Development of Influenza B Universal Vaccine Candidates Using the “Mosaic” Hemagglutinin Approach

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ABSTRACT Influenza B viruses cause seasonal epidemics and are a considerable burden to public health. However, protection by current seasonal vaccines is suboptimal due to the antigenic changes of the circulating strains. In this study, we report a novel universal influenza B virus vaccination strategy based on “mosaic” hemagglutinins. We generated mosaic B hemagglutinins by replacing the major antigenic sites of the type B hemagglutinin with corresponding sequences from exotic influenza A hemagglutinins and expressed them as soluble trimeric proteins. Sequential vaccination with recombinant mosaic B hemagglutinin proteins conferred cross-protection against both homologous and heterologous influenza B virus strains in the mouse model. Of note, we rescued recombinant influenza B viruses expressing mosaic B hemagglutinins, which could serve as the basis for a universal influenza B virus vaccine.

IMPORTANCE This work reports a universal influenza B virus vaccination strategy based on focusing antibody responses to conserved head and stalk epitopes of the hemagglutinin. Recombinant mosaic influenza B hemagglutinin proteins and recombinant viruses have been generated as novel vaccine candidates. This vaccine strategy provided broad cross-protection in the mouse model. Our findings will inform and drive development toward a more effective influenza B virus vaccine.

KEYWORDS antibody-dependent cell-mediated cytotoxicity, broad cross-protection, conserved epitopes, nonhemagglutination inhibition antibody responses, nonneutralizing antibody responses, sequential vaccination

Influenza B viruses almost exclusively infect humans and can cause severe disease and even death (1–3). Children who lack preexisting immunity to influenza viruses are more susceptible to type B virus infection than adults (4–6). Globally, about 25% of all influenza virus-positive clinical cases are caused by influenza B viruses (7), and occasionally, influenza B viruses dominate influenza seasons (8–11). For example, in the 2017–2018 season in Europe, ~65% of influenza cases were caused by influenza B virus, which resulted in severe disease in adults 40 years of age or older (11). Even in influenza A virus-dominated seasons, influenza B viruses can cause a second wave of infection when cases of influenza A viruses wane (12, 13).

Two lineages of influenza B virus are currently cocirculating, the B/Yamagata/16/88-like lineage and the B/Victoria/02/87-like lineage, which diverged in the 1980s from a common ancestor (14). These two lineages are antigenically different from each other, as determined by hemagglutination inhibition reactivity of ferret antisera (14, 15). There is evidence that a seasonal trivalent vaccine containing influenza B antigens from one lineage elicits cross-protection against the other lineage in some vaccine recipients, possibly due to the complex exposure history of humans (16, 17). To achieve more

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effective protection, seasonal vaccines that include antigens from both influenza B lineages are often recommended to prevent influenza B disease. However, due to the antigenic drift of the viral glycoproteins, seasonal vaccines need to be updated frequently according to surveillance recommendations (18). Vaccine efficacy substantially decreases if the vaccine strains do not match the circulating strains (19, 20). To overcome these limitations, influenza B virus vaccines that afford broader protection than the current seasonal vaccines are in development. Examples include a peptide-based vaccine derived from the highly conserved cleavage site and fusion peptide of the hemagglutinin 0 (HA0) polypeptide which protected against influenza B viruses from both lineages in mice (21, 22). Another protein-based vaccine strategy is the chimeric influenza B HA approach, in which the head domains of the influenza B virus HAs are replaced with head domains of different exotic influenza A virus HAs that humans typically are not exposed to. Sequential immunization of different chimeric B HAs (B cHAs) successfully elicited stalk-specific antibody responses that conferred broad cross-protection against multiple influenza B virus strains in mice (23–27). Importantly, the identification of broadly cross-protective monoclonal antibodies targeting the HA (28) in animal models or humans is encouraging for the development of a universal influenza B virus vaccine. The HA-specific antibodies include human monoclonal antibodies, such as CR8033, CR8071, CR9114, 5A7, and 46B8 (29–33), as well as broadly cross-reactive mouse monoclonal antibodies that have been isolated recently (34, 35).

In this study, we report a novel vaccination strategy focusing on the HA of influenza B virus to induce broadly reactive antibody responses against both the head and the stalk domain. The influenza B virus HA contains four major antigenic sites in the head domain, the 120 loop, the 150 loop, the 160 loop, and the 190 helix (36). These major antigenic sites play an immunodominant role in eliciting antibody responses in the host (37, 38). However, these major antigenic sites are subject to antigenic drift under immune selection pressures. Other subdominant conserved epitopes in the head and stalk domains are recognized by the above-mentioned cross-reactive monoclonal antibodies and are auspicious targets for a universal influenza B virus vaccine. To overcome the dominance of the major antigenic sites and redirect the immune system to the more conserved epitopes to achieve cross-protective antibody responses, we developed a vaccination strategy based on “mosaic” influenza B virus HAs (B mHA). Within the B mHAs, the major antigenic sites were replaced with corresponding sequences from exotic influenza A HAs, such as H5, H8, H11, and H13. From our proof-of-principle animal study using B mHA recombinant proteins as immunogens, we have shown that sequential vaccination with three different B mHAs successfully elicited broadly reactive antibody responses that protected mice from challenge with both homologous and heterologous influenza B viruses.

RESULTS

Design of the B mHA viruses. To redirect the antibody responses to the subdominant epitopes of the HA, we previously reported a strategy designated the chimeric HA (cHA) approach. We have shown that sequential immunization with cHAs that contain different exotic head domains on a constant stalk domain could focus the immune system to the conserved stalk domain and elicit broadly cross-reactive immunity. This cHA vaccine approach was applied to both influenza A and influenza B viruses and successfully elicited cross-protection against multiple influenza virus strains in animals (23, 24, 26, 27, 39). In the case of influenza B HAs, the head domains were derived from exotic avian influenza A HAs and combined with B stalks (23) (Fig. 1A). This was necessary because influenza B virus does not have subtypes, and humans are the sole natural reservoir of influenza B virus (with rare exception of cases in seals) (1, 40). Although the influenza B cHAs largely maintained the conformation of the B stalk, they did not allow for the rescue of viable viruses, possibly because the A virus head domain is functionally incompatible with the B virus stalk. To obtain viable viruses in which the immunodominance of the head domain is ablated, we decided to replace only the

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immunodominant major antigenic sites instead of the entire head domain. These sites have been previously characterized as the 120 loop, the 150 loop, the 160 loop, and the 190 helix (36), which have also been shown to elicit most of the antibody responses in infected mice and ferrets (37). We replaced these major hemagglutination inhibition (HI) reactive antigenic sites with sequences from different exotic HAs of influenza A viruses while retaining noncanonical epitopes in the head and stalk domains. We

**FIG 1** Illustration and design of the B mHAs. (A) Illustration of the B cHA and B mHA approaches. The upper panel shows the monomeric B cHAs with head domains from HAs of the influenza A virus. Different colors represent different head domains. Similarly, in the lower panel, the monomeric B mHAs with major antigenic sites replaced with sequences from HAs of the influenza A virus are shown. Different colors represent corresponding sequences from different influenza A HAs. The monomeric HAs were based on the B/Yamanashi/66/98 HA (PDB accession no. 4M44). Arrows represent sequential immunizations. (B) Amino acid sequence (1 to 300) alignment of the B mHAs with the wild-type HA. The amino acid sequence alignment was performed with the HAs of B/Yamagata/16/88, mH5/BYam, mH8/BYam, mH11/BYam, and mH13/BYam using Clustalw2 (H5, A/Vietnam/1203/04 H5N1-PR8-IBCDC-RG/GLP; H8, A/mallard/Sweden/24/02 H8N4; H11, A/shoveler/Netherlands/18/99 H11N9; H13, A/black-headed gull/Sweden/1/99 H13N6). The major antigenic sites of B HA and corresponding H5, H8, H11, and H13 sequences, as well as compensatory mutations, are highlighted (120 loop, cyan; 150 loop, green; 160 loop, blue; 190 helix, red; compensatory mutations, yellow). The putative N-linked glycosylation sites are indicated by the red letter “N.”
hypothesized that sequential immunization with different mHAs will focus the immune responses to the subdominant conserved epitopes in both head and stalk domains (41) (Fig. 1A). Since influenza B HAs present high structural similarity between the two lineages and the amino acid differences among influenza B HAs mainly locate within the major antigenic sites, we do not expect significant differences in using different B HAs as the backbone, because the antigenic sites will be replaced. Here, we selected the B/Yamagata/16/88 HA as the backbone of the mosaic HAs. We constructed four different B mHAs in which the major antigenic sites were replaced by corresponding sequences from H5, H8, H11, or H13. Viruses with B mHAs were successfully rescued in the B/Malaysia/2506/04 mouse-adapted (MA) strain backbone. They were designated mosaic H5/BYam (mH5/BYam), mosaic H8/BYam (mH8/BYam), mosaic H11/BYam (mH11/BYam), and mosaic H13/BYam (mH13/BYam) viruses. The amino acid sequence alignment of the wild-type (wt) B/Yamagata/16/88 HA with four mosaic HAs is shown in Fig. 1B. Several compensatory mutations which are not in the major antigenic sites were also identified in the B mHA viruses (Fig. 1B). Of note, putative N-linked glycosylation sites were found within the 150 loop and the 160 loop (Asn-X-Ser/Thr) (Fig. 1B). Removal of the glycanshield resulting from sequence replacement might also significantly affect the antigenicity of these sites (42–44). Our B mHA approach should eliminate all four antigenic sites (rather than keeping the two putative glycosylation sites).

**The B mHA viruses grow robustly in eggs and are attenuated in mice.** To evaluate the fitness of the B mHA viruses, we determined the growth kinetics of the four B mHA viruses compared to that of the wt B/Malaysia/2506/04 MA virus in embryonated chicken eggs. We inoculated 10-day-old embryonated chicken eggs with 500 PFU of each virus per egg. Allantoic fluids were collected at 8 h, 24 h, 48 h, and 72 h postinoculation, and infectious virus titers were determined by plaque assays on Madin-Darby canine kidney (MDCK) cells. All of the B mHA viruses grew to robust peak titers (48 h) above 10^8 PFU/ml and were only mildly attenuated compared to the wild-type virus, suggesting that the B mHAs were functional (Fig. 2A). We next examined the pathogenicity of the mosaic viruses in mice. Six- to 8-week-old female BALB/c mice were infected intranasally with each virus at 10, 10^2, 10^3, 10^4, or 10^5 PFU per mouse. Weight loss and survival were monitored to calculate median lethal doses (LD_{50}), and mice were euthanized when exceeding 25% weight loss. One mouse infected with 10^3 PFU and all mice infected with 10^4 and 10^5 PFU of the wild-type virus succumbed to infection, while all the B mHA viruses caused little or no weight loss within the dose range. Only the mice infected with mH5/BYam virus at the highest dose showed limited weight loss, but they quickly recovered (Fig. 2B). These results indicated that the B mHA viruses displayed functional HAs that convey attenuated pathogenicity in BALB/c mice.

**The HI reactive major antigenic sites of the B HA were ablated, while the conserved cross-reactive epitopes were preserved in the B mHAs.** We next determined if known conserved epitopes were retained in the B mHAs by examining the binding of four cross-reactive human monoclonal antibodies (MAbs) against the stalk domain (MAbs CR9114 and 5A7) (29, 31) and against the head domain (MAbs CR8033 and CR8059) (29). We also tested mouse antisera generated by sequentially immunizing BALB/c mice with the B/Lee/40 strain, the B/Malaysia/2506/04 strain, and the reassortant A/Puerto Rico/08/34 virus displaying the B/Yamagata/16/88 HA (35), which presumably contain substantial amounts of cross-reactive antibodies directed to the more conserved regions. To examine if the changes we made abrogated the binding of the cross-reactive antibodies to the B mHAs, an immunofluorescence assay (IFA) using MDCK cells infected with wild-type B/Malaysia/2506/04 virus or B mHA viruses was performed. This assay is used to identify the presence or absence of binding of the antibodies to the B mHAs. Uninfected cells served as negative controls. All the MAbs and mouse sera bound equally well to the B mHAs compared to that of the wild-type HAs, except for CR8033, which showed slightly weaker binding to the mH11/BYam HA.
This result suggested that the cross-reactive epitopes targeted by the MAbs and sera were well preserved in the B mHA viruses.

We next examined if the major antigenic sites of the influenza B HA were eliminated in the B mHA viruses using the HI assay with mouse and ferret antisera raised against the wild-type B/Yamagata/16/88 virus through infection or immunizations. The major antigenic sites of the HA are responsible for most of the HI active antibody responses, as they are relatively close to the receptor binding site (RBS) (37). Therefore, in the presence of the original antigenic sites, the HI active antibody raised against the wild-type B HA would bind to those sites, resulting in detectable HI titers. On the contrary, as the major antigenic sites have been replaced in the B mHAs, we expect that most HI active antibodies raised against wild-type HA would not bind to the B mHAs, resulting in decreased HI titers. We raised mouse and ferret sera against the wild-type B/Yamagata/88 virus through infection or immunizations. We performed HI assays to measure HI titers of the sera against the wild-type B/Yamagata/88 virus and the four B mHA viruses. As we expected, the mouse sera showed a high HI titer of 1:1,280 against the wild-type virus, while the HI titers against the mH5/B_Yam, mH8/B_Yam, and mH11/B_Yam viruses were undetectable. Even though a weak HI titer of 1:20 was observed for the mH13/B_Yam virus, the reduction of the HI titer compared to that of the wild-type virus was 64-fold (Fig. 3B). These findings were confirmed with ferret antiserum, where the HI titers against the mH5/B_Yam, mH6/B_Yam, and mH13/B_Yam viruses were decreased 16-fold (mH5/B_Yam, mH11/B_Yam, and mH13/B_Yam) or 32-fold (mH8/B_Yam) compared to that of the wild-type virus (Fig. 3C). These results demonstrated that the original major

(Fig. 3A). This result suggested that the cross-reactive epitopes targeted by the MAbs and sera were well preserved in the B mHA viruses.
antigenic sites have been ablated in the B mHA-expressing viruses. In addition, we examined if the mutated major antigenic sites of the B mHAs were antigenically distinct. To this end, we generated mouse antisera against mH5/BYam, mH8/BYam, and mH13/BYam through sublethal infection and measured HI titers of each antiserum against wild-type B/Yamagata/16/88 and the other mosaic viruses. None of the antisera against the B mHA viruses showed detectable HI titers against the wild-type virus, further confirming that the original HI reactive major antigenic sites were eliminated. The HI titers of antisera against the other mosaic viruses all showed at least an 8-fold reduction (Fig. 3D to F).

In summary, we have confirmed that several known cross-reactive epitopes were preserved in the B mHAs, which could elicit cross-reactive antibody responses. In addition, the major antigenic sites of the B HA were largely ablated in the B mHAs, while the mutated major antigenic sites in each B mHA were antigenically distinct from each other.

Sequential immunization with B mHAs protects mice against lethal challenge with influenza B viruses. To further investigate the potential of B mHA constructs as universal vaccine candidates, we performed an immunization study using recombinant trimeric B mHA proteins as immunogens, whose amino acid sequences are identical to the corresponding viral B mHA sequences, including the compensatory mutations. We selected mH13/Byam, mH5/BYam, and mH8/BYam as immunogens, since we expected three immunizations to be sufficient to redirect the antibody responses toward conserved epitopes based on our previous studies with cHA antigens (23, 26, 27, 45). We determined the sequence of each B mHA in the viruses and expressed the corresponding trimeric B mHAs using a previously reported baculovirus/insect cell expression system (46, 47). We employed a vaccination regimen that included a plasmid DNA prime followed by two protein boosts in 3-week intervals, which is similar to the regimen that was used for B cHA vaccination in our previous study (23). Four weeks after the last boost, mice were either bled for serological studies or challenged with influenza B viruses using 5 doses of the murine LD50 (mLD50) intranasally (i.n.). The vaccination groups and regimens are shown in Fig. 4A. Each group contained five
animals. The B mHA group received mH13/Byam plasmid DNA prime followed by mH5/Byam and then mH8/Byam recombinant protein boosts. The prime-only group was primed with mH13/Byam plasmid DNA and boosted twice with an irrelevant protein, bovine serum albumin (BSA). A B cHA group was also included to compare with the B mHA group. The B cHA group was primed with cH5/Byam plasmid DNA and boosted with cH8/Byam protein, followed by cH7/Byam protein. A standard-of-care control group was vaccinated with trivalent inactivated vaccine (TIV) that contained an influenza B antigen matched to the challenge virus. A mock-vaccinated group that received BSA protein and a naive mouse group were included as negative controls. All the protein boosts were performed with poly(I:C) as the adjuvant (Fig. 4A). To examine the

![Image of vaccination regimen and groups](Fig_4_A)

**FIG 4** Vaccination with influenza B mHA constructs protects mice from lethal challenge with influenza B viruses. (A) Schematic of the vaccination regimen and groups. (B) Weight loss and survival of vaccinated groups challenged with the lineage-matched B/Florida/04/06 virus. (C) Weight loss and survival of vaccination groups challenged with the lineage-mismatched B/Malaysia/2506/04 virus. (D) Day 3 and day 6 lung virus titers in vaccinated mice (n = 3). Vaccination groups in panels B, C, and D include a B mHA group, a prime-only group, a positive-control group, a mock group, and a naive group. (E) Weight loss and survival of groups of naive mice pretreated with postvaccination sera and challenged with the lineage-mismatched B/Malaysia/2506/04 virus. Postvaccination sera include B mHA sera, positive-control (matched TIV) sera, mismatched TIV sera, and naive sera. (F) Comparison of the B mHA vaccination with the B cHA vaccination in mice challenged with the lineage-mismatched B/Malaysia/2506/04 virus. Vaccination groups in panel F include a B mHA group, a B cHA group, a positive-control group (matched TIV), a mock group, and a naive group.
vaccination efficacy against the HA lineage-matched influenza B viruses, we used the mouse-adapted homologous B/Florida/04/06 (B/Yamagata/16/88-like) as the challenge virus. Mice were infected i.n. with 5 mL50 of the virus, and weight loss and survival were monitored for 2 weeks. The mosaic vaccination group showed almost no weight loss and was fully protected from lethality, while most of the mice in the prime-only group and all mice in the mock and naive groups succumbed to the infection (Fig. 4B). This demonstrated that the mosaic HA vaccination approach successfully conferred protection against the HA lineage-matched influenza B virus in mice. To examine cross-protection against the lineage-mismatched influenza B virus, we challenged mice with 5 mL50 of mouse-adapted B/Malaysia/2506/04 (B/Victoria/02/87-like) strain. Again, vaccination with B mHAs conferred full protection against the heterologous strain in mice (Fig. 4C), suggesting that cross-reactive antibodies have been elicited using this approach. To evaluate virus clearance in the lungs by cross-reactive antibodies, we repeated the vaccination/lineage-mismatched virus challenge study using the B/Malaysia/2506/04 strain. At day 3 and day 6 postchallenge, we collected lungs from each group (n = 3). Lungs were homogenized in phosphate-buffered saline (PBS), and infectious virus titers were determined by plaque assay on MDCK cells. At both day 3 and day 6, B mHA-vaccinated mice cleared the virus similarly to the matched positive control, whereas at day 6 postchallenge, almost no infectious titers were observed in either group. In contrast, the prime-only group, the mock group, and the naive group all showed comparably high lung virus titers (Fig. 4D). These findings supported that vaccination with B mHAs cross-protects mice against a heterologous influenza B virus strain, accompanied by viral clearance in the lungs.

To confirm that the cross-protection provided by B mHA vaccination was antibody mediated, we performed a serum passive transfer challenge study with a lineage-mismatched virus. Sera were harvested from vaccinated mice 4 weeks after the 2nd boost as described above. Equal amounts of serum from each mouse were pooled within each group. Four groups (n = 5) were included, the B mHA group, the positive-control (matched TIV) group, the mismatched TIV (containing heterologous virus antigens) group, and the naive group. Each mouse received 200 l of pooled sera intraperitoneally. Two hours after serum transfer, mice were infected with 5 mL50 of B/Malaysia/2506/04 virus i.n. Weight loss and survival were monitored for 2 weeks. As expected, the sera raised against B mHAs fully protected mice from virus challenge similarly to the TIV positive control and only resulted in mild weight loss. In contrast, sera against the mismatched TIV did not provide any antibody-mediated protection, as all mice succumbed to infection similarly to the naive serum group (Fig. 4E). These results confirmed that serum antibodies elicited by the B mHA vaccination conferred cross-protection against the heterologous influenza B virus infection.

We have previously shown that vaccination with recombinant B cHAs protected mice from lethal challenge with influenza B viruses (23). We next sought to compare the protective efficacy afforded by the B mHAs and the B cHAs in a lineage-mismatched virus challenge study. Mice (n = 5) in the B mHA group received the same vaccination regimen as that described above. Mice in the B cHA group were primed with cH5/BYam DNA and boosted with cH8/BYam protein and then cH7/BYam protein. Matched TIV containing the B/Malaysia/2506/04 antigen was used as the positive control. The mock vaccination group and naive group were included as negative controls (Fig. 4F). Four weeks after the second boost, mice were challenged with 5 mL50 of B/Malaysia/2506/04 virus, and weight loss and survival were monitored for 2 weeks. We observed that protection conferred by B mHAs was comparable to that of B cHAs (Fig. 4F), suggesting that the B mHA strategy could be used as an alternative to the B cHA strategy. More importantly, since we were able to rescue viable recombinant viruses with B mHAs, the B mHA approach would be suitable for production of inactivated and live-attenuated vaccines, which is currently not possible with B cHA constructs.

Vaccination with B mHAs induced broadly reactive antibody responses toward influenza B virus HA. To characterize the breadth of antibody responses induced by the B mHA approach toward multiple influenza B virus strains, we measured antibody
binding to various influenza B viruses by enzyme-linked immunosorbent assay (ELISA). Specifically, we tested three B/Yamagata/16/88-like strains, including B/Yamagata/16/88, B/Florida/04/06, and B/Phuket/3073/13, as well as three B/Victoria/02/87-like viruses, including B/Malaysia/2506/04, B/Victoria/02/87, and B/Brisbane/60/08. We first measured the binding of serum IgG to the matched B/Yamagata/16/88 and the lineage-mismatched B/Malaysia/2506/04 viruses using ten serum samples from each group. Sera raised against matched-lineage TIV were used as positive controls. We did not include the mismatched-lineage TIV sera to compare cross-reactivity with B mHA and B cHA sera, since TIV serum antibody responses target multiple viral components (including the HA, NA, and nucleoprotein). Binding signals of both B cHA sera and B mHA sera toward both viruses were comparable (Fig. 5A and D). Using pooled sera from the same ten mice within each group, we observed similar trends of binding to the other two B/Yamagata/16/88-like (Fig. 5B and C) and B/Victoria/2/87-like viruses (Fig. 5E and F), where both sera raised with B cHA and B mHA also showed high binding reactivity comparable to that of the positive-control sera. This demonstrated that the B mHA approach induced broadly reactive antibody responses toward multiple lineage-matched and mismatched influenza B virus strains.

The B mHA approach conferred Fc-mediated protection that was nonneutralizing and non-HI active. To further examine the function of antibody responses induced by B mHA immunizations, we performed HI and microneutralization (MN) assays to identify HI active and neutralizing serum antibodies, respectively. For both assays, receptor-destroying enzyme (RDE)-treated sera were pooled from 10 mice, and B/Yamagata/16/88, B/Florida/04/06, and B/Malaysia/2506/04 strains were tested. Again,
the matched TIV sera were used as positive controls. We observed that both the B cHA
and B mHA vaccination sera showed no detectable HI titers against all three viruses
(Fig. 6A). Similarly, in the MN assay, both B cHA and B mHA vaccination sera showed
negligible MN titers against all three viruses (Fig. 6B). These results showed that similar
to B cHAs (23), B mHAs mainly generated non-HI active and nonneutralizing antibodies.
This suggested that the mechanism of protection conferred by the B mHA vaccin-
ation approach is not through hemagglutination inhibition or neutralization of the
virus. Previous studies have shown that HA-specific non-HI active and nonneutral-
izing antibodies could confer protection through Fc-mediated effector functions,
such as antibody-dependent cell-mediated cytotoxicity (ADCC) (30, 48–54), antibody-
dependent cellular phagocytosis (ADCP) (55), and complement-dependent cytotoxicity
(CDC) (56). Therefore, we next tested whether serum antibodies elicited Fc-mediated
effector functions using an ADCC reporter assay. Six groups of sera were tested against
the three different viruses (Fig. 6C). As we expected, the B cHA group showed a higher

**FIG 6** Vaccination with influenza B mHA constructs generates antibodies that are non-HI active and nonneutralizing but induced high ADCC. (A) HI activity of
postvaccination sera against influenza B viruses, including B/Yamagata/16/88, B/Florida/04/06, and B/Malaysia/2506/04 strains. Vaccination sera tested include
the B mHA group, the B cHA group, the positive-control group, and the naive group. (B) Neutralizing activity of vaccination sera against influenza B viruses.
The same three viruses as those in panel A were tested. To perform the microneutralization (MN) assay, MDCK cells were infected with 100 TCID50 of each
virus for multicycle replication. The same groups of sera as those shown in panel A were tested. (C) Induction of ADCC activity of postvaccination sera in a
reporter assay. The same three viruses as those shown in panels A and B were tested. To perform the ADCC reporter assay, MDCK cells were infected with each
virus at an MOI of 5 for single-cycle replication. Sera tested include the B mHA group, the B cHA group, the prime-only group, the positive-control group, the
mock group, and the naive group. Fold induction of the reporter signals from the sera over those from the blank were analyzed. Pooled serum samples of each
group were used for all three assays. Sera were RDE treated to perform HI and MN assays shown in panels A and B.
induction of ADCC than the positive-control group, whereas the prime-only, mock, and naive groups showed almost no induction of ADCC. More interestingly, the B mHA sera appeared to exhibit a stronger induction of ADCC reporter activity than the B cHA group toward all three viruses. We speculate that this is due to the fact that a different repertoire of antibodies was induced by the B cHA group from the B mHA groups, as we did not observe differences in total antibody binding between the B mHA and the B cHA vaccination sera (Fig. 5A, B, and D). In summary, the B mHA vaccination approach likely conferred protection mainly through non-HI active, nonneutralizing antibodies that engage Fc-mediated effector functions.

**DISCUSSION**

Although there are generally fewer cases of influenza B virus infections than influenza A virus (H1N1 and H3N2) infections, the severity of disease in adults is comparable (2, 7, 57). Importantly, a universal influenza B virus vaccine, if used by a high percentage of the global population, may help to eradicate these viruses, as evolutionary rates are much lower and the animal reservoir is more restricted compared to that of influenza A viruses (22). Here, we report a universal influenza B virus vaccination strategy using mosaic influenza B virus hemagglutinins in which the major antigenic sites of HA were replaced with exotic influenza A virus HA sequences (H5, H8, H11, and H13). We have shown that the B mHAs viruses grew robustly in eggs. Importantly, the B mHA viruses express ablated major HI reactive antigenic sites while preserving several important head and stalk cross-reactive epitopes in the B HA. In a proof-of-principle study using B mHA recombinant proteins as immunogens in mice, we have shown that sequential B mHA vaccination protected mice from challenge with both lineage-matched and mismatched viruses, resulting in the clearance of virus from the lung. Serum transfer studies confirmed that this cross-protection was antibody mediated, and that sera raised with the B mHAs were superior to sera raised with mismatched TIV. Importantly, the stalk- and head-based B mHA approach conferred cross-protection equally well as the stalk-based B cHA approach.

Similar to the B cHA approach, the B mHA approach induced broadly reactive antibodies without detectable HI or in vitro neutralizing activities. Interestingly, we observed a trend of the B mHA approach to induce antibodies with stronger ADCC activity than the B cHA approach, even though the total binding signals were comparable. We speculate that the B mHA approach is able to elicit a different antibody repertoire which contains cross-reactive, non-HI active, and nonneutralizing head antibodies that induce stronger ADCC than the anti-stalk antibodies. However, further studies are required to characterize the exact epitopes recognized by these antibodies induced by the B mHAs. Importantly, we were able to rescue influenza B viruses with the B mHAs, whereas viruses with B cHAs have not been generated. Therefore, the B mHA vaccine approach is more versatile for a universal influenza virus vaccine (than the B cHA strategy), as it is suitable for multiple vaccine production platforms, including recombinant HA, inactivated, and live attenuated constructs. Finally, the B mHA vaccine constructs express immune subdominant conserved epitopes in the HA head.

Previous studies have shown that in humans, the abundance of serum antibodies toward subdominant epitopes of both influenza A and influenza B HA increases with age (37, 58, 59). These antibodies include HA stalk-specific antibodies as well as HI active head-specific antibodies that are directed to the nonclassical antigenic sites (37, 58, 59). These observations may be explained by immunological imprinting during first exposure to influenza during childhood and repeated exposures to later drifted strains whose major antigenic sites change over time, whereas subdominant epitopes remain relatively constant (60–68). As a result, seasonal vaccination did not appear to effectively increase protective antibody responses to the vaccine strain (37). These observations also suggest that repeated vaccination with current seasonal vaccines requires a long period of time to elicit high titers of cross-reactive antibody responses targeting subdominant epitopes. Therefore, a vaccine that lacks the major antigenic sites while retaining the conserved noncanonical epitopes was hypothesized to overcome immu-
nological imprinting. It may elicit antibodies to subdominant conserved epitopes, including HI active head epitopes and stalk epitopes, more efficiently if administered at a young age. The mosaic approach for a universal influenza B virus vaccine candidate described here is potentially more effective at boosting antibody responses toward conserved noncanonical epitopes than current seasonal vaccines.

**MATERIALS AND METHODS**

**Cells, recombinant proteins, and influenza B viruses.** Human embryonic kidney 293T (HEK 293T) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% (vol/vol) fetal bovine serum (FBS) and 100 U/ml of penicillin-streptomycin (PS; Gibco) at 37°C with 5% CO₂.

MDCK cells were grown in minimum essential medium (MEM; Gibco) supplemented with 10% (vol/vol) FBS, 2 mM l-glutamine (Gibco), 0.15% (w/vol) sodium bicarbonate (Corning), 20 mM 2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid (HEPES; Gibco), and 100 U/ml of PS at 37°C with 5% CO₂. Sf9 cells for baculovirus rescue and propagation were grown in TNM-FH insect cell medium (GemiBioproducts) supplemented with 10% FBS and antibiotics mix. BTI-TN-SB1-4 (High Five) cells for protein expression were grown in serum-free Express Five SFM medium (Thermo Fisher Scientific) supplemented with PS. Virus preparations for influenza B virus strains, including B/Yamagata/16/88, B/Victoria/02/87, B/Florida/04/06, B/Malaysia/2506/04, B/Brisbane/60/08, and B/Phuket/3073/13, were generated as follows. Viruses were grown in 10-day-old embryonated chicken eggs (Charles River) for 72 h at 33°C. Eggs were then cooled to 4°C overnight, and allantoic fluids were harvested and cleared by low-speed centrifugation at 2,000 x g for 10 min at 4°C. Titers of each virus stock were determined by plaque assay on MDCK cells for infection. To purify viruses, they were first inactivated with 0.03% formaldehyde at 37°C for 1 h and then at 4°C. Viruses were then pelleted through a 30% sucrose cushion in NTE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) by centrifugation in a Beckman L7-65 ultracentrifuge at 25,000 rpm for 2 h at 4°C using a Beckman SW28 rotor. Pellets were collected in PBS (pH 7.4), and protein content was quantified using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific).

**Plasmids.** H5, H8, H11, and H13 sequences were introduced to B/Yamagata/16/88 HA through overlapping PCR, or modified HA segments were directly ordered as synthetic double-stranded DNA fragments (gBlocks; Integrated DNA Technologies). The HA segments were subsequently cloned into pD2 ambisense vector (69) through In-Fusion HD cloning (Clontech). The recombination products were transformed into Escherichia coli DH5α competent cells (Invitrogen), and plasmids were purified using a Qiagen spin miniprep kit (Qiagen). Sequences of HA segments were confirmed by Sanger sequencing (Mcrogen). The pRS-B/Mal04 7 segment plasmid has been described previously (70). Briefly, bidirectional transcription cassettes containing B/Malaysia/2506/04 (B/Mal04) mouse-adapted strain genomic segments PB1, PB2, PA, NP, NA, M, and NS have been amplified from corresponding pD2 plasmids through PCR. Each cassette was cloned to a modified pBBr322 backbone with unique restriction enzyme sites flanking each cassette by In-Fusion cloning. The recombination products were transformed into MAX Efficiency sb12 competent cells (Invitrogen), and plasmids were purified using a Qiagen spin miniprep kit (Qiagen). After insertion of each cassette, the plasmid was linearized by Ascl digestion, and the next cassette was cloned using a similar approach, except the unique restriction sites were different each time. The maxiprep of pRS-B/Mal04 7 segment plasmid was prepared using a Nucleobond Xtra Maxi plus kit (Clontech) for transfection.

**Rescue of B mHA viruses.** Each well of 6-well plates of HEK293T cells was transfected with 2.8 μg of pRS B/Mal04 7 segment, 0.7 μg of modified pD2 HA segment, and 0.5 μg of pCAGGS B/Mal04 HA helper plasmid using TransIT LT1 transfection reagent (Mirus). Transfected cells were incubated at 37°C for 16 h and then were incubated in a 33°C incubator to achieve optimal rescue efficiency. Forty-eight h posttransfection, cell supernatants together with scraped cells were collected and briefly homogenized through several syringe strokes. Two hundred microliters of cell and supernatant mixture was injected into the allantoic cavity of 8-day-old embryonated chicken eggs (Charles River). Injected eggs were incubated at 33°C for 3 days and then cooled at 4°C overnight. Allantoic fluids were subsequently collected, and an HA assay was used to examine the presence of rescued virus. HA-positive allantoic fluid samples were used to plaque-purify virus on MDCK cells. Plaques grown on MDCK cells were picked, collected, and an HA assay was used to examine the presence of rescued virus. HA-positive allantoic fluid samples were used to plaque-purify virus on MDCK cells. Plaques grown on MDCK cells were picked, resuspended in PBS, and further amplified again in 10-day-old embryonated eggs. RNA was extracted from allantoic fluids containing the plaque-purified virus using a QiAamp viral RNA minikit (Qiagen). One-step reverse transcription-PCR (RT-PCR) was performed to amplify DNA of HA segments using the SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen) and B/Mal04 HA-specific primers. DNA was gel purified and sequenced by Sanger sequencing (Genewiz).

**IFA.** MDCK cells were seeded onto 96-well plates at 10⁴ cells per well the day before infection. The next day, cells were infected with each virus at a multiplicity of infection (MOI) of 5 for single-cycle replication. Sixteen to 18 h postinfection, cells were washed 3 times with warm PBS and fixed for 15 min at room temperature using 4% methanol-free formaldehyde without permeabilization. Fixed cells were then washed 3 times with PBS and incubated in blocking buffer (5% BSA in PBS) for 1 h at room temperature. Cells were then incubated with 10 μg/ml human monoclonal antibodies CR8033, CR8059, CR9114, and SAT or mouse serum (1:200) in 1% BSA in PBS for 2 h at room temperature. Cells were then washed again with PBS 3 times and incubated with anti-human or anti-mouse IgG Alexa 488 (Thermo Fisher Scientific) secondary antibody (diluted 1:2,000 in PBS containing 1% [wt/vol] BSA) for 1 h at room temperature. Cells were washed with PBS, and pictures were taken on a Zeiss LSM 880 Airyscan laser.
scanning confocal fluorescence microscope at the Microscopy Core of the Icahn School of Medicine at Mount Sinai.

**Virus growth in eggs and pathogenicity in mice.** Five hundred PFU of wild-type B/Malaysia/2506/04 mouse-adapted virus or B mHA viruses was injected into 10-day-old embryonated eggs in triplicate for each time point (8 h, 24 h, 48 h, and 72 h) and then incubated at 33°C. Eggs at each time point were moved to a cold room and cooled to 4°C overnight. Allantoic fluids were then collected and virus titers were determined by plaque assay on MDCK cells. To examine virus pathogenesis in mice, 6- to 8-week-old female BALB/c mice were infected with wild-type or B mHA viruses, including the h5H/Bשמע, h8H/Bשמע, h11H/Bשמע, and h13H/B莳 viruses. For each virus, mice were divided into 5 groups (n = 3) and infected intranasally with 10, 10^4, 10^5, 10^6, or 10^7 PFU of virus per mouse. Weight loss and survival were monitored for 2 weeks, and mice were scored dead and euthanized when they lost more than 25% of their body weight. Growth curves in eggs and weight loss in mice for wild-type and B mHA viruses were graphed using GraphPad Prism 7.0.

**Generation of mouse and ferret antisera.** To generate mouse antisera, 6- to 8-week-old female BALB/c mice were anesthetized with ketamine-xylazine and infected intranasally with a sublethal dose (10^4 PFU per mouse) of the B/Yamagata/16/88 strain. Three weeks postinfection, mice were boosted once with 10 μg of purified inactivated B/Yamagata/16/88 virus with 25 μl of AddaVax adjuvant (Inovigen) per mouse. Three weeks after the boost, mice were terminally bled by cardiac puncture according to IACUC protocol. Sera were separated by low-speed centrifugation and stored at −80°C for further use. Mouse antisera against the mosaic viruses were generated similar to those infected with sublethal doses of h5H/B莳 (10^4 PFU per mouse), h8H/B莳 (10^5 PFU per mouse), or h13H/B莳 (10^6 PFU per mouse) intranasally. Three weeks postinfection, mice that were infected with h5H/B莳 and h13H/B莳 were bled and sera were isolated as described above. Mice that were infected with h8H/B莳 were boosted 3 weeks postinfection with 10 μg of inactivated purified h8H/B莳 virus together with 25 μl of AddaVax per mouse. Those mice were then terminally bled 3 weeks after the boost. The generation of cross-reactive B HA mouse sera was described previously (35). Briefly, one 6- to 8-week-old female B/αl/c mouse was intranasally infected with 10^4 PFU of B/Lee/40. Approximately 4 weeks later, the mouse was intranasally infected with 10^5 PFU of B/Malaysia/2506/04. Four weeks later, the mouse was injected intraperitoneally with 100 μg of purified PR8 virus expressing B/Yamagata/16/88 HA adjuvanted with 10 μg of poly(I·C). Three days postboost the serum was collected. To generate ferret antisera, 6- to 9-month-old male Fitch ferrets (Triple F Farms, Sayre, PA) were anesthetized with ketamine-xylazine and intranasally infected with 5 × 10^4 PFU of B/Yamagata/16/88 virus diluted in 1 ml PBS per ferret. Three weeks after infection, ferrets were bled and sera were isolated and stored as described above.

**HI assay.** Animal antisera were treated with cholera filtrate (Sigma) that contained receptor-destroying enzyme (RDE). Briefly, one volume of serum was mixed with four volumes of RDE working dilution (1 part RDE and 9 parts calcium saline solution). The mixture was incubated at 37°C for 1 h, and then the reaction was stopped with three volumes of 2.5% sodium citrate solution, which was then heat treated for 30 min at 56°C. Two volumes of PBS were added to reach a final dilution of 1:10. To perform HI assays, HA titers of tested virus stocks were first measured by HA assay. Virus stocks were then diluted to 8 HA units (4 wells of HA) with PBS. Twenty-five μl of PBS was added to each well, except the first well, of a 96-well V-bottom plate. Fifty μl of RDE-treated serum was added to the first well, and serial 2-fold dilutions were prepared until the second-to-last well, leaving the last well with PBS only. Twenty-five μl of B HA-subunit virus dilution was added to each well and incubated for 30 min at room temperature. Fifty μl of 0.5% turkey red blood cells was then added to each well, and HI titer was determined as the last well in which hemagglutination inhibition occurs.

**Animal immunizations.** Six- to 8-week-old female BALB/c mice (Jackson Laboratories) were used for all animal experiments. Experiments were performed in accordance with protocols approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee (IACUC). Mice were divided into six groups (n = 5): the B mHA vaccination group, the B mHA prime-only group, the B cHA vaccination group, the TIV (positive-control) group, the mock vaccination group, and the naive group. A prime-boost-boost regimen was used for B mHA and B cHA vaccination groups. The B mHA group was primed with h13H/B莳 DNA and boosted by h5H/B莳 protein, followed by another boost with h8H/B莳 protein, while the B cHA group was primed with h5H/B莳 DNA and boosted by h13H/B莳 protein, followed by a second boost with cH7/B莳 protein. For the DNA prime, each mouse received 80 μg of DNA by electroporation intramuscularly. All the protein boosts were administered half intranasally and half intramuscularly for a total of 10 μg of protein with 10 μg of poly(I·C) as adjuvant in 100 μl of PBS, since it was unclear which route would result in better immunity. An irrelevant protein, BSA, was used in the prime-only group and the mock group for protein boosts. For the TIV groups, each mouse was vaccinated intramuscularly with 1 μg of influenza B HA antigen; TIV was given twice in concert with the DNA prime and the 2nd protein boost. Matched TIV preparations, either Fluzone 2006-2007 (includes the B/Malaysia/2506/04 strain; Sanofi Pasteur) or Flulaval 2008-2009 (includes the B/Florida/04/06 strain; GlaxoSmithKline), were used as the positive control depending on the challenge virus (B/Malaysia/2506/04 or B/Florida/04/06). Vaccinations were given in 3-week intervals. Four weeks after the last vaccination, animals were anesthetized with a ketamine-xylazine cocktail and then were challenged intranasally with 5 murine LD₅₀ (mLD₅₀) of mouse-adapted B/Malaysia/2506/04 or mouse-adapted B/Florida/04/06 virus in a volume of 50 μl. Mouse adaptation of B/Florida/04/06 and B/Malaysia/2506/04 was performed before the U.S. government 2014 gain-of-function moratorium and was described previously (33). Animals were monitored for survival and weight loss for 2 weeks postchallenge and were scored dead and euthanized if they lost more than 25% of their initial body weight. To determine lung...
virus titers, groups of animals were challenged as described above with 5 mLDtox of B/Malaysia/2506/04 virus. Lungs of challenged animals were harvested on days 3 and postchallenge (n = 3 per group, per time point). Lungs were then homogenized in PBS using a BeadBlaster homogenizer (Benchmark), and lung virus titers were determined by plaque assay on MDCK cells. For passive transfer experiments, groups of animals (n = 10) were terminally bled 4 weeks after the last vaccination. Sera were harvested, pooled within each group, and transferred intraperitoneally (200 µl per mouse) into groups of naive mice (n = 5). Two hours posttransfer, animals were anesthetized and challenged with 5 mLDtox of B/Malaysia/2506/04 virus as described above. Again, weight loss and survival were monitored to determine serum antibody-mediated protection.

**Microneutralization assay.** MDCK cells were plated at a concentration of 1.8 × 10⁴ cells per well of a 96-well plate and left to grow overnight at 37°C in 5% CO₂ until they reached 80% to 90% confluence. The pooled RDE-treated serum was 2-fold serially diluted in 1 × MEM (10% 10× MEM, 1.6% of a 7.5% sodium bicarbonate stock solution (pH 7.5), 1% of PS antibiotic cocktail (Gibco), 1% 200 mM L-glutamine, 1% of a 1 M HEPES stock solution, 0.6% of a 35% BSA stock solution) containing 1 µg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin. Median tissue culture infective doses (TCID₅₀; 10⁰) of each virus was incubated with half the volume of the serial dilutions at a 1:1 volume ratio for 1 h at room temperature with shaking. MDCK cells were washed one time with sterile PBS, and then the virus-serum mixture was added to the cells and incubated for 1 h at 33°C. The remaining half of the serial serum dilutions was supplemented with a 1:1 addition of 1 × MEM with 1 µg/ml TPCK-treated trypsin. After 1 h of incubation, the virus-serum mixture was removed and cells were washed again with PBS. The serial serum dilutions were added to the MDCK cells and incubated for 3 days at 33°C. An HA assay was used as the readout for the microneutralization assay using 0.5% chicken red blood cells. The microneutralization titer was determined as the last well in which hemagglutination inhibition occurred.

**ELISA.** A quantitative ELISA using the area under the curve (AUC) value as the readout was used to evaluate total IgG response to influenza B viruses. Briefly, Immulon 4 HBX 96-well ELISA plates (Thermo Fisher Scientific) were coated with 5 µg/ml of inactivated purified whole virus (50 µl per well) in coating buffer (SeraCare Life Sciences, Inc.) overnight at 4°C. The next day, all plates were washed 3 times with 225 µl PBS containing 0.1% (vol/vol) Tween 20 (PBST), and 220 µl blocking solution (3% goat serum, 0.5% nonfat dried milk powder, 96.5% PBST) was added to each well and incubated for 1 h at room temperature. Mouse sera were 3-fold serially diluted in blocking solution starting at 1:100, followed by 2 h of incubation at room temperature. ELISA plates were washed 3 times with PBST, and 50 µl of anti-mouse IgG-horseradish peroxidase (HRP)-conjugated antibody (GE Healthcare) was added. The plates were incubated for 1 h at room temperature. Plates were washed 3 times with PBST, and 100 µl of o-phenylenediamine dihydrochloride (SigmaFast OPD; Sigma) substrate was added per well. After 10 min, 50 µl of 3 M HCl was added to each well to stop the reaction, and the optical density (OD) was measured at 492 nm on a Synergy 4 plate reader (BioTek). An average of OD values for blank wells plus three standard deviations was used to set a cutoff for plate blank outliers. A cutoff value was established for each plate that was used for calculating the AUC. AUC of serum IgG responses was graphed using GraphPad Prism 7.0.

**ADCC reporter assay.** The ADCC reporter bioassay kit (Promega Life Sciences) was used to measure the induction of ADCC by serum antibodies. MDCK cells were seeded on flat-bottom white 96-well tissue culture plates (Costar) at 50,000 cells per well the day before infection. Twenty-four hours later, cells were washed three times with PBS and infected with influenza B viruses at an MOI of 5 for single-cycle replication. The next day, infected cells were washed with 100 µl of PBS and supplemented with 25 µl of Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific). Pooled mouse sera were diluted 1:3 (from a starting dilution of 1:30) in RPMI medium and added (25 µl per well) to the virus-infected cells in triplicates. ADCC mouse effector cells (Promega Life Sciences) were added at a concentration of 75,000 cells per well and incubated for 6 h at 37°C. At the end of the incubation, 75 µl of Bio-Glo luciferase assay substrate (Promega Life Sciences) was added to each well and incubated at room temperature for 5 min. Luminescence was read using a Synergy 4 microplate reader (BioTek) and Gen5 2.09 software. Fold induction over the baseline was calculated and graphed using Prism 7.0.

**Statistics.** Statistical analysis was performed using Prism 7.0 (GraphPad). The statistical difference in ELISA was determined using Kruskal-Wallis one-way analysis of variance (ANOVA) corrected for Dunn’s multiple comparisons.

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