M2-Deficient Single-Replication Influenza Vaccine–Induced Immune Responses Associated With Protection Against Human Challenge With Highly Drifted H3N2 Influenza Strain

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   Background. Current influenza vaccines are strain specific and demonstrate low vaccine efficacy against H3N2 influenza disease, especially when vaccine is mismatched to circulating virus. The novel influenza vaccine candidate, M2-deficient single replication (M2SR), induces a broad, multi-effector immune response.

   Methods. A phase 2 challenge study was conducted to assess the efficacy of an M2SR vaccine expressing hemagglutinin and neuraminidase from A/Brisbane/10/2007 (Bris2007 M2SR H3N2; clade 1). Four weeks after vaccination, recipients were challenged with antigenically distinct H3N2 virus (A/Belgium/4217/2015, clade 3C.3b) and assessed for infection and clinical symptoms.

   Results. Adverse events after vaccination were mild and similar in frequency for placebo and M2SR recipients. A single dose of Bris2007 M2SR induced neutralizing antibody to the vaccine (48% of recipients) and challenge strain (27% of recipients). Overall, 54% of M2SR recipients were infected after challenge, compared with 71% of placebo recipients. The subset of M2SR recipients with a vaccine-induced microneutralization response against the challenge virus had reduced rates of infection after challenge (38% vs 71% of placebo recipients; *P* = .050) and reduced illness.

   Conclusions. Study participants with vaccine-induced neutralizing antibodies were protected against infection and illness after challenge with an antigenically distinct virus. This is the first demonstration of vaccine-induced protection against a highly drifted H3N2 challenge virus.

   Keywords. Influenza; vaccine; challenge; H3N2; drift.

Influenza virus causes serious respiratory illness and death each year, with excess mortality and higher hospitalization rates in older adults during influenza seasons dominated by H3N2 compared with other influenza subtypes [1]. Annual vaccination remains the most widely used intervention for preventing seasonal influenza, and vaccine strains are updated annually in attempts to account for continuing antigenic drift. Despite annual updates to vaccine composition, vaccine-induced protection against H3N2 viruses remains low, especially in years when there is a mismatch between the vaccine and circulating strains or when multiple clades

of H3N2 circulate simultaneously. Vaccine effectiveness against H3N2 viruses in the United States ranges from 39% in 2012–2013 when vaccine matched the circulating strain, to as low as 6% in 2014–2015, during a mismatch season [2]. Thus, there is a need for improved influenza vaccines that induce broader, cross-clade immune responses. Such novel vaccines would be less subject to antigenic changes of circulating viruses and could potentially be administered less frequently than currently licensed vaccines.

   M2-deficient single replication (M2SR) is an investigational intranasal vaccine that has shown homologous, heterologous and heterosubtypic protection in animal models [3]. Vaccine virus is produced in cells that produce the essential viral M2 protein, but M2 is absent from the virus genome. The resulting virus mimics wild-type influenza when intranasally administered, but only for a single replication cycle, and it does not produce infectious virus. Vaccine virus infection stimulates protection by inducing broad-spectrum immunity similar to that induced by wild-type influenza infection [3]. An M2SR vaccine expressing hemagglutinin (HA) and neuraminidase (NA) glycoproteins from A/Brisbane/10/2007 (H3N2, clade 1; hereafter Bris2007 M2SR) was shown to protect against a

drifted H3N2 virus from 2015 (clade 3C.3b) in the ferret model [4]. In a phase 1 clinical trial, Bris2007 M2SR was shown to be safe and immunogenic in humans at the highest dose tested (10^8 median tissue culture infectious dose [$TCID_{50}$]) and to induce detectable hemagglutination inhibition (HAI) antibody titers against highly drifted H3N2 strains from different clades, including recent modern strains [5].

In this phase 2 study, we assessed the safety and efficacy of the Bris2007 M2SR vaccine against challenge with a highly antigenically drifted H3N2 wild-type virus strain isolated in 2015. Bris2007 M2SR encoded the H3N2 HA and NA from the 2009–2010 influenza vaccine, whereas the challenge virus was related to the H3N2 component of the 2015–2016 influenza vaccine. Between 2009 and 2015, the World Health Organization updated the H3N2 component of the seasonal influenza vaccine 4 times owing to antigenic changes in HA (≥4-fold difference in HAI) [6]. Thus, the level of antigenic mismatch between our vaccine and the challenge virus was far greater than that typically seen in mismatch influenza seasons that generally demonstrate low vaccine effectiveness [7, 8]. We believe this is the first human H3N2 challenge study with such a large antigenic difference between vaccine- and challenge-strain HA.

MATERIALS AND METHODS

Vaccine and Challenge Viruses

Bris2007 M2SR was generated as described elsewhere [4]. The influenza challenge strain, A/Belgium/4217/2015 (H3N2, clade 3C.3b) (Belg2015), was isolated from a nasopharyngeal swab (NPS) specimen from a patient sample [9]. Belg2015 is antigenically equivalent to the A/Switzerland/9715293/2013 (H3N2) (Swiss2013) vaccine strain [9]. Vaccine and challenge strains and the antigenically equivalent strains are shown in Figure 1. Additional details about vaccine production can be found in the Supplementary Methods.

HA clade designations and the phylogenetic relationships between the viruses shown in Figure 1 were taken from nextstrain.org [10]. There is >64-fold difference in HAI reactivity between Bris2007 and Swiss2013 strains using human reference serum samples collected after vaccination with inactivated Northern Hemisphere 2009–2010 or 2014–2015 vaccine; that is, the years when Bris2007 or Swiss2013 were the H3N2 component of the vaccine [11].

Study Population and Trial Design

This was a blinded, randomized, placebo-controlled human challenge study conducted in Antwerp, Belgium (EudraCT no. 2017–004971–30; https://www.clinicaltrialsregister.eu/ctr-search/trial/2017-004971-30/results) in May–November 2018, in compliance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Note for Guidance on Good Clinical Practice (CMPM/ICH/135/95) and with the applicable regulatory requirement(s). All participants provided written consent. Healthy adults (18–55 years old) were screened for low baseline
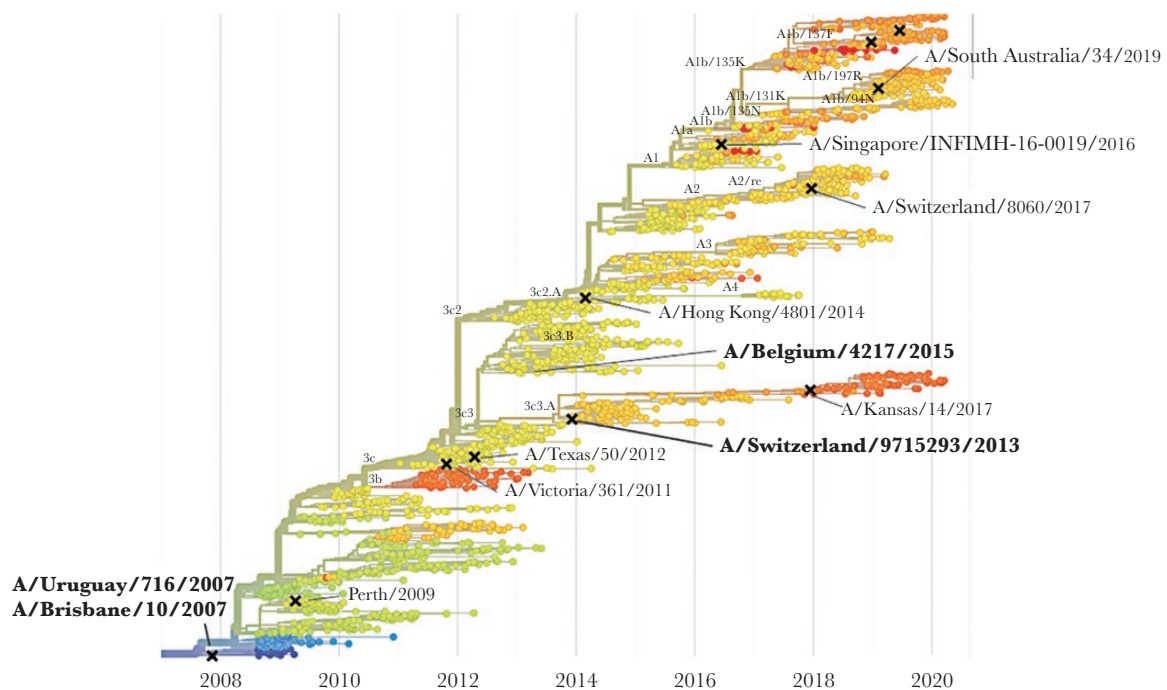
Figure 1. H3N2 hemagglutinin (HA) phylogenetic tree and clades. The World Health Organization–recommended vaccine strains for inclusion in annual vaccines are marked with X's. The HA from the vaccine strain (A/Brisbane/10/2007, A/Uruguay/716/2007) and that from a strain (A/Switzerland/9715293/2013) that is antigenically equivalent to the challenge strain (A/Belgium/4217/2015) are shown in larger type. Color coding (antigenic advance, tree model) and clade designations are taken from Nextstrain.org [10].

serum microneutralization antibody titers (MNT ≤20) against the challenge virus. Microneutralization, instead of HAI, was used to define eligibility and predefined subgroup analysis because of the reduced ability of modern H3N2 strains to cause agglutination of red blood cells [12].

The study was designed as a proof-of-concept study. Its objectives were to confirm the safety and tolerability of M2SR, evaluate the protective effect of M2SR against a significantly drifted challenge virus, evaluate challenge virus shedding, and assess correlations between vaccine immunogenicity results and challenge virus infection. Study participants received 1 intranasal dose of saline or 10^8 TCID$_{50}$ of M2SR Bris2007 vaccine using the VaxINator™ mucosal atomizer device (Teleflex). NPS and blood specimens were collected at regular intervals after vaccination. Solicited adverse events (AEs) were monitored for 7 days after vaccination using a memory aid (see Supplementary Methods). Unsolicited AEs were collected for 28 days after vaccination. Two days before challenge (day −2), participants were tested for infection with adventitious agents and excluded if found to be infected. Four weeks after vaccination (day 1), eligible participants were challenged intranasally with 10^6 TCID$_{50}$ Belg2015 and quarantined for 10 days. NPS specimens were collected twice daily for 10 days, and blood specimens on day 1 (before challenge) and day 28 after challenge. Symptoms and solicited local and systemic reactions were evaluated daily for 10 days after challenge. AEs were monitored for 28 days, and serious AEs for 180 days after challenge.

Laboratory Evaluations

Microneutralization and HAI assays were conducted at Viroclinics, as described elsewhere [13, 14]. Serum immunoglobulin A (IgA) antibody titers were measured at FluGen using enzyme-linked immunosorbent assay (ELISA), as described elsewhere [15] using recombinant HA antigens (Immune Tech). HA-specific secretory IgA antibodies in NPS specimens were evaluated with ELISA at Saint Louis University [16]. Total IgA antibodies were evaluated using the Abnova IgA (Human) ELISA Kit (Fisher Scientific), according to the manufacturer's instructions.

MNT, HAI, and mucosal IgA to Bris2007, and MNT and IgA against the challenge virus (Belg2015 or Swiss2013) were determined 28 days before challenge (baseline), 1 day before (28 days after vaccination) and 28 days after challenge. The challenge virus (like other recent H3N2 viruses) does not cause agglutination of avian red blood cells and therefore was not evaluated using HAI. For microneutralization and HAI-based assessment of responses to Bris2007 M2SR, a Bris2007-like strain, A/Uruguay/716/2007, was used. To measure IgA in serum and secretory IgA (sIgA) in NPS specimens, HA-specific ELISA was performed, using Bris2007 HA or a Belg2015-like HA, A/Switzerland/9715293/2013. Influenza HA-specific sIgA titers were normalized to total IgA in each specimen.

To evaluate cell-mediated immunity, peripheral blood mononuclear cells were collected at baseline and 14 days later. They were then tested for interferon γ and granzyme B secretion with enzyme-linked immunospot assay by Cellular Technology Limited, after stimulation with an influenza-specific nucleoprotein peptide pool (JPT Peptide Technologies). Briefly, enzyme-linked immunospot plates coated with anti–human interferon γ and anti–granzyme B antibodies were overlaid with 4×10^5 peripheral blood mononuclear cells per well, in triplicate, along with stimulating nucleoprotein peptide pool. The next day the plates were developed, dried, and scanned using an Immunospot analyzer.

After challenge with Belg2015, viral shedding was monitored daily by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) at Viroclinics. The lower limits of quantification and detection of the assay are 150 and 113 viral RNA-containing particles per milliliter, respectively. Infection was defined as ≥2 consecutive NPS samples positive for Belg2015 H3N2 RNA by qRT-PCR on day 3 or later [9, 17].

Clinical Evaluations After Challenge

Safety evaluations in challenge virus recipients are described in detail in the Supplementary Methods, along with definitions of symptom categories and assessment methods. Illness was defined as ≥1 respiratory symptom on 2 consecutive days, or ≥1 respiratory and ≥1 general symptom over 2 consecutive days during the 10-day challenge period [17].

Statistical Tests

Statistical tests were performed using GraphPad Prism 7 and SAS 9.4 software. Frequencies were compared using Fisher exact tests, and distributional outcomes using nonparametric tests. Areas under the curve (AUCs) were log-transformed and compared using t tests. Significant results are reported at $P < .05$.

RESULTS

Vaccine Safety

Of 108 randomized participants, 52 received Bris2007 M2SR vaccine and 56 received placebo intranasally in a single dose (Supplementary Figure 1). Baseline characteristics of participants were similar across treatment groups (Supplementary Table 1). Most treatment-emergent AEs (TEAEs) were mild in severity. The proportion of participants with ≥1 TEAE during the 28 days after vaccination did not differ significantly ($P < .99$) between groups: 61% (34 of 56) in the placebo group, compared with 62% (32 of 52) for Bris2007 M2SR (Supplementary Table 2). The most frequently reported TEAEs in the Bris2007 M2SR group were headache (21%), rhinorrhea (15%), nasal congestion (13%), and throat irritation (12%) (Supplementary Table 4). The incidence of each of these events

was similar to or less than that in the placebo group. There were no deaths, serious AEs, or TEAEs that led to study withdrawal. No halting rules defined in the study protocol were met. No vaccine virus shedding was detected in any of the participants (see Supplementary Data). Details of AEs can be found in Supplementary Tables 2–6.

Serum and Mucosal Antibody Responses to Vaccination

Anti-influenza antibody titers were measured in serum and NPS specimens collected 28 days after vaccine or placebo administration, using HAI, MNT, serum IgA, and mucosal sIgA ELISA assays with HA antigens representing the vaccine or challenge strains. Responses are presented as the proportion of participants with ≥2-fold increases 28 days after vaccination (prechallenge titers on day of challenge) compared with baseline (Figure 2A), since 2-fold increases in responses are a sensitive measure of influenza exposure [18, 19].

Nearly half of Bris2007 M2SR recipients (23 of 48 [48%]) showed ≥2-fold increases in serum MNT to Bris2007, the vaccine virus (Figure 2A). As expected, very few (0–3) of the 51 placebo recipients had a ≥2-fold rise in HAI or MNT compared with baseline. Furthermore, 13 of 48 M2SR recipients (27%) also demonstrated vaccine-induced MNT increases (≥2-fold) with the Belg2015 challenge virus (Figure 2A) (Fisher exact test; $P < .001$ for both MNT comparisons), referred to as M2SR Belg MNT responders. Notably, 4 of the 13 vaccine recipients with such responses were seropositive to Bris2007, the vaccine strain, at baseline (MNT, >10; geometric mean titer, 52). The proportions of M2SR recipients with ≥4-fold increases from baseline were 25%, 20.8%, and 14.5% for MNT Bris2007, HAI Bris2007, and MNT Belg2015, respectively, and 0% for placebo.

The geometric mean MNTs against the Belg2015 challenge strain at baseline and day 28 after vaccination were 7.7 and 11.9 in the Bris2007 M2SR group, compared with 7.2 and 6.6 for the placebo group.

Nasal and systemic anti-HA IgA antibodies were evaluated with ELISA, as IgA is associated with protection against influenza [20–22]. Seven of the vaccine recipients, but none of the placebo recipients, had a ≥2-fold increase in serum IgA to the vaccine-strain HA ($P = .005$; Fisher exact test), while 3 of the vaccine recipients had a response to the challenge-strain HA (Figure 2B) after vaccination but before challenge. Mucosal sIgA responses in NPS specimens (≥2-fold increases) from the vaccinated participants (16 of 48 [33%]) were more frequent than in the placebo group (8 of 51 [16%]; $P = .06$) (Figure 2B). Of the 16 vaccine recipients with sIgA response against Bris2007, 7 had baseline sIgA titers higher than the median value (2.8) in all trial participants (geometric mean, 6.7). Fourteen days after vaccination, 50% of M2SR recipients (23 of 46) and 10% of placebo recipients (5 of 49) displayed a 2-fold increase in interferon-γ–positive cell frequency over baseline ($P < .001$, Fisher exact test) (Supplementary Figure 2).

Vaccine Efficacy After Challenge

While the proportion of participants with qRT-PCR–confirmed influenza virus infection after challenge was lower in the Bris2007 M2SR group (54%) than in the placebo group (71%) (Table 1), the difference was not statistically significant. M2SR Belg MNT responders demonstrated the lowest proportion of infected participants (38%) compared with the placebo group ($P = .050$ for vaccine vs placebo; Fisher exact test).
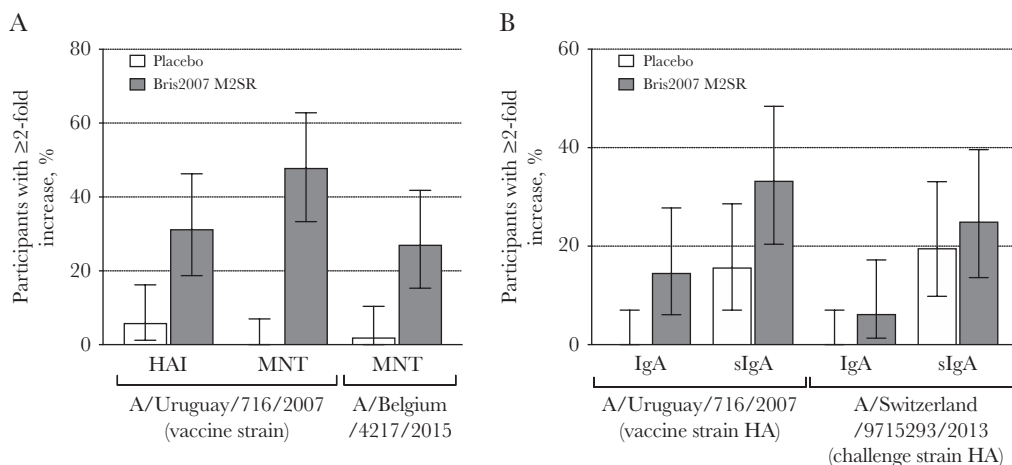
Figure 2. Post-vaccination antibody responses. *A,* Proportions of participants with ≥2-fold increases in serum hemagglutination inhibition (HAI) and microneutralization titer (MNT) measured on day 28 after vaccination with M2-deficient single-replication vaccine expressing hemagglutinin and neuraminidase from A/Brisbane/10/2007 (Bris2007 M2SR) compared with baseline. *B,* Proportions of participants with ≥2-fold increases in serum and nasal swab mucosal immunoglobulin A (IgA) responses measured on day 28 after vaccination with Bris2007 M2SR compared with baseline. IgA responses include serum IgA titers, determined with enzyme-linked immunosorbent assay, and secretory mucosal IgA (sIgA) titer in nasopharyngeal swab samples, normalized to the total IgA content. Error bars represent 95% confidence intervals. A/Uruguay/716/2007 is antigenically equivalent to the vaccine strain, and A/Switzerland/9715293/2013 is antigenically equivalent to the challenge strain.

Table 1. Infection and Influenza Illness After Challenge With Drifted A/Belgium/4217/2015

| | Study Participants, No. (%) | | |
| | | Bris2007 M2SR Group | |
Outcome	Placebo Group (n = 51)	All Recipients (n = 48)	M2SR Belg MNT Responders (n = 13)
Infection[a]	36 (71)	26 (54)	5 (38)[b]
Infection and illness[c]	25 (49)	16 (33)	3 (23)

Abbreviation: Bris2007 M2SR, M2-deficient single-replication vaccine expressing hemagglutinin and neuraminidase from A/Brisbane/10/2007; M2SR Belg MNT responders, Bris2007 M2SR recipients with microneutralization titer response to challenge strain.

[a]Infection was defined as 2 consecutive quantitative reverse-transcription polymerase chain reaction–positive swab specimens starting later than day 3 after challenge.

[b]$P = .050$ (Fisher exact test).

[c]Illness was defined as ≥2 consecutive days of general or respiratory symptoms, including ≥1 respiratory symptom.

The proportion of participants who were infected and ill was lower in the Bris2007 M2SR group (33%) than in the placebo group (49%). The proportion of participants who were infected and ill was lowest (23%) in M2SR Belg MNT responders (Table 1).

The impact of vaccination with M2SR Bris2007 on clinical symptoms after challenge was evaluated by symptom scoring, as described in Methods. Composite symptom scores (all symptoms) over time after challenge are shown in Figure 3. The placebo group had higher symptom scores than the M2SR cohort. M2SR Belg MNT responders displayed significantly lower symptom scores than the placebo and M2SR cohorts. Moreover, no lower respiratory tract or cough symptoms were reported in M2SR Belg MNT responders (Table 2).

Table 2. Composite Symptom Scores

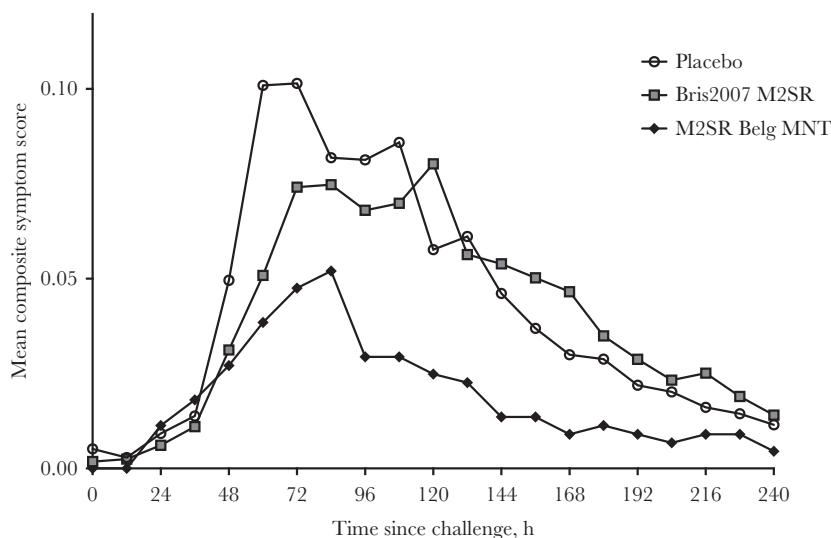| | Symptom Score, Mean (SD) | | |
| | | Bris2007 M2SR Group | |
Symptom Group	Placebo Group (n = 51)	All Recipients (n = 48)	M2SR Belg MNT Responders (n = 13)
Upper respiratory tract	0.059 (0.096)	0.058 (0.107)	0.064 (0.118)
Lower respiratory tract	0.029 (0.082)	0.027 (0.064)	0.000 (0.000)[a]
Cough	0.045 (0.12)	0.037 (0.080)	0.000 (0.000)[b]
General	0.035 (0.076)	0.033 (0.079)	0.011 (0.015)

Abbreviations: Bris2007 M2SR, M2-deficient single-replication vaccine expressing hemagglutinin and neuraminidase from A/Brisbane/10/2007; M2SR Belg MNT responders, Bris2007 M2SR recipients with microneutralization titer response to challenge strain; SD, standard deviation.

[a]$P = .10$ vs placebo (Mann-Whitney test).

[b]$P = .09$ vs placebo (Mann-Whitney test)

Virus Replication After Challenge

qRT-PCR was used to measure the viral burden in NPS specimens after challenge with Belg2015. Compared with placebo recipients, a reduced amount of challenge virus replication was observed in Bris2007-vaccinated participants (Figure 4A). The largest reduction in challenge virus replication was observed in M2SR Belg MNT responders ($P = .02$). The mean time to first detection of challenge virus replication and time to peak virus replication were longer in vaccine recipients, and the time to last detection of challenge virus replication was shortened, compared with placebo (Table 3). Virus replication after challenge was evaluated by quantitating the qRT-PCR AUCs defined by influenza RNA copies per milliliter versus time after

Figure 3. Mean composite symptom scores after challenge with A/Belgium/4217/2015 in recipients of M2-deficient single-replication vaccine expressing hemagglutinin and neuraminidase from A/Brisbane/10/2007 (Bris2007 M2SR; *squares*), placebo recipients (*circles*), and vaccine recipients with microneutralization titer (MNT) response to the challenge virus (M2SR Belg MNT; *diamonds*).
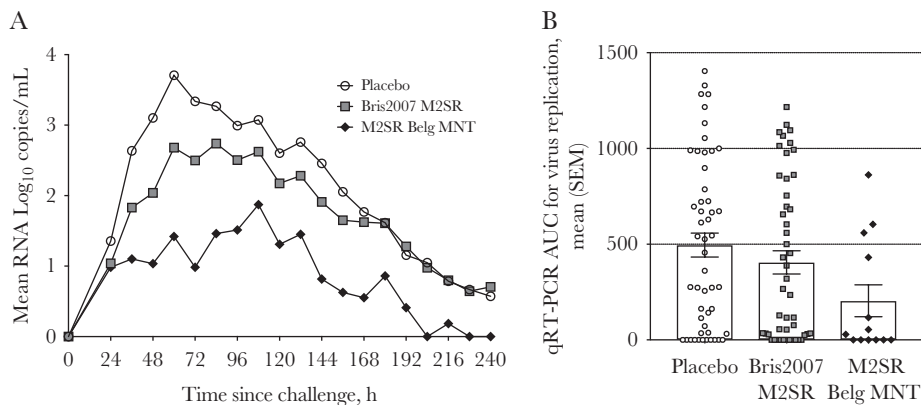
Figure 4. Challenge virus shedding after A/Belgium/4217/2015 (Belg2015) challenge in recipients of M2-deficient single-replication vaccine expressing hemagglutinin and neuraminidase from A/Brisbane/10/2007 (M2SR Bris2007) or placebo recipients. *A*, RNA levels over time after challenge. The difference between M2SR Bris2007 recipients with a microneutralization titer response to Belg2015 (M2SR Belg MNT responders) and placebo recipients was significant (P = .02; Wilcoxon test). *B*, Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) area under the curve (AUC) for viral replication (RNA copies per mL vs time) after challenge. The difference in AUC between M2SR Belg MNT responders and placebo recipients was significant (P = .03; Mann-Whitney test). Circles represent placebo recipients; squares, M2SR Bris2007 vaccine recipients; and diamonds, M2SR Belg MNT responders. Abbreviation: SEM, standard error of the mean.

challenge (Figure 4B). The mean AUC for the entire M2SR cohort was 405 $\log_{10}$ copies-hours/mL compared with 495 $\log_{10}$ copies-hours/mL for placebo. The mean AUC for M2SR Belg MNT responders was significantly lower (204 $\log_{10}$ copies-hours/mL) compared with that in placebo recipients (*t* test, P = .03).

DISCUSSION

Influenza H3N2 virus continues to be a major cause of disease and death, especially for the >65-year age group. Although seasonal influenza vaccines are evaluated annually for the need to update the vaccine with antigenically contemporary strains, H3N2 vaccine effectiveness with currently licensed vaccines continues to be lower than for H1N1 or B viruses [2],

highlighting the need for vaccines with broad-spectrum protection. In this human challenge study, we show that M2SR vaccine expressing HA and NA antigens that circulated more than a decade ago (2007) can provide protection against challenge with a highly drifted, more contemporary H3N2 virus (2015) in individuals who demonstrate a cross-reactive serum response to the vaccine. The magnitude of drift (HAI titer, >64-fold) between the vaccine and challenge virus was substantially greater than that seen in a typical mismatch year (≥4-fold HAI differences).

After challenge with drifted H3N2 virus, protection from infection and influenza illness was most prominent in the subset of M2SR recipients who demonstrated vaccine-induced prechallenge increases in MNT to the challenge strain. This M2SR subset demonstrated a 46% reduction in infection and a 53% reduction in infection and illness compared with the placebo cohort. In addition, this subset demonstrated overall reduced virus shedding and reduced clinical symptoms relative to placebo. Moreover, this M2SR subset did not have any lower respiratory tract symptoms, such as cough, compared with the placebo cohort. The intranasal M2SR vaccine elicited serum and mucosal responses in addition to cellular responses, demonstrating that M2SR generates a multi-effector immune response. Serum and mucosal antibody responses were observed against both the vaccine and the challenge strain, demonstrating the potential of M2SR to generate broad-spectrum cross-reactive responses.

Intranasal administration of 10^8 TCID$_{50}$ of M2SR Bris2007 was well-tolerated, confirming previous observations of this intranasal, single-replication virus vaccine [5]. AEs after dosing were mild, with no significant differentiation of vaccine from placebo. Furthermore, there were no safety concerns related

Table 3.   Time to Event After Challenge With Heterologous Wild-Type Virus

| | Time to Event, Mean (SD), h | | |
| | | Bris2007 M2SR Group | |
Virus Shedding Event[a]	Placebo Group (n=40)	All Recipients (n = 37)	M2SR Belg MNT Responders (n = 7)
First virus detected	34.5 (13.9)	41.5 (31.4)	49.7 (40.1)
Peak virus detected	75.8 (27.6)	91.1 (47.8)	92.6 (47.4)
Last virus detected	173.7 (58.4)	166.7 (69)	145.7 (59.4)

Abbreviations: Bris2007 M2SR, M2-deficient single-replication vaccine expressing hemagglutinin and neuraminidase from A/Brisbane/10/2007; M2SR Belg MNT responders, Bris2007 M2SR recipients with microneutralization titer response to challenge strain; SD, standard deviation.

[a]Time of quantitative polymerase chain reaction (PCR)–positive test (hours after challenge) of events listed among those who shed virus. Participants who never had a PCR-positive nasopharyngeal swab specimen are excluded (n = 11 each in placebo and Bris2007 M2SR groups; n = 6 in M2SR Belg MNT responders).

to challenge with influenza virus in individuals vaccinated with M2SR.

These results demonstrate the potential of M2SR to provide broad protection, because the vaccine and challenge strains used here belonged to different H3N2 phylogenetic clades. Bris2007, the H3N2 vaccine component from 2007, is clade 1, while Belg2015 belongs to the more modern clade 3C.3b and is related to the 2013 H3N2 vaccine strain. Current licensed inactivated influenza vaccines (IIVs) do not demonstrate such cross-reactivity across highly drifted strains. For example, the IIV from 2009–2010 containing the Bris2007 H3N2 component elicited strong HAI titers against the Bris2007 vaccine strain but did not cross-react against the A/Switzerland/9715293/2013 vaccine strain [11]. Similarly, H3N2 cross-reactivity for IIV from 1997 provided some seroprotection for the next season when the strain changed, but not for the following year's strain [7]. In addition, IIV generally does not induce mucosal and cellular immune responses that provide benefit against drifted influenza strains [17, 23, 24]. In contrast, wild-type H3N2 infection seems to confer longer-term protection, as indicated by ever-decreasing attack rates with aging and acquisition of infection-induced immunity [25–30].

The current study identified an important signal for M2SR protection. While a serum HAI titer of 40 is an accepted surrogate of protection for IIV [31, 32], the results from this study show that a $\geq$2-fold increase in MNT from baseline, in the context of additional stimulation of mucosal and cellular immune responses, was associated with protection for the intranasal M2SR vaccine. This serves as a marker representing the cumulative M2SR immune response that includes mucosal antibodies and cellular immunity in addition to serum neutralizing antibody. Thus, an important outcome of this study is to direct future efforts toward increasing the frequency of such responses. To this end, doses up to 10^9 TCID$_{50}$ are being evaluated in a phase 1b dose escalation study, with an aim to induce protective immune responses among a higher proportion of recipients than observed with 10^8 TCID$_{50}$ in the challenge study described here (https://clinicaltrials.gov/ct2/show/NCT03999554). Preliminary results from the phase 1b study indicate that the 10^9 TCID$_{50}$ dose was well tolerated, with no appreciable increase in reactogenicity, and induced 2-fold and 4-fold increases in MNTs against the challenge strain in a significantly higher proportion of participants, 81% and 58%, respectively, suggesting the potential for significantly increased protection with the 10^9 dose compared with the 10^8 dose [33].

We believe this is the first human challenge study to demonstrate protection against challenge with an influenza strain that has such a substantial antigenic difference from the vaccine strain, and it indicates the potential for M2SR to provide improved breadth of protection compared with currently licensed vaccines. This study also identified a potential serum marker of protection for the intranasal M2SR vaccine. The mild AE profile of M2SR vaccine indicates the potential for higher vaccine dose levels to provide further enhancements of protection by M2SR against highly drifted H3N2 influenza strains.

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