diluted with 50 µl of PBS. Following abdominal skin disinfection with 70% ethanol, mice were intraperitoneally injected with CT149, CT149mGlG1 or CT149mGlG2a at a dose of 3 mg kg \(^{-1} \) of body weight. For therapeutic efficacy, groups of 10 mice were injected with only CT149 (H3) to achieve 100% body doses (7.5 mg kg \(^{-1} \) at 4 h). The mice were monitored for 4 days before conducting the experiment. Housing and care of the animals were performed in the Animal BSL-3–4 facility located within Biolab.

Structure determination of the CT149/HA complex. Initial sparse-matrix crystallization screening was carried out using a Topaz Free Interface Diffusion Crystallizer system (Fluidigm Corporation, San Francisco, CA, USA). Preliminary crystallization conditions for the CT149/H3 complex were obtained after 24 h in several conditions containing 200–250 mM ammonium sulfate, 15–50 mM Tris-HCl (pH 8.5). The CT149/H3 complex data set was collected from a single crystal at 3.5 Å resolution at the Advanced Photon Source SER CAT 23–2–1 of the sitting drop method with 1.0 µl drops containing CT149/H3 in 20% PEG 3000 and 100 mM Na citrate (pH 5.5). The CT149/H3 complex data set was collected from a single crystal at 3.5 Å resolution at the Advanced Photon Source SER CAT 23–2–1 of the sitting drop method with 1.0 µl drops containing CT149/H7 in 20% PEG 3000 and 100 mM Na citrate (pH 5.5). The CT149/H7 complex data set was collected from a single crystal at 2.8 Å resolution at the Shanghai Synchrotron Radiation Facility beamline 17U.

Data collection and refinement statistics are presented in Table 3. Data were processed and scaled using HKL2000 and Denzo24. The structures were solved by molecular replacement using Phaser25 from the CCP4 programme suite26. Initial rigid body refinement was performed using REFMAC5 (ref. 57), and extensive model building was performed using COOT58. Further rounds of refinement were...
carried out using the phenix.refine programme implemented in the PHENIX package\(^9\) with energy minimization, isotropic ADP refinement and bulk solvent modelling. The structures were then adjusted using COOT and were refined with PHENIX. The stereochemical quality of the final model was assessed with the programme PROCHECK\(^9\).

The structure of CT149/H3 complex contains an HA trimer and three antibody molecules in the asymmetric unit, and the structure of CT149/H7 complex contain a HA protomer and one antibody molecule in the asymmetric unit. Portion of well-ordered electron density is showed in Supplementary Figure 7.

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**Author contributions**
G.F.G., R.O.D., S.J.C. and C.-F.Q. designed and coordinated the study. Y.W., M.Cho, D.S., M.S., J.C., T.I., Y.-Q.D., M.B., L.A., H.Y., L.-M.C., A.L., M.Chang J.S.B., H.L., J.S. and S.H. conducted the experiments. J.Q. and J.Stevens collected the data sets and solved the structures. G.F.G, R.O.D., Y.S. and K.S.Y. wrote the manuscript. Y.W., M.Cho and S.J.C. and C.-F.Q. participated in the manuscript-editing and discussion. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention or the Agency for Toxic Substances and Disease Registry.

**Additional information**

**Accession codes:** The structures for CT149/H3 and CT149/H7 were deposited in the Protein Data Bank under accession codes 4UBD and 4R8W, respectively.

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