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Single-replication BM2SR vaccine provides sterilizing immunity and cross-lineage influenza B virus protection in mice

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ABSTRACT

Both influenza A and B viruses cause outbreaks of seasonal influenza resulting in significant morbidity and mortality. There are two antigenically distinct lineages of influenza B virus, Yamagata lineage (YL) and Victoria lineage (VL). Since both B lineages have been co-circulating for years, more than 70% of influenza vaccines currently manufactured are quadrivalent consisting of influenza A (H1N1), influenza A (H3N2), influenza B (YL) and influenza B (VL) antigens. Although quadrivalent influenza vaccines tend to elevate immunity to both influenza B lineages, estimated overall vaccine efficacy against influenza B is still only around 42%. Thus, a more effective influenza B vaccine is needed.

To meet this need, we generated BM2-deficient, single-replication (BM2SR) influenza B vaccine viruses that encode surface antigens from influenza B/Wisconsin/01/2010 (B/WI01, YL) and B/Brisbane/60/2008 (B/Bris60, VL) viruses. The BM2SR-WI01 and BM2SR-Bris60 vaccine viruses are replication-deficient *in vitro* and *in vivo*, and can only replicate in a cell line that expresses the complementing BM2 protein. Both BM2SR viruses were non-pathogenic to mice, and vaccinated animals showed elevated mucosal and serum antibody responses to both Yamagata and Victoria lineages in addition to cellular responses. Serum antibody responses included lineage-specific hemagglutinin inhibition antibody (HAI) responses as well as responses to the stem region of the hemagglutinin (HA). BM2SR vaccine viruses provided apparent sterilizing immunity to mice against intra- and inter-lineage drifted B virus challenge. The data presented here support the feasibility of BM2SR as a platform for next-generation trivalent influenza vaccine development.

1. Introduction

Seasonal human influenza epidemics impose a heavy burden on society with 3–5 million cases and 250,000–500,000 deaths worldwide every year [1]. Influenza B often co-circulates with influenza A virus and accounts for approximately 20% of total influenza cases globally [2]. Recent public health surveillance strongly indicates that the impact of influenza B infection is especially significant as it was responsible for an estimated 22–44% of pediatric influenza mortality between 2004 and 2011 [3]. Since 1983, two antigenically distinct lineages of influenza B viruses have been co-circulating: B/Victoria/2/1987-like and B/Yamagata/16/1988-like strains [4]. These two distinct influenza B lineages co-exist and alternate in prevalence in a so far unpredictable pattern [5]. Trivalent influenza vaccines (TIV) provide little or no

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; BAL, bronchoalveolar lavage; BRISC, Biomedical Research Institute of Southern California; BSA, bovine serum albumin; CDC, Centers for Disease Control and Prevention; DMEM, Dulbecco's minimal essential medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HA, hemagglutinin; HAI, hemagglutination inhibition; IN, intranasally; BM2SR, BM2-deficient single-replication vaccine virus; MDCK, Madin-Darby canine kidney; BM2CK, Madin-Darby canine kidney cells expressing BM2 protein; MEM, minimal essential medium; MLD_{50}, 50% mouse lethal dose; MOI, multiplicity of infection; NA, neuraminidase; OD, optical density; PBS, phosphate-buffered saline; PFU, plaque-forming unit; QIV, quadrivalent inactivated influenza vaccine; RBC, red blood cell; RDE, receptor-destroying enzyme; S.D., standard deviation; SR, single replication; $TCID_{50}$, 50% tissue culture infectious dose; TIV, trivalent inactivated influenza vaccine; TMB, tetramethylbenzidine; TPCK, Tosyl phenylalanyl chloromethyl ketone; VL, Victoria lineage; YL, Yamagata lineage.

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cross-reactive protection between the two influenza B lineages resulting in low efficacy against strains from the omitted lineage [6]. Prediction of the predominant strain often fails, and so little or no protection was provided by the trivalent vaccines against the circulating influenza B virus in 5 of the 10 seasons between 2001 and 2010 in the USA [7]. The impact of the mismatch of the B vaccines was shown to be substantial in children and adolescents providing a strong rationale for the inclusion of both influenza B lineages in seasonal influenza vaccines [8]. Since 2012, influenza vaccine manufacturers have included both lineages in vaccine formulations resulting in quadrivalent influenza vaccines [9]. An immunogenicity benefit as well as improved efficacy against the influenza B viruses was seen with the quadrivalent vaccines compared to the trivalent vaccines [10–12].

Prophylactic vaccines remain the best countermeasure against influenza disease. Yet, current vaccines targeting both seasonal influenza A and B are only about 10–60% effective in young healthy adults and are considerably less effective in vulnerable populations such as the elderly [13]. Unlike in natural influenza infection, inactivated influenza vaccines do not elicit mucosal or T cell immunity and have limited duration of about 6 months before measurable efficacy begins to wane in man [14–17]. These vaccines are viral strain-specific and must be updated almost annually, based upon which strains are predicted to be circulating. There remains an urgent need for safe, more-effective influenza vaccines that offer broad-spectrum protection.

We have recently described a novel single-replication (SR) vaccine for influenza A virus that is an otherwise wild-type virus that does not express the essential M2 ion channel protein [18]. The M2-deficient single-replication (M2SR) vaccine viruses grow in cells that stably express M2, but do not replicate in normal cells. We previously showed that M2SR vaccine provided effective protection against both homologous and heterosubtypic influenza A virus infections, including highly pathogenic avian H5N1 virus, in both mouse and ferret models [18,19]. Here, we extend the promising SR vaccine platform to include an SR vaccine against influenza B virus. We modified a previously described replication-deficient BM2 knock-out (BM2KO) virus expressing the HA and NA from B/Lee/1940 [20] to generate BM2SR vaccines that express influenza B HA and NA from current circulating Victoria and Yamagata lineage viruses. The BM2SR vaccines were evaluated for efficacy against heterologous influenza B virus challenge in mice. We show that the BM2SR vaccines elicit systemic and mucosal immune responses that block detectable virus replication in the lung and provide effective cross-lineage protection against lethal influenza B challenge.

2. Materials and methods

2.1. Cells and viruses

Madin-Darby Canine Kidney (MDCK) cells were maintained in MEM, supplemented with 10% Fetal Bovine Serum (FBS). BM2CK cells (MDCK cells that stably express influenza BM2 protein [20]) were maintained in MEM, supplemented with 10% FBS and 150 μg/mL hygromycin B at 37 °C, 5% CO_2. The 293T (human embryonic kidney) cells were grown in DMEM supplemented with 10% FBS.

Wild-type influenza B strains were grown in MDCK, and BM2SR strains were grown in BM2CK cells in MEM, 0.3% bovine serum albumin (BSA) (Thermo Fisher Scientific, Waltham, MA) with 1 μg/mL Trypsin-TPCK (Worthington Biochemical Corporation, Lakewood, NH) at 35 °C, 5% CO_2. Viral culture supernatants were clarified by low-speed centrifugation and stored at <−65 °C until use.

2.2. Virus generation

BM2SR viruses were generated as previously described [20]. Briefly, influenza B viral RNA (vRNA) segments 1, 2, 3, 5, 8 and segment 7 lacking entire BM2 open reading frame (ORF) from influenza B/Lee/1940, and the HA and NA vRNA (segments 4, 6) of influenza B/Brisbane/60/2008 (B/Bris60, Victoria Lineage, VL) and B/Wisconsin/01/2010 (B/WI01, Yamagata Lineage, YL), respectively, were cloned into a RNA Polymerase I expression cassette as described [21,22]. Resulting plasmids were transfected along with viral polymerase subunit and NP (from B/Lee/1940) expression plasmids into 293T cells and viruses released into supernatant were amplified in BM2CK cells. The resulting viruses were called BM2SR-Bris60 and BM2SR-WI01, respectively. Control replication competent 6:2 reassortant viruses possessing the six internal segments from B/Lee/1940 and HA and NA from wild-type strains were generated using a wild-type B/Lee/1940 segment 7 cDNA clone.

2.3. Mice

Female BALB/c mice 7–8 weeks old (Envigo, East Millstone, NJ) were used in experiments previously approved by the FluGen Institutional Animal Care and Use Committee (IACUC) and 8–10 weeks old female C57BL/6 mice (Jackson ImmunoResearch, Sacramento, CA) were used in experiments previously approved by Biomedical Research Institute of Southern California (BRISC) IACUC. All experiments were performed according to National Institutes of Health guidelines for the care and use of laboratory animal subjects.

2.4. Immunization and challenge

Mice were anesthetized (N = 26) and vaccinated intranasally (IN) twice (days 0 and 28) with BM2SR virus (10^6 $TCID_{50}$/mouse), as previously described [18]. Mock-vaccinated control mice received PBS vehicle alone. In some experiments, additional groups of mice were vaccinated subcutaneously with 1 μg/mouse recombinant B/Wisconsin/01/2010 HA protein (rHA-WI01; Immune Technology Corp., Tarrytown, NY) for comparison. Twenty-one days post-final immunization, 4 mice per group were euthanized and sera and bronchoalveolar lavage (BAL) were collected for determination of antibody responses. Six weeks after final vaccination, mice were challenged IN with 20 MLD_{50} influenza B/Malaysia/2506/2004 (B/Malaysia, VL). Mouse body weight and survival were monitored for 14 days following challenge. Lungs and nasal turbinates were obtained from 3 mice per vaccine group on day 4 post-challenge. Virus load in the organs was determined in MDCK cells by plaque assay as previously described [23].

2.5. Virus-specific antibody detection

Immunoglobulin G (IgG) and IgA titers were measured in sera and BAL as previously described by ELISA against recombinant HA protein from B/WI01 and B/Bris60 (Immune Technology Corp., Tarrytown, NY) [24]. Functional antibody levels were assessed by hemagglutination inhibition (HAI) assay as described previously [18].

For immunoblotting, recombinant proteins encoding HA0 of B/Bris60 and B/WI01 (amino acids 16–548, Immune Technology Corp.), HA1 of B/Bris60 and B/WI01 (amino acids 6–303 and 6–301, respectively, International Reagent Resource, IRR, Manassas, VA); and HA2 of influenza B/Florida/4/2006 (YL) -Human Fc fusion (HA amino acids 362–555, Sino Biological Inc., Wayne, PA) were subjected to denaturing 4–12% acrylamide SDS-PAGE, electroblotted to PVDF membranes and then probed with sera from BM2SR-Bris60- and BM2SR-WI01-immunized or from PBS-Mock control mice, followed by HRP-conjugated goat anti-mouse IgG

(Kirkegaard & Perr Laboratories Inc., Gaithersburg, MD). Bands were visualized using chromogenic TMB membrane peroxidase substrate (Thermo Fisher, Waltham, MA).

2.6. Lymphocyte subsets in the BAL

Lymphocyte or T cell subsets in the bronchoalveolar lavage (BAL) were analyzed by staining with fluorochrome-conjugated antibodies to cell surface markers followed by flow cytometric analysis as previously described [18,23].

2.7. Histopathology

Tissue samples were formalin-fixed after harvesting from euthanized mice. Tissues were embedded in paraffin wax and sectioned. Haematoxylin and eosin staining was performed for histopathological assessment.

3. Results

3.1. BM2SR viruses are replication-deficient in vitro

BM2-deficient, single-replication influenza B virus (BM2SR) that lacks the coding capacity for the BM2 ion channel protein from B/Lee/1940 strain, is replication-deficient in normal MDCK cells, but does generate infectious progeny in complementing MDCK cells that stably express the BM2 protein (BM2CK) [20]. We used this virus backbone and updated the HA and NA to represent the two modern influenza B lineages: Victoria lineage (VL) and Yamagata lineage (YL). Thus, BM2SR vaccine viruses expressing HA and NA proteins from B/Brisbane/60/2008 (BM2SR-Bris60, VL) or from B/Wisconsin/01/2010 (BM2SR-WI01, YL) were generated. Control cognate viruses expressing the same HA and NA (6:2 reassortants in B/Lee/40 background encoding functional BM2) were also generated.

To demonstrate that BM2SR viruses encoding modern HA and NA on the B/Lee/40 backbone do not generate progeny virus in normal cells, growth kinetics of the BM2SR viruses were evaluated in permissive (BM2CK) and non-permissive cells (MDCK). Cells were infected with viruses (MOI = 0.001) and culture supernatant aliquots taken at indicated time points after infection were analyzed for virus titers by $TCID_{50}$. As expected, the BM2SR viruses did not replicate in normal MDCK cells but grew as well as their cognate 6:2 viruses in BM2CK cells (Fig. 1). The 6:2 reassortant control viruses grew to similar high titers in both cell types. No replication of BM2SR is seen in normal MDCK cells even under high MOI infection conditions, (data not shown). These data demonstrate that although BM2SR virus replication is indeed restricted in normal cells, high-titer virus can be produced in cells that stably express the BM2 protein.

The genetic stability of the BM2SR virus backbone was tested by serial passage in BM2CK cells. The BM2SR-Bris60 was passaged 13 times in BM2CK cells, and sequence analysis of the complete virus genome was performed. Analysis of the BM2SR M segment sequence revealed that the virus still possessed a complete deletion of the BM2 ORF as expected. Only a single nucleotide mutation (a406c change in segment 7) was observed resulting in a BM1 conservative amino acid substitution, L128I (data not shown). No other changes were observed in any of the other segments (data not shown). These data indicate that the BM2SR virus backbone is genetically stable and thus suitable for construction of vaccine candidates that express WHO-recommended, modern influenza B virus HA and NA surface antigens.

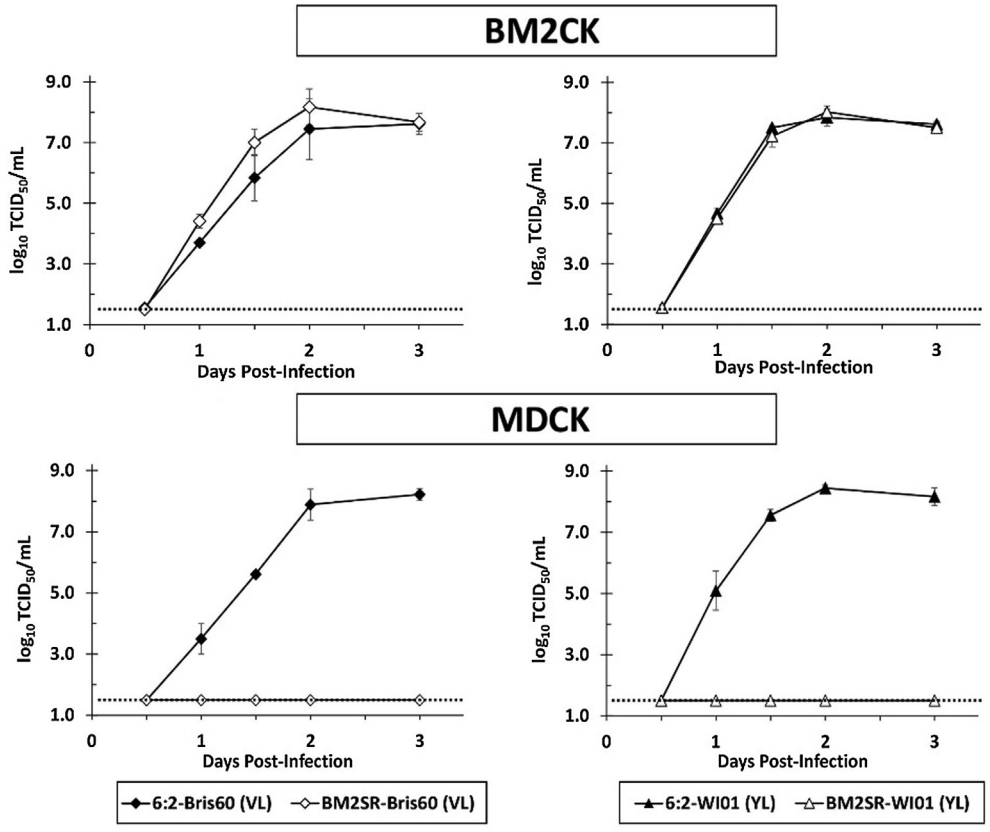

Fig. 1. BM2SR is replication-deficient *in vitro*. **BM2CK** cells (top panels) or **MDCK** cells (bottom panels) were infected with **BM2SR-Bris60** (open diamond) and reassortant **6:2-Bris60** (filled diamond) or **BM2SR-WI01** (open triangle) and reassortant **6:2-WI01** (filled triangle) at MOI = 0.001. Culture supernatants were sampled at 0.5, 1, 1.5, 2 and 3 days after inoculation and tested for virus titer by $TCID_{50}$ assay in BM2CK cells. Mean value of triplicate determination ± standard deviation (SD, error bars) are reported. Samples with no detectable titer are plotted at assay limit of detection $\log_{10}$ $TCID_{50}$ = 1.67 shown as dotted line.

3.2. BM2SR is non-pathogenic in vivo

Virus attenuation is critical for development of a live vaccine; therefore, we examined the pathogenicity of the BM2SR virus in mice. BALB/c mice (N = 26 per group) were dosed intranasally (IN) with BM2SR-WI01 or BM2SR-Bris60 (10^6 $TCID_{50}$/mouse), or PBS vehicle and monitored for weight loss for 14 days. Mice did not exhibit any loss in body weight or display any signs of illness after inoculation. There was no significant difference in body weights of BM2SR and PBS inoculated mice at any time-point, suggesting that BM2SR vaccines are non-pathogenic *in vivo* (Fig. 2).

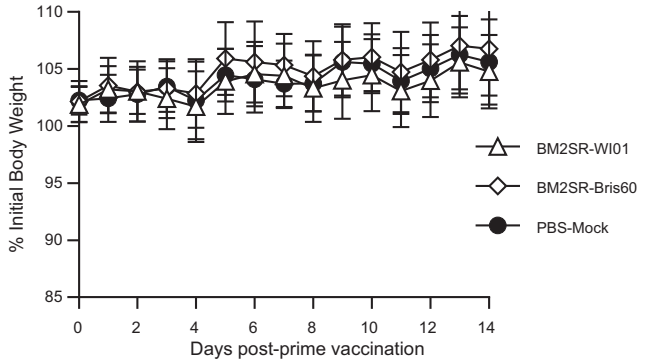

Fig. 2. BM2SR is non-pathogenic *in vivo*. Three groups of BALB/c mice (N = 26) were vaccinated with 10^6 $TCID_{50}$ of **BM2SR-WI01** (open triangle), **BM2SR-Bris60** (open diamond) or **PBS** as mock (filled circle). Mouse body weights were measured daily from days −3 to 14 post-vaccination. The mean body weight of the individual mice measured for four days prior to vaccination was used as 100% body weight to normalize the data. Mean % body weight and SD for each group is shown.

Moreover, no BM2SR virus was detected in lungs or nasal turbinates harvested on days 1 and 4 post-inoculation and tested by plaque assay in permissive BM2CK cells (data not shown). These results indicate that BM2SR viruses do not replicate in the mouse respiratory tract and that the virus is not pathogenic.

3.3. BM2SR induces moderate increases in cell number in the bronchoalveolar lavage

Cellular infiltration was evaluated in the lungs of C57BL/6 mice after priming and boosting 28 days later with BM2SR-WI01 (10^6 $TCID_{50}$/mouse IN). After priming, the total number of live cells in bronchoalveolar lavage (BAL) increased 2 to 3-fold peaking at day 7 (Fig. 3A). However, the live cell number in the BAL changed very little after boosting (Fig. 3B). The distribution of leukocyte subsets in the BAL was evaluated using DiffQuik (Thermo-Fisher, Carlsbad, CA) stained cytospin preparations. Most of the cells in the BAL after both prime and boost inoculations were macrophages/monocytes, with smaller numbers of lymphocytes and very few neutrophils (data not shown). No basophils or eosinophils were observed.

Cytokine profiles in the cell-free BAL fluid collected after BM2SR prime boost were evaluated by ELISA. Only low levels of IFN-γ, TNF, and IL-10 and no IL-4 could be detected (data not shown). The levels of the cytokines were not statistically significantly higher than those in control, unvaccinated mice.

A measurable influx of T cells was observed in BAL after both priming and boosting with BM2SR, although the magnitude of the response was lower after boosting. Most of the T cells detected were αβ TCR^+ $CD8^+$ T cells (Fig. 3C and D), although low numbers of $CD4^+$ γδ T cells were also found.

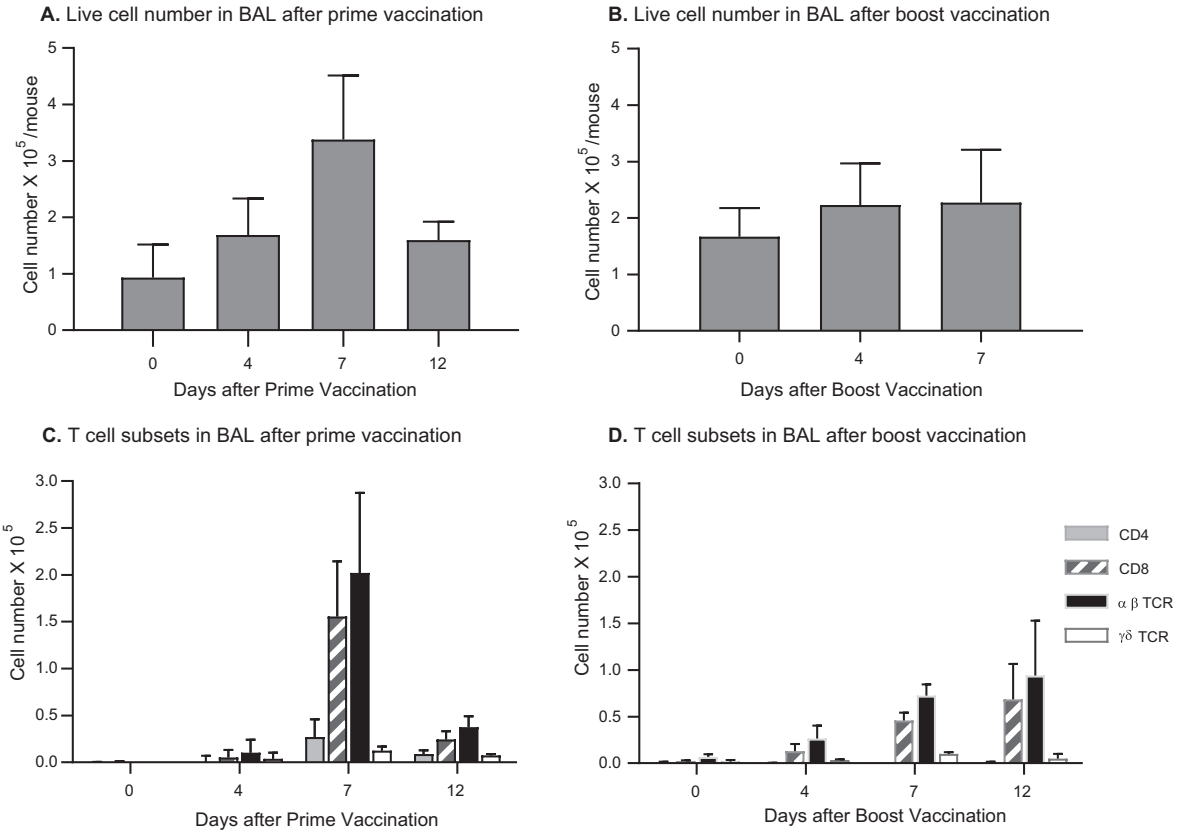

Fig. 3. Cellular responses to BM2SR. C57BL/6 mice were primed intranasally on day 0 and boosted 28 days later with 10^6 $TCID_{50}$ of BM2SR-WI01. Bronchoalveolar lavage (BAL) was harvested from groups of 5 mice before (day 0) and at the specified intervals after prime (A. and C.) or boost (B. and D.). Live cell counts (A. and B.), shown as mean cell number/mouse + SD, were determined by Trypan Blue exclusion. T cell subsets were analyzed by flow cytometry (C. and D.), shown as mean cell numbers/mouse + SD.

Taken together, these data indicate that BM2SR vaccination does induce an increase in cellular infiltration, but does not induce excessive inflammation.

3.4. BM2SR induces mucosal and serum antibody responses in mice

The immunogenicity of the BM2SR vaccines was evaluated in mice following intranasal vaccination in a prime and boost regimen. Three weeks after the last immunization, serum and BAL were collected from the mice and anti-HA IgG and IgA antibody titers against both influenza B lineages (YL and VL) were measured by ELISA. Both the BM2SR-WI01 and BM2SR-Bris60 vaccines induced mucosal IgA and IgG antibody responses to both HA proteins in BAL (Fig. 4A and C). Similarly, both vaccines induced serum IgA and IgG antibody titers against both B/WI01 (YL) and B/Bris60 HA (VL) proteins (Fig. 4B and D). Antibody responses to the B/WI01 HA in the BM2SR-Bris60-vaccinated mice and conversely to the B/Bris60 HA within

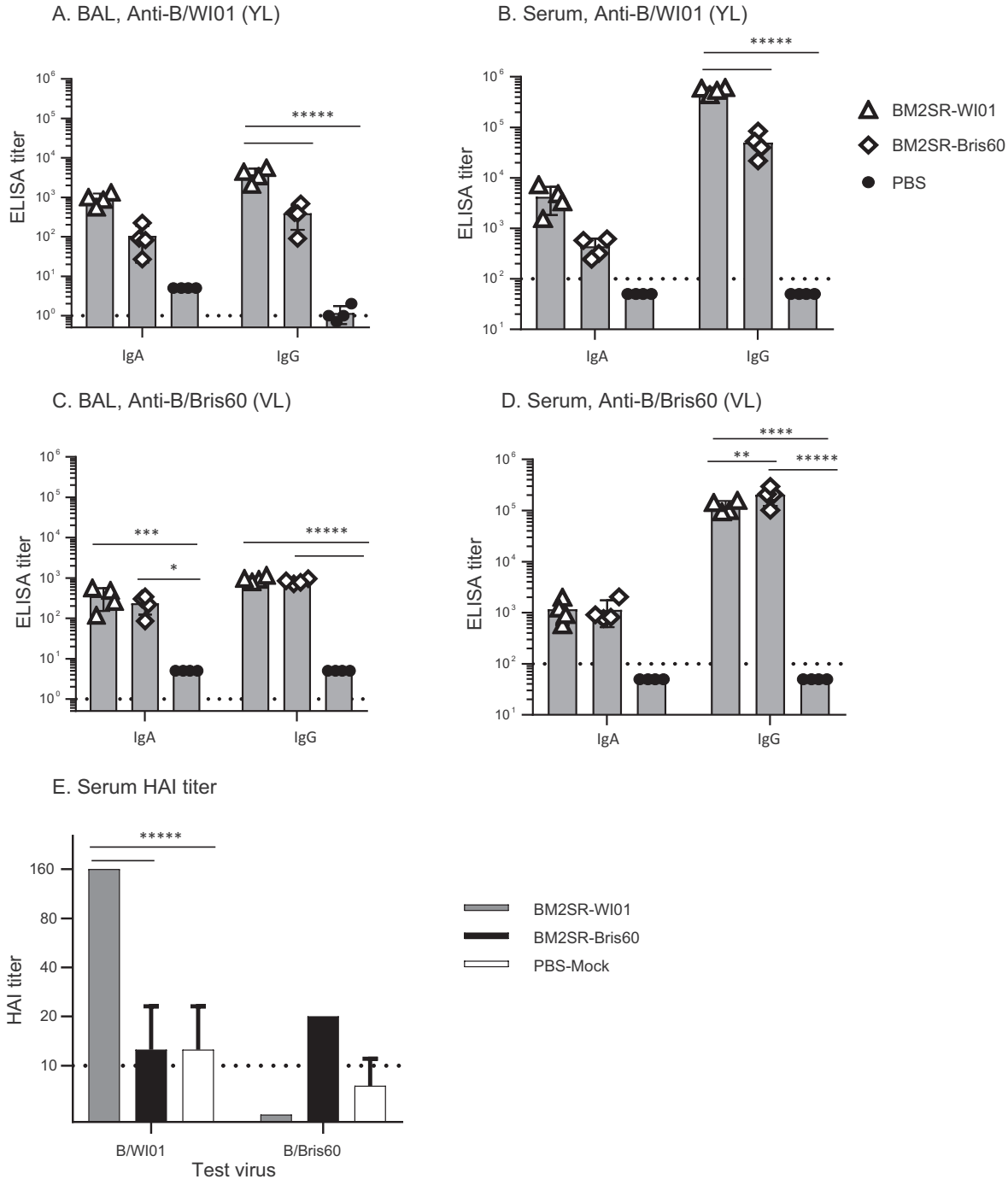

Fig. 4. BM2SR induces mucosal and serum antibody responses in mice. BALB/c mice were vaccinated with either BM2SR-WI01 (open triangle), BM2SR-Bris60 (open diamond), or PBS (Mock, filled circle). Sera and BAL were obtained from four mice per vaccine group 21 days post-boost vaccination. A. Anti-WI01 IgA and IgG ELISA titers in BAL. B. Serum anti-WI01 IgA and IgG ELISA titers. C. Anti-Bris60 IgA and IgG ELISA titers in BAL. D. Serum anti-Bris60 IgA and IgG ELISA titers. For panels A through D, symbols are titers for individual animals, bars represent the mean titer for the group. E. Serum HAI titers. Mean ± SD from duplicate vaccinations is shown. Dashed lines show lower limit of detection for each assay. Reference ferret antisera to WI01 (IRR, Cat # FR-810) and to Bris60 (IRR, Cat # FR-392) showed HAI titer 320 and 160 against homologous antigens in the same assay (data not shown). $^{*}P = 0.035$; $^{**}P = 0.0131$, $^{***}P = 0.0018$, $^{****}P = 0.0003$, $^{*****}P < 0.0001$, Two-way ANOVA, Tukey's Multiple Comparison Test.

BM2SR-WI01-vaccinated mice indicate substantial cross-reactivity to the influenza B VL and YL HA proteins in the BM2SR immune response yet each BM2SR vaccine demonstrated statistically significant ELISA titer differences in serum against its cognate antigen (Fig. 4B and D). These data demonstrate that BM2SR viruses elicit cross-reactive systemic and mucosal immune responses in mice.

Functional antibody titers in serum were measured by HAI assay. BM2SR-WI01 elicited strong HAI titers against the WI01 virus that were significant ($P < 0.0001$) and not cross-reactive with the Bris60 virus (Fig. 4E). Although BM2SR-Bris60 induced high ELISA titers against Bris60 HA, the HAI titers against Bris60 virus were low (Fig. 4E). Thus, although strong cross-reactive antibodies were detected by ELISA, BM2SR elicited HAI antibodies were lineage-specific.

To further understand the nature of the cross-reactive antibody responses to HA, we evaluated whether BM2SR induces antibodies to the HA2 stem region. Sera collected three weeks after boosting from the mice immunized above were analyzed by Western analysis for reactivity against recombinant influenza B HA0, HA1 or HA2 proteins. Recombinant HA0 (full-length including head and stem), HA1 (head region) and HA2 (stalk region) were separated by gel electrophoresis and analyzed by immunoblot for reactivity with sera from BM2SR-Bris60, BM2SR-WI01 or mock-immunized mice. As expected, the sera from mice vaccinated with BM2SR-Bris60 or BM2SR-WI01 recognized both Bris60 and WI01 HA0 proteins, consistent with the ELISA results (Fig. 5, lanes 2, 4, 7, 9). The BM2SR-Bris60 and the BM2SR-WI01 sera appeared to yield slightly higher signal on cognate HA1 antigens (Fig. 5, lanes 3, 5, 8, 10) consistent with the ELISA and HAI results. More importantly, sera obtained from BM2SR-Bris60- or BM2SR-WI01-vaccinated mice reacted strongly with the HA2 protein from B/Florida/06/2004, VL (Fig. 5, lanes 6 and 11). The immunoblot signal from the HA2 antigens did not appear to differ between the two types of sera. Sera from the mock-immunized mice did not react with any of the recombinant proteins (Fig. 5, lanes 12–16). These results indicate that BM2SR vaccines induce cross-reactive antibodies against the highly conserved HA2 stem region of the HA.

3.5. BM2SR protects mice against intra-lineage and inter-lineage drifted influenza B virus challenge

BALB/c mice prime-boost vaccinated with BM2SR-WI01 (YL), BM2SR-Bris60 (VL) or PBS-mock were challenged with 20 MLD_{50} of B/Malaysia/2506/2004 (B/Malaysia, VL), which is antigenically distinct from the Victoria-lineage vaccine virus, BM2SR-Bris60. No PBS-mock immunized mice survived the lethal B/Malaysia (VL) challenge (Fig. 6A). Meanwhile all mice vaccinated with BM2SR-Bris60 survived the challenge with an antigenically drifted VL virus (Fig. 6A) and no weight loss was observed (Fig. 6B). Importantly, vaccination with BM2SR-WI01 (YL) also protected against challenge with the B/Malaysia (VL) virus, demonstrating inter-lineage protection (Fig. 6A and 6B). No virus replication was detected in lungs or in nasal turbinates of either BM2SR-WI01 or BM2SR-Bris60-vaccinated animals in contrast to the PBS-mock-immunized mice that had viral load exceeding 1×10^6 PFU/g of lung at 4 days post-challenge, (Fig. 6C). These results indicate that VL BM2SR vaccine viruses induced intra-lineage sterilizing immunity against an antigenically drifted strain and, more importantly, inter-lineage sterilizing immunity by YL BM2SR vaccine viruses.

3.6. BM2SR reduces inflammatory responses and lung pathology after inter-lineage challenge

To further investigate the mechanism of BM2SR protection, we examined immune cell infiltration following challenge by enumerating cell populations in BAL obtained from mice that were vaccinated and then subjected to challenge. Consistent with the lack of viral replication observed in the lungs after challenge, there was very little influx of cells into the airways of BM2SR-WI01 (YL)-immunized mice after B/Malaysia (VL) challenge (Fig. 7A). In contrast, a large influx of cells was observed in the lungs of rHA-WI01- and PBS-mock-immunized mice 7 days after wild-type virus challenge. Most infiltrating cells were macrophages and neutrophils, although significant numbers of lymphocytes were also observed at day 7 post-challenge (Fig. 7B, C, D). BM2SR vaccinated mice showed a significant reduction in weight loss between days 4 and 9 after challenge, compared with unvaccinated mice. In contrast there was no significant difference in weight loss between unvaccinated and rHA-WI01 vaccinated groups (Fig. 7E). Increased pulmonary inflammation was still apparent in the lungs of mock-immunized mice as late as day 21 after challenge, although all of the C57BL/6-background mice survived the challenge (data not shown). Meanwhile BM2SR-vaccinated mice showed a much lower level of inflammation at day 21 (Fig. 7F, G).

4. Discussion

Most influenza vaccines today are formulated in a quadrivalent format necessitated by low cross-protection between the two

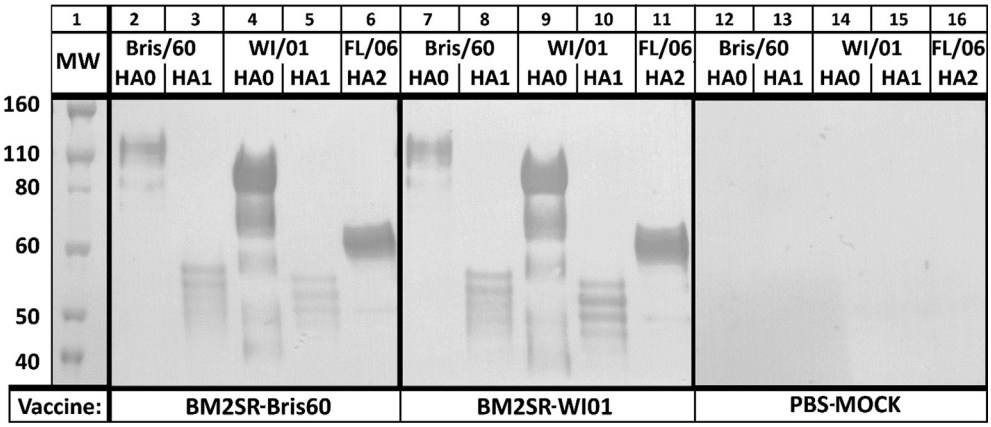

Fig. 5. BM2SR induces serum antibody responses to both HA1 and HA2**.** An immunoblot was performed as described in Materials and Methods. Recombinant antigens were HA0 as well as HA1 from both Bris60 and WI01, and an HA2 fusion protein from B/Florida/2006. Primary antibodies were pooled sera from BM2SR-Bris60 and BM2SR-WI01 vaccinated mice used at 1:1000 dilution and naïve sera from the PBS-Mock group used at 1:200 dilution.

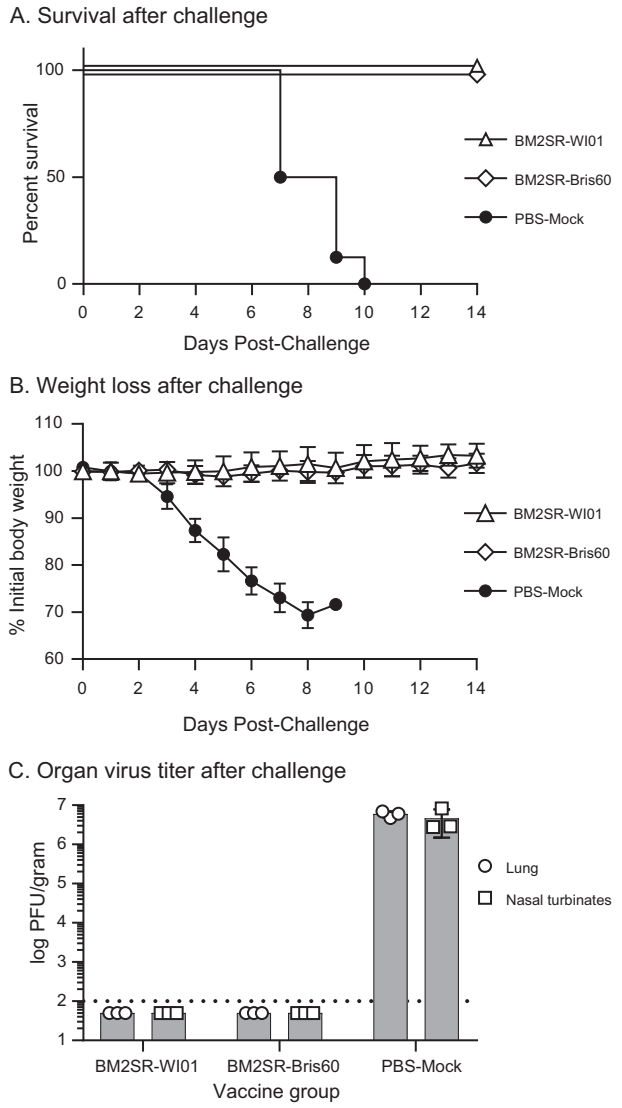

Fig. 6. BM2SR protects mice against influenza B challenge by ablation of viral replication. Three groups of BALB/c female mice were vaccinated twice at 4-week intervals with BM2SR-WI01 (open triangle), BM2SR-Bris60 (open diamond) or with PBS Mock (filled circle). Six weeks after boost immunization, mice were challenged with 20 MLD_{50} of wild-type B/Malaysia/2506/2004 (VL). Animals (N = 8) were observed daily for 14 days after challenge: A. Percentage survival. B. Body weight. Body weight was normalized to 100% of the average of the individual weights measured for three days before challenge. C. Lungs and nasal turbinates (N = 3) were harvested from each group on day 4 post-challenge and viral load in organs was determined by plaque assay. Lower limit of detection was approximately 100 PFU/g (dashed line). Individual virus titers (circle or square) with mean (bar) and SD (error bars) are shown.

human influenza A strains and between the two influenza B lineages. Previously, trivalent vaccine formulations contained a single influenza B lineage vaccine. However, divergence of influenza B into antigenically-distinct B/Yamagata/16/1988-like and B/Victoria/2/1987-like lineages has resulted in frequent mismatch of trivalent influenza vaccine towards the circulating influenza B [25]. In addition, sequential administration of TIV with alternate lineages on occasion would result in a poor response, as B/Victoria-containing boost vaccination strongly recalled antibody to the dominant B/Yamagata priming antigen but induced only low B/Victoria antibody responses in a published clinical trial [26,27]. Thus, manufacturers now include both influenza B lineages in the preferred quadrivalent influenza vaccine formulation. Although data support improved efficacy of QIV vs TIV for the B lineage not included in TIV [28], some cross-lineage protection has been observed with TIV containing mismatched B/Victoria antigen during the 2017–2018 epidemic [29]. We show here that monovalent BM2SR vaccines provide strong protection against mismatched influenza B virus challenge. Intranasal administration of BM2SR, a live single-replication influenza vaccine virus, encoding HA and NA from either lineage provided sterilizing immunity against challenge with a drifted influenza B virus belonging to the B/Victoria lineage. These results suggest that BM2SR may circumvent the need for quadrivalent formulations allowing a future trivalent formulation that is highly effective against all circulating influenza B virus lineages.

Mice vaccinated with either BM2SR-WI01 (YL) or with BM2SR-Bris60 (VL) were protected against lethal challenge with a drifted strain, B/Malaysia/2506/2004 (VL). All (100%) of vaccinated mice survived and little, if any weight loss or overt signs of illness were observed post-challenge. Inflammation and lung pathology, after challenge, were greatly reduced in BM2SR-vaccinated mice compared to mock-vaccinated subjects. In accordance with this observation, no viral replication could be detected in the lungs or nasal turbinates after challenge. These data show that BM2SR vaccination provides sterile immunity against drifted intra-lineage and cross-lineage influenza B virus infections in a murine challenge model. This cross-protection is in line with data from wild-type influenza B infection of ferrets with virus from either lineage that were partially protected against subsequent infection with the opposite lineage [30]. Similarly, live attenuated influenza B vaccination of ferrets, provided some cross-lineage protection against challenge in the absence of cross-lineage serum antibody responses suggesting that local and cellular immune response mechanisms may play a role in cross-protection [31]. Indeed cross-reactivity of $CD8^+$ cytotoxic T-lymphocyte populations obtained from human subjects has been shown between the two influenza B lineages [32].

BM2SR stimulated a broad immune response including serum and mucosal antibodies and cellular responses as described previously for influenza A M2SR [18]. The response to Bris60 HA (VL) antigen as measured by ELISA was remarkably similar in both BM2SR-Bris60 and BM2SR-WI01 vaccinated mice, while in contrast immune specificity was seen in the response against BM2SR-WI01 (YL), as ELISA IgA and IgG ELISA titers were at least 10-fold higher than for the Bris60-BM2SR vaccine response to WI01 HA. The response includes antibodies specific towards both HA1 and the more-conserved HA2 domains by immunoblot analysis. The signal from WI01 HA1-specific IgG in the serum from BM2SR-WI01-vaccinated mice was more intense than that from BM2SR-Bris60 animals, which suggests the 10-fold higher ELISA titer was caused by head-specific response to the YL vaccine. This observation is consistent with B/Yamagata immunodominance observed with trivalent vaccines [26,27].

Specific functional response was also seen in serum HAI titer to both BM2SRs tested, but even though serum and mucosal antibodies elicited by BM2SR-Bris60 reacted strongly to HA from WI01 and vice-versa, only the BM2SR-WI01 HAI titers reached a level considered to be protective (HAI titer $\geq$ 40). The BM2SR-WI01 HAI results are consistent with the observation that response to WI01 HA was more specific and higher in overall IgA and IgA ELISA titers. However, both BM2SRs appear to provide complete protection from challenge, thus the serum HAI assay was not a reliable immune correlate for the protection afforded by the SR vaccine platform in this study. As suggested for other intranasal live influenza vaccines [31], the mechanism of protection for BM2SR may not be serum neutralizing antibodies. Recently, others have proposed that the HA2 stem is important to universal immunity against both influenza A and B viruses [33,34]. In this study, a strong response

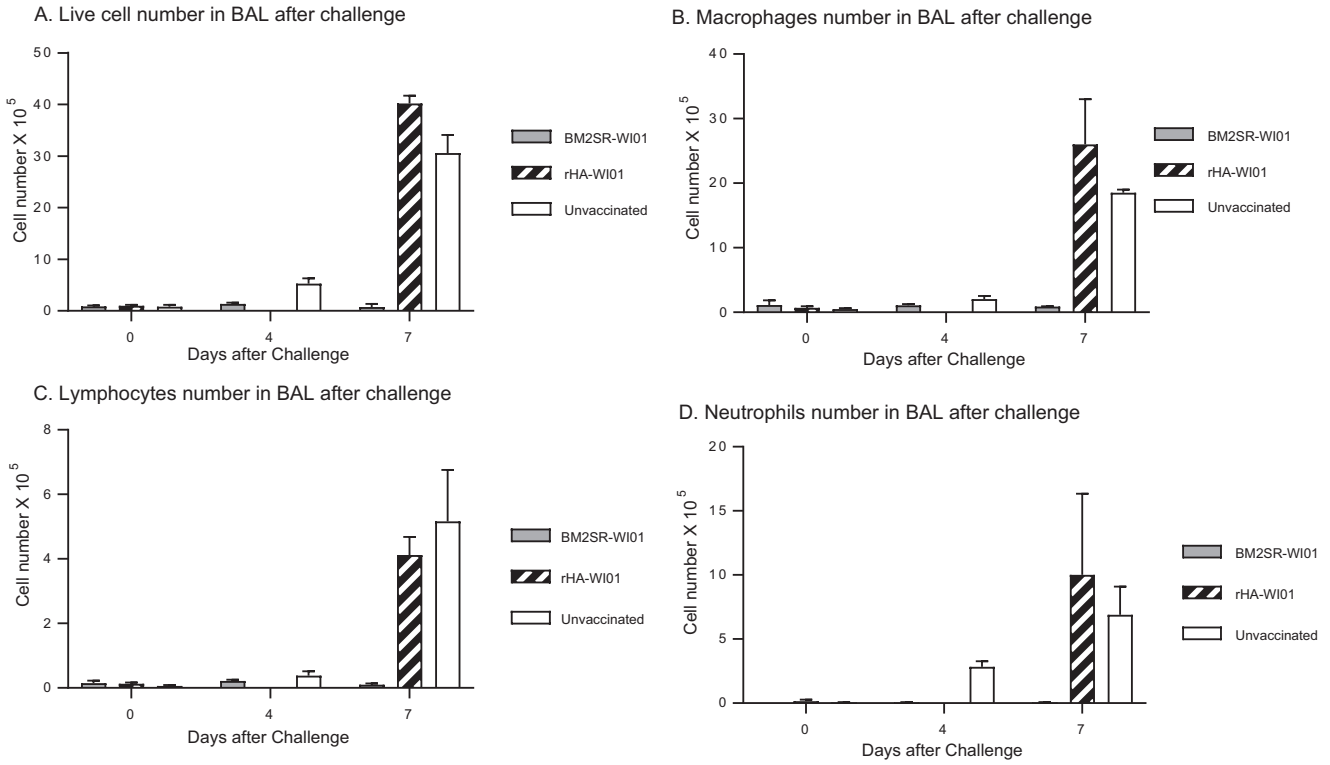

Fig. 7. BM2SR reduces inflammatory responses after challenge. C57BL/6 mice were primed intranasally on day 0 and boosted on day 28 with 10^6 $TCID_{50}$ BM2SR-WI01. Additional groups of control unvaccinated mice and mice vaccinated with 1 μg recombinant HA from influenza B/WI01 (rHA-B/WI01) subcutaneously were included for comparison. All groups were challenged with B/Malaysia on day 28 after the boost immunization. Cells were stained and enumerated in BAL samples collected on day 0 before challenge and at 4 and 7 days after challenge. A. Total live cell counts by Trypan Blue exclusion. Leukocyte subsets in the BAL were stained with Diffquik to facilitate differential cell counting of: B. macrophages, C. lymphocytes, and D. neutrophils. E. Weight loss post-challenge. *Significantly different from unvaccinated and recombinant HA-vaccinated between days 4 and 9 post-challenge ($p < 0.05$, Paired *t* test). F. Histopathology score measuring severity of inflammation was classified as 1 (none), 2 (moderate), 3 (significant), 4 (severe) or 5 (very severe). ****Significantly different from unvaccinated and recombinant HA vaccinated ($p < 0.0001$, *T* test). G. Representative H&E stained sections of lung tissue at day 21 after challenge at low (×10) and high (×100) magnification.

to the highly conserved influenza B HA2 antigen following BM2SR immunization was seen on immunoblot. Human monoclonal antibodies that bind influenza B HA2 stem antigens have been identified [35,36]. While neutralizing activity has been demonstrated for some, the majority were non-neutralizing that exert protective effects via mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) [37–39]. In addition, a universally-conserved and immunodominant influenza B HA2 $CD8^+$ T cell epitope capable of inducing a strong cellular response and ADCC has been identified [40]. Perhaps the mechanism of protection with BM2SR is partly driven by a response towards more universal epitopes found in the influenza B HA2 stem.

Our previous findings with influenza A M2SR vaccine showed that M2SR vaccination provided complete protection against homologous and heterosubtypic challenge with live influenza A. Yet, unlike the BM2SR vaccine, M2SR only provided sterilizing immunity against a homologous strain, while some viral replication was observed in the lungs of mice after challenge with heterosubtypic influenza A virus [18,19]. The difference in the strength and breadth of cross-protection observed here may in part be due to the greater conservation between the influenza B lineages as compared to the influenza A strains. Further studies will be required to provide insight into differences in the mechanisms of protection afforded by the M2SR and BM2SR vaccines.

Severe or lethal influenza, including that caused by infection with highly pathogenic avian influenza (H5N1) viruses, is associated with an acute respiratory distress syndrome characterized by high levels of inflammatory cell infiltration, cytokine production (often referred to as "cytokine storm") and subsequent pulmonary damage. In addition, significant increases in the numbers of neutrophils and in TNF production have been reported [41]. Administration of BM2SR vaccine virus did not result in respiratory distress or lung damage in the vaccinated mice. The vaccination induced only low levels of cell infiltration and cytokine production and significantly reduced lung pathology after high dose challenge. Very few neutrophils were observed in BM2SR-vaccinated animals after priming and boosting, or after challenge. This supports the hypothesis that since BM2SR vaccine virus does not replicate in the lung, it induces only a limited inflammatory response.

Historically, some vaccines for respiratory viruses like RSV have resulted in asthma-like symptoms after natural infection [42], while some, but not all, studies have shown that LAIV may induce wheezing in children [43–45]. If this were the case for BM2SR vaccines, we would expect to see an influx of eosinophils and neutrophils and high levels of TH2 cytokines like IL-4. However, this type of inflammatory immune response profile was not observed in the present study of BM2SR or previously with M2SR [18].

Overall, our data demonstrate that BM2SR vaccination provides safe and effective protection against infection with drifted and heterologous influenza B virus in mice by inducing systemic and mucosal immune responses. The cross-protection provided by BM2SR may in part be due to cross-reactive antibodies directed against the highly conserved stem region of the HA.

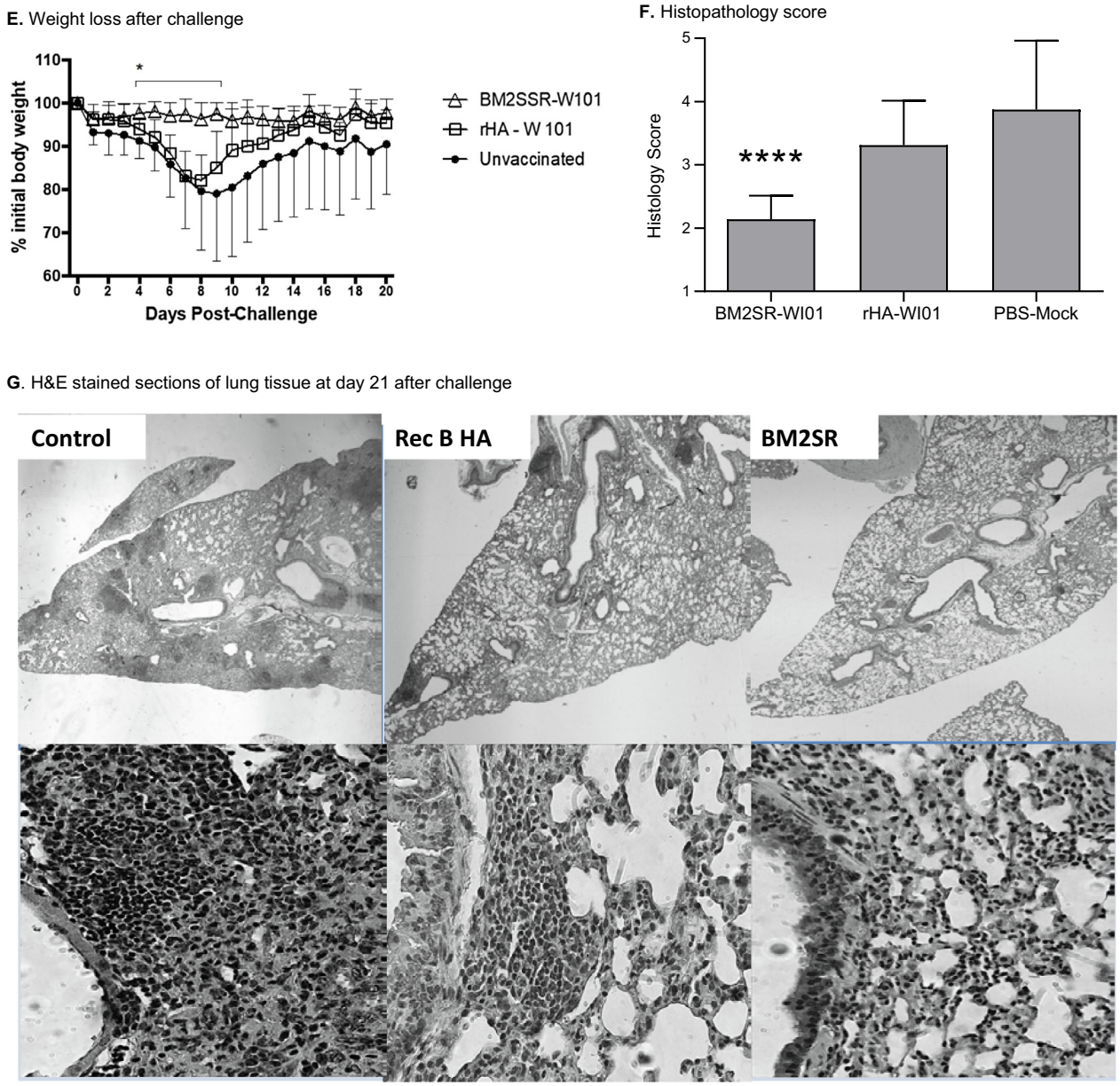

Fig. 7 (*continued*)

Declaration of Competing Interest

S.S., C.G., A.S., P.D. and M.H. have no conflicts of interest. G.N. and Y.K. are founders of FluGen. MJM, Y.H. and P.B. are employees of FluGen.

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