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Immunogenicity and efficacy of replication-competent recombinant influenza virus carrying multimeric M2 extracellular domains in a chimeric hemagglutinin conjugate

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Abstract

Current influenza vaccines provide hemagglutinin (HA) strain-specific protection. To improve cross protection, we engineered replication-competent influenza A virus to express tandem repeats of heterologous M2 extracellular (M2e) domains in a chimeric HA. M2e epitopes conjugated to HA glycoproteins (M2e4x-HA) were found to be expressed on the surfaces of a replicable influenza virus as examined by electron microscopy. The recombinant influenza virus containing M2e4x-HA was moderately attenuated but superior to the parental virus in inducing M2e specific antibodies without compromising HA immunogenicity. Recombinant influenza virus immune mice showed better cross protection than parental virus immune mice. Immune sera from the mice with inoculation of live recombinant influenza virus expressing M2e4x-HA were effective in conferring protection against H1, H3, and H5 subtype influenza viruses. This study indicates that recombinant influenza virus expressing conserved protective epitopes in an HA chimeric form can provide a new approach for improving the efficacy of influenza vaccines.

Keywords

recombinant influenza virus; viral vector; chimeric M2e4x-HA protein; universal influenza vaccine

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1. Introduction

Influenza virus causes respiratory diseases in humans, with significant medical and economic burdens. While the current vaccine confers strain-specific protection by inducing antibodies specific for hemagglutinin (HA), vaccine formulations do not provide sufficient protection against variant strains with an antigenically distinct HA. In addition to the seasonal epidemic burden, there is an emerging risk of a new pandemic to which the majority of the world population is immunologically naïve. Influenza viruses continuously mutate, circulate, and evolve in diverse natural hosts including wild birds, chickens, pigs, and humans. Since 1997, new types of influenza A viruses, H5, H7 and H9 serotypes, have crossed the species barrier from birds to human causing infections on multiple occasions (Fouchier et al., 2004; Gao et al., 2013; Peiris et al., 1999; Wong and Yuen, 2006). The emergence of the 2009 pandemic H1N1 virus is another example of the generation of a new strain with distinct antigenic properties (Hancock et al., 2009; Smith et al., 2009).

Vaccinations are effective in reducing the impact of epidemic if a chosen vaccine strain is well matched to the HA antigenicity of circulating strains. It is desirable to develop influenza vaccine with broader cross protection, which would require less frequent updating and induce longer lasting immunity. In contrast to variable HA and neuraminidase (NA) surface proteins, the ectodomain (M2e) of the membrane ion channel M2 protein, is highly conserved across human influenza A subtypes, and thus viewed as a promising target to develop universal influenza vaccine (Subbarao and Matsuoka, 2013). However, M2 is poorly immunogenic due to its small size, its low level of incorporation into virions, and possible shielding effects by larger viral surface proteins of HA and NA. In contrast, HA is highly immunogenic and the most dominant protein representing approximately 30% of total proteins in virions (Hutchinson et al., 2014). The efficacy of influenza vaccine candidates based on conserved antigenic targets such as M2e and HA stalk domains was less effective in conferring homologous protection compared to HA-based influenza vaccines (Jegerlehner et al., 2004; Lee et al., 2016; Steel et al., 2010). In our recent study, we reported that M2e immunity alone by tandem repeat M2e virus-like particle (M2e5x VLP) vaccine shows insufficient protection against HA homologous virus compared to HA-based split vaccine (Lee et al., 2016). Nonetheless, M2e immunity was better than HA split vaccine in conferring protection against heterosubtypic rgH5N1 virus (Lee et al., 2016). Therefore, it would be desirable to induce HA strain-specific as well as M2e antibodies by incorporation of multiple M2e epitopes into HA. We hypothesized that incorporating cross-protective M2e epitopes into HA of replication competent influenza virus in a chimeric HA molecule would induce both HA and M2e antibodies, contributing to better protection against influenza viruses with different antigenicities.

2. Methods

2.1 Cells and viruses

293T cells were obtained from ATCC. The following influenza A viruses used in this study were grown in 10-day-old chicken eggs: A/PR/8/34 (A/PR8, H1N1), A/California/04/09 (A/California, pdm2009flu, H1N1), A/Philippines/2/82 (A/Philippines, H3N2), A/Vietnam (rgH5N1) which contains HA (polybasic residues removed) and NA from A/Vietnam/

1203/2004 and 6 internal genes from A/PR8 (Song et al., 2011) and A/Mandarin Duck/Korea/PSC24-24/2010 (A/Mandarin duck, avian rgH5N1 containing HA with polybasic residues removed, NA and M from A/Mandarin Duck, and the remaining backbone genes from A/PR8 virus). The viruses were inactivated using formalin (Quan et al., 2008).

2.2 Generation of recombinant virus

Replication competent recombinant viruses (rg/M2e4x-HA) were rescued using the pHW2000-based eight-plasmid system described by Hoffmann et al. (Hoffmann et al., 2000). In brief, the sequence encoding 4× tandem repeats of heterologous M2e sequences derived from human, swine, and avian influenza viruses (Fig. 1A) was designed and synthesized (Genscript) to be inserted between the HA signal peptide sequence and N-terminal domain of the HA1 ectodomain (M2e4x-HA) of wild-type A/PR8 virus (Fig. 1A) using a previous strategy by Li et al. (Li et al., 2005). A M2e4x-HA gene fragment was inserted into pHW2000 plasmid and the sequence information is provided (Supplementary Fig. 1). To generate recombinant virus, 293T cells were co-transfected with an M2e4x-HA pHW2000 plasmid and seven other pHW2000 plasmids containing A/PR8 virus backbone genes. After 48 hours, the supernatant was inoculated into 10-day-old chicken eggs. After 72 hours, the rescue of recombinant viruses was confirmed in allantoic fluids from inoculated eggs by a hemagglutination assay.

2.3 Immunogold electron microscopy of purified recombinant M2e4x-HA virus

Sucrose gradient purified wild-type A/PR8 and recombinant rg/M2e4x-HA viruses (1 µg) were adsorbed onto formvar/carbon-coated copper grids (Electron Microscopy Sciences, Fort Washington, PA). After 15 min incubation, 4 µg of primary M2e monoclonal antibody (14C2, Abcam) was applied to grid for 1 h at room temperature. After washing with PBS (phosphate buffered saline), secondary 6 nm gold-conjugated anti-mouse antibody (Abcam) was added for 15 min at room temperature. The grids were then washed with PBS and negatively stained with 1.5% phosphotungstic acid (pH 7.0) for 30 sec. The images of stained wild-type A/PR8 or recombinant M2e4x-HA viruses were captured using a JEOL JEM 2100 transmission electron microscope (TEM).

2.4 Characterization and pathogenicity of recombinant viruses

Rescued recombinant viruses were exposed to multiple passages from 2 to 10 times in eggs and the passaged recombinant viruses (passage #2, #6, and #10) were characterized. Influenza A virus M2 monoclonal antibody (14C2 mAb, Abcam Inc., Cambridge, MA) or HA specific monoclonal antibody (IC5-4F8, BEI) was used for detection of HA or M2e protein by western blot or ELISA. To assess viral growth kinetics, embryonated chicken eggs were infected with 15 times of 50% egg infectious dose (EID₅₀) of recombinant (rg/M2e4x-HA) or wild-type influenza viruses and incubated for 48 hours. The allantoic fluids were harvested every 12 hour, and virus titers were assayed as EID₅₀/ml.

To verify pathogenicity of recombinant viruses compared to wild-type virus, 6–8-week-old female BALB/c mice (N=6 or 3, respectively; Harlan Laboratories) were intranasally inoculated with 2,000 or 4,000 EID₅₀ of recombinant or wild-type viruses. At day 7, lung

samples were collected from three mice of each group to titer viruses. Lung extracts were prepared and the viral titers were determined (Kim et al., 2013a).

2.5 Immunizations of mice

Six to eight-week-old female BALB/c mice (N=8; Harlan Laboratories) were intranasally inoculated with 500 EID₅₀ for prime, and 5,000 EID₅₀ of recombinant or wild-type viruses for boost 5 weeks later. Blood samples were collected at 4 weeks after each inoculation. All animal experiments were approved by the Georgia State University IACUC review boards. All experiments including blood and tissue collections were performed in accordance with relevant guidelines and regulations.

2.6 Antibody and hemagglutinin inhibition (HAI) assays

Virus-specific antibody responses were determined by ELISA using A/PR8 virus as a coating antigen (2µg/ml). M2e-specific antibody responses were determined using human, swine, and avian M2e sequences (Fig. 1B) (Kim et al., 2013a; Kim et al., 2013b; Quan et al., 2008) of chemically synthesized 23 amino acids (aa) polypeptides. Antibody concentrations were calculated using standard IgG and IgA antibodies. Hemagglutination inhibition (HAI) assay were determined against homologous, heterologous, and heterosubtypic influenza viruses as described (Kim et al., 2014; Quan et al., 2009).

2.7 In Vivo T-cell depletion and challenge

Groups of mice after boost inoculation with recombinant or wild-type viruses were treated with anti-CD4 and anti-CD8 monoclonal antibodies (CD4 clone GK1.5 200 µg/mouse, CD8 clone 53.6.7 150 µg/mouse, BioXCell, West Lebanon, NH) through intraperitoneal injection on days - 3, -1 relative to the date of challenge. The levels of CD4 and CD8 T cell depletion were confirmed by flow cytometry of blood samples and over 95% of T cells were depleted. The isotype control antibody (Clone LTF-2, rat IgG2b, BioXCell) did not affect the levels of CD4 and CD8 T cells compared to the PBS control (data not shown). All groups (N=4) were challenged with a lethal dose (8×LD₅₀) of A/Philippines/2/82 (H3N2) influenza virus. Mice were monitored daily to record weight changes and mortality.

2.8 Preparation of bronchoalveolar lavage fluids (BALFs) and antibody responses

BALFs were obtained by infusing 1 ml of phosphate-buffered saline (PBS) into lungs (Kim et al., 2013b). Virus or M2e-specific antibodies in sera or BALFs were determined by ELISA (Kim et al., 2013a).

2.9 Cross-protective efficacy tests of immune sera

A modified passive transfer assay of immune sera with virus was applied as previously described (Kim et al., 2014; Kim et al., 2013b; Song et al., 2011). Immune sera were collected at 4 weeks after boost inoculation. In brief, sera were heat-inactivated at 56°C for 30 min and the serum samples were mixed with 2×LD₅₀ of influenza virus, A/California (pdmH1N1), A/Philippines (H3N2), A/Mandarin duck (avian rgH5N1) or A/Vietnam (rgH5N1), and incubated at room temperature for 1 hour. Naive mice (N = 3, BALB/c) were

infected with a mixture of virus and sera, and body weight and survival rates were daily monitored for 14 days.

2.10 Statistical analysis

A two-tailed Student's t-test and a Mann-Whitney test were used to determine the statistical significance when comparing two different conditions. *P*-values of less than or equal to 0.05 were considered significant.

3. Results

3.1 Replicable recombinant virus carrying chimeric M2e4x-HA protein expresses M2e epitopes at high levels

Avian and swine influenza viruses have few residue differences in the M2e domain compared to human influenza A viruses (Fiers et al., 2004; Liu et al., 2005). Recently a construct with multiple heterologous M2e epitopes from human, swine, and avian viruses was shown to be effective in inducing cross protection when delivered via virus-like particles (Kim et al., 2013a). In an attempt to test the proof-of-concept of improving the intrinsic capacity of influenza virus to induce cross protection, chimeric HA constructs (M2e4x-HA) were generated, containing a tandem repeat of heterologous M2e (M2e4x) at the N-terminus of HA (Fig. 1A). M2e4x is composed of human M2e (2×), swine M2e, and avian M2e (Fig. 1B). This tandem repeat M2e domain was conjugated to HA by either GGGGS (Li et al., 2005) or AAAPGAA connector, to facilitate the proper folding of the inserted polypeptides as an independent domain (Fig. 1C). Replication-competent recombinant influenza viruses were recovered using the reverse genetic system. Incorporation of M2e epitopes into rg/M2e4x-HA virus in a chimeric HA conjugate was determined in comparison with wild-type PR8 virus (wt/HA) after sucrose-gradient purification and inactivation of virus in ELISA using M2e 14C2 mAb (Fig. 1D). The M2e epitope of wt/HA was detected at a minimal level (Fig. 1D). In contrast, two recombinant viruses showed high reactivity to M2e mAb (14C2) and the reactivity of recombinant virus containing AAAPGAA (connector 2) was approximately two times higher than that of recombinant virus containing GGGGS (connector 1). M2e epitopes are covalently linked to HA and M2e4x-HA expression levels were similar in terms of HA between the connectors C1 and C2 (data not shown). Thus, a difference in 14C2 reactivity is likely due to conformational changes in the M2e4x-HA C1 and C2 constructs. In the following experiments, we focused on the C2 recombinant virus (connector 2: AAAPGAA) that showed higher reactivity to M2e 14C2 mAb.

3.2 Recombinant virus maintains morphological integrity of virus presenting M2e epitopes on the surfaces

The morphological integrity of recombinant viruses carrying chimeric M2e4x-HA proteins and M2e epitope presentation were examined using an electron microscope. The overall structural morphology of membrane enveloped recombinant virus was similar to that of wild-type influenza virus, and both spherical virus particles showed an average size of 100 nm (Fig. 2A and B). To probe the M2e epitope presentation on the virus particles, M2e specific primary and 6 nm gold particle-conjugated secondary antibodies were incubated

with the virus particles. M2 proteins of wild-type influenza virus were not exposed to the envelope surfaces being inaccessible to antibodies (Fig. 2C). In contrast, many gold particles were clearly visible on the surfaces of recombinant virus with M2e4x-HA, suggesting that M2e epitopes are sufficiently well exposed to M2e 14C2 mAb reactive to gold particle secondary antibodies (Fig. 2D). Frilly spherical morphology with spike projections surrounding the virus envelope on the recombinant virus might be due to gold-conjugated antibodies bound to the M2e4x-HA molecules on the surfaces. These results suggest that the recombinant virus maintains structural and morphological integrity of virus particles with substantial M2e epitopes accessible to antibodies in HA conjugate molecules on the spherical surfaces.

3.3 Recombinant virus stably expresses M2e4x-HA proteins

To test the stability of chimeric M2e4x-HA proteins, recombinant viruses at 2, 6, 10 passages in eggs were harvested and the expression of chimeric M2e4x-HA proteins was determined (Fig. 2E and F). The incorporation of M2e4x-HA and HA proteins was determined in recombinant and wild-type viruses (Fig. 2E). Wild-type and recombinant viruses showed a similar level of HA protein expression (Fig. 2E, top). When probed with M2e specific 14C2 mAb, only recombinant viruses containing chimeric M2e4x-HA showed M2e reactivity at the corresponding HA position. In quantitative determination of chimeric M2e4x-HA protein at the passage #2, #6, and #10, the M2e reactivity in recombinant virus at passage #10 was maintained approximately 80% of M2e reactivity compared to the passage #2 (Fig. 2F).

3.4 Recombinant virus is replication-competent and shows an attenuated phenotype

To determine *in vitro* viral growth kinetics, 15 EID₅₀ of wild-type (wt/HA) or recombinant viruses (rg/M2e4x-HA) of passages #2 and #10 were inoculated into chicken embryonated eggs and viral titers were quantified by EID₅₀ (Fig. 3A). A similar growth kinetics was observed in both the recombinant and wild-type virus. These results indicate that the recombinant virus is replication-competent and well propagated in chicken eggs without compromising growth properties in eggs. Mice that received 2,000 EID₅₀ of rg/M2e4x-HA did not show weight loss whereas mice that were infected with wt/HA displayed significant weight loss up to 13% (Fig. 3B). A high dose (4,000 EID₅₀) inoculation with rg/M2e4x-HA virus was moderately pathogenic, causing approximately 10% loss of body weight, but wt/HA led to more severe disease with over 22% weight loss (Fig. 3C). Lung viral titers are also an important criterion for determining replication and attenuated phenotypes. At day 7 after inoculation, viral titers in mice with rg/M2e4x-HA were approximately 10 times lower comparing those in mice with wt/HA (Fig. 3D). These results indicate that rg/M2e4x-HA is moderately attenuated in pathogenicity and replication compared to the parental wt/HA in mice.

3.5 Recombinant virus is effective in inducing M2e antibodies without compromising virus immunogenicity

Four weeks after each immunization, we measured serum antibody titers to virus and M2e peptide antigens. Both prime and boost immune sera from wild-type and recombinant viruses showed high levels of antibody responses specific for virus (Fig. 4A). Recombinant

rg/M2e4x-HA immune sera showed high HAI titers comparable to those in wt/HA immune sera (Fig. 4B). Thus, the recombinant virus retains viral immunogenicity without a defect in inducing immune responses to virus compared to wt/HA. The group of mice with rg/M2e4x-HA induced significantly higher levels of M2e antibodies reactive to human, swine, and avian M2e antigens after prime, compared to the wt/HA group (Fig. 4C). After boost inoculation, immune sera with recombinant virus showed further enhanced levels of M2e antibodies. These results suggest that rg/M2e4x-HA is superior to wt/HA wild type virus in inducing M2e specific antibodies without compromising virus immunogenicity.

3.6 Recombinant but not wild-type virus is effective in inducing cross-protection and M2e antibodies at mucosal sites

Mice that survived influenza virus primary infection were reported to acquire a certain level of heterosubtypic cellular immunity against secondary infection (Guo et al., 2011; Nguyen et al., 1999; Nguyen et al., 2000; Nguyen et al., 2007; O'Neill et al., 2000). Mice that received wt/HA parental virus inoculation two times were protected against H3N2 virus (A/Philippines) without displaying weight loss (data not shown). Therefore, to compare the efficacy of cross protection in mice inoculated with wild-type and recombinant H1N1 rg/M2e4x-HA virus, CD4 and CD8 depleting antibodies were treated in these immune mice before and during challenge infection with a heterosubtypic H3N2 virus, at 12 weeks after immunization (Fig. 5A). The mice that were inoculated with rg/M2e4x-HA showed a slight loss (approximately 5–7%) in body weight post challenge. In contrast, mice inoculated with wt/HA virus showed a significant loss (approximately 16%) in body weight as well as a substantial delay in recovering weight despite 100% survival rates (Fig. 5B). The naïve group did not survive and all died of infection (Fig. 5B). Thus, these results demonstrate that rg/M2e4x-HA is superior to wt/HA virus in conferring protection against H3N2 virus.

We determined virus- or M2e-specific antibody responses in BALFs after boost inoculation. Significant levels (12.63 ± 3.2 ng/ml, 5.8 ± 1.77 ng/ml, and 5.14 ± 1.5 ng/ml, respectively) of IgG antibodies specific for human, swine, and avian M2e antigens, respectively, were induced with rg/M2e4x-HA, but not wt/HA (Fig. 5C). Furthermore, the recombinant virus induced IgA antibodies specific for human, swine, avian M2e peptides at substantial levels (2.46 ± 0.42 ng/ml, 2.05 ± 0.41 ng/ml, and 2.23 ± 0.49 ng/ml, respectively) (Fig. 5D). These results indicate that recombinant virus with M2e4x-HA is highly effective and superior to wt/HA in eliciting M2e specific IgG and IgA antibodies at mucosal sites.

3.7 Immune sera from recombinant virus confer better protection against different viruses

It is important to determine the potential capability of immune sera with M2e antibodies to confer protection against antigenically different viruses. A modified passive transfer assay was applied by infecting naïve mice with a mixture of a lethal dose of virus and immune sera as described (Kim et al., 2014; Kim et al., 2013b; Song et al., 2011). Recombinant rg/M2e4x-HA immune sera conferred naïve mice with better protection against A/California/4/2009 (pdmH1N1) and A/Philippines/2/1982 (H3N2), as shown by less than 5% weight loss and quick recovery (Fig. 6A, B). On the other hand, naïve mice that were infected with a mixture of heterologous H1N1 or heterosubtypic H3N2 virus and wt/HA immune sera showed approximately 13% and 17% loss in body weight, respectively, experiencing severe

morbidity. Naïve serum-treated mice exhibited most severe morbidity by showing approximately 18–20% losses (Fig. 6A, B). At day 5 after infection with H3N2 virus, the group of naïve mice that received rg/M2e4x-HA immune sera showed approximately 5-fold lower replication than that of wt/HA, as shown in the results of lung viral titers determined by infectivity assay in embryonated chicken eggs (Fig. 6C). We further determined the efficacy of protection against H5N1 influenza viruses, A/Mandarin duck (avian rgH5N1) with avian M2 and A/Vietnam (rgH5N1) (Fig. 6D, E). The groups of naïve mice that received M2e antibody-containing immune sera showed protection against both rgH5N1 viruses without significant weight loss. In contrast, the groups of naïve mice that were treated with wt/HA immune sera showed significant weight loss of 10–12%. The naïve serum-treated mice exhibited more severe weight loss of 15–17%. These results suggest that rg/M2e4x-HA can induce antibodies that confer better protection against H1, H3, and H5 subtype influenza viruses. Therefore, effective induction of antibodies cross-reactive to various M2e antigens is important for conferring protection against antigenically diverse HA subtype influenza A viruses.

4. Discussion

The levels of M2e antibodies were very low in influenza-infected mice and not often detected in humans (Black et al., 1993; Feng et al., 2006). Several strategies were reported in an attempt to overcome poor immunogenicity of M2e, including fusion of M2e peptides to immunogenic carrier proteins (De Filette et al., 2005; Fan et al., 2004; Neiryneck et al., 1999) with some adjuvants or delivery of VLPs containing M2e epitopes (Alvarez et al., 2013; Bessa et al., 2008; Hashemi et al., 2012; Jegerlehner et al., 2004; Kim et al., 2013b; Turley et al., 2011). A previous study reported that HA2 stalk domain-based vaccines conferred survival protection in vaccinated mice after challenge (Steel et al., 2010). Recent studies demonstrated that novel vaccine constructs of stabilizing trimeric stalk domains could significantly improve the cross protection against a broad range of influenza viruses (Impagliazzo et al., 2015; Lu et al., 2014; Yassine et al., 2015). M2 is not N-glycosylated even though it contains a potential glycosylation site (Zebedee et al., 1985). M2e5x VLP vaccine was found to be superior to tandem repeat M2e protein vaccines in inducing M2e antibodies and M2e protein vaccines did not induce M2e antibodies in the absence of adjuvants (data not shown). Efficacy of M2 immunity alone was insufficient for protection against HA-matched vaccine virus compared to inactivated influenza vaccines (Jegerlehner et al., 2004; Lee et al., 2016). M2e5x VLP and carrier protein conjugate vaccines are considered as new drugs and might encounter more regulatory hurdles during translation to clinical trials. In this study, we generated recombinant influenza viruses expressing conserved M2e epitopes in a chimeric HA conjugate form on the surface of influenza virus. The results in this study support a proof-of-concept that expressing M2e epitopes as a chimeric HA in recombinant influenza virus could be a new approach to effectively induce M2e antibodies increasing the breadth of protection without compromising HA-based vaccine efficacy.

Influenza viruses can be manipulated using reverse-genetics and attenuated live vaccines are administered using needle-free sprayers. The majority of neutralizing antibodies generated by influenza vaccines recognizes the HA glycoprotein on the viral surface. N-terminal

regions of HA can be flexible in length, possibly allowing to accommodate extra polypeptide segments without interfering with essential HA functions (Nobusawa et al., 1991). The size of insertions between the HA signal peptide and the HA1 N-terminus can be as large as 246 aa while maintaining the HA functionality (Hatzioannou et al., 1999). In this study, the size of inserted tandem repeat heterologous M2e peptides is 137 aa. We could successfully rescue recombinant viruses expressing tandem repeat containing heterologous M2e epitopes. The recombinant viruses showed high levels of reactivity to M2e antibody compared to wild-type virus, which implicates that chimeric M2e4x-HA proteins were well incorporated and exposed. In addition, the recombinant virus seemed to be moderately attenuated in mice, as evidenced by less weight loss and lower lung viral titers compared to those of wild-type virus.

Immune sera with M2e antibodies from mice inoculated with recombinant virus were found to confer improved protection against H1, H3, and H5 subtype influenza viruses. M2e sequences of influenza viruses from different host origins have few mutations in the core residues such as the 2009 pandemic swine-origin H1N1 influenza virus although M2e is highly conserved among human influenza A viruses (Liu et al., 2005). Nonetheless, the efficacy of protection against A/Mandarin duck rgH5N1 and A/California/2009 pandemic virus with avian and swine M2e respectively was observed in a similar pattern as in the protection against A/Vietnam rgH5N1 with human M2e. A tandem repeat human M2e was shown to induce similar levels of antibodies reactive to human M2e, but lower levels of antibodies reactive to swine or avian M2e compared to heterologous tandem repeats of M2e vaccine (Kim et al., 2013b). These results support the added importance of incorporating M2e from multiple origins. Since M2e antibodies do not neutralize the virus directly, several mechanisms for cross protection by M2e antibodies have been suggested. Using a clodronate-mediated depletion approach, alveolar macrophages and dendritic cells were shown to be important in conferring protection by M2 immune sera (El Bakkouri et al., 2011; Song et al., 2011). Also, using a model of FcR γ chain deficient mice, Fc receptors were found to play a critical role in conferring cross protection by M2e antibodies (Kim et al., 2013a; Lee et al., 2014). Further studies will be needed to better understand the underlying mechanisms of improved protection by recombinant influenza virus vaccine.

In previous studies, mice that survived influenza virus infection were shown to have heterosubtypic immunity at a certain level through the induction of cross-reactive T cell and B cell responses (Guo et al., 2011; Nguyen et al., 1999; O'Neill et al., 2000). Although mice with wild type virus-mediated immunity showed significant weight loss, they survived against lethal infection, even under the condition of CD4 and CD8 T cell depletion, suggesting the induction of cross protective antibodies in immune sera by infection with influenza virus. In CD4 and CD8 T-cell depleted mice, the protective efficacy of recombinant M2e4x-HA influenza viruses was higher, as evidenced by significantly lower weight loss compared to that of wild-type virus immune mice. These results support a proof-of-concept of improving the protective efficacy by recombinant influenza virus after vaccination.

Wild-type virus is a pathogen and cannot be considered as a vaccine candidate. Therefore, it is needed to test the concept of utilizing recombinant virus as a potential vaccine in the

backbone of live attenuated influenza vaccine (LAIV) strain. With regards to developing recombinant LAIV inducing cross protection, it is important to note that the growth capacity in eggs and virus immunogenicity in mice were not compromised. LAIV has several advantages, including cell-mediated immunity and mucosal immune responses, thereby providing long-lasting immunity and broad antigenic coverage (Cox et al., 2004; He et al., 2006; Horimoto and Kawaoka, 2009). LAIV was also demonstrated to be superior to inactivated vaccines in providing protection for children in clinical trials (Osterholm et al., 2012; Tricco et al., 2013).

In a recent comparison study of M2e5x VLP and inactivated split influenza virus vaccine, the protective efficacy of M2e5x VLP vaccine was lower, resulting in over 10 % body weight loss compared to the inactivated split vaccine (0% weight loss) when the HA homologous virus to the inactivated vaccine was challenged (Lee et al., 2016). The immune sera of rg/M2e4x-HA showed a higher survival rate (100%) than M2e5x VLP immune sera (50%) but both displayed a similar pattern of weight loss (data not shown). However, detail comparative experiments with normalized vaccine amounts should be carried out in future studies. The results in this study using a model system provide evidence that vaccination with recombinant virus carrying M2e epitopes in an HA conjugate can be an approach to enhance the efficacy of cross protection by current vaccine platforms. The major benefit of this approach is that rg/M2e4x-HA is expected to provide protection against the vaccine strains as supported by high HAI titers similar to the levels of wild type virus immune sera. In the cross-protection experiments, the rg/M2e4x-HA immune sera did not show cross protective HAI activity. M2e4x-HA live virus vaccine confers better protection (<5% weight loss) compared to wild type virus immune mice showing over 15% weight loss under a lethal challenge dose (8 LD₅₀) where all naïve mice died of infection. Despite of conferring better protection, M2e4x-HA live virus vaccine immune sera could not prevent weight loss completely, representing limitation in this approach. Further studies are required to enhance the efficacy of cross protection.

In conclusion, this study provides a proof-of-concept for developing a novel approach to improve the cross-protective efficacy of live influenza virus vaccines by incorporating a tandem repeat of conserved M2e epitopes into the strain-specific variable HA protein. The tandem repeat of various influenza M2e antigens was incorporated into recombinant viruses in an M2e4x-HA conjugate protein without compromising virus replication in eggs and virus immunogenicity in mice. Actively immunized mice and immune sera from mice that were previously inoculated with recombinant virus expressing M2e4x-HA were found to confer better protection against different subtype influenza viruses. These findings not only demonstrate the efficacy of recombinant influenza virus vaccines expressing additional M2e epitopes, but also support a novel approach of engineering influenza vectors that would be instrumental to improve the efficacy of LAIV vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Generation of replication competent recombinant influenza virus with 4xM2e-HA proteins.

Recombinant influenza virus rg/4xM2e-HA induces HA and M2e antibodies.

Recombinant rg/4xM2e-HA confers better cross protection than the parental influenza virus

Fig 1. Recombinant influenza virus with M2e4x-HA shows high reactivity to M2e epitope specific antibody

The sequence of M2e4x was inserted between the HA signal peptide and ectodomain (M2e4x-HA). Sp: signal peptide, Human, Swine, Avian: human, swine, and avian influenza A type M2e respectively. L: linker (AAAGGAA). C: connector (GGGS, AAAPGAA), Ectodomain: HA ectodomain, TM and CT: transmembrane and c-tail. (A) Structure of chimeric M2e4x-HA proteins. (B) Sequence of human [A/PR8(H1N1), A/Philippines(H3N2), A/Vietnam(rgH5N1)], swine [pdm2009flu(H1N1)], and avian M2e sequences [A/Mandarin duck (rgH5N1)]. (C) Sequences of connectors between M2e4x domain and HA ectodomain. The recombinant rg/M2e4x-HA viruses were coated and M2e reactivity was analyzed using M2e 14C2 mAb. (D) Comparison of M2e reactivity in recombinant viruses. The differences between rg/M2e4x-HA (C1) and rg/M2e4x-HA (C2) of recombinant viruses were measured from each representative.

Fig 2. Electron microscopy and stability of rg/M2e4x-HA

(A) Wild-type of A/PR8 (H1N1) virus. (B) Recombinant A/PR8 virus (rg/M2e4x-HA). (C–D) (C) Immunogold-labeled wild type of A/PR8 (H1N1) virus. (D) Immunogold-labeled rg/M2e4x-HA. Mouse 14C2 mAb and 6 nm gold-conjugated goat anti-mouse antibodies were used as primary and secondary antibodies. (E) Western blot of M2e and HA proteins. The incorporation of M2e and HA proteins into recombinant and wild-type viruses was determined by western blots using M2e monoclonal antibody (14C2) and HA monoclonal antibodies (IC5-4F8, BEI), respectively. Lane wt/HA: wild-type virus (A/PR8, 8ug), Lane #2: egg passage #2 of rg/M2e4x-HA (#2, 8ug), Lane #6: egg passage 6 of rg/M2e4x-HA (#6, 8ug), Lane #10: egg passage 10 of rg/M2e4x-HA (#10, 8ug). (F) Contents of M2e epitopes in the passaged rg/M2e4x-HA viruses. Reactivity of rg/M2e4x-HA to 14C2 mAb was determined by ELISA. The differences between rg/M2e4x-HA(#2) and rg/M2e4x-HA(#10) of recombinant viruses were measured from each representative.

Fig 3. Recombinant rg/M2e4x-HA virus maintains high growth property in eggs but shows attenuation in mice

(A) *In vitro* growth kinetics. Eggs were inoculated with 15 EID₅₀ of wt/HA (A/PR8), rg/M2e4x-HA #2, or rg/M2e4x-HA #10 virus. (B–C) Body weight changes of mice after inoculation with 2,000 EID₅₀ (B) and 4,000 EID₅₀ (C) of rg/M2e4x-HA, respectively. BALB/c mice (N=3) were intranasally inoculated with 2,000 EID₅₀ or 4,000 EID₅₀ of wild-type or recombinant virus and monitored for their weight changes. All mice survived virus infection with rg/M2e4x-HA or wt/HA. (D) Viral titers were determined in lung samples (N=3) at day 7 post-challenge. The viral titer in lung extracts was determined by an EID₅₀ assay. Error bars indicate mean $\pm$ SEM and statistical significances are indicated.

Fig 4. Recombinant rg/M2e4x-HA is superior to wild-type virus in inducing M2e antibodies
BALB/c mice were intranasally inoculated with 500 EID (prime, p) and 5,000 EID (boost, b) of the wild-type (A/PR8, N=8) or recombinant (rg/M2e4x-HA, N=8) viruses. Sera were collected at 4 weeks after prime (p) or boost (b) immunization. (A) Serum IgG antibody response to A/PR8 virus. (B) Hemagglutinin inhibition (HAI) assay. The dotted line denotes the limit of HAI detection. (C) Serum IgG antibody responses to M2e peptides. Asterisk indicates significant differences between wild-type and recombinant virus prime or boost sera (**: <0.01). Error bars indicate mean $\pm$ SEM. EID, egg infectious dose.

Figure 5. Recombinant rg/M2e4x-HA immune mice show enhanced cross protection and mucosal M2e antibodies

(A) Protection against heterosubtypic H3N2 virus. Groups of mice (N=4) that were immunized with wt/HA or rg/M2e4x-HA were treated with CD4 and CD8 depleting antibodies prior to lethal infection (8xLD₅₀) with H3N2 virus (A/Philippines/2/82). (B) Survival curve. (C) M2e specific IgG in BALF. (D) M2e specific IgA in BALF. Levels of IgG and IgA antibodies to M2e peptide antigens were determined at 6 weeks after boost immunization (N=4). Error bars indicate mean $\pm$ SEM. Asterisk indicates significant differences between wild-type and recombinant virus groups or BALF samples (*: <0.05, **: <0.01). BALF, bronchoalveolar lavage fluids; ng, nanogram.

Fig 6. Roles of immune antisera in conferring protection against different influenza virus subtypes

(A) Body weight changes in naïve mice after infection with a mixture of A/California (H1N1) virus and immune or naïve sera. (B) Serum-mediated protection against A/Philippines (H3N2) virus. (B–C) All naïve mice that were inoculated with virus and sera survived. (C) Viral titers in lungs after infection with A/Philippines (H3N2) virus and immune sera. Lung viral titers (N=4) were determined at day 5 after infection with $2 \times \text{LD}_{50}$ of A/Philippines (H3N2) mixed with sera from rg/M2e4x-HA or wt/HA immune or naïve mice. Embryonated chicken eggs were inoculated with diluted lung extracts and tested for hemagglutination activity to determine viral titers (Wen and Wang, 1959). (D) Serum-mediated protection against A/Mandarin duck (rgH5N1 with avianM2e) virus. (E) Serum-mediated protection against A/Vietnam (rgH5N1) virus. Body weight changes and survival rates were monitored for 14 days. (D–E) All naïve mice that were inoculated with virus and sera survived. P values indicate significant differences between the immunized sera of recombinant or wild-type viruses. Data represent mean $\pm$ SEM. EID, egg infectious dose. rg/M2e4x-HA a/s: infection of naïve mice with virus plus rg/M2e4x-HA immune antisera (a/s), wt/HA a/s: infection with virus plus wt/HA immune antisera, Naïve sera: infection of naïve

mice with virus plus naïve sera. Statistical significances are indicated between the rg/M2e4x-HA a/s and wt/HA a/s.

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