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Thermostable H1 hemagglutinin stem with M2e epitopes provides broad cross-protection against group1 and 2 influenza A viruses

Jeeva Subbbiah,[1] Judy Oh,[1] Ki-Hye Kim,[1] Chong Hyun Shin,[1] Bo Ryoung Park,[1] Noopur Bhatnagar,[1] Yu-Jin Jung,[1] Youri Lee,[1] Bao-Zhong Wang,[1] Baik-Lin Seong,[2,3] and Sang-Moo Kang[1]

[1]Center for Inflammation, Immunity & Infection, Institute for Biomedical Sciences, Georgia State University, 100 Piedmont Avenue, PSC 718 P.O. Box 5035, Atlanta, GA 30303, USA; [2]Department of Microbiology, College of Medicine, Yonsei University, Seoul 03722, Republic of Korea; [3]Vaccine Innovative Technology Alliance (VITAL), Seoul 03722, Republic of Korea

Hemagglutinin (HA) stem-based vaccines have limitations in providing broad and effective protection against cross-group influenza viruses, despite being a promising universal vaccine target. To overcome the limited cross-protection and low efficacy by HA stem vaccination, we genetically engineered a chimeric conjugate of thermostable H1 HA stem and highly conserved M2e repeat (M2e-H1stem), which was expressed at high yields in *Escherichia coli*. M2e-H1stem protein presented native-like epitopes reactive to antisera of live virus infection. M2e-H1stem protein vaccination of mice induced strong M2e- and HA stem-specific immune responses, conferring broadly effective cross-protection against both antigenically distinct group 1 (H1N1, H5N1, and H9N2 subtypes) and group 2 (H3N2 and H7N9 subtypes) seasonal and pandemic potential influenza viruses. M2e-H1stem vaccination generated $CD4^+$ and $CD8^+$ T cell responses and antibody-dependent cytotoxic cellular and humoral immunity, which contributed to enhancing cross-protection. Furthermore, comparable broad cross-group protection was observed in older aged mice after M2e-H1stem vaccination. This study provides evidence warranting further development of chimeric M2e-stem proteins as a promising universal influenza vaccine candidate in adult and aged populations.

INTRODUCTION

Effectiveness of seasonal influenza vaccines is in low ranges from 10% to 60%,[1] due to the continuous antigenic changes in the variable immunodominant head domain of hemagglutinin (HA), a major antigenic target.[2] HA is composed of the immunodominant antigenic HA1 head domain of receptor binding and the relatively conserved HA2 stem domain mediating viral fusion. Identification of broadly neutralizing antibodies recognizing the stem domain provided rationales for developing stem-based universal vaccines.[3–5] Based on structural and antigenic phylogeny, the HA subtypes are divided into group 1 (H1, H2, H5, H6, H8, H9, H11–H13, H16, and H17) and group 2 (H3, H4, H7, H10, H14, and H15).[6] Prior studies demonstrated that headless stem protein vaccine immunogens stabilized with a trimer-forming motif provided protection in mice.[7–9] Sequential vaccinations with the H1 stem-based vaccines conferred protection against homologous or heterosubtypic virus within the same group HA, but the protective efficacy of cross-group HA viruses was low or not tested.[7–10]

The ectodomain (M2e) of influenza A virus M2 ion channel protein is highly conserved in human influenza A viruses despite few residue changes among the avian and swine origin viruses.[11] Previous animal studies have proven that M2e immunity could provide broad protection against both group 1 and 2 viruses, regardless of HA subtypes.[12–15] Clinical trials reported safety of M2e-based recombinant vaccines.[13,16,17] However, the efficacy of M2e immunity alone was lower than that of inactivated virus vaccination inducing neutralizing antibodies, when vaccinated mice were challenged with HA-matching homologous virus, probably due to non-neutralizing immune-mediated protection and limited B cell and T cell epitopes.[11,13,18–20] Chemically cross-linked double-layer nanoparticles of combined M2e repeat and head-removed H1 plus H3 HA2 stem proteins could provide enhanced efficacy and breadth of cross-protection against group 1 and group 2 viruses, but each H1 or H3 HA2 stem protein nanoparticle vaccination induced protection in a group-specific manner.[21] A similar concept was also reported, demonstrating that intranasal vaccination of flagellin carrier conjugate vaccine of HA2 domain (amino acids [aas] 76–130) and 4xM2e repeat induced survival cross-protection against various influenza H2N2, H3N2, and H5N1 viruses but could not prevent severe weight loss in mice.[22]

To overcome the limited efficacy of cross-group protection by HA stem-based vaccines, we designed a headless H1 stem chimeric construct by genetically linking to M2e repeat (M2e-H1stem). Vaccination with

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Correspondence: Sang-Moo Kang, PhD, Center for Inflammation, Immunity & Infection, Institute for Biomedical Sciences, Georgia State University, 100 Piedmont Avenue, PSC 718 P.O. Box 5035, Atlanta, GA 30303, USA.
E-mail: skang24@gsu.edu

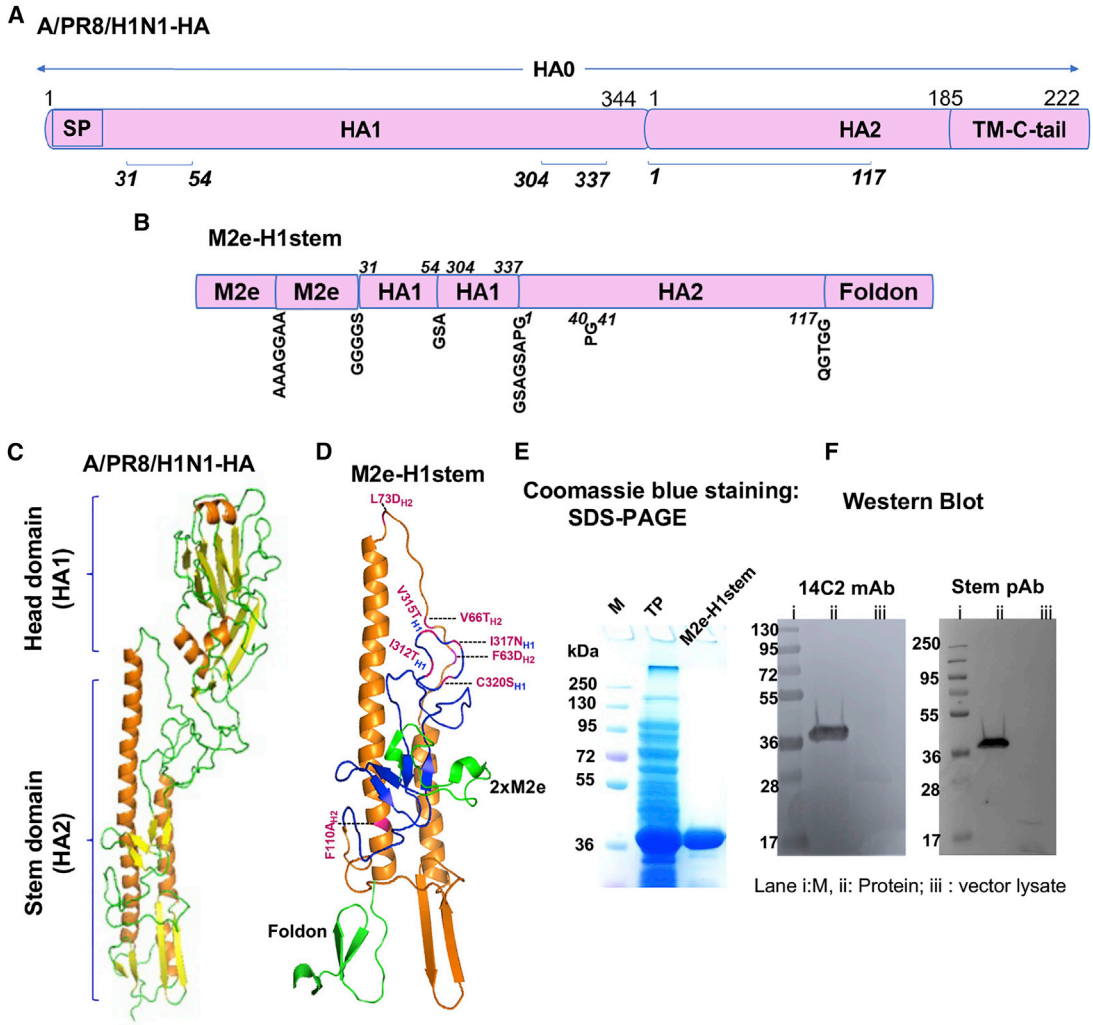

Figure 1. Design and characterization of chimeric M2e-H1stem protein vaccine construct

(A) Schematic diagram of full-length HA (A/PR8). The regions that were selected as a vaccine target are numbered in amino acid (aas 31–54, 304–337, and 1–117) residues. (B) Chimeric M2e-H1stem construct with linker sequences is shown (AAAGGAA, GGGGS, GSA, GSAGSAPG, PG, and QGTGG). M2e sequence is as follows: MSLLTEVETPIRNEWGSRSNDSSD (Cys to Ser changes underlined). (C and D) Structural modeling of HA and M2e-H1stem construct is shown. A/PR8/1934 HA (ID: YP_163,736) was used as a template to predict the structure of HA and M2e-H1stem by SWISS-Model (Expasy web server); the 3D cartoon structure was generated by PyMOL. The structure of M2e and foldon was derived from PDB: 4N8C and 1RFO, respectively. In (D), the locations of the point mutations are marked in pink, whereas the HA1 region is colored in blue. (E) Coomassie Blue staining of M2e-H1stem protein is shown. M, protein size marker (kDa); M2e-H1stem, purified M2e-H1stem protein (20 μg); TP, total cell lysates (30 μg). (F) Western blot of M2e-H1stem protein is shown. 14C2, M2e-specific mAb; stem, anti-stem polyclonal antibodies (pAbs) recognizing HA2 aas 103–116 peptide. i, protein ladder kDa; ii, purified M2e-H1stem protein; iii, empty vector transformed *E. coli* cell lysate.

M2e-H1stem induced heterologous and heterosubtypic broad cross-group protection against group 1 and 2 seasonal and pandemic potential influenza viruses in adult and older aged mice, which provides evidence supporting a promising universal vaccine candidate.

RESULTS

Rational design and generation of chimeric M2e-H1stem fusion construct

HA comprises HA1 globular, receptor-binding, variable head domain, and fusion-inducing, comparatively conserved stem domain (Figures 1A and 1C). N- and C-terminal of the HA1 parts contribute to stabilizing the HA2 stem domain,[7,9,10,21] providing the rationale for including HA1 parts in the M2e-H2stem construct. To overcome the low efficacy of stem-based vaccines in conferring cross-group protection due to HA stem sequence variations (Figure S1), we designed a chimeric M2e-stem fusion construct (Figures 1B and 1D). Two M2e domains were genetically linked to the N-terminal of the stem domain derived from A/PR8 HA (M2e-H1stem). The cysteine residues at position 17 and 19 in M2e were changed to serine to avoid unwanted cross links. To improve the immune response targeting stem

and fusion domain epitopes and to minimize potential protein aggregation due to the exposure of hydrophobic residues on the surface of HA2, we deleted the C-terminal of the HA2 domain. The M2e-H1 stem construct contains 2xM2e, HA1 parts (aas 31–54; aas 304–337), and HA2 stem in α helix (aas 1–117) (Figures 1B and 1D). Point mutations known to improve expression[8] were introduced in the hydrophobic patches in both HA1 (I312T, V315T, and I317N) and HA2 stem domains (F63D, V66T, L73D, and F110A). We also mutated cysteine residue into serine (C320S) to avoid intra-molecular disulfide bond formation (Figure 1D). Each domain is connected via flexible linkers to facilitate its domain-independent native-like conformation. Foldon was shown to be important for stabilizing the recombinant proteins and proteolytic resistance.[8] The β-rich trimeric foldon[23] is linked to the C terminus of M2e-H1stem. Codon-optimized M2e-H1stem gene was synthesized and cloned into pCold II vector to induce protein expression at a low temperature in *Escherichia coli* (*E. coli*). The M2e-H1stem protein was expressed at high levels (>50% of cell lysates in SDS-PAGE Coomassie Blue stain) in Rosetta (DE3) pLysS cells and affinity purified (Figure 1E), confirmed by western blot using M2e-specific monoclonal antibody (mAb) (14C2) and stem-specific polyclonal antibody (pAb) (Figure 1F).

M2e-H1stem protein displays cross-reactive antigenicity

We investigated the antigenic epitope properties of M2e-H1stem protein with M2e-specific, HA2 stem-specific, and virus-specific antibodies (Figure 2). M2e-H1stem was found to be reactive with M2e mAb (14C2) and pAbs raised against highly conserved HA2 aas 1–13 fusion peptide, HA2 aas 14–27, and HA2 aas 103–116 (Figures 2A–2D). HA2 stem-specific pAbs also recognized a full-length HA, supporting the specific binding of these pAbs to native stem epitopes (Figures 2B–2D). In addition, M2e-H1stem was cross recognized by pAbs induced by different HA proteins derived from diverse subtypes, including H1N1, H5N1, H3N2, and H7N9 (Figures 2E–2H). Notably, M2e-H1stem was antigenically cross-reactive and recognized by antisera from infection with different subtypes of influenza viruses, such as H5N1, H3N2, and H7N9 at significant levels (Figures 2I–2K), suggesting the presence of native-like epitopes. Taken together, these results suggest that M2e-H1stem protein exposes native-like, conserved epitopes recognized by different pAbs against group 1 and 2 HA subtypes of influenza A viruses.

Antisera of M2e-H1stem vaccination recognize M2e, stem, and cross-group viruses

Liposome adjuvant AS01 (QS-21 + MPL) is licensed for use in herpes zoster vaccination.[24,25] Therefore, we determined whether the inclusion of AS01-like adjuvant (QS-21 + MPL) would enhance vaccine antigen-specific immunoglobulin G (IgG) antibody responses at 2 weeks after intramuscular (IM) vaccination of BALB/c mice with M2e-H1stem protein (20 μg). After prime, even in the absence of adjuvant, M2e-specific IgG Abs were induced at substantial levels, whereas stem peptide (HA2 aas 74–98) and stem protein-specific IgG levels were very low (Figures S2A–S2C). With adjuvanted prime vaccination, IgG Abs specific for M2e and stem protein, as well as stem peptide, were induced at significantly higher levels than non-adjuvanted counterpart (Figures S2A–S2C). After boost dose, the levels of IgG Abs specific for M2e and HA2 stem protein as well as HA2 stem peptide (HA2 aas 74–98) were significantly increased in the adjuvanted M2e-H1stem group, compared with unadjuvanted vaccination (Figures 3A–3C). Adjuvanted M2e-H1stem prime or prime-boost vaccination also induced IgG, IgG1, and IgG2a isotype Abs specific for M2e-H1 stem vaccine antigen at significantly higher levels than the unadjuvanted vaccination (Figures S3A–S3F). The level of IgG antibodies binding to the foldon-linked spike protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was close to the background, suggesting that adjuvanted M2e-H1stem vaccination induced IgG responses specific for M2e-H1stem, but not for foldon domain (Figure S3H). Consistently, when viruses were used as a coating antigen, the adjuvanted M2e-H1stem-vaccinated group showed significantly higher levels of IgG Abs recognizing group 1 (rgA/Viet/H5N1 and rgA/HK/H9N2) and group 2 (A/HK/H3N2 and rgA/Shanghai/H7N9) viruses than those from the unadjuvanted vaccine group (Figure S3G). IgG Ab responses specific for the group 1 (H1N1, H5N1, and H9N2) viruses were higher than those against the group 2 (H3N2 and H7N9) viruses in antisera from adjuvanted M2e-H1stem vaccination (Figures 3D and 3E).

Antisera of M2e-H1stem recognize cell-surface-expressed viral antigens and exhibit ADCC

Immune sera of adjuvanted M2e-H1stem vaccination were highly reactive to the Madin-Darby canine kidney (MDCK) cell surface viral antigens after infection with group 1 (H1N1 [A/WSN, A/Cal, A/PR8, and A/FM], A/HK/H9N2, and A/Viet/H5N1) and group 2 viruses (H3N2 [A/Phil, A/HK, and A/Nanchang] and A/Sha/H7N9) (Figures 3F, 3G, S4A, and S4B). Consistently, antibody-dependent cell-mediated cytotoxicity (ADCC) surrogate assay in influenza-A-virus-infected MDCK cells showed strong reporter signals in group 1 (Figure 3H) and group 2 (Figure 3I) virus-infected cells, suggesting that M2e-H1stem immune sera function via ADCC. Altogether, these results indicate that adjuvanted M2e-H1stem vaccination effectively induced antibodies recognizing M2e, stem, and group 1 and 2 viruses as well as engaging in Fc-mediated ADCC, contributing to cross-protection.

M2e-H1stem provides cross-group protection against diverse group 1 and 2 viruses

The inclusion of adjuvant (QS-21 + MPL) was found to significantly enhance the efficacy of M2e-H1stem vaccination by preventing severe weight loss and promoting recovery after lethal challenge with A/PR8/H1N1 virus (Figure S5); we focused adjuvanted vaccine on testing the breadth of cross-protection. Groups of mice with M2e-H1stem prime-boost vaccination were challenged with a lethal dose of antigenically different group 1 viruses (Figures 4A–4F). Effective protection against A/WSN/1933 H1N1 (Figure 4A), rgA/Viet/2004 H5N1 (Figure 4B), and rgA/HK/1999 H9N2 (Figure 4C) was observed in vaccinated mice with minimum weight loss (∼2%–5%); mock control mice did not survive. Moderate weight loss (6%–8%) was observed in M2e-H1stem-vaccinated mice after lethal challenge with pathogenic H1N1 viruses, including A/PR8/1934 (Figure 4D), A/Cal/2009 (Figure 4E), and A/FM/1947 (Figure 4F). Moreover, we

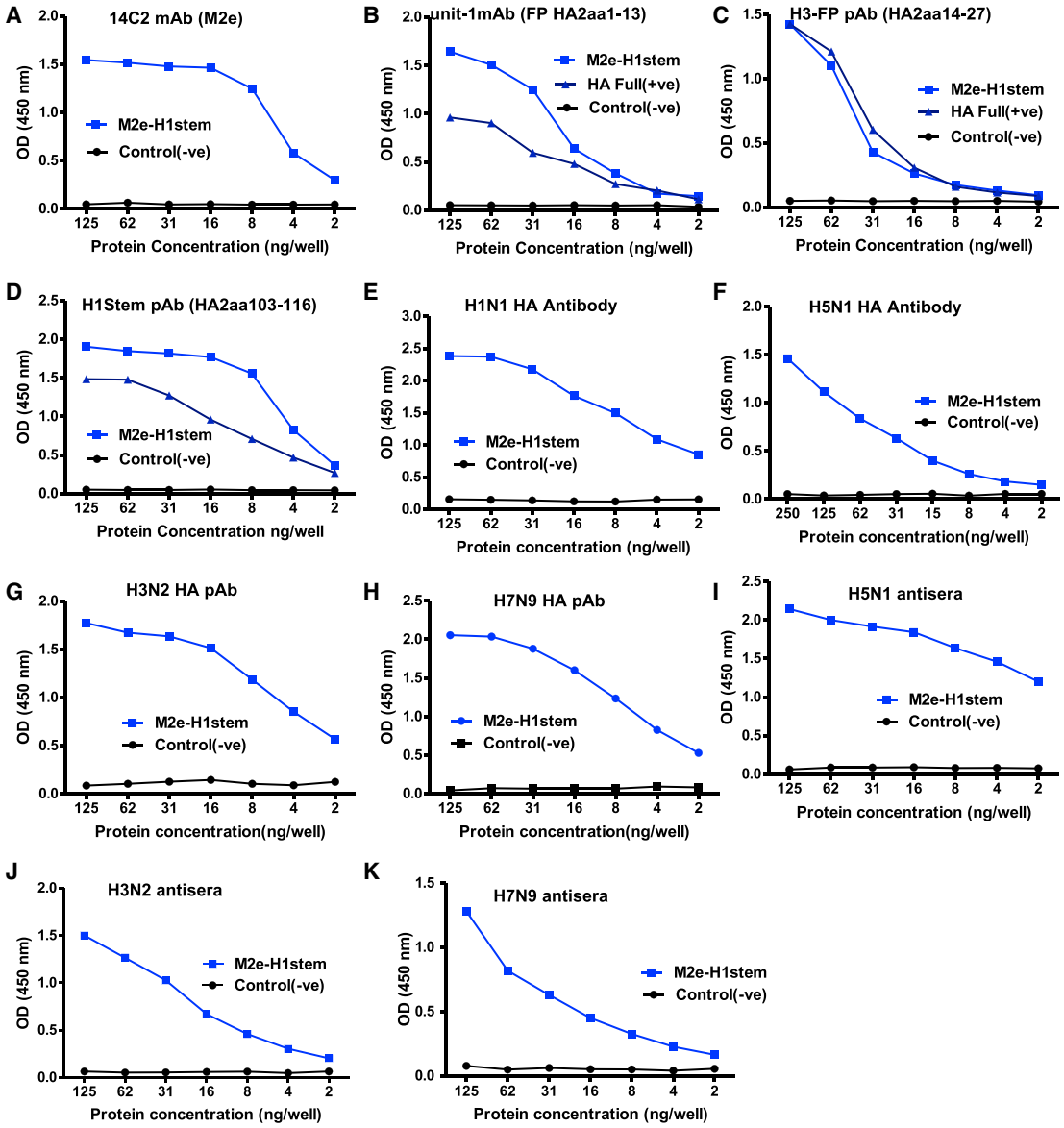

Figure 2. Antigenic characterization of M2e-H1 stem protein

The antigenicity of M2e and stem epitopes in the M2e-H1stem protein was determined by ELISA using epitope-specific antibodies. (A) M2e-specific monoclonal antibody (mAb 14C2) is shown. OD, optical density. (B) Unit-1 mAb: rabbit mAb specific for fusion domain (GLFGAIAGFIEGGW) is shown. (C) H3-FP pAb (HA2 aas 14–27): purified rabbit pAbs specific for HA2 aas 14–27 (WEGMVDGWYGFRHQ) are shown. (D) H1stem pAb: purified rabbit pAbs specific for HA2 aas 103–116 (ENERTLDYHDSNVK) are shown. (E–H) Antigenicity of M2e-H1stem to pAbs specific for recombinant HA is shown. (E) pAb to H1 HA: antisera for H1 HA (A/California/04/2009 H1N1), (F) pAb to H5 HA: antisera for H5 HA (A/Vietnam/1203/04/H5N1), (G) pAb to H3 HA: antisera for H3 HA (A/Hong Kong/1/1968/H3N2), and (H) pAb to H7 HA: antisera for H7 HA (A/Anhui/1/2013/H7N9) are shown. (I–K) Antigenicity of M2e-H1stem to antisera from live virus infection is shown. (I) H5N1 antisera (A/Viet/H5N1), (J) H3N2 antisera (A/Phil/1982 H3N2), and (K) H7N9 antisera (A/Sha/2013 H7N9). Control(−ve): BSA; HA Full(+ve): full HA protein of A/Cal/H1N1 virus.

determined whether M2e-H1stem vaccination could provide cross-protection against group 2 viruses. Remarkably, the groups of mice vaccinated with M2e-H1stem were well protected against lethal challenges with A/Nanchang/1995 H3N2 (Figure 4G) and rgA/Shanghai/2013 rgH7N9 (Figure 4H), as evidenced by no and minimum (∼5%) weight loss, respectively. When challenged with A/Phil/1982 H3N2 (Figure 4I) and A/HK/1968 H3N2 (Figure 4J) virus, M2e-H1stem-vaccinated mice showed low to moderate (∼7%) weight loss and recovered. Mock control mice experienced severe weight loss and died of each virus infection (Figures 4A–4J). These results suggest that M2e-H1stem vaccination conferred broad cross-protection against antigenically different group 1 and 2 influenza A viruses.

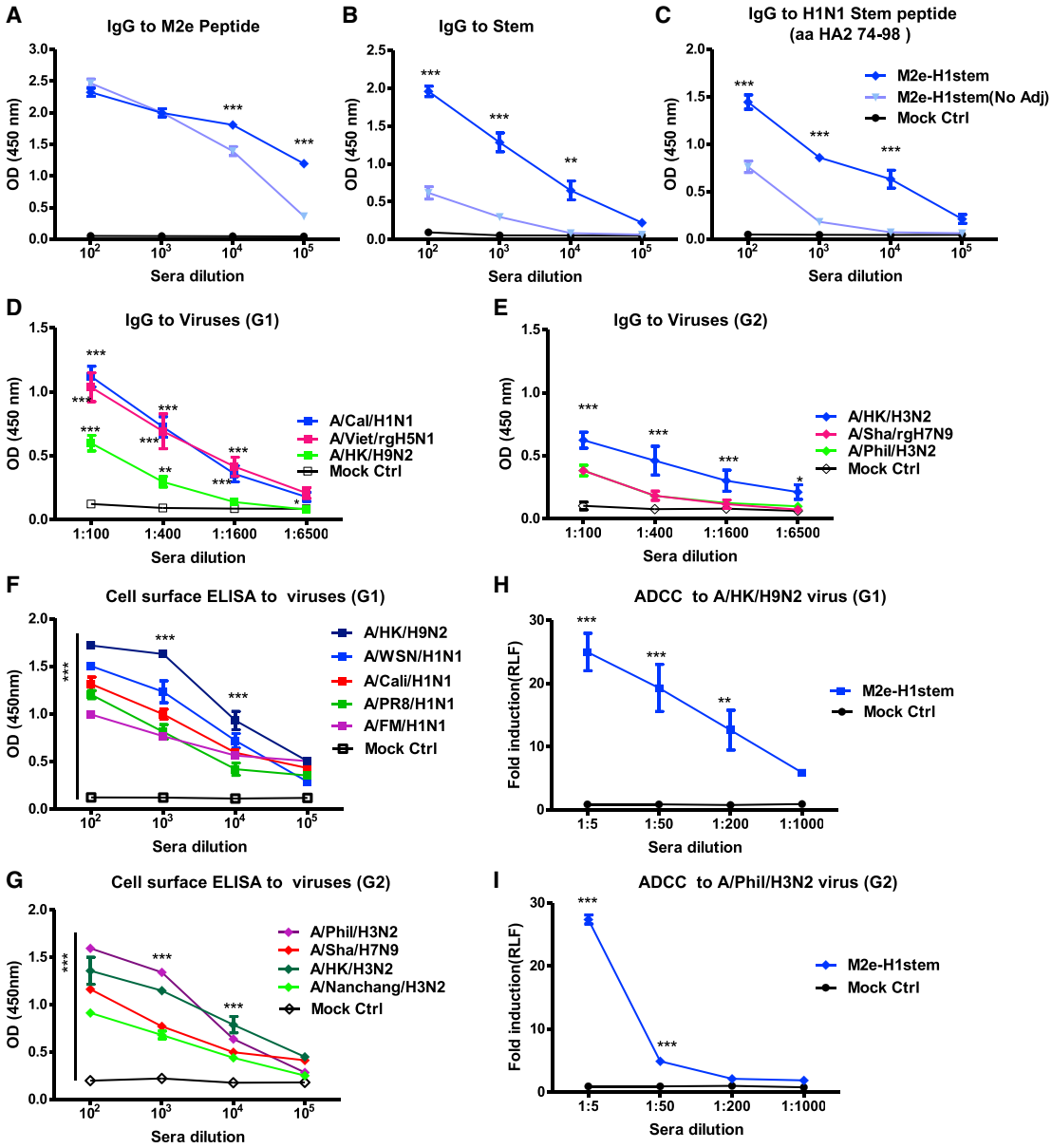

Figure 3. Vaccination with adjuvanted M2e-H1stem induces antibodies recognizing M2e, stem, and group 1 and 2 viral antigens

BALB/c mice (n = 10) were intramuscular prime-boost immunized with M2e-H1stem (20 μg) with or without adjuvants (QS21-MPL), and sera were collected 2 weeks after vaccination. (A–C) IgG antibodies specific for M2e (A), stem protein (B), and H1 stem peptide HA2 aas 74–98 (C) are shown. (D and E) IgG antibodies specific for group 1 and 2 influenza A viruses are shown. Group 1 (G1) HA viruses (D; A/Cal/09/H1N1, A/Viet/rgH5N1, and A/HK/rgH9N2) and group 2 (G2) HA viruses (E; A/HK/H3N2, A/Phil/H3N2, and A/Sha/H7N9) are shown. Mock Ctrl, adjuvanted naive sera. (F and G) M2e-H1stem vaccination induced IgG antibodies recognizing both group 1 (F) and 2 (G) viral antigens on the surface of virus-infected MDCK cells. (H and I) Antibodies induced by M2e-H1stem vaccine engage in Fc-mediated activation of Jurkat effector cells, mimicking a surrogate ADCC activation pathway. The statistical significance was determined by using two-way ANOVA; error bars indicate mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001.

M2e-H1stem vaccination promotes lung-viral clearance and protective humoral and cellular immunity

To further assess the effectiveness of cross-group protection, we determined lung viral titers as well as humoral and cellular immune responses. Compared with the unvaccinated mock control group after infection, M2e-H1stem vaccination led to rapid recovery of weight loss (Figure 5A) and effective lung viral clearance by 1,000-fold lower upon rgH7N9 infection (Figure 5B). A modified passive transfer assay was reported as an effective and sensitive method to determine the roles and contribution of antisera in conferring protection in naive mice, which we demonstrated in previous studies.[14,26] To determine the roles of antiserum humoral immune responses in conferring

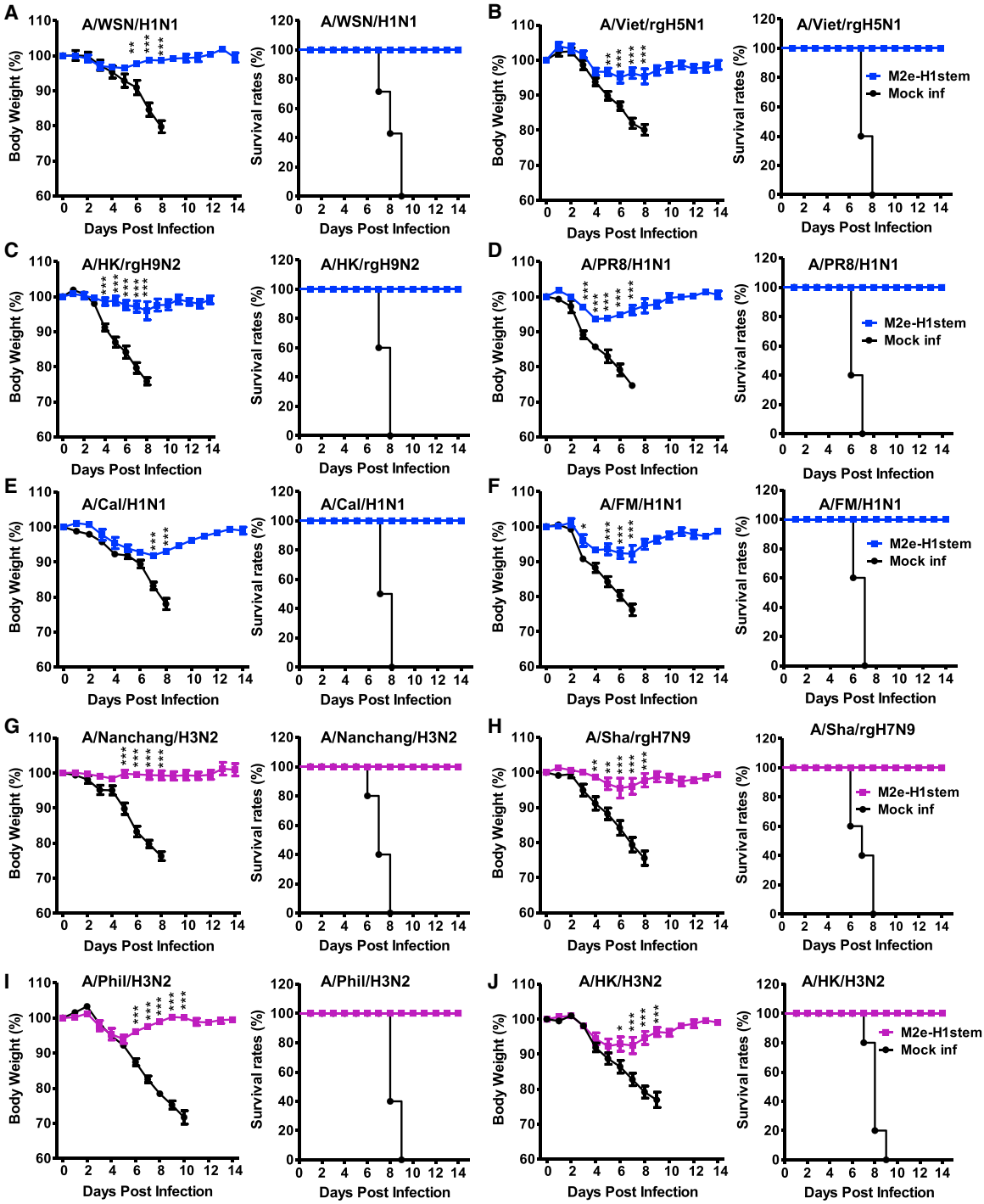

Figure 4. Adjuvanted M2e-H1stem vaccination provides cross-protection against both group 1 and 2 viruses

M2e-H1stem (20 μg + adjuvant) prime-boost-vaccinated BALB/c mice (n = 5 per group) were intranasally challenged with influenza A viruses. Body weight changes and survival rates were monitored for 14 days. Group 1 viruses were as follows: (A) A/WSN/1933 H1N1(2x$LD_{50,}$ 1.5 × 10^2 EID_{50}), (B) A/Viet/2004 rgH5N1 (3xLD_{50}, 2.6 × 10^4 EID_{50}), (C) A/HK/1999 H9N2 (4xLD_{50}, 7.8 × 10^1 EID_{50}), (D) A/PR8/1934 H1N1 (4x$LD_{50,}$1.2 × 10^3 EID_{50}), (E) A/Cal/2009 H1N1 (3xLD_{50}, 2 × 10^3 EID_{50}), and (F) A/FM/1947 H1N1 (3xLD_{50}, 8 × 10^3 EID_{50}). Group 2 viruses are as follows: (G) A/Nanchang/1995 H3N2 (2xLD_{50}, 3 × 10^6 EID_{50}), (H) A/Sha/2013 H7N9 (3xLD_{50}, 1.1 × 10^4 EID_{50}), (I) A/Phil/1982 H3N2 (3xLD_{50}, 2.3 × 10^2 EID_{50}), and (J) A/HK/1968 H3N2 (3xLD_{50}, 4 × 10^1 EID_{50}). The statistical significance was determined by using two-way ANOVA; error bars indicate mean ± SEM; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

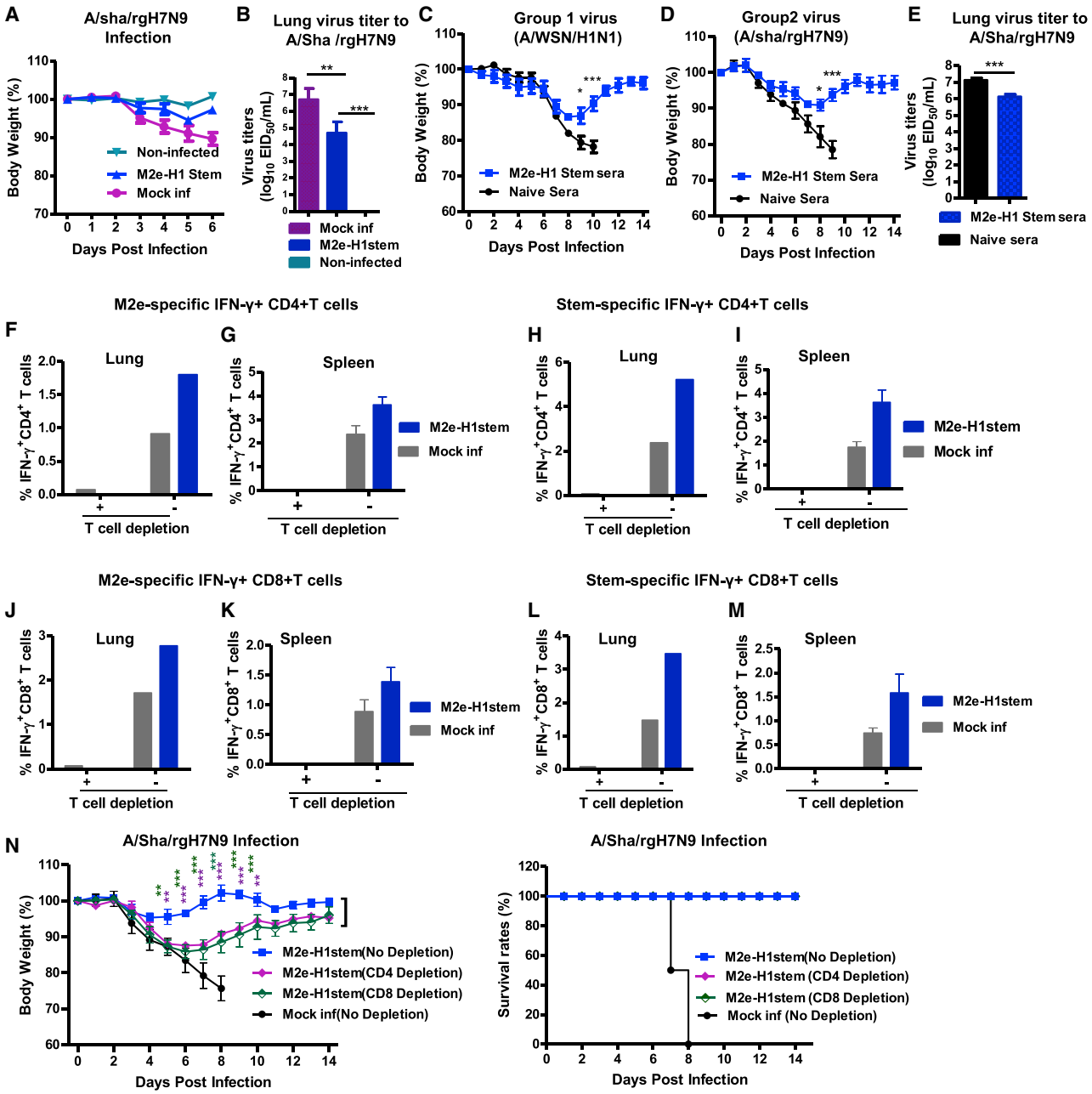

Figure 5. Adjuvanted M2e-H1stem vaccination bestows cross-protection by lowering lung viral loads and inducing protective humoral and cellular immune responses

(A) Body weight changes in M2e-H1stem (20 μg) prime-boost vaccinated BALB/c mice for 6 days after challenge with A/rgH7N9 virus. (B) Lung viral titers at 6 days post-infection as in (A) are shown. Mock inf, mock group with virus infection; non-infected, naive group with no virus infection. (C and D) Body-weight changes in naive mice after intranasal inoculation with a mixture of M2e-H1stem immune or naive sera and virus (4.2xLD_{50}). A/WSN (H1N1 in C) and A/rgH7N9 viruses (A/Sha/2013 in D) are shown. (E) Lung viral titers in naive mice at 6 days post-infection with a mixture of sera and A/rgH7N9 virus as in (D) are shown. (F–M) IFN-γ^+ T cell responses analyzed by flow cytometry of intracellular cytokine staining are shown; lung and spleen cells were collected at 5 days post-infection with rgH7N9 virus. (F–I) IFN-γ^+ $CD4^+$ T cells responses are shown. Lung (F) and spleen (G) IFN-γ^+ $CD4^+$ T cells upon M2e stimulation are shown. Lung (H) and spleen (I) IFN-γ^+ $CD4^+$ T cells upon stem protein stimulation are shown. (J–M) IFN-γ^+ $CD8^+$ T cell responses are shown. Lung (J) and spleen (K) IFN-γ^+ CD8 T cells upon M2e stimulation are shown. Lung (L) and spleen (M) IFN-γ^+ $CD8^+$ T cells upon stem protein stimulation are shown. +/−, with or without CD4/CD8 depletion. (N) Impact of $CD4^+$ or $CD8^+$ T cell depletion on protection in adjuvanted M2e-H1stem (20 μg) vaccinated mice before challenge with A/rgH7N9 virus is shown. Statistical significance was determined using the one- or two-way ANOVA followed by Tukey's multiple comparison or Bonferroni posttest. Error bars indicate means ± SEM; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

cross-protection, naive mice were intranasally infected with a mixture of virus and immune sera collected from M2e-H1stem-vaccinated mice (Figures 5C–5E). Naive sera did not protect against rgH7N9 or A/WSN (H1N1) virus, as evidenced by severe weight loss and 0% survival. In contrast, M2e-H1stem immune sera conferred protection in naive mice with moderate weight loss (~10% and 13%, respectively) (Figures 5C and 5D). M2e-H1stem immune sera lowered lung viral titers by 10-fold at day 5 post-infection of rgH7N9 (Figure 5E). To further determine the contribution and roles of M2e and HA stem antibodies in protection, naive mice were intranasally infected with a mixture of group 1 virus (A/WSN/H1N1) or group 2 virus (A/Phil/H3N2) with antisera of M2e or group 1 HA2 stem or a mixture of M2e and group 1 HA2 stem (Figure S6). The group 1 HA2 stem group and the combination group of M2e-RBD and HA2 stem sera showed better protection (~10% weight loss) against A/WSN/H1N1 compared with M2e alone antisera (~20% weight loss). Importantly, the combination group of M2e and group 1 HA2 stem antisera more effectively prevented weight loss (~6%) against group 2 virus A/Phil/H3N2, suggesting significantly improved cross-protection, compared with the M2e or group 1 HA2 stem alone antiserum group displaying approximately 13% weight loss (Figure S6).

In addition, the mediastinal lymph nodes (MLNs) and spleens collected from the M2e-H1stem vaccination group were effective in secreting M2e-specific IgG and chimeric M2e-H1stem-specific IgG Abs after *in vitro* culturing, compared with the unvaccinated control group (Figures S7A and S7B). These data suggest the presence of B cells (memory-like) rapidly responding to the induction of IgG antibodies specific for M2e and HA2 stem antigens upon antigenically different virus exposure. Furthermore, M2e-H1stem group induced significantly high levels of interferon (IFN)-γ^+-secreting T cells in spleen and lung after *in vitro* stimulation with M2e, M2e-H1stem, stem, and inactivated rgA/Sha/H7N9 virus (Figures S7C–S7F); these data implicated that M2e-H1stem vaccination induced the generation of T cells rapidly responding to secret IFN-γ^+ upon challenge. Consistently, flow cytometry of intracellular cytokine staining showed that M2e or stem-specific IFN-γ^+CD4$^+$ T and IFN-γ^+CD8$^+$ T cells were increased in the lung and spleen cells in the adjuvanted M2e-H1stem group compared with the unvaccinated control (Figures 5F–5M and S8). To further investigate whether T cell immunity would contribute to protection, each of CD4$^+$ and CD8$^+$ T cells was depleted from the mice immunized with M2e-H1stem prior to rgA/Sha/H7N9 challenge (Figure 5N). Significant weight loss (~13%) and delayed recovery were observed in M2e-H1stem-vaccinated mice after CD4$^+$ or CD8$^+$ T cell depletion compared with the non-depleted M2e-H1stem-vaccinated mice displaying minimum weight loss (3% to 4%) (Figure 5N). Taken together, these data demonstrate that humoral and cellular immunity induced by M2e-H1stem vaccination have contributed to cross-protection against different subtypes of influenza A viruses.

M2e-H1stem retains thermostable universal vaccine features

The thermostability of M2e-H1stem protein was determined after storage for 10 days at different temperatures (4°C, 20°C, 37°C, and 50°C). There was no significant difference of M2e-H1stem in retaining antigenic epitopes, based on the reactivity against mAb 14C2 specific for M2e, pAbs specific for fusion peptide and stem, and antisera of rgH5N1 virus infection after 10 days storage at 50°C (Figures S9A–S9D). Adjuvanted vaccination of mice with M2e-H1stem protein pre-stored for 10 days at 50°C induced comparable IgG Abs specific for M2e-H1stem (Figure 6A) and protection against lethal challenge with A/WSN H1N1 virus (Figure 6B), retaining M2e-H1stem vaccine immunogenicity and efficacy even after storage at 50°C temperature.

M2e-H1stem vaccine provides comparable cross-protection in aged mice

We further evaluated the efficacy of adjuvanted M2e-H1stem vaccination in aged mice. Prime-boost vaccination of 14 months old mice with M2e-H1stem induced high levels of serum IgG Abs specific for M2e, stem, and M2e-H1stem antigens (Figure 6C). The M2e-H1stem-vaccinated aged mice were protected against lethal challenge with A/WSN H1N1 virus, as evidenced by 100% survival rates and minimum weight loss (~3.5%; Figure 6D). Remarkably, adjuvanted M2e-H1stem vaccination of aged mice provided cross-group protection against lethal challenge with group 2 virus (A/Sha/H7N9) by conferring 100% protection with minimal weight loss (~4%; Figure 6E). In addition, M2e-H1stem vaccination led to significantly lowering lung viral titers by 100-fold in aged mice after challenge with rgH7N9 virus (Figure 6F) than mock control. In addition, M2e-H1stem vaccination in aged mice induced the generation of B cells, which rapidly differentiated to plasma cells secreting IgG Abs and IFN-γ^+-secreting T cells in splenocytes upon *in vitro* stimulation with M2e peptide or stem protein (Figures 6G–6J). These results suggest that M2e-H1 stem vaccination might be effective in providing cross-group protection in elderly populations.

DISCUSSION

Vaccination-inducing antibodies against the conserved HA stem were considered as a promising strategy for cross-protection against influenza virus infection. However, substantial challenges exist in developing stem-based universal vaccines, including difficulty in preparing stem immunogens, their nature of being immune-subdominant epitopes, and low efficacy of cross-protection. In this study, to overcome the limited breadth and efficacy of stem-based vaccines, we uniquely designed a chimeric M2e-H1stem construct, which was successfully expressed in *E. coli*. The M2e-H1stem antigen was found to be highly reactive to antisera of different subtype HA protein immunization and group 1 and group 2 live virus infection, implicating the presentation of native-like epitopes. M2e-H1stem was immunogenic, inducing IgG antibodies specific for M2e and stem domains as well as group 1 and 2 HA viruses and virus-infected cell surface antigens. Vaccination of young adult and aged mice with adjuvanted M2e-H1stem protein induced broad cross-protection against both group 1 and 2 HA subtype viruses. Therefore, our chimeric M2e-H1stem represents a potential strategy of developing a surpassed universal influenza A vaccine conferring cross-group protection in aged populations.

The HA stem domain has high sequence conservation within the same subtype but moderate variations among the different subtypes

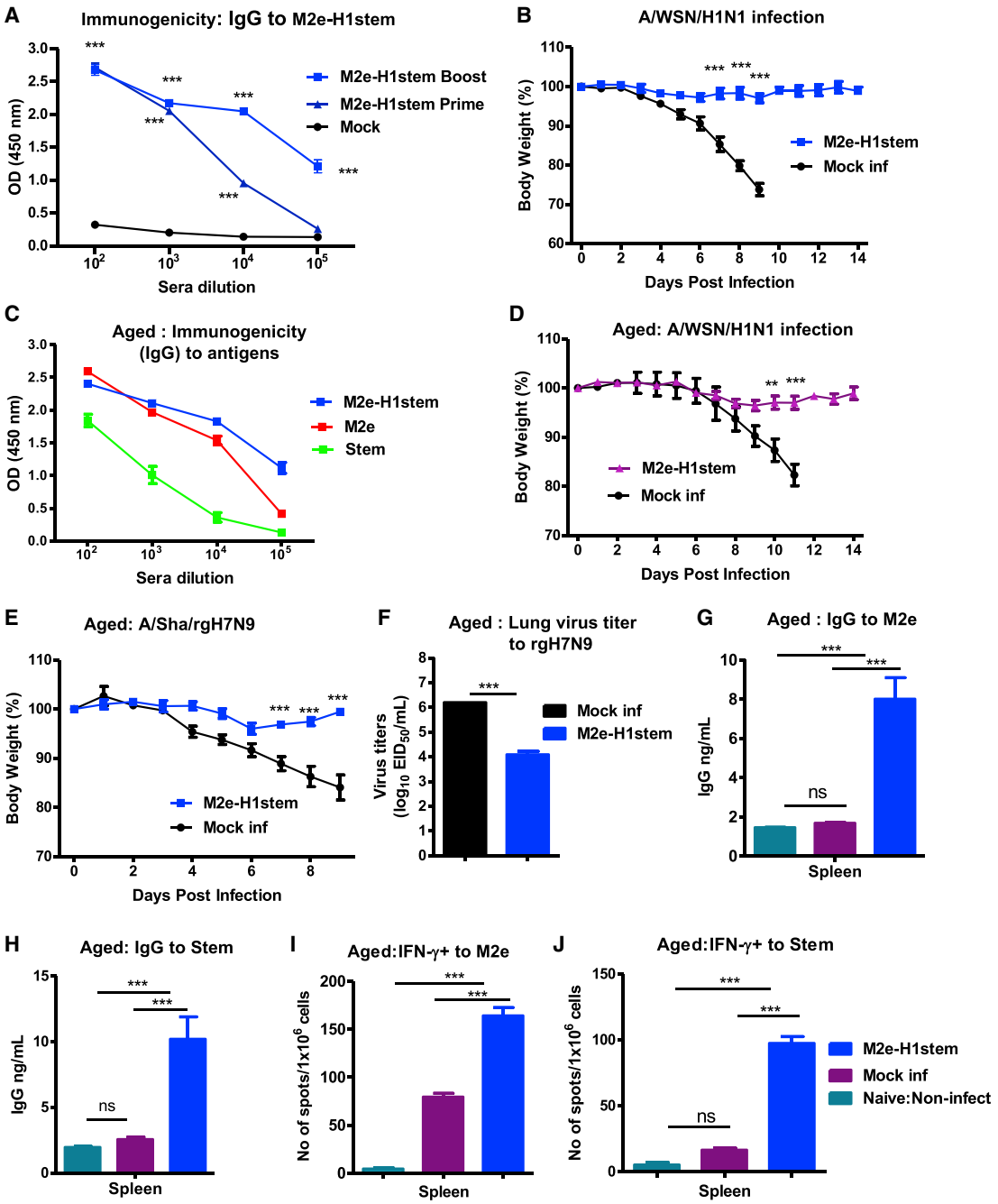

Figure 6. Evaluating the thermostability of adjuvanted M2e-H1stem vaccine and protective immunity in aged mice

(A and B) The impact of high-temperature storage of M2e-H1stem vaccine on inducing protective immunity is shown. (A) M2e-H1 stem-specific serum IgG antibody responses to vaccination of 8-week-old mice (n = 5) with adjuvanted M2e-H1stem pre-stored at 50°C for 10 days are shown. Mock inf, mock (no vaccine) group with virus infection. (B) Body-weight changes in vaccinated mice with 50°C pre-stored M2e-H1 stem after challenge with A/WSN/1933 H1N1 (2xLD_{50}, 1.5 × 10^2 EID_{50}) are shown. (C–J) Determination of immunogenicity and protection efficacy of M2e-H1stem vaccine in 14-month-old mice (n = 6) is shown. (C) M2e- and stem-specific IgG antibodies are shown. (D and E) Body-weight changes in vaccinated mice after challenge with H1N1 virus are shown (D; A/WSN/1933, 2xLD_{50}, 1.5 × 10^2 EID_{50}) or rgH7N9 (E; A/Sha/2013, 3xLD_{50}, 1.1 × 10^4 EID_{50}). (F) Lung viral titers of (E) are shown. (G and H) *In vitro* IgG production from spleen cells is shown. IgG antibodies specific for M2e (G) and stem (H) in spleen cells. (I and J) IFN-γ-secreting splenocytes stimulated with M2e peptide (I) or stem (J) are shown. Statistical significance was determined using the one- or two-way ANOVA followed by Tukey's multiple comparison or Bonferroni posttest. Error bars indicate means ± SEM; **p < 0.01; ***p < 0.001; ns, no significance between two compared groups.

and substantial differences across the other group HA viruses (Figure S1). Headless H1 and H3 HA full-length stem protein vaccines, which were structurally stabilized with trimeric GCN4 or ferritin nanoparticles expressed in mammalian cells, were reported to provide protection against homosubtypic virus in mice, ferrets, or non-human primates but significantly less effective in conferring protection against different subtype viruses, even within the same group.[7,9,27,28] These and other studies[8,29] suggest limitations on developing a stem only universal vaccine. Our previous study reported enhanced cross-protection by inducing immunity to both M2e and stem upon vaccination of mice with double-layered nanoparticles composed of three separate proteins expressed in insect cells, where H1 and H3 stem proteins layered onto the M2e cores were physically mixed.[21] A chemical cross linker was used to conjugate M2e particles and full-length H1 stem-GCN4 trimer protein with top helix C deleted, conferring HA-group-specific, but not cross-group, virus protection.[21] Multi-step preparations of nanoparticles likely raise concerns on scale-up vaccine manufacturing and chemical modifications of the potential epitopes. Here, we successfully designed a genetic fusion chimeric M2e-H1stem protein construct to be expressed in *E. coli* with the retention of M2e and stem epitopes, overcoming low efficacy of cross-group protection by stem vaccine.

Previous studies identified mAbs that recognize the epitopes located in the HA2 stem helix A and C domains as well as HA1 N- and C-terminal parts, which were structurally designed to stabilize the stem structure with point mutations.[4,5,7,9,27,30] An earlier study reported near full-length stem (HA2 aas 1–172) stabilized with HA1 (aas 1–41; aas 290–325) domain, which conferred low efficacy protection against homologous virus after CpG adjuvanted vaccination in mice.[31] Prior mini-stem construct[8] lacks the highly conserved HA2 fusion peptide domain (aas 1–40), which was shown to be a universal antigenic target inducing antibodies that recognize and cross-neutralize multiple subtypes of influenza A viruses.[32,33] In contrast, M2e-H1stem construct contains HA2 fusion domain (aas 1–40), major stem helix (aas 41–117), and M2e tandem repeats. M2e-H1stem was immunogenic and highly reactive to antibodies for M2e and fusion peptide epitopes, inducing M2e and stem-specific antibodies recognizing viral particles and antigens expressed on cell surfaces.

There were no significant differences in the levels of IgG Abs among the different vaccine doses (5 μg, 10 μg, and 20 μg) (Figures S10A–S10D). The 20-μg and 10-μg vaccine groups showed slight weight loss (∼5%), whereas the 5-μg vaccine group displayed 8% weight loss (Figures S10E and S10F). M2e-specific, but not stem-specific, antibodies were induced after prime vaccination of M2e-H1stem, even without adjuvant, suggesting that M2e was more immunogenic than stem within the M2e-H1stem protein. Notably, addition of adjuvant (QS-21 + MPL) in M2e-H1stem vaccination enhanced immune responses to M2e, stem, and viruses as well as protective efficacy. Previous studies reported the use of adjuvants, such as oil-in-water emulsion[28,29,34] or CpG,[8,31] in stem protein (20 μg) vaccination.[27] A different strategy to enhance stem-specific Abs was employed by multiple and sequential vaccinations with DNA, protein, and inactivated viruses with chimeric HAs containing the non-seasonal globular head and seasonal stem domains.[35–37] While these prior approaches of stem target vaccines protected animals from homologous and heterologous virus within the same group, our study represents significant advancement where M2e-H1stem vaccination provided broad cross-group protection against antigenically different, cross-group subtype viruses.

Several mechanisms contribute to enhancing cross-protection by inducing immunity to both M2e and stem, in the absence of receptor-blocking neutralizing antibodies. Binding of stem antibodies interferes with subsequent membrane fusion, eventually preventing the infection.[38] Passive transfer of M2e-specific or stem-specific antisera was shown to provide protection in mice.[14,34] Antibody binding to the fusion domain prevents cleavage of the HA precursor (HA0) into HA1 and HA2.[38,39] Stem antibodies can bind to HA0 on the infected cells, interfering with viral release.[40] Natural killer (NK) cells can kill virus-infected cells via interaction between Fc receptors (FcR) on NK cells and Fc of antibodies bound to the M2e and HA stem.[41–44] In addition, macrophages or neutrophils engulf M2e and stem IgG-bound influenza viral particles or virus-infected cells via interaction of FcR and Fc.[26,45] We found that M2e-H1stem antisera recognized viral antigens expressed on the surface of MDCK cells infected with group 1 or 2 viruses and enabled the activation of Jurkat cells endogenously expressing a transcription factor involved in the signaling events of ADCC, suggesting a protective mechanism. Antisera of M2e-H1stem protected naive mice from both group 1 (H1N1) and 2 (rgH7N9) viruses, further supporting cross-protective roles of humoral immune responses. Mice immunized with adjuvanted tandem repeat M2e protein vaccine (no stem) could not prevent severe weight loss,[46] in line with low efficacy of M2e alone immunity. Consistently, M2e-H1stem vaccination induced high levels of IFN-γ^+ secreting $CD4^+$ and $CD8^+$ T cells in spleen and lung. Either $CD4^+$ or $CD8^+$ T cell depletion in M2e-H1stem-vaccinated mice led to lower protection efficacy, implicating an important role of T cells in bestowing protection by M2e-H1stem vaccination.

It has been shown that the pigs that were vaccinated with inactivated virus vaccine (H1N2) induced antibodies binding to the HA2 domain but displayed enhanced pneumonia and disease after heterologous pandemic virus H1N1 infection.[47] Promoting viral infection and disease might be due to the induction of antibodies binding close to the fusion peptide in the absence of neutralizing antibodies or the use of a swine animal model prone to this possibility. Vaccination inducing broader humoral and cellular immunity to M2e and stem domains or supplementing cross-protective immunogens to seasonal vaccination might provide a strategy of attenuating or preventing respiratory disease against heterologous virus infection. Nonetheless, a possibility of disease enhancement by anti-HA2 stem antibodies requires cautious awareness in developing universal vaccines.

In summary, our chimeric M2e-H1stem protein immunogen was unique in presenting multi-cross-protective epitopes and inducing IgG antibodies specific for M2e, fusion peptide, stem, and cross-group

viral antigens on the virion particles and the surfaces of virus-infected cells. Adult and aged mice vaccinated with adjuvanted M2e-H1stem could provide a broad range of cross-group protections against lethal challenge with group 1 and 2 viruses. Mechanisms, such as cellular and humoral immunity, including ADCC, to both M2e and stem might be contributing to broad cross-group protection. Conjugation of M2e and stem domain as a single antigen would have some benefits of enabling the induction of M2e and HA2 stem-specific immune responses and broadening cross-protection. Importantly, *E. coli* expressed M2e-H1stem protein was thermostable, enabling rapid scale-up during a pandemic outbreak, even in low-resource countries. Further studies in more relevant animal models, such as ferrets, are warranted.

MATERIALS AND METHODS

Chimeric M2e-H1stem construct, expression, and protein purification

Influenza A virus (A/PR8/1934 [H1N1]) HA gene sequence was obtained from GenBank (NC_002,017) and used for the design of H1 stem protein construct. The HA2 region of group 1 HA was multiply aligned using Clustal Omega, and aa residues 31–54 and 304–337 of HA1 as well as 1–117 of HA2 region were selected as a vaccine target based on the conserved region of the HA stem and stabilizing domains, without altering the structure of the HA. The hydrophobic residues were modified by introducing polar aa mutations. Tandem 2xM2e repeat was genetically connected to the N-terminal of the HA1 via flexible linkers. The C terminus of chimeric M2e-H1stem construct was connected to the β-rich, trimer-stabilizing foldon. The M2e and foldon structures were derived from PDB: 3BKD and 4NCB, respectively. The 3D structure of HA was predicted using SWISS model and visualized in PyMOL.

The nucleotide sequence of the M2e-H1stem construct was codon optimized for expression in *E. coli* and synthesized by GenScript (USA). The synthesized gene was ligated into the pCold II cold expression vector (Takara Bio) containing N-terminal 6× His-tagged and subsequently transformed into *E. coli* (*DH5-α*) and Rosetta (DE3) pLysS cells (Novagen, USA). The expression of M2e-H1stem was induced in transformed DE3 bacteria by 1 mM isopropyl-β-D-1-thiogalactopyranoside and cultured at 16°C for 14 h. To obtain higher yields of protein preparations, we harvested and solubilized *E. coli* cell pellets containing both soluble and insoluble forms in 8 M urea lysis buffer (20 mM HEPES [pH 8.0], 300 mM NaCl, 2 mM CHAPS, 8 M urea, and 10 mM imidazole). After sonication, cleared lysates were applied to His tag affinity Ni-NTA beads. The bound M2e-H1stem protein was eluted with lysis buffer containing 250 mM imidazole and refolded by step dialysis in 20 mM HEPES (pH 8.0), 200 mM NaCl, 5% glycerol, and 1 mM dithiothreitol (DTT) with a gradual decrease in urea. The final refolded M2e-H1stem protein was further dialyzed against PBS, quantified, and stored at −80°C until further use. The purified M2e-H1stem protein was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and further evaluated by western blot using M2e-specific 14C2 mAb and H1-stem specific rabbit pAbs. The M2e and stem epitopes in the purified M2e-H1stem protein were determined by standard ELISA using M2e and stem specific antibodies and live virus antisera. The level of endotoxin in M2e-H1stem vaccine formulation was within a low range (0.5 units/20 μg/mL) as tested by chromogenic LAL Endotoxin Assay Kit (GenScript), which is below the allowable level (20 endotoxin units/mL) for recombinant protein subunit vaccines.[48]

Antibodies and protein

M2e specific mAb (14C2) was purchased from Santa Cruz Biotechnology (USA). Unit-1 mAb was kindly provided by Xuguang Li (University of Ottawa). The following rabbit pAbs specific for stem were generated by GenScript (USA) via hyper-immunizations with HA2 stem peptide epitope conjugates and purification by using the peptide-linked affinity column: anti-H3-FP specific for HA2 aas 14–27, anti-fusion peptide (HA2 aas 1–13), and anti-H1stem (HA2 aas 103–116). Goat pAbs specific for recombinant HA were acquired from BEI Resources (ATCC/NIH): anti-H1 HA pAbs (NR 15696), anti-H5 HA pAbs (NR 2705), anti-H3 HA pAbs (NR-48597), and anti-H7 HA pAbs (NR-48597). Preparation of the stem protein (without M2e) was previously described[49] and used as an ELISA coating antigen for measuring HA stem-specific IgG antibodies.

Immunization and virus challenges

Mouse experiments were approved by Georgia State University Institutional Animal Care and Use Committee (IACUC) (A21004). BALB/c female mice (6–8 weeks old; Jackson Laboratories) were intramuscularly immunized in a prime-boost schedule with a 3-week interval in the hind legs. M2e-H1stem protein (5, 10, and 20 μg) vaccine in 100 μL PBS (50 μL in each leg) was used for prime with adjuvants (10 μg QS-21 [Desert King International] plus 1 μg monophosphoryl lipid A [MPL] [Sigma Aldrich]) and boost vaccination with adjuvants (5 μg QS-21 + 0.5 μg MPL). Immunized mice were anesthetized with isoflurane prior to blood collection, and approximately 100 μL of blood samples were collected through retro-orbital sinus at 2 weeks after prime or prime and boost immunization. For aged mice, 14-month-old BALB/c mice were intramuscularly immunized with adjuvanted M2e-H1stem vaccine (20 μg). Blood samples were collected after 2 weeks of prime and boost immunization. Eight weeks after boost, mice were challenged intranasally with a lethal dose of influenza A virus in 50 μL PBS. Weight loss $\geq$ 20% was considered as the IACUC endpoint. Group 1 influenza A viruses were as follows: A/Puerto Rico/8/1934/H1N1 (A/PR8/H1N1), A/California/04/2009/H1N1 (A/Cal/H1N1), A/WSN/1933/H1N1 (A/WSN/H1N1), A/Hong Kong/1073/1999 H9N2 (A/HK/H9N2), mouse-adapted A/Fort Monmouth/1/1947 (A/FM/H1N1), and reassortant A/Vietnam/1203/2004/H5N1 with A/PR8 backbone (A/Viet/H5N1). Group 2 viruses used include A/Philippine/2/1982/H3N2 (A/Phil/H3N2), A/Hong Kong/1/1968/H3N2 (A/HK/H3N2), reassortants A/Shanghai/11/2013/H7N9 with A/PR8 backbone (A/Sha/H7N9), and A/Nanchang/933/1995/H3N2 with A/PR8 backbone (A/Nanchang/H3N2).

Enzyme-linked immunosorbent assay

M2e-H1stem protein vaccine antigenicity using antibodies specific for known epitopes, IgG antibody responses in sera, and *in vitro* cultures

were determined by standard ELISA as previously described.[14] ELISA coating antigens included M2e peptide (100 ng/well), M2e-H1stem or group 1 stem protein (50 ng/well) prepared as previously described,[49] foldon-linked spike protein (BEI NR-52396, 50 ng/well) of SARS-CoV-2, H1 stem peptides (100 ng/well), or inactivated viruses (200 ng/well). In brief, we performed a serial dilution of pAbs to find an optimal concentration for M2e-H1stem protein vaccine antigenicity assay. Then we determined the antigenicity of the M2e-H1stem protein vaccine to determine the antigenic affinity and exposure to the epitope-specific pAbs by testing different protein-coating concentrations. IgG antibody levels were determined with serial dilutions of immune sera from vaccinated mice on a specific antigen-coated ELISA plate.

In vitro detection of IgG antibody

MLN and spleen cells were isolated from young adult (6-week-old) or aged (14-month-old) BALB/c mice at 6 days post-infection. The cells were cultured on the plates pre-coated with antigens (M2e peptide, M2e-H3 stalk, or stalk protein) for 5 days at 37°C. The quantitation of antigen-specific IgG production in ng/mL after *in vitro* culture was determined by ELISA using mouse standard IgG antibody (cat no. 1010–01; Southern Biotech), as previously described.[50,51]

Determination of lung viral titers

The lung tissues collected at day 5, 6, and 9 post-infection were homogenized, and serially diluted (10-fold) lung extracts (200 μL) were injected into 10-day-old embryonated chicken eggs. The viral titers were presented in median embryo infectious dose (EID_{50}) after incubation for 3 days by hemagglutination assay in allantoic fluids as described.[14]

Interferon-γ ELISpot and flow cytometry

IFN-γ^+-secreting cells were evaluated on the 96-well ELISpot plates pre-coated with IFN-γ-capture antibody. ELISpot plates were seeded with splenocytes (5×10^5 cells) or lung cells (2×10^5 cells) and incubated with the stimulators: M2e peptide (5 μg/mL), M2e-H1stem protein (2 μg/mL), stem protein (2 μg/mL), or inactivated A/Sha/H7N9 influenza virus (4 μg/mL). IFN-γ-secreting cell spots were visualized with color-developing 3,3′-diaminobenzidine substrates and counted as described.[14] *In vitro* cultures of the isolated lung and spleen cells were stimulated with 5 μg/mL of M2e peptide and/or stem protein with brefeldin A (20 μg/mL). After 5 h culture, the cells were stained with mouse anti-CD3 (clone 17A2; BD Pharmigen), anti-CD4 (clone 553,051; BD Biosciences), and anti-CD8 (clone 25-0081-82; eBiosciences), followed by fixation and permeabilization using a Cytofix/Cytoperm kit (BD Biosciences) and then staining of intracellular cytokine IFN-γ using IFN-γ mAb (anti-mouse IFN-γ; clone XMG1.2; BD Biosciences). The Table S1 contains the source and working dilutions of antibodies used for flow cytometry and intracellular cytokine staining listing. Lymphocytes were gated to exclude dead cell-like events in flow cytometry experiments (Figure S8). The IFN-γ^+ T cells were analyzed by Becton-Dickinson LSR-II/Fortessa flow cytometer (BD Biosciences) and FlowJo software (FlowJo V10; Tree Star) as described.[52]

In vivo protection of immune sera and *in vivo* T cell depletion

Sera were heat inactivated, diluted (4-fold), and mixed with a lethal dose of A/Sha/H7N9, A/Phil/H3N2, or A/WSN/H1N1 virus. Body weight and survival rates were monitored after intranasal inoculation of naive mice with the mixture of sera and virus. Antisera were obtained from the groups of mice that were boost vaccinated with adjuvanted M2e-H1stem. T cell depletion in mice was performed with anti-CD4 (GK1.5) and anti-CD8 (53.6.7) mAbs (BioXCell) as previously described.[53] The levels of $CD4^+$ and $CD8^+$ T cells were determined by flow cytometry.

Cell surface ELISA and ADCC assay

The MDCK cells were seeded on 96-well cell culture plates (3×10^4 cells) and then infected with virus. Day 1 post-infection, cells were fixed with 4% paraformaldehyde and incubated with diluted sera, followed by standard ELISA to determine IgG antibodies bound to the viral antigens on the MDCK cell surface. The ADCC Reporter Kit (cat no: M1215, Promega Life Sciences, USA) was used to measure the activation of Jurkat cells mimicking NK cells[54] as a surrogate indicator for ADCC by serum antibodies bound to the virus-infected MDCK cells (3×10^4 cells). After incubation with Jurkat (7×10^4) effector cells for 6 h, luminescence was read to calculate fold increases using a Cytation 5 imaging reader (BioTek).

Statistical analyses

Data analyses were performed using Prism software (GraphPad Software). The statistical significance was determined by either one- or two-way ANOVA followed by Tukey's multiple comparison or Bonferroni posttest. All the data were represented as the mean ± the standard errors of the mean (SEM). $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.05.007.

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AUTHOR CONTRIBUTIONS

S.-M.K. and J.S. conceptualized and designed the research. J.S. designed the vaccine construct and generated the M2e-H1stem vaccine. J.S., J.O., and K.-H.K. characterized the vaccine antigens. J.S., J.O., K.-H.K., C.H.S., B.R.P., N.B., Y.-J.J., and Y.L. performed research. B.-Z.W. and B.-L.S. contributed to providing critical reagents. J.S.,

C.H.S., and S.-M.K. wrote the paper. All authors discussed the results and approved the final version of the manuscript.

DECLARATION OF INTERESTS

Some data presented in this manuscript will be included in the patent application. The authors declare no competing interests.

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