Glycan Shielding of the Influenza Virus Hemagglutinin Contributes to Immunopathology in Mice

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Rationale: Pandemic influenza viruses historically have had few potential sites for N-linked glycosylation on the globular head of the hemagglutinin (HA) on emergence from the avian reservoir. Gain of glycans within antigenic sites of the HA during adaptation to the mammalian lung facilitates immune evasion.

Objectives: In this study, we sought to determine in mice how exposure to highly glycosylated viruses affects immunity to poorly glycosylated variants to model the emergence of a novel pandemic strain of a circulating subtype.

Methods: We engineered the 1968 H3N2 pandemic strain to express an additional two or four potential sites for glycosylation on the globular head of the HA. Mice were infected sequentially with highly glycosylated variants followed by poorly glycosylated variants and monitored for immune responses and disease.

Measurements and Main Results: The mutant with four additional glycosylation sites (+4 virus) elicited significantly lower antibody responses than the wild-type or +2 virus and was unable to elicit neutralizing antibodies. Mice infected with the +4 virus and then challenged with wild-type virus were not protected from infection and experienced significant T-cell-mediated immunopathology. Infection with a recent seasonal H1N1 virus followed by infection with the 2009 pandemic H1N1 elicited similar responses.

Conclusions: These data suggest that sequential infection with viral strains with different surface glycosylation can prime the host for immunopathology if a neutralizing antibody response matching the T-cell response is not present. This mechanism may have contributed to severe disease in young adults infected with the 2009 pandemic virus.

Keywords: influenza virus; glycosylation; pandemic; immunopathology; pneumonia

N-linked glycosylation is a common post-translational modification of viral glycoproteins (1). Glycans are added during transit of the endoplasmic reticulum and Golgi apparatus at the glycosylation site motif Asn-X-Ser/Thr, where X may represent any amino acid except proline (2). A diverse repertoire of glycans can result from this process, and these modifications may have substantial effects on the biology of the proteins. Accumulation of oligosaccharide chains near antigenic sites is an evolutionarily advantageous adaptation of viruses, such as influenza virus and HIV, as these glycans can disrupt binding of neutralizing antibodies (3, 4). Glycosylation of the influenza virus surface glycoprotein hemagglutinin (HA) also affects folding, entry, and innate recognition (4–8). Because of enhanced recognition and clearance by collectins, the benefit provided to influenza viruses through immune evasion is balanced by attenuation

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

It is unclear why a subset of young adults experience severe viral pneumonitis during influenza infections.

What This Study Adds to the Field

This study proposes a novel mechanism for influenza-mediated pulmonary disease. Data from a mouse model demonstrate that T-cell-mediated immunopathology can contribute to severe influenza pneumonitis in the setting of infection with a poorly glycosylated virus in a host who has been previously exposed to a highly glycosylated variant.

of infection (9). It has also been proposed that glycosylation of the HA can interfere with T-cell responses by disrupting glycopeptide-major histocompatibility complex (MHC) interactions (10), but the relevance of this to immunity is unclear at present.

Historically, accumulation of sites of potential N-linked glycosylation has been a linear, step-wise progression during adaptation of influenza A viruses to humans (9, 11). The three 20th century pandemic strains all contained HA proteins derived from the avian reservoir and had little to no glycosylation of their surface proteins. Each lineage gained several glycosylation sites in or near antigenic regions over time, such that recent seasonal H1N1 and H3N2 strains are highly glycosylated (9, 11). Along with the four conserved sites in the stalk, the HA of most H1N1 influenza viruses circulating between 1949 and 2008 had at least two additional sites of potential glycosylation in the vestigial esterase domain, and another two in the sialic acid binding (Sa) site of the receptor-binding domain (11, 12). These latter two are predicted to interfere with antibody-mediated neutralization (13). Similarly, the HA proteins of recent seasonal H3N2 viruses contain up to 12 sites of potential glycosylation, with only 5 of them in the stalk (9).

We undertook the present study to determine the impact of exposure to highly glycosylated variants of influenza viruses on adaptive immunity to native, poorly glycosylated variants of the same subtype. The hypothesis to be tested was that glycosylation would interfere with antibody recognition of the HA, leaving the host vulnerable to subsequent infection with a poorly glycosylated variant, such as a nascent pandemic strain. During the course of this work, a new pandemic of the H1N1 subtype emerged (14), providing a direct test of this hypothesis in the human population. This pandemic has been characterized by sparing of elderly persons, who have cross-reactive antibodies from exposure to antigenically similar and poorly glycosylated H1N1 viruses in childhood (11, 15, 16), but high clinical attack rates and unusual, severe lower respiratory tract infections in healthy children and young adults (11, 17, 18). Our data reported here suggest that T-cell-mediated immunopathology en-

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gendered by prior exposure to highly glycosylated H1N1 variants may contribute to serious pulmonary disease during pandemic H1N1 infection in this latter age group.

METHODS

Viruses

Three influenza viruses were generated by reverse genetics (19) based on a 2:6 reassortant (wild-type [WT] virus) containing the HA and neuraminidase (NA) of A/Hong Kong/1/68 (H3N2) and the six internal gene segments of A/Puerto Rico/8/34 (H1N1). Before virus rescue, the HA gene segment was modified using site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA) by previously described methods (9) to generate two variants with additional sites of potential glycosylation at positions 63 and 126 (+2 virus), or at positions 63, 126, 248, and 135 (+4 virus). These sites were selected as they represent changes that appeared naturally during evolution at periods of significant antigenic drift in 1975 (+2 variant) and 1995 (+4 variant). H1N1 subtype viruses A/Beijing/359/95 and A/California/4/09 were acquired from Drs. Robert Webster and Richard Webby (St. Jude Children's Research Hospital [SJCRH]). All viruses were passaged a single time in eggs for stocks and were fully sequenced to ensure no inadvertent mutations occurred during rescue or passage.

Infectious Model

Eight-week-old BALB/c or C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME), were maintained in a Biosafety Level 2 facility in the Animal Resources Center at SJCRH. All experiments were approved by the Institutional Animal Care and Use Committee of SJCRH and were done under anesthesia with inhaled isoflurane (2.5%). B-cell deficient μ MT mice were obtained from Dr. Paul Thomas (SJCRH). All described experiments were done with BALB/c mice except those involving μ MT mice, which were done with the C57BL/6 strain. For primary infections, influenza viruses were inoculated intranasally into anesthetized mice at a dose of 1×10^4 50% tissue culture infectious dose (TCID₅₀) in 100- μ l sterile phosphate-buffered saline (PBS) (50 μ l in each nostril). Challenge viruses were administered by the same route at a dose of 1×10^6 TCID₅₀, equivalent for each virus to about three 50% lethal doses (MLD₅₀) in naive animals. All mice were followed for at least 21 days after infection or challenge. Depletion of CD4⁺ or CD8⁺ T cells or both were accomplished as described (20) by intraperitoneal administration of antibodies obtained from Dr. Paul Thomas (SJCRH) on Days -3, -1, 1, 3, and 5 relative to virus challenge. Depletion efficiency was confirmed by flow cytometric analysis to be greater than 90% in representative mice pulled from the groups as described (20). Viral titers were determined on Madin-Darby canine kidney (MDCK) monolayers from homogenized lungs as described (20). Serum from convalescent mice was derived from blood obtained by puncture of the retroorbital venous plexus with a sterile glass pipette.

Immune Assays

Serum was separated from blood collected from the retroorbital plexus of mice 20 or 120 days after primary viral infection, heat inactivated and treated with receptor-destroying enzyme, and then analyzed for influenza-specific antibodies by hemagglutination-inhibition, micro-neutralization, and ELISA assays as described (20). Bronchoalveolar lavage fluid (BALF) cell counts were determined by flow cytometry as described (21).

Histopathology

Microscopic evaluation of lungs was performed by an experienced veterinary pathologist (K.L.B.) blinded to study purpose and design and to group composition. A semiquantitative grading scheme was used to score two parameters: the overall character of the pneumonic process and the pathology specifically observed in the interstitium and terminal airways as described (21). For detection of T cells by immunohistochemistry, sections were stained with rabbit anti-human CD3 antibodies (sc-1127; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1,500 followed by biotinylated donkey anti-goat antibody (sc-2042; Santa Cruz) at 1:200 for 30 minutes. Slides were then incubated with the chromogen 3,3'-diaminobenzidine (DAB, cat # K3466; DAKO,

Carpenteria, CA) for 5 minutes and were counterstained with hematoxylin.

Statistical Analysis

Comparison of survival between groups of mice was done with the log rank chi-square test on the Kaplan-Meier survival data. Weight loss, cell counts, antibody titers, histopathology scores, and viral titers were compared between groups by analysis of variance using SigmaStat for Windows (SysStat Software, Inc., V 3.11). A *P* value of less than 0.05 was considered significant for these comparisons.

RESULTS

Glycosylation Affects Induction of Neutralizing Antibodies

To test the hypothesis that glycosylation can prevent development of neutralizing antibodies, we infected mice with three viruses that differed only in the number of potential glycosylation sites on the globular head of the HA and assessed the resulting immune responses. These were otherwise isogenic viruses derived from the pandemic strain A/Hong Kong/1/68 (H3N2) that were engineered by reverse genetics to have no additional sites of potential glycosylation (WT), two extra sites at positions 63 and 126 (+2), or four extra sites at positions 63, 126, 248, and 135 (+4). The HA of these three viruses exhibited different electrophoretic mobility patterns as previously described (9). In addition, they exhibited similar replication efficiency *in vitro* and for several days after infection *in vivo* but were cleared more rapidly from the lungs of mice as described (9).

Antibody titers were measured from mice 21 days after primary infection. Significantly lower responses directed against the WT and +2 viruses were demonstrated from mice infected with the +4 virus by the hemagglutination inhibition, micro-neutralization, and ELISA assays (Figure 1). Neutralization capacity was particularly affected. Sera from mice infected with WT or +2 viruses recognized and neutralized both the WT and +2 viruses, but demonstrated poor reactivity against the +4 virus. None of the sera recognized the +4 virus very well in comparison with WT or +2 recognition. By ELISA, decreasing total antibody responses were seen with increasing glycosylation from WT to +2 and +2 to +4, but the decrease in reactivity by HA inhibition and neutralization was seen only in the +4 variant. Both IgG1 and IgG2a subclasses could be detected after primary infection, with IgG2a predominating (*see* Figure E1 in the online supplement). As with total IgG, weaker responses of both subclasses were observed from mice infected with +4 compared with those infected with WT. These data support the hypothesis that highly glycosylated variants elicit poor antibody responses, such that neutralization of lesser glycosylated viruses is impaired.

Glycosylation Impairs Immunity to Challenge

In accord with prior data (9), the addition of glycosylation sites attenuated the viruses in mice on primary infection such that only the WT virus caused significant weight loss at a TCID₅₀ of 1×10^4 (Figure 2A). On challenge of convalescent mice 21 days later with a lethal dose of WT virus, mice previously infected with the WT and +2 viruses were not productively reinfected and did not lose weight (Figures 2B and 2C). Mice previously infected with the +4 virus, however, could be reinfected (Figure 2B), lost significant weight (Figure 2C), and only one of six (17%) of the animals followed for mortality survived. In the inverse experiment, mice infected initially with WT virus did not lose weight on challenge with +4 virus or experience significant illness (Figure E2A). In addition, mice infected first

Figure 1. Antibody titers against influenza virus hemagglutinin (HA) glycosylation mutants. Sera from groups of six mice infected with wild-type (WT) virus or viruses with an additional two (+2) or four (+4) potential sites for glycosylation were tested for antibodies to the WT, +2, or +4 viruses by (A) HA inhibition (HAI) assay, (B) microneutralization (MN) assay, and (C) ELISA. HAI and MN titers are expressed on a $\log_2$ scale as the geometric mean $\pm$ SD. ELISA titers are expressed as the mean of total IgG (γ -fraction) $\pm$ SD. An asterisk (*) indicates a significant difference ($P < 0.05$) by analysis of variance (ANOVA) compared with the corresponding titers directed against the WT and +2 viruses (within-group comparison). A double asterisk (**) indicates a significant difference ($P < 0.05$) by ANOVA against the specified virus compared with the corresponding titers from mice infected with the WT or +2 viruses (between-groups comparison).

with the +2 virus were protected from reinfection with the +2 virus (Figure E2B). To demonstrate that these effects on immunity to challenge were mediated by differences in adaptive, not innate, immunity, the experiment was repeated with the secondary challenge occurring 121 days after primary infection with similar results. The mice initially infected with +4 could be reinfected by WT, lost significant weight, and had deficient antibody responses compared with WT-primed mice (Figure E3). We conclude that a deficient adaptive immune response to the highly glycosylated variant allows reinfection with a poorly glycosylated variant.

Morbidity after Challenge Is T-Cell Mediated

Because virus was cleared by Day 3 after reinfection (Figure 2B), it was unlikely that viral replication accounted for the

Figure 2. Outcomes after primary and secondary infections with influenza virus hemagglutinin glycosylation mutants. Groups of mice were infected with 1×10^4 50% tissue culture infectious dose (TCID₅₀) of the wild-type (WT) virus or viruses with an additional two (+2) or four (+4) potential sites for glycosylation and then challenged 21 days later with 1×10^6 TCID₅₀ of the WT virus. Mean weight loss $\pm$ SD after the (A) primary infections and (C) secondary infection is pictured. An asterisk (*) indicates a significant difference ($P < 0.05$) by analysis of variance at that time point compared with the other two viruses. (B) Viral lung titers 1 and 3 days after reinfection with the WT virus in mice infected 21 days earlier by the WT, +2, or +4 viruses or mock-infected with phosphate-buffered saline.

progressive illness and deaths seen in mice reinfected with WT virus after primary infection with +4 virus. To determine the arm of the immune system responsible for this effect, we repeated the challenges in B-cell-deficient and T-cell-depleted models. B-cell-deficient μ MT mice could be reinfected with WT virus after initial infection with either +4 or WT virus (Figure 3), and although both sets of mice lost some weight initially on reinfection, mice initially infected with WT virus recovered rapidly, whereas mice initially infected with +4 virus lost significant weight (Figure 4A), and again only one out of six (17%) of the mice survived. Mice initially infected with the +2 virus showed the same pattern of disease as WT-infected mice. Mice depleted of both CD4⁺ and CD8⁺ T cells at the time of secondary challenge could be reinfected by WT virus only if they were initially infected with +4 (Figure 3) and showed similar viral titers as had nondepleted mice (Figure 2B). Strikingly, +4 primed/T-cell-depleted mice did not lose weight on reinfection with WT virus (Figure 4B). Both CD4⁺ and CD8⁺ T cells contributed to this immunopathology as depletion of either subset alone was intermediate in phenotype to depletion of both subsets (Figure 4C).

Figure 3. Lung titers in immunodeficient mice infected with influenza virus hemagglutinin glycosylation mutants. Groups of three to four B-cell-deficient μ MT mice or wild-type (WT) C57Bl/6 mice were infected with WT virus or virus with an additional four (+4) potential sites for glycosylation and then challenged 21 days later with WT virus. WT C57Bl/6 mice were depleted of both CD4⁺ and CD8⁺ T cells before secondary challenge. Lung titers 1 and 3 days after secondary challenge are pictured. The horizontal bar indicates the mean titer at that time point.

To confirm that the T-cell-mediated pathology was the cause of illness and death in mice primed with the highly glycosylated +4 virus then challenged with the poorly glycosylated WT virus, cell counts in BALF and lung histopathology were examined. Three days after WT challenge, after clearance of virus (Figure 2B), significantly more T cells were found in BALF of mice initially infected with +4 virus than in uninfected mice or mice initially infected with WT or +2 viruses (Figure 5E). This difference was seen in both CD4⁺ and CD8⁺ subsets of T cells (Figure E4). Analysis of lung sections demonstrated immunopathologic changes in mice primed with the +4 virus compared with other groups. The lungs of mice initially infected with WT showed scattered lymphoid nodules in the parenchyma with no significant changes evident in the airways (Figure 5C). Lungs from mice initially infected with +4 virus and challenged with WT had prominent inflammatory changes in a perivascular and peribronchiolar distribution (Figures 5A and 5D), whereas mice sequentially infected and challenged with the +4 virus had normal-appearing lungs (Figures 5A and 5B). Hemorrhage and edema were more commonly observed in +4-primed mice than in those initially infected with the WT virus. Confirming the results from BALF studies, there was a marked infiltration of T cells evident in the lungs of mice challenged with WT after +4 infection. These T cells were seen to form cuffs around vessels and extensively infiltrate airway walls (Figure 5H). Only scattered CD3⁺ cells were evident in lungs from mice initially infected with WT, with most of these found within the lymphoid nodules (Figure 5G). Mice primed only with PBS then challenged with WT virus showed few T cells in the lungs and changes in the airways typical of primary viral infection (Figure 5F).

To rule out a contribution of the antibodies themselves to disease (e.g., through antibody-dependent enhancement of infection [22]), we repeated infection and challenge studies in μ MT mice and provided serum derived from recovered wild-type mice previously infected with either the WT or the +4 virus by adoptive transfer. These mice did not experience enhanced disease and in fact saw protection from death and modest improvements in weight loss on WT challenge with antibodies from either set of mice (Figure E5). We conclude that a mismatched adaptive immune response characterized by poor neutralizing antibody production but robust T-cell responses contributes to severe disease on reinfection with a poorly glycosylated influenza virus by allowing immunopathology.

Figure 4. Outcomes in immunodeficient mice infected with influenza virus hemagglutinin glycosylation mutants. Groups of four to six B-cell-deficient μ MT mice or wild-type (WT) C57Bl/6 mice were infected with WT virus or viruses with an additional two (+2) or four (+4) potential sites for glycosylation and then challenged 21 days later with WT virus. Groups of WT C57Bl/6 mice were depleted of either CD4⁺ or CD8⁺ T cells, both, or neither before secondary challenge as indicated in the figures. Mean weight loss $\pm$ SD after secondary challenge in (A) μ MT mice, (B) CD4⁺ and CD8⁺ depleted or nondepleted mice, and (C) mice depleted of CD4⁺ or CD8⁺ T cells, both, or neither is pictured. An asterisk (*) indicates a significant difference ($P < 0.05$) by analysis of variance at that time point compared with the other groups.

The Pandemic H1N1 Strain Causes Immunopathology in Mice Primed with a Seasonal H1N1 Virus

To determine whether this finding in an H3N2 model system of immunopathology after sequential exposure to highly glycosylated then poorly glycosylated viruses extended to H1N1 subtype viruses, we infected mice with a recent seasonal H1N1 virus and then challenged them with the pandemic H1N1 strain. The seasonal strain has five sites of potential glycosylation on the globular head at positions 63, 94, 129, 163, and 271 (H1 numbering), whereas the pandemic H1N1 strain only has a single glycosylation site at position 94 (11). Mice initially infected with the pandemic H1N1 were protected from infection and weight loss on challenge with pandemic H1N1, whereas mice primed with the seasonal H1N1 were infected and lost significant weight on challenge (Figures 6A and 6E). Histopathologic findings with the pandemic H1N1 challenge also paralleled the work in the H3N2 system; increased inflammatory changes were seen in mice preinfected with the seasonal H1N1 (Figure 6C) compared with mice initially infected with the pandemic H1N1 (Figure 6B). In addition, an increased influx of T cells into the lungs of mice primed with seasonal H1N1 (Figure 6G) was demonstrated on challenge with the pandemic

Figure 5. T-cell mediated immunopathology contributes to poor outcomes. (A) Mean $\pm$ SD semi-quantitative histopathology scores of groups of mice infected with wild-type (WT) virus or virus with an additional four (+4) potential sites for glycosylation or mock-infected with phosphate-buffered saline (PBS) and then challenged 21 days later with WT or +4 virus. An asterisk (*) indicates a significant difference ($P < 0.05$) by analysis of variance (ANOVA) compared with the other groups. Lungs from mice infected with (B) +4 virus followed by +4 challenge, (C, E) WT virus followed by WT challenge, (D, H) +4 virus followed by WT challenge, or (F) mock-infected with PBS before WT challenge. Hematoxylin and eosin staining of lungs (20 $\times$) shows (C) formation of lymphoid

nodules (large arrowheads) and (D) extensive perivascular and peribronchiolar infiltration of lymphocytes. (E) Mean number of CD3⁺ T cells/mL of bronchoalveolar lavage fluid $\pm$ SD recovered from groups of four mice infected with WT, +2, or +4 viruses or mock-infected with PBS and then challenged 21 days later with WT virus. An asterisk (*) indicates a significant difference ($P < 0.05$) by ANOVA compared with the other groups. (H) Staining for CD3⁺ of lungs (20 $\times$) from +4-primed mice challenged with WT shows extensive infiltration of T cells into the walls of airways (arrows) compared with other groups (F) primed with PBS followed by WT, or (G) primed with WT followed by WT.

H1N1 compared with mice preinfected with pandemic H1N1 (Figure 6F). This was not merely a difference in the ability to be reinfected, because the lung pathology and T-cell infiltration was worse with reinfection of primed mice (Figures 6C and 6G) than that observed in naive mice infected with a lethal dose of pandemic H1N1 (Figures 6D and 6H). We conclude from these data that immunopathology can contribute to severe lower respiratory tract disease from the pandemic H1N1 strain in hosts previously infected with highly glycosylated seasonal H1N1 viruses.

DISCUSSION

A central puzzle regarding the age distribution of pandemic influenza is why severe disease is sometimes disproportionately

seen in young adults, resulting in the most extreme case in the “w-shaped” mortality curve of the 1918 pandemic (23). The prevailing hypothesis at present is that immunological memory from prior exposure to related viruses protects older adults, whereas damaging immune responses in young adults that are not seen in children account for the increased disease (24). The immunologic memory argument appears to hold true for the current H1N1 pandemic, with solid evidence suggesting that persons exposed to poorly glycosylated, antigenically related viruses in the early 20th century retain immunity that is protective from the novel 2009 pandemic strain (11, 12, 15, 16). The mechanism for increased immunopathology has not been adequately explained, however, nor has the sparing of younger children relative to older children and young adults. Based on these preclinical data, we propose the novel hypothe-

Figure 6. Outcomes in mice infected with seasonal or pandemic H1N1 strains. Groups of six mice were infected with either a recent seasonal H1N1 strain (sH1N1) or the 2009 pandemic strain (pH1N1) and then were challenged 21 days later with a lethal dose of the pandemic H1N1. (A) Viral lung titers 3 days after reinfection with the pH1N1 virus in mice infected 21 days earlier by sH1N1 or pH1N1. (B–D) Hematoxylin and eosin and (F–H) anti-CD3⁺ staining of lungs (20 $\times$) from mice infected with the (B, F) pH1N1, (C, G) sH1N1, or (D, H) mock-infected with phosphate-buffered saline and then chal-

lenged with pH1N1. (E) Mean weight loss $\pm$ SD after challenge with pH1N1 21 days after primary infection with pH1N1 or sH1N1. An asterisk (*) indicates a significant difference ($P < 0.05$) by analysis of variance at that time point compared with the other group. TCID₅₀ = tissue culture infectious dose.

esis that the severe disease seen in this age group is mediated in part by robust T-cell responses in the setting of inadequate antibody neutralization. This would explain both the enhanced disease in young adults, who will have experienced influenza several times in their lives and developed T-cell memory, and the sparing of children, who are susceptible to infection but do not experience immunopathology because their cellular immunity has not been adequately primed. A similar scenario is unlikely for the 1968 pandemic, because only those older than 50 years of age would have immunological memory of the proposed H3N8 virus that might have circulated until 1917 (25). Thus, the lack of a peak of illness in young adults during the 1968 pandemic fits with our hypothesized mechanism.

In this report we demonstrate that glycosylation of the globular head of influenza virus HA interferes with development of protective immune responses to reinfection with similar viruses. Serum antibody levels to a highly glycosylated virus were globally diminished as measured by three different assays, with neutralization being most markedly affected. This lack of neutralization allowed productive reinfection by a nearly identical virus, although earlier clearance was achieved than is seen with infection of the naive host. The differences in the magnitude of the T-cell responses between WT- and +4-primed animals on WT challenge likely relates to the difference in total antigen load derived from this period of productive replication. The ability of glycans to mask antibody epitopes has been previously demonstrated using monoclonal antibody and vaccine approaches (4, 26), but the consequences of sequential infections as happen in humans over time had not been explored. In this model, the mismatch between the humoral and cellular arms of the immune response, with poor neutralization yet robust T-cell-mediated responses, resulted in immunopathology despite achieving viral control. Although it has been proposed that glycosylation can alter T-cell responses in a negative manner by interfering with binding of MHC to peptides carrying retained glycans (10), this does not appear to be the case in our model as T-cell responses were more prominent after infection with the heavily glycosylated virus. The alternate hypothesis that lack of neutralization actually enhances T-cell responses by allowing prolonged replication of the virus during primary infection also seems unlikely, because the highly glycosylated viruses are cleared slightly earlier through a surfactant protein D (SP-D)-mediated mechanism (9). Interestingly, both CD4⁺ and CD8⁺ T cells contributed to the immunopathology seen with reinfection in this report. It has been previously shown that many CD4⁺ and CD8⁺ epitopes are conserved between recent seasonal and the novel pandemic H1N1 strains (27–29), suggesting that both cell types might contribute to immunopathology in humans by this mechanism. A potential role for NA was not assessed in this study, but is possible because NA is also a glycoprotein and accumulates additional glycosylation sites over time (9). Because NA immunity alone can provide protection from influenza in animal models (30), the contribution of NA to this process should be assessed.

The dual nature of protective and pathologic T-cell responses to influenza virus is well appreciated in laboratory models (31) but has not been conclusively shown to impact disease in humans. Autopsy studies from the current pandemic suggest that pathologic findings in fatal disease are similar to those of past pandemics. Tracheitis, bronchitis, and diffuse alveolar damage have been the most common findings, often in conjunction with characteristic pathology related to superimposed secondary bacterial infections (32, 33). Hemorrhage has been a common finding in human cases, as it was in our study. Interestingly, Mauad and colleagues noted a prominence of CD8⁺ T cells throughout the lungs in infected cases relative

to controls, suggesting that an aberrant immune response contributed to the pathology (33) and providing a potential clinical link to our hypothesized mechanism. However, these case series may be of limited usefulness for this analysis as the majority (16/21 [76%] [33] and 31/34 [91%]) of the subjects in each study had significant comorbidities that likely predisposed to development of severe lower respiratory tract disease. Further autopsy study of fatal cases in previously healthy young adults is indicated to address our hypothesis and determine whether the pathology in those cases differs significantly from that of routine cases. One important caveat for this discussion is that the distinction between the contributions of virus-induced cytotoxicity and immunopathology is likely to be blurred and difficult to separate in human autopsy cases, particularly with more highly pathogenic viruses that can efficiently access the lower respiratory tract. It is also likely that this mechanism is not the sole reason for severe disease in this age group, as differences in prior exposure, vaccination history, and genetic variability likely all contribute to an individual's response to a particular virus.

The impact of glycosylation on immune responses has implications for both pandemic preparation and vaccine design. Would a similar scenario occur if a virus expressing a poorly glycosylated H3 were to cross over from the avian reservoir? Persons born after the mid-1980s may not produce functional neutralizing antibodies to poorly glycosylated strains, but are likely to have robust T-cell memory if they have experienced influenza virus infections previously. Because H2N2 viruses only circulated for a short time before being replaced in 1968, and accumulated minimal additional glycosylation sites during that period, a new H2 pandemic is unlikely to exhibit this unique age distribution of severe disease. Should vaccines be designed with or without glycosylation of the HA? Our data suggest that highly glycosylated viruses are poor immunogens, so perhaps the larger risk is infection with a pandemic strain with low glycosylation, not drift mutants that are successively attenuated with the addition of glycans to the globular head (9). Bright and colleagues showed that matching glycosylation pattern had little effect on induction of immunity to an H5 HA using a DNA-based vaccine (34), but the most glycosylated HA tested had only three potential sites on the globular head, and these were sites naturally occurring in avian viruses, not those selected in mammalian lungs. If limited glycosylation or glycosylation at specific sites does not significantly impair immunity, then a strategy of adding those sites predicted to accumulate during antigenic drift to vaccine strains might prevent emergence of escape variants (13). However, this might also have the unintended effect of decreasing virus neutralization allowing the immunopathologic responses discussed in this paper.

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