INTRODUCTION

Seasonal influenza caused by influenza A H3N2 and H1N1 subtypes and influenza B Yamagata and Victoria lineages remains a serious threat to health worldwide (1). Although influenza A has garnered much attention because of its pandemic association, the clinical presentations and complications of seasonal influenza A and B virus infections are clinically indistinguishable (2). A recent increase in the rate of influenza B infections has resulted in higher morbidity and mortality worldwide compared to that observed for influenza A H1N1 (3, 4). This situation highlights the limitations of current influenza vaccines and antiviral drugs in combating influenza B. Currently circulating influenza B viruses originated in the 1940s and evolved into two genetically and antigenically distinct lineages in the 1980s, the Victoria lineage and the Yamagata lineage (5). The continuous cocirculation of both influenza B virus lineages with influenza A/H3N2 and A/H1N1 viruses during seasonal epidemics has prompted the development of quadrivalent vaccines that include strains from both influenza B lineages (6). However, current influenza vaccines struggle to induce sufficient levels of cross-reactive neutralizing antibodies, and vaccine strains frequently become mismatched from continuously evolving influenza variants (7, 8). In addition, the effectiveness of existing antiviral drugs for the treatment of influenza infection is limited because of short treatment windows and emerging antiviral drug resistance (9). Thus, there is an unmet medical need to develop more effective universal prophylactic and therapeutic approaches against influenza infection.

Passive immune protection using broadly neutralizing antibodies (bnAbs) that target vulnerable conserved epitopes is a promising approach for treating highly variable viral infections (10). The hemagglutinin (HA) protein, which is the major influenza viral surface glycoprotein responsible for binding cellular receptors, is a common target of influenza bnAbs. This protein can be bound by neutralizing antibodies to prevent the propagation of influenza virus via distinct mechanisms (11). Most previously described anti-HA bnAbs are specific for conserved epitopes in the HA stem region (12–14). In contrast, only a small number of HA bnAbs targeting the HA head region have been characterized due to the higher variability in this region. Because epitopes on the HA head are critical for virus infection and more accessible than those on the HA stem, the development of HA head–specific bnAbs targeting the vulnerable receptor binding site (RBS) is desirable. Although most anti-HA bnAbs have targeted influenza A (15), the recent isolation of anti–HA bnAbs targeting influenza B revealed the presence of similar conserved HA head and stem epitopes on two phylogenetically and antigenically distinct influenza B virus lineages (16–18). The CR8033 bnAb described by Dreyfus et al. (17), which is directed at the RBS epitope on the HA head, showed better prophylactic effects in vivo than bnAbs directed at HA epitopes near the stem, such as CR8071
and CR9114. Mechanistic analysis indicated that CR8033 prevented viral entry and egress, whereas CR8071 only prevented viral egress; antibodies having multiple neutralizing mechanisms understandably provide more comprehensive antiviral activities (17). Notably, whereas CR8033 targets a conserved site in the influenza B virus HA, this epitope is not identical in function for both lineages of influenza B viruses, with CR8033 only inhibiting viral entry of Yamagata lineage viruses. Therefore, generation of novel bnAbs directed at conserved HA head epitopes associated with viral entry in both influenza B lineages is necessary.

The difficulty in developing bnAbs against the HA head of influenza lies in the diverse antigenicity of the different subtypes or lineages of influenza viruses and the highly variable epitopes on the HA head. To address this, we trialed various immunization regimens to induce cross-reactive antibodies in mice against highly conserved epitopes in the HA protein of influenza B and used a functional screening strategy to select antibodies that have multiple inhibiting mechanisms. Using these optimized immunization and screening protocols, one antibody, designated 12G6, was identified, and a chimeric version containing a human immunoglobulin G1 (IgG1) Fc fragment was generated (C12G6). The potent and broad-spectrum antiviral effects of C12G6 were characterized in vitro and in vivo, indicating that C12G6 may be a promising candidate for the development of a high-efficacy universal prophylactic or therapeutic agent against influenza B.

RESULTS

A multimechanistic bnAb against influenza B was generated by sequential immunization

To generate bnAbs against conserved functional epitopes in the HA head of influenza B, we implemented eight distinct immunization regimens in mice. The mice were sequentially immunized intranasally or subcutaneously with two representative influenza B live viruses, FL/2006 (B/Florida/4/2006, Yamagata) and BR/2008 (B/Brisbane/60/2008, Victoria) (fig. S1A). Enzyme-linked immunosorbent assay (ELISA) screening against FL/2006 and BR/2008 identified 10 cross-lineage reactive antibodies from a total of 318 influenza B–specific antibodies generated from mice of groups 3 (12G6, 3G8, 4E7, 5C9, 7G8, 10F6, and 10H6 antibodies), 4 (13E6 and 13C2 antibodies), and 7 (13D8 antibody) (fig. S1B). Then, to generate efficient bnAbs that are able to block infection of both lineages of influenza B virus, we performed the hemagglutination inhibition (HI) assay in the second screening round. Three of the 10 antibodies (12G6, 3G8, and 10F6) showed cross-lineage HI activity against both lineages (fig. S1C). We further determined the activity of these three antibodies against BR/2008 by three other functional assays. As expected, 12G6, 3G8, and 10F6 antibodies effectively neutralized the BR/2008 (fig. S1D). Of these, 12G6 and 10F6 antibodies efficiently inhibited low pH–induced viral fusion with endosomal membranes (fig. S1E), and specifically, only 12G6 exhibited antibody–dependent cell-mediated cytotoxicity (ADCC) activity against BR/2008 (fig. S1F). Because the 12G6 cross-reacted with both lineages of influenza B viruses and inhibited the viruses through multiple mechanisms, it was studied further.

12G6 was purified from mouse ascites and demonstrated reactivity against 18 virus strains representing the three distinct influenza B lineages in ELISA, HI, and MN (microneutralization) assays (fig. S2 and table S1). The DNA sequences of the VH (variable region of immunoglobulin heavy chain) and VL (variable region of immunoglobulin light chain) regions of 12G6 were obtained and compared with the closest germline sequences using the VBASE2 database (www.vbase2.org/), and the mutation rates of the nucleotides in the DNA sequences of the VH and VL are 7.14% (21 of 294) and 2.48% (7 of 282), respectively (fig. S3). To further evaluate the potential clinical use of 12G6, a chimeric 12G6 monoclonal antibody (mAb), designated C12G6, which contains the variable region of mouse 12G6 and the human IgG1 Fc region, was constructed and characterized in subsequent in vitro and in vivo experiments.

C12G6 broadly neutralizes all available influenza B viruses isolated since 1940

To determine the breadth of C12G6 activity against influenza B viruses, we tested purified C12G6 for the activity against a panel of 18 available influenza B virus strains from distinct lineages (table S2). The diversity of these representative strains is illustrated by a dendrogram of the full-length nucleotide sequences of their respective HA genes (fig. 1A). In a primary binding test, 10 representative purified influenza B viruses were used, and C12G6 reacted with all of them, with half maximal effective concentration (EC50) values ranging from 7.68 to 60.39 ng/ml. In contrast, the control antibody C5G6 (a chimeric mAb against 2009 pandemic H1N1 influenza A viruses) did not bind to any influenza B viruses but did bind A/California/04/2009 control (fig. 1B and table S3). In addition, binding of C12G6 IgG to recombinant HA (rHA) proteins of two representative influenza B strains was measured by surface plasmon resonance, with the rHAs of the two strains both being strongly bound by C12G6: Kd (dissociation constant) = 0.858 nM for Yamagata rHA; Kd = 2.26 nM for Victoria rHA (fig. S4 and table S4). Consistent with its ability to bind to a panel of influenza B viruses, C12G6 also binds to B/Florida/4/2006 (Yamagata)– and B/Brisbane/60/2008 (Victoria)–infected Madin-Darby canine kidney (MDCK) cells, when tested by immunofluorescence assay, flow cytometry, or Western blotting against the B/Florida/4/2006 HA proteins (figs. S5 to S7).

To further compare the functional activities of C12G6 with those of four previously described cross-lineage neutralizing influenza B HA–specific bnAbs (16, 17), chimeric versions of them were constructed and prepared, being designated CR8033-like, CR8071-like, CR9114-like, and 5A7-like, respectively (fig. S8A). We confirmed that these chimeric mAbs all showed binding activity against both influenza B virus lineages (fig. S8B and table S5). Next, we directly compared the in vitro HI and neutralization activities and breadth of reactivity of C12G6 with those of the four reported antibodies. CR8033-like antibody displayed HI activity against Yamagata and earlier lineage strains, but not Victoria virus strains. The CR8071-like antibody neutralized all influenza B virus strains (fig. S8C and table S6). Of these antibodies, C12G6 exhibited the highest breadth of reactivity, being designated C12G6 5A7-like, and C5G6 antibodies did not exhibit HI activity against any influenza B strain. In contrast, C12G6 showed specific HI activity against all 18 influenza B viruses tested (fig. 1C). In the MN assay, C12G6, CR8033-like, CR8071-like, and 5A7-like antibodies neutralized both lineage virus strains, although there were differences in both potency and breadth of reactivity (Fig. 1D, fig. S9, and tables S6 to S8). C12G6 was the only antibody tested that had neutralizing activity against all the representative viruses, with a median IC50 of 1.40 µg/ml. The CR8033-like antibody failed to neutralize the Victoria virus strain B/Rhode Island/01/2012–like, whereas the CR8071-like antibody was unable to neutralize the three earlier lineage strains (B/Lee/1940, B/Great Lakes/1739/1954, and B/Singapore/3/1964) or the Victoria lineage strain (B/New York/1352/2012–like). The 5A7-like antibody weakly neutralized only one of the five Yamagata lineage virus strains tested (B/Florida/4/2006) and 3 of the 10 Victoria lineage strains tested (B/Hong Kong/330/2001, B/Malaysia/2506/2004, and B/Brisbane/60/2008). IC50 values are detailed in fig. S9. Median IC50 values were 1.40, 1.97, 10.42, and 34.22 µg/ml for C12G6, CR8033-like, CR8071-like, and 5A7-like antibodies, respectively, when non-neutralized viruses were excluded from the evaluation. Thus,
in addition to greater neutralization breadth, C12G6 also exhibits greater neutralization potency than the four reported influenza B bnAbs.

**C12G6 shows broad prophylactic and therapeutic activity in mice and ferrets**

Three mouse-adapted (MA) influenza B viruses, MA-B/Florida/4/2006 (Yamagata lineage), MA-B/Brisbane/60/2008 (Victoria lineage), and MA-B/Lee/1940 (earlier lineage) generated in our laboratory were used to evaluate the prophylactic and therapeutic antiviral activities of C12G6 in vivo. For the evaluation of cross-protection of C12G6 in mice, a dose-ranging study was carried out by intravenously administering various single doses of each antibody 1 day before (prophylactic groups) or after (therapeutic groups) intranasal virus challenge. C12G6 showed considerable prophylactic and therapeutic efficacies against lethal challenge with all three representative influenza B viruses (Fig. 2). For the prophylactic groups, C12G6 doses greater than 1 mg/kg fully protected mice against infection with all three viruses, with animals in these groups all gaining weight by the end of the study (fig. S10, A to C). Even C12G6 (0.06 mg/kg) still partially protected mice from lethal infection with the three virus strains (Fig. 2, A to C). For the therapeutic groups, mice treated with a single dose of C12G6 (5 or 10 mg/kg) 1 day after infection all survived lethal challenge with each of the three representative MA influenza B viruses. A single dose as low as 0.2 mg/kg still provided partial protection against all viruses (Fig. 2, D to F). Reduced weight loss was also observed, compared to the IgG control (fig. S10, D to F).
Florida/4/2006 or MA-B/Brisbane/60/2008 infections were considerably reduced in mice receiving C12G6 compared to those given control IgG (Fig. 2, G and H). Hematoxylin and eosin staining results and immunohistochemical staining analysis (using an anti–influenza B nucleoprotein mAb, 4D5, generated by our laboratory and validated in fig. S11) indicated that prophylactic and therapeutic treatment with C12G6 also decreased the lung damage caused by both influenza B virus lineages, compared to the control IgG–treated group (figs. S12 and S13).

To extend the evaluation, we directly compared the in vivo therapeutic efficacy of C12G6 in mice with that of the four reported bnAbs and the anti-influenza drug oseltamivir. All mice receiving C12G6 survived lethal challenge with both influenza B virus strains. In contrast, 80, 60, 0, 20, or 0% of mice survived infection with the Yamagata virus strain MA-B/Florida/4/2006 1 day before receiving CR8033-like (2 mg/kg), CR8071-like (2 mg/kg), CR9114-like (2 mg/kg), 5A7-like (2 mg/kg), or C5G6 (2 mg/kg) antibody, respectively (Fig. 3A). For the Victoria strain MA-B/Brisbane/60/2008, the same doses of CR8033-like, CR8071-like, CR9114-like, 5A7-like, or C5G6 antibody protected 60, 40, 0, 20, or 0% of mice, respectively (Fig. 3B). Reduced weight loss in C12G6–treated mice also reflected the better protective potency of C12G6 when compared to the other influenza B–specific bnAbs (Fig. 3, C and D). Consistent with the survival and body weight data, at day 3 after infection, viral titers of both MA-B/Florida/4/2006 and MA-B/Brisbane/60/2008 were considerably lower in the lungs of C12G6–treated mice than in those treated with the four reported bnAbs (Fig. 3, E and F).

For the comparison with oseltamivir, we administered a single dose of C12G6 (10 mg/kg) or two doses of oseltamivir (25 mg/kg) a day for 4 consecutive days at different time points after infection with MA-B/Florida/4/2006 or MA-B/Brisbane/60/2008 virus. As expected, all control animals died by day 10 after infection. When administered 1 day after infection, C12G6 treatment resulted in 100% survival and little weight loss for both virus lineage strains, whereas oseltamivir only partially protected animals, with survival rates of 60 and 40% with marked weight loss for the Yamagata and Victoria strains, respectively (Fig. 4, A and B, and fig. S14). Survival rates of 80% were achieved even when treatment with C12G6 was delayed until 3 days after infection with either influenza B virus lineage.

Encouragingly, more than 50% of mice survived after treatment with C12G6 at 5 days after infection with either virus. In contrast, all mice treated with control IgG or with oseltamivir at 3 or 5 days after infection died by 10 days after infection (Fig. 4, A and B).
Despite the efficacy of oseltamivir being limited, especially when the treatment is delayed, severely ill influenza patients are generally given oseltamivir upon hospital admission. Therefore, we tested whether coadministration of C12G6 and oseltamivir provided better protective efficacy than either treatment alone. Mice infected with MA-B/Florida/4/2006 or MA-B/Brisbane/60/2008 were treated, starting at 48 hours after infection, with C12G6, oseltamivir, or a combination of these two therapies. Mice receiving oseltamivir plus control C5G6 antibody exhibited 100% mortality by day 9 after lethal challenge with both lineages of influenza B virus, similar to the control group (C5G6 plus water). Administration of C12G6 plus water only partially protected animals, with survival rates of 40 and 60% for the Yamagata and Victoria strains, respectively. In contrast, coadministration of C12G6 with oseltamivir completely protected mice after lethal challenge with the two influenza B virus lineages (Fig. 4, C and D). Coadministration also resulted in reduced weight loss in mice, compared to treatment with either active agent alone (Fig. 4, E and F). Finally, consistent with the survival and body weight data, combined treatment considerably reduced lung viral titers at days 4 and 6, compared with C12G6 or oseltamivir alone (Fig. 4, G and H).

To further estimate the protective potential of C12G6 in vivo, we determined the prophylactic and therapeutic windows for treating ferrets infected with B/Florida/4/2006 (Yamagata lineage) or B/Brisbane/60/2008 (Victoria lineage). As expected, nasal wash viral titers for ferrets treated with C12G6 either prophylactically or therapeutically were considerably lower than for those treated with control antibody, for both virus infections (Fig. 5, A to D). In addition, C12G6 treatment resulted in fever reduction after infection with each of the two viruses, in comparison to control antibody–treated animals (Fig. 5, E to H). Moreover, infected animals administered C12G6 only experienced slight body weight loss; in contrast, considerable body weight loss was observed in control antibody–treated animals (fig. S15). Consistent with the data above, all control ferrets showed clinical signs of infection, including nasal discharge, sneezing, and inactivity, whereas a lesser proportion of ferrets treated with C12G6 displayed clinical signs (fig. S16).

Fig. 3. Comparison of therapeutic efficacies of C12G6 and other bnAbs in mice. (A to F) Survival curves (A and B), body weight change (C and D), and lung viral titers (E and F) for BALB/c mice (n = 5 per group) treated intravenously with antibodies (2 mg/kg), indicated 24 hours after lethal challenge with 25 MLD50 of MA-B/Florida/4/2006 or B/Brisbane/60/2008. Virus titers in the lungs were determined on day 3 after infection. The black bars indicate mean values. The body weight curves represent mean ± 95% confidence interval of the mean. For (A) and (B), statistical analysis was performed by log-rank test. For (C) and (D), comparisons are by area under the curve (AUC) analysis. For (E) and (F), statistical analysis was performed by t test. *P < 0.05, **P < 0.01, and ***P < 0.001, compared to the control IgG–treated group.
**C12G6 recognizes a highly conserved epitope that overlaps with the RBS domain of influenza B HA**

The strong HI activity of C12G6 suggests that the epitope targeted by C12G6 is located at the top of the HA head. To further understand the molecular basis underlying the C12G6 recognition of HA proteins in the influenza B viruses and identify key residues recognized by this antibody, we generated C12G6-induced escape mutants of influenza B viruses by culturing the viruses in the presence of C12G6. Viruses that grew in such conditions were harvested, and the entire HA sequence of these viruses was determined. Two escape mutants of B/Singapore/3/1964 (G156R and G156E) and three escape mutants of the B/Hong Kong/537/2009-like virus (G156W, P176Q, and T183K) were generated (Fig. 6A). All of the mutant residues are located either in or near the HA RBS of influenza B (19). G156 and P176 are highly conserved (both 100%) among all available HA sequences of influenza B in the NCBI database. The residue at position 183 in all influenza B viruses is either T or N. The T183 variant occurs in 32.4% of influenza B virus strains, whereas all other virus strains, including B/Florida/4/2006 and B/Massachusetts/02/2012-like, exhibit N183; both T183 and N183 variants are sensitive to neutralization by C12G6 (Fig. 1D). In contrast, the T183K mutation has not been observed in any naturally arising influenza B isolates. Because escape mutants raised against anti–HA head antibodies may affect the viral fitness in vivo (20), we also compared the pathogenicity of wild-type (WT) and escape mutant B/Hong Kong/537/2009-like viruses in vivo. Whereas WT B/Hong Kong/537/2009-like virus infection caused 100% mortality, considerable body weight loss, and high lung viral titers, all mice infected with the three mutant viruses survived, with only slight body weight loss and lower lung viral titers (fig. S17).

We next determined the epitope targeted by C12G6 using a molecular docking strategy. The candidate epitope residues were determined on the basis of three-dimensional HA trimer models, which indicated the presence of the epitope at the top of the HA head and its overlap with the RBS region (Fig. 6B). On the basis of the conservation analysis, most of the amino acids within the epitope are more than 98% conserved (in green), and the remainder is 75 to 98% conserved (in yellow) in all 2000 full-length influenza B HA sequences in the NCBI database (Fig. 6B). To further characterize the C12G6 epitope, we selected nine potential C12G6-contacting residues on B/Hong Kong/537/2009-like virus HA for single-point mutational analysis based on the escape mutant results and calculation and filtering for surface interaction (table S9). We expressed these HA mutants on human embryonic kidney (HEK) 293T cells and tested for C12G6 binding by flow cytometry. Three HA mutants (P159A, N163A, and Q249A) bound C12G6 similarly to the WT HA, whereas six of the nine HA mutants (G156W, P176Q, T183K, K86A, G254A, and S258A) showed reduced C12G6 binding (Fig. 6C). Of these, G156W, K86A, and G254A almost abolished binding, whereas the other three mutations decreased binding by less than fourfold. Consistent with the flow cytometry results, G156W, P176Q, and T183K escape mutant viruses also revealed reduced C12G6 binding when tested by ELISA (fig. S18). These results identified
We then compared the differences between the epitopes recognized by C12G6 and CR8033 antibodies (17); the two epitopes are distinct but overlap to a certain degree in the RBS domain (Fig. 6E). As shown in competition ELISA assay, C12G6 overlapped with the RBS-targeted CR8033-like antibody epitope by competing for binding with the CR8033-like antibody but did not compete for binding to HA with the CR9114-like antibody (Fig. 6F).

**C12G6 targets influenza B through multiple inhibition mechanisms**

Consistent with the HI activity exerted by C12G6 against both influenza B lineages, cross-lineage inhibition of viral infection by C12G6 was observed after pre-incubation with either B/Florida/4/2006 (Yamagata) or B/Brisbane/60/2008 (Victoria) virus (Fig. 7A), indicating that C12G6 has the ability to prevent influenza B virus entry into cells. To determine whether C12G6 inhibits influenza B viral egress from infected cells, we measured influenza B virus antigens (using polyclonal rabbit sera against the respective virus) present in the supernatants and lysates of MDCK cells infected with either B/Florida/4/2006 (Yamagata) or B/Brisbane/60/2008 (Victoria) virus, where different concentrations of antibodies were added 4 hours after infection, before further incubation. Viruses were present in all cell lysates but were only detected in the supernatants of infected MDCK cell cultures incubated with a control antibody or a low concentration of C12G6. No virus was detected in supernatants incubated with C12G6 (2 μg/ml) despite a strong virus band in the lysate (Fig. 7B), indicating that C12G6 inhibits viral egress from infected cells for both influenza B lineages.

We next tested whether C12G6 has the inhibition mechanisms generally observed in stem-binding antibodies. Activation of HA-mediated membrane fusion requires trypsin-mediated cleavage of the precursor, HA0, and exposure of the cleaved HA to the low pH of endosomes. For the HA0 activation inhibition assay, HA0 protein was incubated with the antibody before exposure to TPCK (tosyl phenylalanyl chloromethyl ketone)–treated trypsin for 0, 5, 10, 20, or 40 min. Western blot analysis showed that C12G6 did not block trypsin-mediated HA0 activation because the HA0 protein was rapidly cleaved by trypsin in the presence of C12G6 (fig. S19).

We also performed a pH-induced protease sensitivity immunoblot assay to determine whether C12G6 inhibits membrane fusion in both lineages of influenza B viruses. Exposure to low pH converts the HAs...
to the postfusion state, rendering them sensitive to trypsin digestion. Notably, C12G6 binding to FL/2006 HA or BR/2008 HA prevented the low pH–induced conformational change, which is required for membrane fusion, by stabilizing the prefusion conformation (Fig. 7C). Because it is very rare for an anti-head antibody to inhibit membrane fusion, we conducted cell-cell fusion and red blood cell fusion assays to validate this C12G6 neutralization mechanism. These two assays confirmed that C12G6 blocked the membrane fusion of viruses from both lineages (figs. S20 and S21).

Because protective efficacy of HA bnAbs largely depends on antibody effector functions (12, 14), we finally evaluated the ADCC, complement-dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP) activities of C12G6 against B/Lee/1940-, B/Florida/4/2006-, or B/Brisbane/60/2008-infected cells. For the ADCC and CDC assay, we produced an Fc mutant of C12G6 that lacked Fc receptor binding activity (C12G6-LALA) (12) as a negative control antibody. C12G6-LALA exhibited the same binding and in vitro neutralizing properties as C12G6 (fig. S22 and table S10) and a comparable half-life in vivo (fig. S23). To benchmark C12G6 effector functions against antibodies directed toward other sites of the HA, we included CR8033-like, CR8071-like, CR9114-like, and C5G6 antibodies as controls. C12G6 demonstrated weak ADCC activity against B/Lee/1940, no ADCC activity against B/Florida/4/2006, and comparably high ADCC activity against B/Brisbane/60/2008. In contrast, CR8033-like antibody only displayed comparably weak ADCC activity against the Victoria strain, whereas CR8071-like and CR9114-like antibodies exhibited strong ADCC activities against all three viruses, and C12G6-LALA and C5G6 antibodies only showed background levels of activity (fig. S24A and fig. 7, D and E). Unexpectedly, C12G6, along with CR8071-like and CR9114-like antibodies, showed high CDC activity against all three virus strains. In contrast, C12G6-LALA and control IgG did not reveal any CDC activity (fig. S24B and Fig. 7, F and G). The antibody effector functions of C12G6 were further verified in in vivo protective efficacy experiments. The results also indicated that the in vivo efficacy of C12G6 is partially dependent on antibody effector functions (fig. S25). Finally, we demonstrated that 12G6 could not induce any ADCP activity against influenza B in the mouse model (fig. S26). Thus, C12G6 becomes a multimechanistic cross-lineage therapeutic antibody targeting the HA head of influenza B virus (fig. S27 and table S11).
We report an anti–influenza B bnAb, C12G6, directed against the head of HA with higher potency and broader breadth of anti-influenza activity in vitro and in vivo than four other antibodies previously reported (16, 17). Stronger antiviral activity was still observed even when C12G6 treatment was delayed until day 3 or 5 after influenza B infection. Co-administration of C12G6 and oseltamivir improved protective efficacy, compared to either treatment alone, possibly by synergistically targeting distinct viral functions associated with different stages of the viral life cycle. Thus, C12G6 appears to be a promising candidate for the development of broad-spectrum therapeutics against influenza B infection and may inform the design of a universal influenza vaccine.

The development of bnAbs targeting the highly variable epitopes on the HA head is difficult. This study demonstrated that the influenza B bnAbs can be generated from mice sequentially immunized with live B/Florida/4/2006 (Yamagata) and B/Brisbane/60/2008 (Victoria) viruses via the intranasal route. Another study, using intramuscular immunization, reported that priming with Victoria and boosting with Yamagata induced more responses to both lineages than priming with Yamagata and boosting with Victoria (21). It has been shown that the route of
administration strongly influences both the quantity and quality of vaccine-induced immunity (22, 23). Thus, a rational design for sequential and mucosal immunization strategies has the capability to produce broadly neutralizing and highly potent immune response.

Four influenza B HA bnAbs (CR8033, CR8071, CR9114, and 5A7), which show cross-lineage neutralization and protective activity, have been previously reported (16, 17). Of these four antibodies, C12G6 is most similar to CR8033 in that both bind to the RBS domain in the HA head. Remarkably, C12G6 exhibited distinct HI activity against influenza B virus strains when compared to CR8033-like antibody, suggesting that it recognizes an epitope distinct from that recognized by the CR8033. C12G6 targets an epitope that overlaps with the RBS domain and is overlapped by the CR8033-like epitope, as determined by epitope mapping of C12G6 with escape and HA mutants. However, the epitope targeted by C12G6 is completely distinct from those targeted by CR8071-like, CR9114-like, and 5A7-like antibodies. It has been previously reported that escape mutants raised against anti–HA head antibodies can gain pathogenicity (23). In contrast, we found that the escape mutants raised against C12G6 showed reduced fitness in vivo, highlighting that the mutant residues of C12G6 epitope are critically important for viral replication. Therefore, the breadth and potency of the activity of C12G6 should be related to a vulnerable, highly conserved HA head epitope in the influenza B virus.

C12G6 showed better in vivo efficacy than CR8033, although they seem to have a similar epitope. C12G6 exerts its neutralization effects by directly inhibiting binding to an epitope that overlaps the RBS domain. Such direct inhibition of viral binding to host receptors is generally critical for virus neutralization (17). C12G6 inhibited the tested viruses even after infection. Specifically, C12G6 blocked low pH–induced viral fusion with endosomal membranes, which is a key event in the viral replication cycle. This function is generally observed among antibodies targeting the HA stem (12, 14) and is seldom seen in CR8033-like antibodies targeting the HA head (16). The C12G6 epitope is somewhat closer to the stem region of HA than CR8033, which may be related to functions generally displayed by HA stem–targeting antibodies. Thus, C12G6 binding may target a truly unusual motif on the HA head, disturbing low pH–dependent structural changes in HA and preventing viral replication. Furthermore, similar to CR8033-like antibody, C12G6 is able to inhibit virus replication by blocking the release of progeny virions from infected cells, an effect resembling that exerted by neuraminidase inhibitors (17). Notably, in addition to directly neutralizing viruses, we observed that C12G6 triggered the Fc-mediated viral clearance mechanisms, ADCC and CDC. Two recent papers found that binding of the HA to sialic acid on the effector cell is crucial for ADCC activity (24, 25); thus, ADCC activity may be blocked by HI active antibodies. For the three viruses tested, C12G6 showed no ADCC activity against B/Florida/4/2006, weak ADCC activity against B/Lee/1940, and comparably high ADCC activity against B/Brisbane/60/2008. Conversely, C12G6 exhibited high HI activity against B/Florida/4/2006, moderately high HI activity against B/Lee/1940, and relatively lower HI activity against B/Brisbane/60/2008. Thus, the ADCC activity of C12G6 against these three viruses revealed an opposite trend to that of its HI activity, consistent with reports that HI active antibodies can block ADCC activity (24, 25). C12G6 also induced strong CDC activity against all three influenza B virus lineages. By contrast, CR8033 displayed very weak ADCC and CDC responses against influenza B viruses. Hence, the better protective efficacy of C12G6 is likely attributable to its more comprehensive antiviral mechanisms, which, together, inhibit influenza B viral infection.

There are some limitations of this study: (i) The relatively few representative influenza B virus strains isolated between 1964 and 2001 are available for the characterization of antibodies, which limit more complete spectrum characterization of the breadth and potency activity of the bnAbs; (ii) the HI and neutralization activity of C12G6 against some representative influenza B viruses was not strong enough, but we could not determine whether the variation in HI and neutralization activity may affect the protective efficacy of C12G6 in vivo; and (iii) the detail structure of the epitope bound by C12G6 has not been revealed. Further studies are necessary to delineate the molecular basis of the binding epitope bound by C12G6 for understanding its potent and broad viral clearance activities.

MATERIALS AND METHODS

Study design

The objective of this study was to develop bnAbs that neutralize multiple lineages of influenza B viruses and potently cross-protect against influenza B virus infection. A variety of immunization approaches using different antigenic lineages of influenza B strains were combined with a panel of functional screening methods to generate multiple cross-reactive mAbs using the murine hybridoma technique. One bnAb, C12G6, was generated and evaluated in vitro and in vivo and verified to be a promising candidate for the development of prophylactics or therapeutics against influenza B. All in vivo studies were performed in accordance with Institutional Animal Care and Use Committee guidelines and were approved by the Ethics Committee of Xiamen University Laboratory Animal Center. Six-week-old female BALB/c mice were purchased from Shanghai Silaike Laboratory Animal Co. Ltd. and were used for all experiments. Ferrets were purchased from Wuxi Sangosho Biotechnology Co. Ltd. The animals were maintained in individually ventilated cages and monitored closely for survival and signs of illness for up to 14 days after challenge. The guidelines for humane end points were strictly followed for all in vivo experiments; animals that lost more than 25% of their initial body weight were immediately euthanized by CO2 asphyxiation and were recorded as nonsurvivors. All animals were randomly assigned to treatment groups using a randomization tool implemented in Microsoft Excel. The pathologists who evaluated the tissue sections were blinded to treatment groups. All in vitro and in vivo experiments were repeated at least three times, unless otherwise stated within the figure legends. The inhibitory mechanisms of C12G6 were further investigated to determine the prophylactic or therapeutic potential of C12G6 for future clinical applications. All HA sequences of influenza B viruses used in this study are listed in table S12. Primary data are located in table S13.

Prophylactic and therapeutic efficacy studies in mice

In a prophylactic setting, groups of five female BALB/c mice aged 6 to 8 weeks were injected intravenously with 200 μl of vehicle control or a dose of C12G6 (5, 0.6, 0.2, or 0.06 mg/kg). One day later, the mice were deeply anesthetized with isoflurane and oxygen and challenged intranasally with 25 MLD50 of MA-B/Florida/4/2006 (Yamagata), MA-B/Brisbane/60/2008 (Victoria), or MA-B/Lee/1940 (earlier) virus, which was grown in MDCK cells using standard viral culturing techniques. In a therapeutic setting, the mice received the antibody or oseltamivir at the indicated doses at 1, 3, or 5 days after infection. The lungs of mice were collected for virus titration at 3 or 6 days after infection. Tissue was collected for histopathological evaluation 4 days after infection. For oseltamivir comparison studies, mice were administered oseltamivir.
data for infected mice and the ADCC and CDC experiments were analyzed using multiple t tests in GraphPad Prism 6.0. P values reported in the figures and figure legends indicate the following significance levels: *P < 0.05, **P < 0.01, and ***P < 0.001.

**SUPPLEMENTARY MATERIALS**

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Materials and Methods

Fig. S1. A schematic diagram showing the 12G6 generation and selection process.

Fig. S2. In vitro binding and neutralization activities of mouse antibody 12G6.

Fig. S3. Nucleotide and amino acid sequences of the V<sub>α</sub> and V<sub>γ</sub> chain regions of C12G6.

Fig. S4. Binding curves for reported IC<sub>50</sub> values for binding of C12G6 to influenza B HAs.

Fig. S5. Immunofluorescence assay activity of C12G6 against both lineages of influenza B viruses.

Fig. S6. Binding of C12G6 to MDCK cells infected with influenza B viruses, detected by flow cytometry.

Fig. S7. C12G6 binds the B/Florida/4/2006 HA subunit in Western blotting.

Fig. S8. Expression, purification, and characterization of influenza B HA bnAbs.

Fig. S9. In vitro neutralization activities (IC<sub>50</sub> values) of C12G6 and other reported influenza B HA bnAbs.

Fig. S10. Body weight change curves of mice treated with C12G6 before or after challenge with influenza B viruses.

Fig. S11. Immunofluorescence assay activity of anti-influenza B nucleoprotein mAb 4D5 against both lineages of influenza B viruses.

Fig. S12. Histological analysis of lungs from mice prophylactically treated with C12G6 before infection with influenza B viruses.

Fig. S13. Histological analysis of lungs from mice therapeutically treated with C12G6 after infection with influenza B viruses.

Fig. S14. Comparison of therapeutic effects of C12G6 and oseltamivir against influenza B infection in mice.

Fig. S15. Body weight change curves of ferrets treated with C12G6 before or after challenge with influenza B viruses.

Fig. S16. Clinical sign of ferrets after prophylactic and therapeutic treatment with C12G6 against influenza B infection.

Fig. S17. In vivo fitness of mutant B/Hong Kong/537/2009-like viruses.

Fig. S18. Reactivity of C12G6 with WT or mutant B/Hong Kong/537/2009-like viruses in ELISA.

Fig. S19. C12G6 does not block HA0 activation.

Fig. S20. C12G6 inhibits syncytia formation.

Fig. S21. Red blood cell fusion assay.

Fig. S22. Comparison of the in vitro binding and neutralizing properties of C12G6 and C12G6-LALA.

Fig. S23. Serum antibody concentrations after C12G6 or C12G6-LALA treatment.

Fig. S24. ADCC and CDC activities of C12G6 against the B/Lee/1940 virus strain.

Fig. S25. Determination of the role of antibody effector functions in the protective efficacy of C12G6.

Fig. S26. ADCP activity of 12G6 against influenza B viruses.

Fig. S27. A schematic diagram showing the inhibition mechanisms of C12G6.

Table S1. ELISA EC<sub>50</sub> and SD values of mouse antibodies 12G6 and 5G6, related to Fig. S2.

Table S2. The full designations and abbreviations of the influenza virus strains used in this study.

Table S3. ELISA EC<sub>50</sub> and SD values of C12G6 and C5G6 antibodies, related to Fig. 1B.

Table S4. IC<sub>50</sub> for binding of C12G6 to HAs of influenza B strains.

Table S5. ELISA EC<sub>50</sub> and SD values of the indicated antibodies, related to Fig. S8B.

Table S6. Neutralization IC<sub>50</sub> and SD values of C12G6 and CR8033-like antibodies, related to Fig. 1D.

Table S7. Neutralization IC<sub>50</sub> and SD values of C8071-like and CR9114-like antibodies, related to Fig. 1D.

Table S8. Neutralization IC<sub>50</sub> and SD values of SA7-like and C5G6 antibodies, related to Fig. 1D.

Table S9. Characterization of interaction surface area.

Table S10. ELISA EC<sub>50</sub> and SD values of C12G6 and C12G6-LALA antibodies, related to Fig. S22.

Table S11. C12G6 reactivity pattern.

Table S12. HA sequences of influenza B viruses used in this study.

Table S13. Primary data.

**REFERENCES AND NOTES**


The specificity of the influenza B virus hemagglutinin receptor binding pocket: What does it bind to?

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A multimechanistic antibody targeting the receptor binding site potently cross-protects against influenza B viruses

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An antibody to battle flu B

Although it circulates globally and is prevalent enough to warrant inclusion in the seasonal influenza vaccine, influenza B is far less well studied than its cousin, influenza A, and therapeutics are lacking. Shen et al. have now generated a potent antibody that inhibits diverse strains of influenza B virus. The antibody recognizes the receptor binding site in hemagglutinin, a region critical to viral entry, and was shown to be therapeutically effective in mice and ferrets. This antibody could be widely deployed to treat or prevent influenza B infection around the world.