Vaccination of monoglycosylated hemagglutinin induces cross-strain protection against influenza virus infections

Juine-Ruey Chen[a,1], Yueh-Hsiang Yu[a,1], Yung-Chieh Tseng[a,b,1], Wan-Ling Chiang[a], Ming-Feng Chiang[a], Yi-An Ko[a,c], Yi-Kai Chiu[a,c], Hsiu-Hua Ma[a], Chung-Yi Wu[a], Jia-Tsrong Jan[a], Kuo-I Lin[a,2], Che Ma[a,2], and Chi-Huey Wong[a,2]

[a]Genomics Research Center, Academia Sinica, Taipei 115, Taiwan; [b]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, Taiwan; and [c]Institute of Microbiology and Immunology, National Yang-Ming University, Taipei 112, Taiwan

Contributed by Chi-Huey Wong, December 24, 2013 (sent for review October 14, 2013)

The 2009 H1N1 pandemic and recent human cases of H5N1, H7N9, and H6N1 in Asia highlight the need for a universal influenza vaccine that can provide cross-strain or even cross-subtype protection. Here, we show that recombinant monoglycosylated hemagglutinin (HA_{mg}) with an intact protein structure from either seasonal or pandemic H1N1 can be used as a vaccine for cross-strain protection against various H1N1 viruses in circulation from 1933 to 2009 in mice and ferrets. In the HA_{mg} vaccine, highly conserved sequences that were originally covered by glycans in the fully glycosylated HA (HA_{fg}) are exposed and thus, are better engulfed by dendritic cells (DCs), stimulated better DC maturation, and induced more CD8+ memory T cells and IgG-secreting plasma cells. Single B-cell RT-PCR followed by sequence analysis revealed that the HA_{mg} vaccine activated more diverse B-cell repertoires than the HA_{fg} vaccine and produced antibodies with cross-strain binding ability. In summary, the HA_{mg} vaccine elicits cross-strain immune responses that may mitigate the current need for yearly reformulation of strain-specific inactivated vaccines. This strategy may also map a new direction for universal vaccine design.

glycoprotein engineering | broadly neutralizing antibody

HA glycoprotein on the surface of influenza virus is a major target for infectivity-neutralizing antibodies. However, the antigenic drift and shift of this protein mean that influenza vaccines must be reformulated annually to include HA proteins of the viral strains predicted for the upcoming flu season (1). This time-consuming annual reconfiguration process has led to efforts to develop new strategies and identify conserved epitopes recognized by broadly neutralizing antibodies as the basis for designing universal vaccines to elicit antibodies with a broad protection against various strains of influenza infection (2–6). Previous studies have shown that the stem region of HA is more conserved and able to induce cross-reactive and broadly neutralizing antibodies (7–9) to prevent the critical fusion of viral and endosomal membranes in the influenza lifecycle (10–14). Other broadly neutralizing antibodies have been found to bind regions near the receptor binding site of the globular domain, although these antibodies are fewer in number (15, 16).

Posttranslational glycosylation of HA plays an important role in the lifecycle of the influenza virus and also contributes to the structural integrity of HA and the poor immune response of the infected hosts. Previously, we trimmed down the size of glycans on avian influenza H5N1 HA with enzymes and showed that H5N1 HA with a single N-linked GlcNAc at each glycosylation site [monoglycosylated HA (HA_{mg})] produces a superior vaccine with more enhanced antibody response and neutralization activity against the homologous influenza virus than the fully glycosylated HA (HA_{fg}) (17). Here, to test whether the removal of glycans from HA contributes to better immune responses and possibly protects against heterologous strains of influenza viruses, we compared and evaluated the efficacy of HA glycoproteins with various lengths of glycans as potential vaccine candidates.

Results

HA_{fg}, HA_{mg}, and unglycosylated HA (HA_{ug}) proteins were prepared using secreted constructs of HA from the A/Brisbane/59/2007 (Bris/07) and A/California/07/2009 (Cal/09) H1N1 strains (Fig. 1*A* and see Fig. S2*A*). Because HA_{ug} is obtained after treatment with the glycosidase PNGase F, each glycosylation site has an amino acid change from asparagine (Asn) to aspartic acid (Asp). The HA proteins are trimeric in solution (Fig. S1 *C–F*), and their compositions and secondary structures can be confirmed by MS and CD (Fig. S1 *G–J*). HA_{fg} and HA_{mg} were found to have the same secondary structure, and HA_{ug} showed a slight difference (Fig. S1 *G* and *I*). The glycan profile of each glycosylation site on HA was next determined by glycan peptide analysis (Table S1). In addition, glycan microarray analysis showed that the HA from either Cal/09 or Bris/07 binds to sialosides with an α2,6 linkage and that shortening the N-glycans on HA increases the binding avidity (Fig. 1*B* and Fig. S2*B*). These HA proteins were used as vaccines in mice to analyze their ability to induce cross-protective immune responses.

Mice antisera were collected and tested for HA inhibition (HI) and microneutralization (MN) against the Cal/09 vaccine strain NIBRG-121 (Cal/09), A/WSN/1933 (WSN/33), and A/Puerto Rico/8/1934 (PR8/34) viruses (Fig. 1). Compared with HA_{fg} and HA_{ug}, the antisera from HA_{mg}-vaccinated mice were found to have higher HI titer (≧40) against all three viruses and exhibit higher neutralizing capacity to WSN/33 and Cal/09 viruses, whereas

Significance

Influenza epidemics continue to be a threat to public health, and the recent human cases of avian viruses of H5N1, H7N9, and H6N1 in Asia raise the possibility of a new disastrous influenza pandemic. Although an effective universal vaccine that can protect from influenza viruses from different subtypes or even both type A and B is still far from reality, our unique findings, showing that monosaccharide glycosylated HA vaccine induces broader protection against different strains, may lead to a better influenza vaccine design that does not require frequent updates and annual immunizations. This strategy may also map out a new direction for development of universal flu vaccines and be applied to vaccine design for other human viruses.

Author contributions: K.-I.L., C.M., and C.-H.W. designed research; J.-R.C., Y.-H.Y., Y.-C.T., W.-L.C., M.-F.C., Y.-A.K., Y.-K.C., H.-H.M., C.-Y.W., and J.-T.J. performed research; J.-R.C., Y.-H.Y., Y.-C.T., W.-L.C., M.-F.C., Y.-A.K., Y.-K.C., H.-H.M., C.-Y.W., J.-T.J., K.-I.L., and C.M. analyzed data; and J.-R.C., Y.-H.Y., Y.-C.T., K.-I.L., C.M., and C.-H.W. wrote the paper.

The authors declare no conflict of interest.

[1]J.-R.C., Y.-H.Y., and Y.-C.T. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: kuoilin@gate.sinica.edu.tw, cma@gate.sinica.edu.tw, or chwong@gate.sinica.edu.tw.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1323954111/-/DCSupplemental.

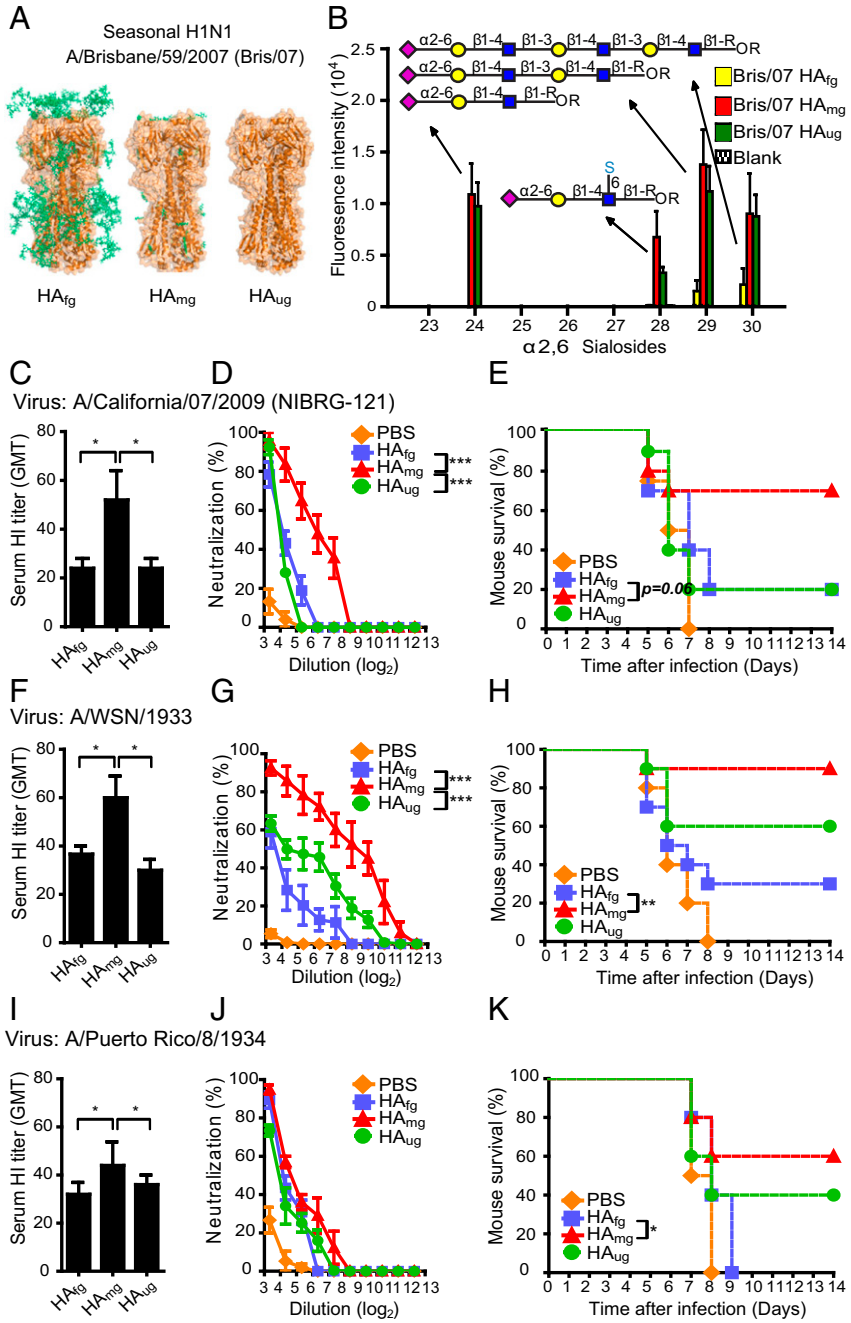

Fig. 1. Bris/07 HA_{mg} vaccine induces cross-protection against Cal/09, WSN/33, and PR8/34 viruses. (*A*) Bris/07 (H1N1) HA proteins with different lengths of glycans at the glycosylation sites: HA_{fg}, HA with the typical complex type N-glycans; HA_{mg}, HA with GlcNAc only at its N-glycosylation sites; HA_{ug}, HA without glycans at its N-glycosylation sites. Models are created with Protein Data Bank ID code 3LZG by adding sialylated complex biantennary N-glycans (HA_{fg}) and GlcNAc (HA_{mg}) and displayed with program PyMOL (www.pymol.org). (*B*) Glycan microarray profiling of HA variants HA_{fg}, HA_{mg}, and HA_{ug}. (*C–K*) H1N1 seasonal influenza Bris/07 HA_{fg}, HA_{mg}, and HA_{ug} were used as vaccines to analyze their ability to induce cross-protective immune response in mice. (*C–E*) Mice vaccinated with Bris/07 HA against NIBRG-121 (Cal/09) virus. (*C*) HI assay. (*D*) MN assay and (*E*) survival rate of virus challenge. (*F–H*) Mice vaccinated with Bris/07 HA against WSN/33 virus. (*F*) HI assay. (*G*) MN assay and (*H*) survival rate of virus challenge. (*I–K*) Mice vaccinated with Bris/07 HA against PR8/34 virus. (*I*) HI assay. (*J*) MN assay and (*K*) survival rate of virus challenge. GMT, geometric mean titer. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

no significant differences were observed among the three protein vaccines against PR8/34 virus. To test whether vaccination with HA_{mg} offered cross-protection against these three H1N1 viruses, the immunized mice were challenged with lethal doses (100 LD_{50}) of Cal/09, WSN/33, and PR8/34 viruses, and the efficacy of vaccine protection was evaluated over 14 d based on survival rate (Fig. 1). After virus challenge, mice vaccinated with PBS died before day 8 (Fig. 1). A previous study using inactivated Bris/07 virus as a vaccine reported that it provided 30% protection against challenge with the 2009 pandemic A(H1N1) virus. In this study, Bris/07 HA_{fg} showed a comparable level of protection (20%) against a lethal Cal/09 challenge. However, to our surprise, immunization with Bris/07 HA_{mg} offered 70% protection against Cal/09 challenge (Fig. 1*E*), and immunization with HA_{ug} only offered partial (20%) protection, probably because of the difference in structure. It should be noted that Bris/07 and Cal/09,

WSN/33, and PR8/34 have HA sequence identities of 80%, 84%, and 86%, respectively. Similarly, HA_{fg}, HA_{mg}, and HA_{ug} from the 2009 pandemic influenza Cal/09 were used as vaccines to analyze their ability to induce cross-protective immune responses (Fig. S2). As expected, Cal/09 HA_{mg} induced antibodies with better neutralizing activity against heterologous virus infection (Fig. S2). The HA_{ug} vaccines were found to be similar or in most cases, worse than HA_{mg} in cross-strain protection. The reason behind this result is not clear and needs to be investigated, but it could be because HA_{ug} contains partial glycans, which are not completely removed by enzyme (Table S1), has slight differences in the secondary structure (Fig. S1 *G* and *I*), and has the amino acid changes from Asn to Asp at each glycosylation site.

We next investigated the underlying immune responses that may contribute to the cross-protectivity of HA_{mg} vaccine. To this end, we first examined whether Bris/07 HA_{fg} and HA_{mg} initiated differential activation of dendritic cells (DCs), the immune sentinels that can detect, process, and present antigens to prime adaptive immune responses. We found that, compared with HA_{fg}, HA_{mg} was engulfed more readily by DCs (Fig. 2*A*) and induced the increased expression of the activation marker (Fig. S3*A*) and the elevated production of cytokines/chemokines (Fig. S3*B*). Also, more splenic CD8+ granzyme B-secreting T cells were induced in Bris/07 HA_{mg}-vaccinated mice challenged with Bris/07 or Cal/09 HA_{fg} (Fig. 2*E*), suggesting that HA_{mg} vaccine induced stronger CD8+ cytotoxicity effects than HA_{fg}. However, the enhanced activation of DCs by HA_{mg} was not linked to better CD4+ T-cell responses, because total splenocytes or CD4+ T cells from Bris/07 HA_{fg} or HA_{mg}-vaccinated mice produced similar levels of IL-2 (Fig. 2*B*), CD4+INFγ+ Th1 cells (Fig. 2*C*), and CD4+IL-4+ Th2 cells (Fig. 2*D*) when challenged with Bris/07 HA_{fg}, Cal/09 HA_{fg}, or DCs loaded with these proteins. Although HA_{mg} did not induce better CD4+ T-helper responses, more HA-specific antibody-secreting cells were produced in Bris/07 HA_{mg}-vaccinated mice, and they showed cross-recognition (Fig. 2*F*), consistent with our findings that HA_{mg} induced higher levels of cross-protective antibodies. Given that CD4+ T-helper responses were not promoted but HA-specific antibody-secreting cells were increased in HA_{mg}-vaccinated mice, we suspect that B cells may functionally and specifically recognize the intact structure of HA_{mg}, because all HA glycoproteins used in this study were in their trimeric forms. Vaccine efficacy had the potential for modulation by the structural integrity of HA proteins, because antisera from the mice vaccinated with the heat-denatured form of Cal/09 HA_{mg} or the monomeric form of Cal/09 HA_{mg} (Fig. S1) showed highly reduced HI titers (Fig. 2*G*); additionally, the vaccines showed decreased protection levels compared with the trimeric forms (Fig. 2*H*). These results suggest that intact trimeric HA_{mg} may reveal extra epitopes that are recognized by B cells and result in the production

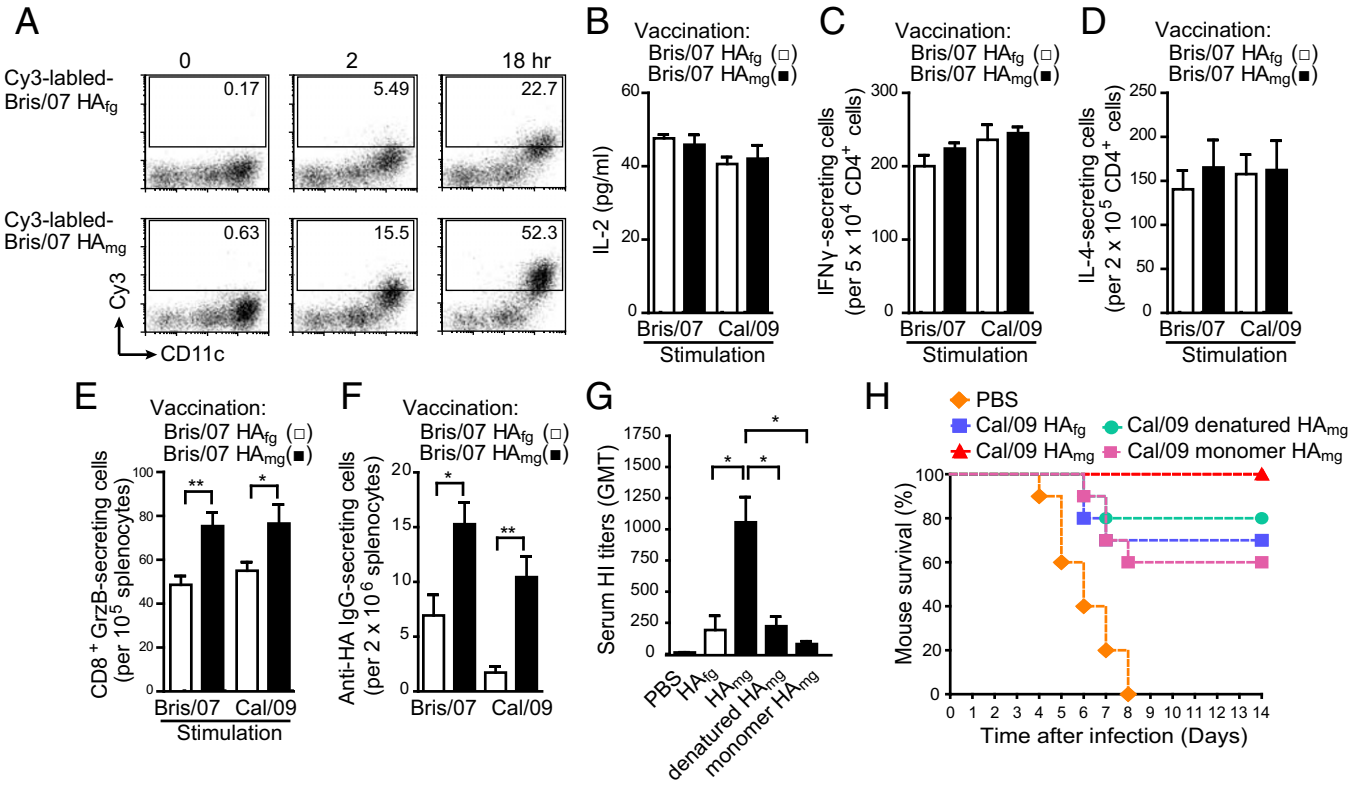

Fig. 2. Recognition of intact structure of HA_{mg} by B cells is crucial for cross-protection. (*A*) DCs uptake HA_{mg} more efficiently than HA_{fg}. Bone marrow immature DCs stimulated with Cy3-labeled Bris/07 HA_{fg} or Cy3-labeled Bris/07 HA_{mg} for 0, 2, and 18 h were monitored for uptake of Cy3-labeled HA by flow cytometry. (*B–F*) Adaptive immune responses elicited by Bris/07 HA_{fg} or HA_{mg} vaccination. Cells from spleens of vaccinated mice were analyzed either (*F*) directly or (*B–E*) after stimulation with Bris/07 or Cal/09 HA_{fg}. (*B*) IL-2 production was analyzed by ELISA using supernatants from total splenocytes stimulated with Bris/07 or Cal/09 HA_{fg} for 2 d. (*C*) IFNγ- and (*D*) IL-4–secreting cells were determined by Elispot analysis using CD4+ T cells cocultured with bone marrow-derived DCs loaded with Bris/07 or Cal/09 HA_{fg} for 24 h. (*E*) The number of CD8+ granzyme B (GrzB) -secreting cells in splenocytes stimulated with HA for 2 d in vaccinated mice was determined by flow cytometric analysis. (*F*) The number of Bris/07 or Cal/09 HA_{fg}-specific IgG antibody-secreting cells was determined by Elispot analysis. (*G*) Neutralizing antisera from mice vaccinated with the denatured and monomeric forms of Cal/09 HA_{mg} were examined and compared with the trimeric forms of HA_{mg} and HA_{fg}. HI results showed that the trimeric forms of HA_{mg} provided more potent neutralizing activity against the Cal/09 virus than the denatured or monomeric forms of HA_{mg}. (*H*) The immunized mice from *G* were challenged with a lethal dose (100 LD_{50}) of RG121 viruses, and the efficacy was evaluated by measurement of survival over 14 d. $*P < 0.05$; $**P < 0.01$.

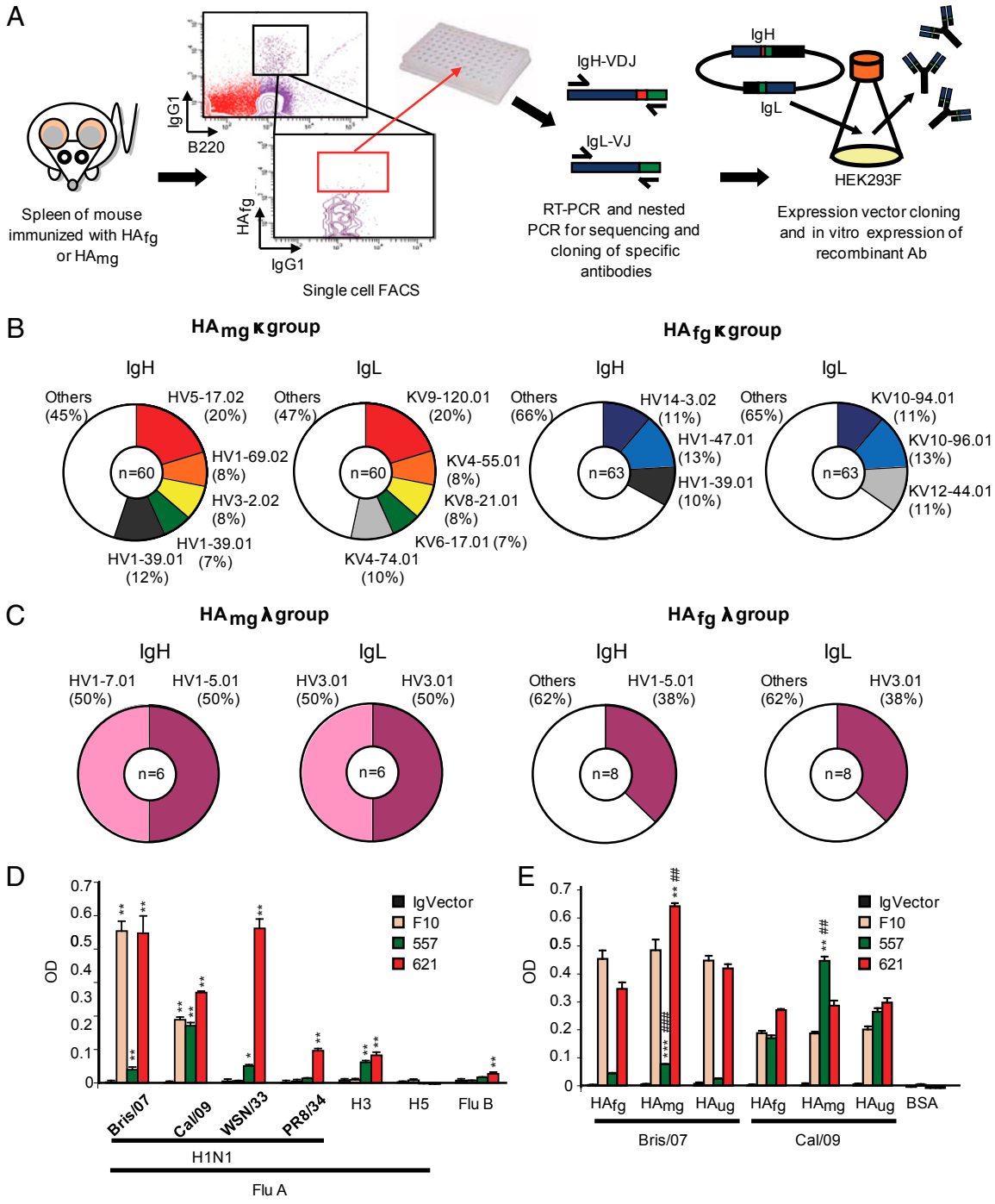

Fig. 3. Single cell RT-PCR revealed that differential HA-specific B-cell repertoires were elicited by HA_{mg} and HA_{fg} vaccination. (*A*) Overview of experimental design. Single HA-specific B cells from the spleen of vaccinated mice were sorted into 96-well plates by FACS. The IgH and IgL gene transcripts of each single B cell were amplified by RT-PCR. After sequencing, the cDNAs from the variable regions of IgH and IgL genes were subcloned into an expression vector containing the human Ig constant region. The resultant recombinant monoclonal antibody, produced by HEK293F cells, was then subjected to ELISA. (*B* and *C*) The pie charts show the distribution of the Bris/07 HA-specific B-cell repertoire on day 29 after immunization with Bris/07 HA_{mg} or HA_{fg} two times two weeks apart. The proportions of all IgH or IgL (κ-chain in *B* and λ-chain in *C*) genes with a frequency greater than 5% in the B-cell population of each vaccinated mouse are indicated by colors. Bris/07 HA-specific B-cell clones with a frequency lower than 5% are shown in white. B cells producing heavy chains and light chains encoded by the same indicated IgH and IgL gene loci are grouped by the same color. Heavy chains (shown in dark gray) or light chains (shown in light gray) indicate that their paired light chains or heavy chains, respectively, were not clonally amplified. (*D*) The recombinant antibodies elicited by vaccination with Bris/07 HA_{mg} show cross-recognition. The OD value in ELISA is shown by using 0.03 μg/mL recombinant antibodies in ELISA plates coated with BSA or indicated HA_{fg}. The Ig vector is the negative control antibody. F10 is an HA-specific broadly neutralizing monoclonal antibody. Clone 557 carries V1-39.01 heavy chain and V6-17.01 κ-light chain. Clone 621 is composed of V5-17.02 heavy chain and V4-55.01 κ-light chain. (*E*) The 557 and 621 recombinant antibodies bind better to HA_{mg} than to HA_{fg} or HA_{ug} from Cal/09 or Bris/07. The statistical significance between HA_{mg} and HA_{fg} or HA_{ug} of each antibody is indicated. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.

of different antibodies or simply, that the trimeric protein is a better activator of B cells than the monomer to induce better antibodies.

Next, the sera from immunized mice were incubated with the HA from a heterologous strain coated on Sepharose beads, and the flow-through sera were then subjected to MN experiments against various H1N1 viruses. A reduction in the MN activity of the flow-through sera would indicate that the antibodies bound to the HA of the heterologous strain contribute to the observed cross-strain neutralization ability. We observed stronger neutralizing activity from the sera of HA_{mg} vaccination, indicating that the compositions of induced antibodies from HA_{mg} and HA_{fg} are different and that the antibodies from HA_{mg} exhibit stronger cross-strain neutralizing activity. If this implication were not the case, we would expect to observe the same percentage of neutralization inhibition (Fig. S4 *A–F*). When HA_{ug} was coated on the beads, the stronger neutralizing activity of HA_{mg} over HA_{fg} immunization was no longer observed (Fig. S4 *G–I*). Because HA_{ug} carries a negative charge (Asp residue) at each glycosylation site that may prevent the binding of neutralizing antibodies, this observation strengthens the idea that the extra cross-strain neutralizing antibodies from HA_{mg} vaccination recognize the conserved epitopes near the glycosylation sites.

To better understand the B-cell repertoires after immunization with HA_{mg} or HA_{fg}, about 200 single splenic B cells capable of binding to Bris/07 HA_{fg} from the spleens of Bris/07 HA_{fg}- or HA_{mg}-vaccinated mice were sorted. By analyzing the amino acid sequences of the RT-PCR products of the variable regions of Ig heavy-chain (IgH) and Ig light-chain (IgL) genes from about 70 individual single B cells that contained detectable PCR signals (Fig. 3*A*), we were able to profile the HA-specific B-cell repertoire elicited by HA_{mg} vaccination and found that HA_{mg} response was more complex than that of HA_{fg}. Some HA-specific B cells with particular compositions of IgH and κ-IgL or λ-IgL loci were clonally amplified in HA_{mg}-vaccinated mice (Fig. 3 *B* and *C*). Particularly, compared with HA_{fg}-vaccinated mice, at least five HA-specific B-cell clones were selectively amplified by the HA_{mg} vaccine (Fig. 3 *B* and *C*). These amplified clones had undergone somatic hypermutation in the germinal center, because each single B cell within the clonally amplified populations carried various frequencies of mutation in the variable region of IgH and IgL genes (Fig. S5 and Table S2).

We next wondered whether these differentially expanded B-cell clones recognize the epitopes exposed after the removal of the glycans on HA. To this end, variable regions of heavy- and light-chain genes from the amplified HA-specific B-cell clones

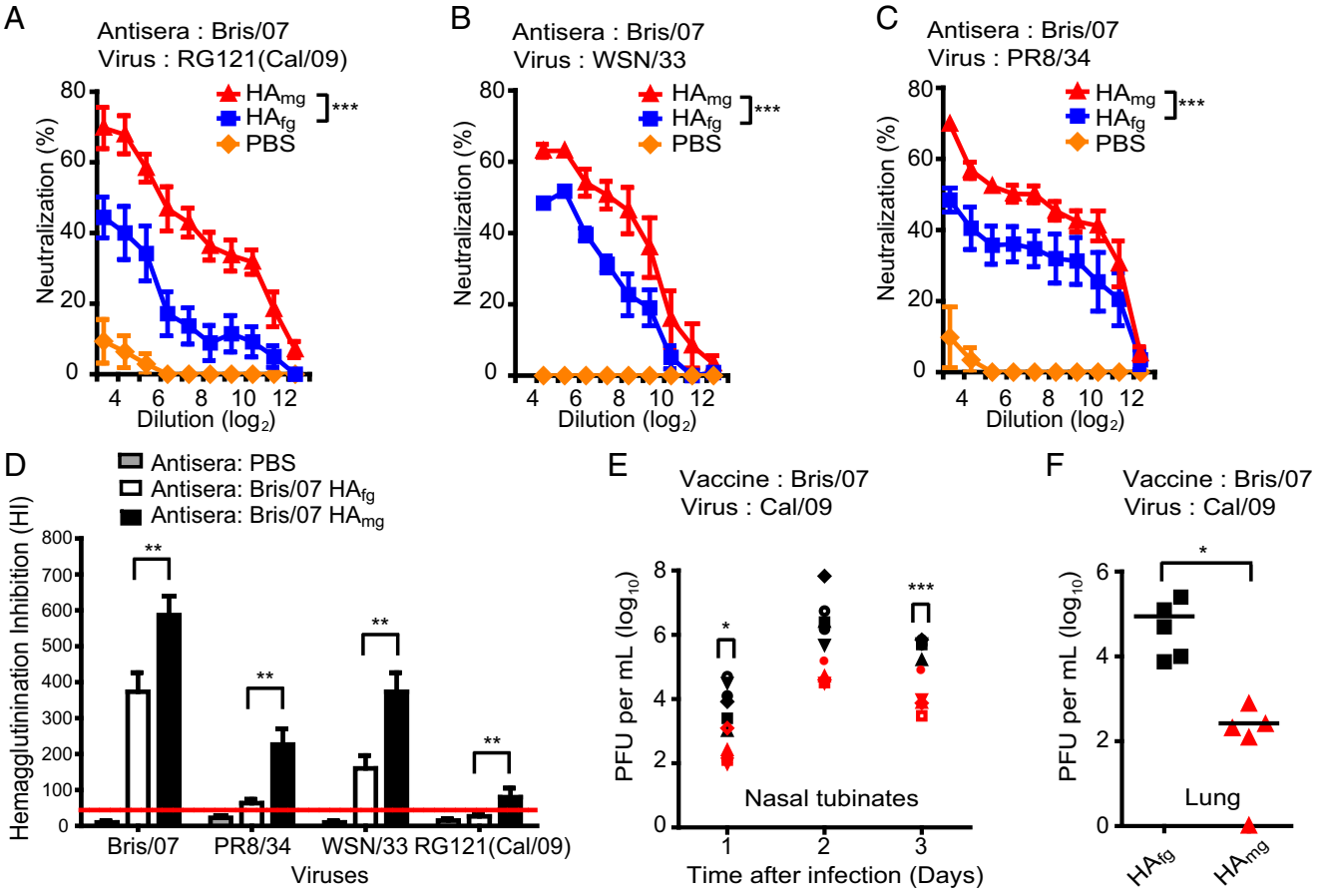

Fig. 4. HA_{mg} vaccination induced cross-strain protection in ferrets. Bris/07 HA_{fg} and HA_{mg} were used as vaccines to determine their ability to induce cross-protective immune response in ferrets. (*A*) Ferrets vaccinated with Bris/07 HA vaccines and assayed against Cal/09 virus. (*B*) Ferrets vaccinated with Bris/07 HA vaccines against WSN/33 virus. (*C*) Ferrets vaccinated with Bris/07 HA vaccines against PR8/34 virus. The antisera from ferrets vaccinated with Bris/07 HA_{mg} neutralize Cal/09, WSN/33, and PR8/34 viruses with a high titer in contrast to Bris/07 HA_{fg}. (*D*) HI assay indicates good neutralizing antibody production from HA_{mg} vaccines against the viruses Cal/09, WSN/33, and PR8/34. (*E*) The vaccinated ferrets were challenged with a lethal dose (100 LD_{50}) of Cal/09. Viral titers (black symbols represent HA_{fg}-vaccinated ferrets and red symbols represent HA_{mg}-vaccinated ferrets) were measured in nasal washes collected on indicated days and after serial titration by using plague forming unit (PFU) assay. (*F*) The inoculated ferrets were euthanized on day 4, and the lung viral titers were also measured with PFU assay. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

were further subcloned into an expression vector to produce recombinant antibodies for analysis of their binding to the HA of diverse influenza virus strains, including Bris/07, Cal/09, WSN/33, PR8/34, H3, H5, and Flu B viruses (Fig. 3*D*). The Ig vector control antibody, which cannot recognize HA, served as a negative control, and the positive control monoclonal antibody F10 with broad neutralizing activity (12) can only bind to the HA from Bris/07 and Cal/09 (Fig. 3*D*) under the same dilution condition used for the other tested antibodies. Two recombinant monoclonal antibodies, clones 557 and 621, generated in this study were further examined. We found that clone 557 binds to the HA from Cal/09 better than to the HA from Bris/07, WSN/33, and H3 but not to the HA from PR8/34 and Flu B; we also found that clone 621 binds to all tested HAs, except the HA from the H5 virus, suggesting that the HA-specific B-cell clones identified here may recognize a broad spectrum of influenza viruses. Moreover, the antibodies produced by clones 557 and 621 bound Bris/07 and Cal/09 HA_{mg} more strongly than HA_{fg} or HA_{ug} (Fig. 3*E*), suggesting that additional epitopes near or at the glycan sites of HA may be recognized by the HA_{mg}-induced antibodies. Sequence similarity search of the glycosylation site sequences (12 aa N- and C-terminal to Asn residue, a total of 25 aa for each glycosylation site) from either Bris/07 or Cal/09 HA was performed against human proteome by using BLAST (http://blast.ncbi.nlm.nih.gov), and no significant hits were found, implying that adverse cross-reactivity with human proteins may not be expected when vaccinated with HA_{mg}.

Cross-strain neutralization and protection induced by HA_{mg} vaccination were also tested in ferrets. Human and avian influenza viruses can replicate efficiently in the respiratory tract of ferrets without prior adaptation (18). The specificity of the resulting immune responses indicated that the antisera from the Bris/07 HA_{mg}-immunized ferrets were able to neutralize Cal/09, WSN/33, and PR8/34 viruses with a significantly higher titer compared with the antisera from HA_{fg} vaccination (Fig. 4 *A*–*C*). Similar cross-reactivity was also observed in the HI assay (Fig. 4*D*). Significantly lower virus titers in the washes of nasal turbinates at days 1 and 3 after infection were observed from the HA_{mg}-vaccinated ferrets (Fig. 4*E*), and virus growth and replication were consistently considerably lower in the lung tissues from the HA_{mg}-vaccinated ferrets (Fig. 4*F*).

Discussion

Antigenic drift, mostly caused by the accumulation of mutations in HA, is the major cause of the frequent failure of influenza vaccines from previous seasons to provide good protection against currently circulating influenza strains (19). Changes in epitopes, including the occasional addition or removal of glycosylation sites, escape host immune recognition (20–22). The complexity and variation of glycosylation on HA are, thus, important factors to be considered in influenza vaccine design. In addition, the 20% difference in the HA sequence in Bris/07 and Cal/09 is mostly on the surface where glycosylation takes place, whereas the sequences in the interior regions are relatively unchanged (23). A consensus HA-based DNA vaccine has been shown to protect against diverse avian influenza viruses (24). This study opens the door to the possibility of design of a consensus HA glycoprotein vaccine in monoglycosylated form that can protect against a broad range of seasonal and pandemic influenza viruses.

Materials and Methods

DNA sequences of recombinant HAs are from H1N1 strains of Cal/09 and Bris/07 and synthesized with codons optimized for human cell expression. HAs with different glycosylations were produced by transient transfection of expression vectors in 293 EBNA or 293S cells (ATCC), purified by affinity chromatography, and treated with Endo H or PNGase F (New England Biolabs). All animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee of Academia Sinica. Mice and ferrets were immunized intramuscularly with HA (20 and 50 μg, respectively) and 50 μg Alum (Sigma) at weeks 0 and 2, blood was collected at week 4, and virus challenges were performed at week 5. Statistical analyses were by the program Prism (GraphPad Software). More details are in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Yueh-Hsuan Chan, Yu-Jou Lu, and Jhih-Jia Liao for technical assistance. This work was supported by Academia Sinica and Taiwan National Science Council Grants 102-2325-B-001-009 (to K.-I.L.) and 102-2325-B-001-016 (to C.M.).

1. Russell CA, et al. (2008) The global circulation of seasonal influenza A (H3N2) viruses. *Science* 320(5874):340–346.
2. Bommakanti G, et al. (2010) Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge. *Proc Natl Acad Sci USA* 107(31):13701–13706.
3. Steel J, et al. (2010) Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio* 1(1):e00018-10.
4. Eckert DM, Kay MS (2010) Stalking influenza. *Proc Natl Acad Sci USA* 107(31):13563–13564.
5. Wang TT, et al. (2010) Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. *Proc Natl Acad Sci USA* 107(44):18979–18984.
6. Kanekiyo M, et al. (2013) Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. *Nature* 499(7456):102–106.
7. Wrammert J, et al. (2011) Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *J Exp Med* 208(1):181–193.
8. Wang TT, et al. (2010) Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. *PLoS Pathog* 6(2):e1000796.
9. Corti D, et al. (2010) Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. *J Clin Invest* 120(5):1663–1673.
10. Ekiert DC, et al. (2009) Antibody recognition of a highly conserved influenza virus epitope. *Science* 324(5924):246–251.
11. Corti D, et al. (2011) A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science* 333(6044):850–856.
12. Sui J, et al. (2009) Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol* 16(3):265–273.
13. Dreyfus C, et al. (2012) Highly conserved protective epitopes on influenza B viruses. *Science* 337(6100):1343–1348.
14. Ekiert DC, et al. (2011) A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science* 333(6044):843–850.
15. Ekiert DC, et al. (2012) Cross-neutralization of influenza A viruses mediated by a single antibody loop. *Nature* 489(7417):526–532.
16. Yoshida R, et al. (2009) Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses. *PLoS Pathog* 5(3):e1000350.
17. Wang C-C, et al. (2009) Glycans on influenza hemagglutinin affect receptor binding and immune response. *Proc Natl Acad Sci USA* 106(43):18137–18142.
18. van Riel D, et al. (2007) Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. *Am J Pathol* 171(4):1215–1223.
19. Boni MF (2008) Vaccination and antigenic drift in influenza. *Vaccine* 26(Suppl 3):C8–C14.
20. Rudneva IA, Ilyushina NA, Timofeeva TA, Webster RG, Kaverin NV (2005) Restoration of virulence of escape mutants of H5 and H9 influenza viruses by their readaptation to mice. *J Gen Virol* 86(Pt 10):2831–2838.
21. Tsuchiya E, et al. (2002) Effect of addition of new oligosaccharide chains to the globular head of influenza A/H2N2 virus haemagglutinin on the intracellular transport and biological activities of the molecule. *J Gen Virol* 83(Pt 5):1137–1146.
22. Abe Y, et al. (2004) Effect of the addition of oligosaccharides on the biological activities and antigenicity of influenza A/H3N2 virus hemagglutinin. *J Virol* 78(18):9605–9611.
23. Chen J-R, Ma C, Wong C-H (2011) Vaccine design of hemagglutinin glycoprotein against influenza. *Trends Biotechnol* 29(9):426–434.
24. Chen M-W, et al. (2008) A consensus-hemagglutinin-based DNA vaccine that protects mice against divergent H5N1 influenza viruses. *Proc Natl Acad Sci USA* 105(36):13538–13543.