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Guiding the Immune Response against Influenza Virus Hemagglutinin toward the Conserved Stalk Domain by Hyperglycosylation of the Globular Head Domain

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Influenza virus hemagglutinin consists of a highly variable and immunodominant head domain and a more conserved but immunosubdominant stalk domain. We introduced seven N-linked glycosylation sites in the hemagglutinin head domain to shield the immunodominant antigenic sites. The hyperglycosylated hemagglutinin enhanced stalk-directed seroreactivity while dampening the head response in immunized mice. Upon influenza virus challenge, mice vaccinated with the hyperglycosylated hemagglutinin were better protected against morbidity and mortality than mice receiving the wild-type hemagglutinin.

Currently, most influenza virus vaccines consist of a combination of three or four seasonal virus strains predicted to circulate in the following season. Influenza virus hemagglutinin (HA) and neuraminidase are the most abundant viral glycoproteins on the surface of the virus. Antibodies elicited by conventional vaccines target primarily the immunodominant globular head domain of HA (1–3). These antibodies generally bind four antigenic sites proximal to the receptor binding site (4, 5), and they neutralize the virus by obstructing its binding to the receptor on the cell surface. The antigenic regions are highly variable, and current vaccines therefore offer limited protection against drifted strains or other HA subtypes.

Recently, broadly neutralizing antibodies have been described that are reactive against a wide variety of strains or subtypes (6–13). Many of these antibodies target the stalk domain of HA, which is more conserved than the highly variable head domain. These antibodies do not block virus attachment but rather inhibit the conformational changes required for membrane fusion, block the cleavage of HA to HA1 and HA2, or prevent the egress of new virions from the plasma membrane of the infected cell (6–8, 10, 12). It has been proposed that a vaccine capable of eliciting high titers of stalk-directed antibodies could confer broader protection than current vaccines and therefore eliminate the need for annual vaccination (14, 15). Such a vaccine could also offer protection against emerging pandemic strains of influenza.

We aimed to redirect the immune response away from the immunodominant and variable head domain in order to focus the immune response on the conserved stalk domain. To this end, we constructed an HA with a hyperglycosylated globular head domain. We hypothesized that by masking the antigenic sites in the globular head with carbohydrates, we could suppress the immune response to the globular head domain and redirect the immune response toward the more conserved stalk domain.

The number of glycosylation sites in the globular head domain of HA has increased over time (16–18). It was shown that the addition of glycosylation in the head domain prevents the binding of monoclonal antibodies (19) or antisera raised against less glycosylated HAs (18, 20) and decreased the need for other escape substitutions in the antigenic sites during antigenic drift (17, 21). These studies suggest that the introduction of additional glycosylation sites in the globular head of the HA alters its antigenicity. Therefore, hyperglycosylation of the HA globular head may be a good way to redirect the antibody response toward the conserved stalk domain.

Using site-directed mutagenesis (primer sequences available upon request), we introduced seven N-linked glycosylation sites in the immunodominant antigenic sites of the HA head domain (4) (Fig. 1). We used the HA of A/Puerto Rico/8/34 virus (PR/8) as our model, because the PR/8 HA contains very few glycosylation sites. Only one of the naturally occurring glycans is situated in the head domain, although it does not seem to cover any of the antigenic sites. Glycans were introduced at the Ca, Cb, Sa, and Sb antigenic sites (Fig. 1A and B). Nearly all N-linked glycosylation sites within different H1 hemagglutinins appear in just two of the four antigenic sites, being Cb (e.g., glycan at position 90) and Sa (e.g., positions 142, 171, and 178), or outside the classical antigenic sites (e.g., positions 71 and 104) (20, 22). Four of the seven introduced glycosylation sites of the hyperglycosylated HA correspond to naturally occurring glycosylation in antigenic sites Cb and Sa (positions 90, 142, 171, and 178). To shield the other two antigenic sites, glycosylation sites were introduced in the antigenic sites Ca and Sb based on the HA structure and, when possible, using existing Asn residues.

The wild-type and mutant genes for full-length and soluble HA with the addition of a trimerization domain and His tag were cloned into the pCAGGS expression vector (23) for transient transfection into mammalian cells using linear polyethylenimine (Polysciences) (24). The hyperglycosylated HA expressed to levels similar to those of the wild-type HA glycoprotein. An increase in molecular weight as demonstrated by SDS-PAGE analysis with subsequent Coomassie staining and Western blot analysis confirms that additional glycans are expressed on the surface of the hyperglycosylated proteins (Fig. 1C). However, because of limitations of the resolution of standard SDS-PAGE gels and expected

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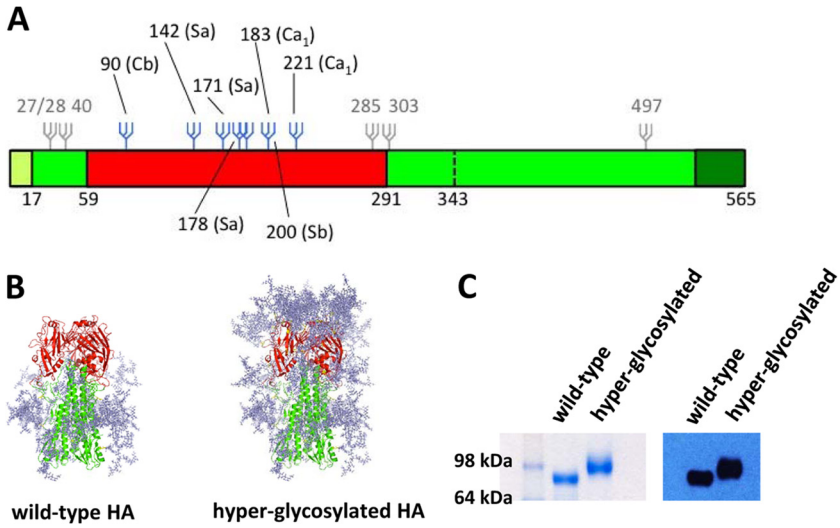

FIG 1 Covering antigenic sites of HA with N-linked glycosylation. (A) Schematic of the influenza HA and the position of introduced glycans. The signal peptide is shown in light green, the head and stalk domain of the ectodomain in red and green, respectively, and the transmembrane and cytoplasmic domains are depicted in dark green. Glycans indicated in gray are present in the wild-type HA. Additional glycans in the hyperglycosylated HA are depicted in blue. (B) Modeling of introduced glycans on the crystal structure of the PR/8 HA (PDB 1RU7). Original and additional glycans were modeled on the crystal structure of the PR/8 HA using GlyProt (37) and drawn using PyMol (DeLano Scientific). Because of modeling limitations, only 6 out of the 7 added potential N-linked glycans are present on the hyperglycosylated HA model. (C) The ectodomain of the wild-type HA and the hyperglycosylated HA containing the T4-foldon trimerization domain and a His tag were expressed in 293T cells, and purified glycoproteins were run on an SDS-PAGE polyacrylamide gel under denaturing and reducing conditions. A clear increase in molecular weight is present for the hyperglycosylated HA compared to the wild-type HA, indicating the presence of additional glycosylation.

variation in glycosylation site usage and glycan chain heterogeneity (25–27), it is not possible to determine whether each of the newly created glycosylation sites is efficiently glycosylated.

Immunofluorescence microscopy showed good binding by conformational antibodies to the stalk domain of the hyperglycosylated HA (7, 11, 28, 29), suggesting that the stalk domain is properly folded (Fig. 2A). On the other hand, antibodies targeting the antigenic sites at the globular head domain of the PR/8 HA did not bind the hyperglycosylated HA (Fig. 2B), suggesting effective shielding of the antigenic sites. This was further confirmed by enzyme-linked immunosorbent assay (ELISA) binding studies (Fig. 2C), showing no binding of five different head-directed antibodies to purified soluble hyperglycosylated HA, while the binding of stalk-directed antibody KB2 was not affected. It is most likely that this loss of binding is caused by the presence of a glycan, though we cannot exclude the effect of the mutation "introducing" the glycosylation site on the binding of these antibodies.

Soluble wild-type and hyperglycosylated HA proteins containing a T4-foldon trimerization domain and His tag were expressed in 293T (ATCC CRL-11268) cells upon transient transfection and purified using Ni-nitrilotriacetic acid (NTA) beads (Qiagen) as described previously (30). A mammalian expression system was chosen because mammalian cells utilize large complex glycans, in contrast to other expression systems like insect cells (25). Naive 6- to 8-week-old BALB/c mice were vaccinated intramuscularly with 10 μg of protein adjuvanted with 5 μg of poly(I·C) (Invivogen) three times in 3- to 4-week intervals. Serum samples were collected 3 weeks after the second and third immunizations. All mouse experiments were performed under the guidelines of the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee (permit LA09-00266).

Seroreactivity induced by either wild-type HA or the hyperglycosylated variant was analyzed by ELISA against purified influenza viruses as described previously (31, 32). First, reactivity to the parental strain, PR/8, was tested. Mice vaccinated with wild-type HA elicited higher titers of antibodies than hyperglycosylated HA, which was especially apparent after two immunizations (Fig. 3A and B). After three immunizations, the difference in reactivity to PR/8 was less apparent. This could be the result of boosting epitopes on the head that are not shielded efficiently by the introduced glycans or due to an increase in stalk antibodies. To assay binding to the stalk domain without interference from head reactivity, we made use of the chimeric HAs that were recently developed in our lab (32, 33). The chimeric HAs used in the described experiments contain the stalk of PR/8 HA (H1) with the head domain of an exotic HA subtype to which no cross-reactivity is expected, like H9 or H5. Thus, chimeric HAs with a PR/8 stalk and an H9 or H5 head are named cH9/1 and cH5/1, respectively. Observed seroreactivity to these chimeric HAs induced by our immunization strategy is therefore solely directed toward the H1 stalk domain, as the mice are naive to both H9 and H5 subtype HAs. Here, we demonstrate that the seroreactivity to the stalk is higher upon vaccination with the hyperglycosylated HA than in mice vaccinated with the wild-type HA. After two immunizations, the difference was 3-fold, while the difference increased to almost 9-fold after three immunizations (Fig. 3C and D). Thus, the enhanced seroreactivity to the stalk domain is accompanied by a dampened response to the globular head domain, suggesting that the introduction of glycans to shield immunodominant epitopes is an effective way to alter the antigenicity of the influenza virus HA.

To further determine whether immunization with the hyperglycosylated HA increased the breadth of the antibody response, heterologous seroreactivity was tested against two distinct H1N1

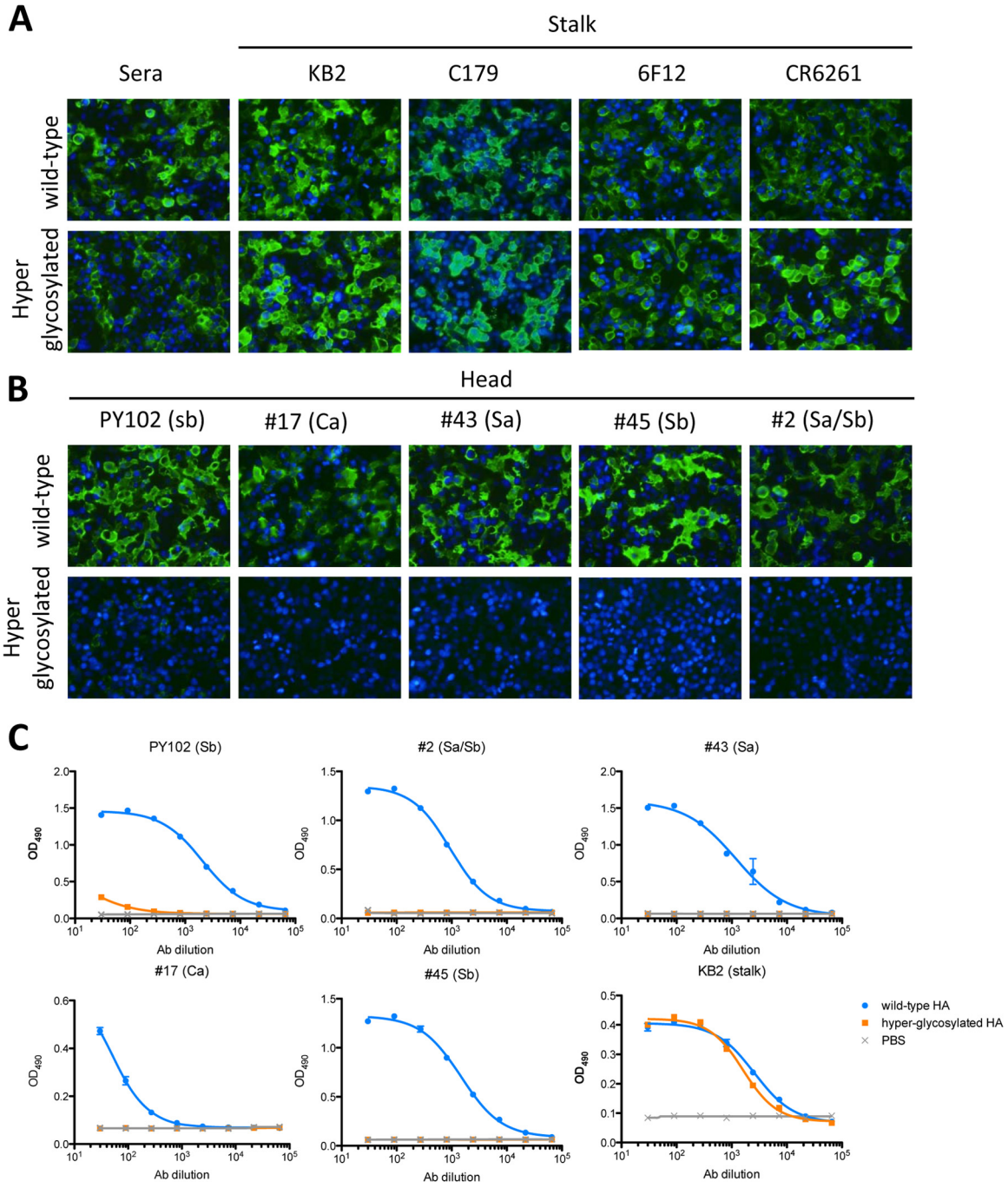

FIG 2 Hyperglycosylated HA expresses well at the surface of transfected mammalian cells and is able to bind stalk antibodies. Plasmids encoding wild-type or hyperglycosylated full-length HA were transfected into 293T cells. The HA was stained 24 h posttransfection with PR8 antiserum or stalk-directed antibodies KB2 (28), C179 (29), 6F12 (11), and CR6261 (7) (A) or head-directed antibodies PY102 (38), 17, 43, 45, and 2 (B). Monoclonal antibodies 17, 43, 45, and 2 were generated for this research as described before (11). The indicated binding epitope at the PR/8 HA was mapped using escape studies in eggs for each newly generated HA head antibody as described previously (4). Binding of stalk-directed antibodies to the hyperglycosylated HA is not changed compared to that of the wild-type HA. In contrast, introduction of glycosylation sites in the antigenic sites of the globular head domain of the HA abrogated binding of head-directed antibodies. (C) Binding of five different head-directed antibodies to purified, soluble HA was tested in ELISAs. Head-directed antibodies did not bind to hyperglycosylated HA, while binding of stalk-directed antibody KB2 was not affected.

strains, A/USSR/92/77 and A/Brisbane/59/07 (Fig. 3E and F). ELISAs show superior reactivity of sera from hyperglycosylated HA-vaccinated mice to these H1N1 viruses compared to that of the wild-type HA-vaccinated mice. In addition, enhanced reactivity was also detected against a low-pathogenic variant of a pandemic human H5N1 strain, A/Vietnam/1203/04 (Fig. 3G). Together, these analyses show that hyperglycosylated HA is able to elicit a robust heterologous and heterosubtypic response directed

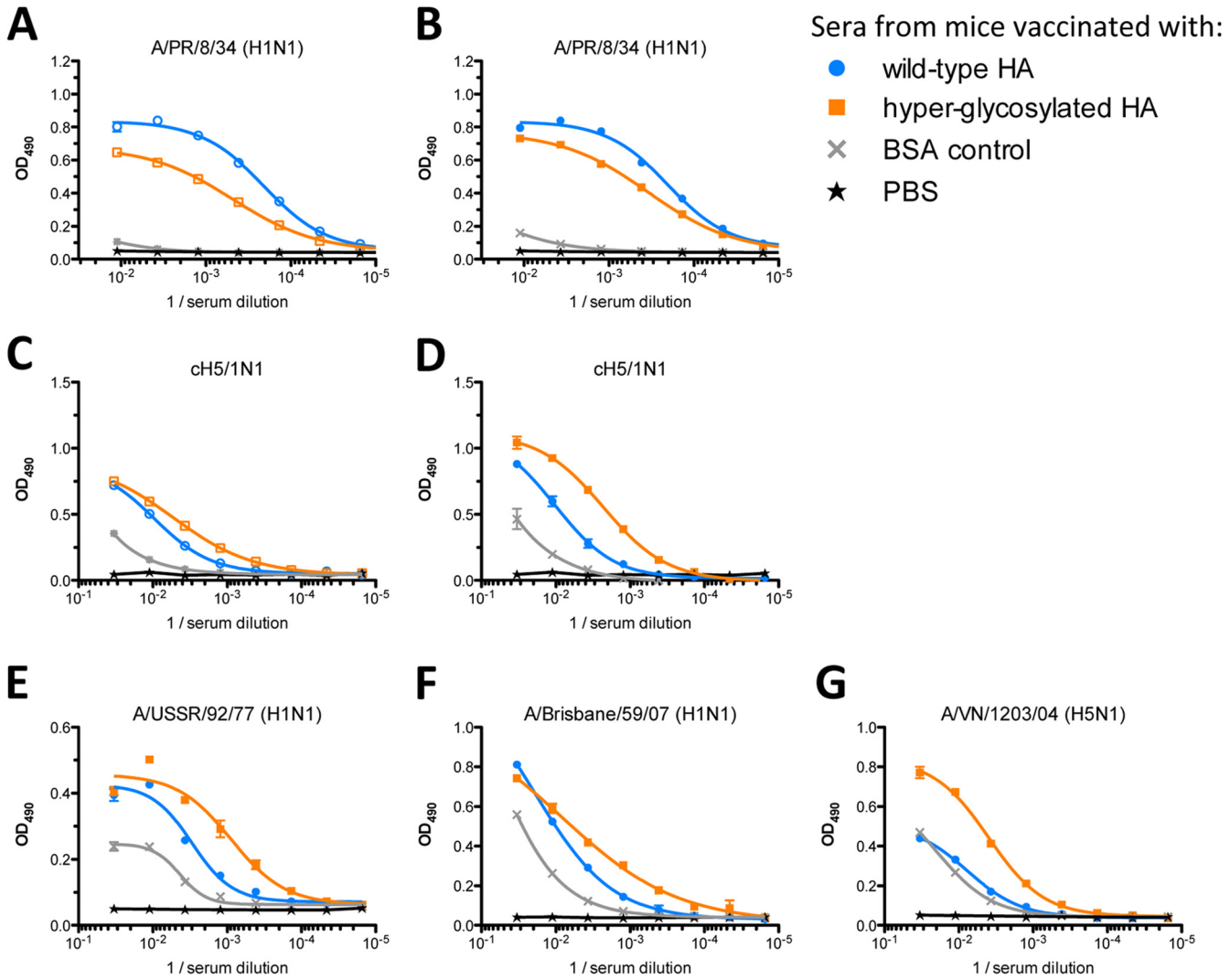

FIG 3 Immunization of mice with the hyperglycosylated HA induces high titers of stalk-directed antibodies against distinct seasonal H1N1 and pandemic H5N1 viruses. Sera originating from mice vaccinated with the wild-type or hyperglycosylated HAs were assayed by ELISA for reactivity against purified viruses: A/Puerto Rico/8/34, cH5/1, A/USSR/92/77, A/Brisbane/59/07, and A/Vietnam/1203/04. Sera originating from mice vaccinated with the hyperglycosylated HA were less reactive to virus containing the full-length PR/8 HA compared to mice vaccinated with the wild-type HA after two (A) and three (B) immunizations. In contrast, seroreactivity to a virus bearing the cH5/1 HA, which consists of a subtype H5 head domain and the PR/8 stalk domain, was higher for sera originating from mice vaccinated with the hyperglycosylated HA than in mice vaccinated with the wild-type HA. This difference in reactivity was observed after two immunizations (C) and became more apparent after three immunizations (D). The seroreactivity to distinct H1N1 viruses (E, F) and an H5N1 virus (G) was also enhanced in mice vaccinated with the hyperglycosylated HA compared to that in mice immunized with the wild-type HA.

against the stalk of HA while reducing the immune response to the globular head compared to wild-type HA.

We hypothesized that the enhanced stalk reactivity induced by the hyperglycosylated vaccine construct would result in improved *in vivo* efficacy against viral infection. To test this hypothesis, mice were infected with 20 mouse median lethal doses (mLD_{50}) of a "7 + 1" reassortant PR/8 virus expressing cH9/1 (32, 34) 4 weeks after the final boost. In addition to mice immunized with either the hyperglycosylated or wild-type HA, control animals vaccinated with bovine serum albumin (BSA) adjuvanted with poly(I·C) were included. Because the mice were naive to the H9 head and all other viral gene products, any observed protection should be attributed solely to the response specific to the HA stalk domain. Weight loss was monitored daily for 14 days after challenge, and animals that lost 20% or more of their initial body weight were scored as dead and euthanized. As shown in Fig. 4A, mice vaccinated with the hyperglycosylated HA lost minimal weight compared to the control animals. Mice vaccinated with wild-type HA lost significantly more weight and began succumbing to infection at day five postinfection. Thus, mice vaccinated with the hyperglycosylated HA were better protected against morbidity (weight loss; Fig. 4A) and mortality (survival; Fig. 4B) than mice vaccinated with the wild-type HA on the basis of enhanced reactivity to the HA stalk. In addition to virus neutralization (11, 34), other mechanisms of action for broadly reactive stalk-directed antibodies, including antibody-dependent cellular cytotoxicity (35) and complement-dependent lysis (36), may have contributed to the *in vivo* protection observed in our animal experiments.

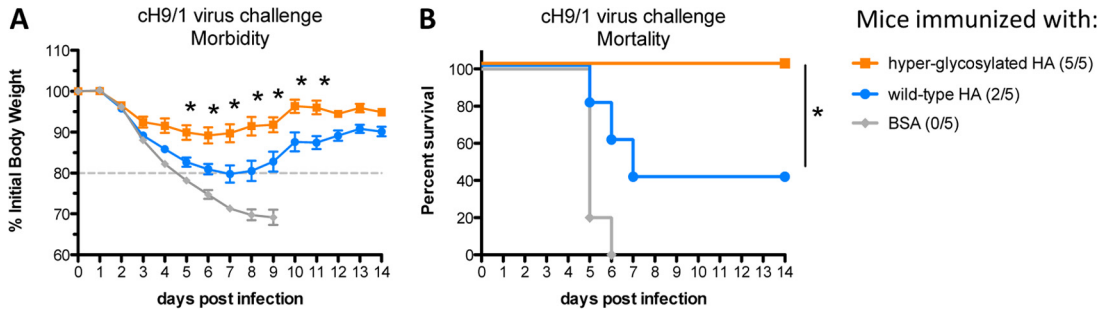

FIG 4 Mice immunized with the hyperglycosylated HA show protection against morbidity and mortality upon challenge with a cH9/1 virus. (A) Mice ($n = 5$) were challenged with 20 mLD_{50} of a virus containing the cH9/1 HA and weighed daily. Vaccination with the hyperglycosylated HA protected mice better from morbidity as measured by weight loss (A) and mortality (B) compared to mice vaccinated with the wild-type HA. Significant differences between the hyperglycosylated HA and the wild-type HA-vaccinated mice were observed at the indicated points. Statistical significance for weight curves was calculated using multiple unpaired *t* tests correcting for multiple comparisons with the Holm-Sidak method (*, $P < 0.001$; Prism version 6.0b; GraphPad Software). Statistical significance for the Kaplan-Meier survival curve was calculated with the Mantel-Cox test (*, $P < 0.05$; Prism version 6.0b; GraphPad Software).

Here, we demonstrate that immunization with the hyperglycosylated HA protein induces higher titers of stalk-directed antibodies, while dampening the immune response to the globular head domain, than immunization with the wild-type HA. The induced humoral immune response is broadly cross-reactive to distinct H1N1 viruses and to a pandemic human H5N1 strain. The hyperglycosylated HA-immunized animals were better protected against a lethal viral infection, solely on the basis of HA stalk-directed immunity, than animals immunized with the wild-type HA. The data suggest that shielding immunodominant epitopes at the antigenic sites shifts the head/stalk antibody balance toward the conserved stalk domain.

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