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In contrast to conventional inactivated influenza vaccines, 4xM2e.HSP70c fusion protein fully protected mice against lethal dose of H1, H3 and H9 influenza A isolates circulating in Iran

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ABSTRACT

Ideal vaccines against influenza viruses should elicit not only a humoral response, but also a cellular response. *Mycobacterium tuberculosis* HSP70 (mHSP70) have been found to promote immunogenic APCs function, elicit a strong cytotoxic T lymphocyte (CTL) response, and prevent the induction of tolerance. Moreover, it showed linkage of antigens to the C-terminus of mHSP70 (mHSP70c) can represent them as vaccines resulted in more potent, protective antigen specific responses in the absence of adjuvants or complex formulations. Hence, recombinant fusion protein comprising C-terminus of mHSP70 genetically fused to four tandem repeats of the ectodomain of the conserved influenza matrix protein M2 (M2e) was expressed in *Escherichia coli*, purified under denaturing condition, refolding, and then confirmed by SDS–PAGE, respectively. The recombinant fusion protein, 4xM2e.HSP70c, retained its immunogenicity and displayed the protective epitope of M2e by ELISA and FITC assays. A prime-boost administration of 4xM2e.HSP70c formulated in F105 buffer by intramuscular route in mice (Balb/C) provided full protection against lethal dose of mouse-adapted H1N1, H3N2, or H9N2 influenza A isolates from Iran compared to 0–33.34% survival rate of challenged unimmunized and immunized mice with the currently in use conventional vaccines designated as control groups. However, protection induced by immunization with 4xM2e.HSP70c failed to prevent weight loss in challenged mice; they experienced significantly lower weight loss, clinical symptoms and higher lung viral clearance in comparison with protective effects of conventional influenza vaccines in challenged mice. These data demonstrate that C-terminal domain of mHSP70 can be a superior candidate to deliver the adjuvant function in M2e-based influenza A vaccine in order to provide significant protection against multiple influenza A virus strains.

Introduction

Influenza A viruses are globally important respiratory pathogen which cause a high degree of morbidity and mortality during annual epidemics (Cox et al., 2004).

The recent emergence of highly virulent avian influenza, H5N1, and swine-origin H1N1 showed a threat not only to the poultry industry, but also to wild birds, several species of mammalians, and humans in contact with such species (Garten et al., 2009; Malik Peiris et al., 2007).

Because of growing evidence for increased incidence of different subtypes of influenza A virus in both animal and human population worldwide and failure of vaccination with conventional vaccines due to frequent antigenicity changes resulting from antigen drift and antigen shift, bring out an urgent need to develop a universal influenza vaccine, which could provide cross-protection against different influenza virus strains (Brett et al., 2005; Ebrahimi et al., 2011a, b; Fan et al., 2007; Johansson et al., 1989). The development of a truly universal vaccine would present the potential opportunity for both reducing the threat of a pandemic and the impact of seasonal influenza.

One candidate protein for such a vaccine is matrix protein 2 (M2), a 97 amino acid (aa) transmembrane and nonglycosylated protein of influenza A virus, whose extracellular domain with 24 aa residues (23 aa after posttranslational removal of the

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N-terminal Met) is called M2e (Fischer and Sansom, 2002). The mature M2 protein forms homotetramers and functions as a proton-selective channel and is important for uncoating of viral particles in endosomes for viral entry, in which the virion releases the vRNP from M1 such that vRNP is free to enter the host cell's cytoplasm (Ciampor et al., 1992; Lamb et al., 1985).

M2e, which consists of N-terminal 24 residues, is remarkably conserved, and the first 9 amino acid residues are almost conserved among all subtypes of influenza A viruses (Fiers et al., 2004; Liu et al., 2005).

One of the concerns about influenza A vaccines based on M2e protein is their limited potency; hence, it seems the immunogenicity of the M2e can be increased by delivery in multimeric form or in combination with complex adjuvants or delivery systems.

Thus, numerous small-scale experimental studies using various M2e-based vaccines, mostly M2e fusion constructs, have reported the value of vaccination in protecting mouse models and occasionally ferret against influenza A virus challenges.

So far, various approaches for increasing the potency of M2e as vaccine candidate were done including M2e peptide has been chemically conjugated to protein carriers such as keyhole limpet hemocyanin (KLH), BSA, or *Neisseria meningitidis* outer membrane protein complex (OMPC), to provide T helper cell epitopes (Fan et al., 2004; Liu et al., 2003).

The other strategies are genetic insertion of M2e into the HBVc open reading frame as M2e-HBc and toll-like receptor 5 (TLR5) ligand salmonella typhimurium flagellin type 2 as M2e-flagellin (Huleatt et al., 2008; Jegerlehner et al., 2002; Neirynck et al., 1999).

Consequently, these studies have shown that immunization of mice with above mentioned M2e-based vaccine can protect them against lethal dose of homosubtypic and heterosubtypic influenza A infection. Protection was measured by reduced host morbidity, host mortality and virus shedding following challenge with human or avian influenza A viruses. M2e-HBc and M2e-flagellin have recently been evaluated for their safety and immunogenicity in human phase I and I/II clinical trials with promising results, respectively (De Filette et al., 2008; Turley et al., 2011).

In this study for the first time, we have produced an *E. coli*-expressed fusion protein, 4xM2e.HSP70c, comprising of four tandem repeats of a consensus M2e sequence genetically fused to the N-terminus of adjuvant fragment of *Mycobacterial* HSP70 ($mHSP70_{359-610aa}$ or mHSP70c). We then evaluated the potency and protectivity of this construction in mouse model against lethal dose of mouse-adapted H1N1, H3N2, or H9N2 influenza A isolates from Iran in comparison with protective effects of conventional influenza vaccines.

Mycobacterial HSP70 was proved to act as adjuvants and augment the immunogenicity of weak antigens. Moreover, proteins or peptides fused with HSP70 when used for immunizations in mice have shown to yield desirable humoral and cellular immune responses (Suzue and Young, 1996; Suzue et al., 1997; Udono et al., 2001).

Suzue and Young (1996) showed that antigen linked covalently with mHSP70 as fusion proteins induce better humoral and cellular responses than the antigen alone.

Cho et al. (2000) showed that HSP70-antigen fusion proteins are capable of inducing specific CD8+CTL response to peptides in the absence of other adjuvants and even without requiring any activation of CD4+T-cells. It was also reported that vaccines containing E7-HSP70 fusion genes, in which E7 was derived from human papillomavirus type 16 E7 and HSP70 from mycobacterium tuberculosis, could increase the frequency of E7-specific $CD8^+$ T cells in mice by at least 30-fold relative to vaccine containing the wild- type E7 gene (Chen et al., 2000). In other studies, the effects of $HSP70_{359-610}$ on foot and mouth virus (FMDV) and Japanese encephalitis virus (JEV) in mice were evaluated separately and the HSP70 markedly enhanced both the humoral and cell-mediated immune responses (Chunxia et al., 2006; Fei-fei et al., 2006).

It was also shown that the C-terminal fragment of HSP70 fused to the malarial antigen EB200 (HSP70-EB200) induced MHC responses considerably (Qazi et al., 2005).

Here, we also showed that a prime-boost administration of 4xM2e.HSP70c formulated in F105 buffer by intramuscular route in mice (Balb/C) provided full protection against lethal dose of mouse-adapted H1N1, H3N2, or H9N2 influenza A isolates from Iran compared to 0–33.34% survival rate of challenged unimmunized and immunized mice with the currently in use conventional vaccines designated as control groups. However, protection induced by immunization with 4xM2e.HSP70c failed to prevent weight loss in challenged mice, but they experienced significantly lower weight loss, clinical symptoms and higher lung viral clearance in comparison with protective effects of conventional influenza vaccines in challenged mice.

Results

Production of the recombinant fusion proteins

Previously we have demonstrated that genetically fusing single copy of M2e derived from the avian influenza A H9N2 virus to the mHSP70c markedly enhances the immunogenicity of the *E.coli*-expressed fusion protein in rabbits (Ebrahimi and Tebianian, 2010).

Consequently, a recombinant fusion protein comprising C-terminus of mHSP70 (mHSP70c), which can interact with CD91, TLR4, TLR2, and CD40 receptors on antigen-presenting cells (APCs), genetically fused to four tandem repeats of the ectodomain of the conserved influenza matrix protein M2 (M2e) was cloned into pQE-60 vector and then expressed in *Escherichia coli* strain M15.

The recombinant fusion protein, 4xM2e.HSP70c, with a C-terminal histidine (His)-tagged was purified under denaturing condition, refolding, and then analyzed in SDS–PAGE. An approximately 40 kDa band corresponding to 4xM2e.HSP70c fusion protein was readily visible by Coomassie Blue and by immunoblot using either of commercial penta-His HRP conjugated antibody (Sigma-Aldrich, USA), anti-HSP70 (Abcam, UK) or anti-M2 (Abcam, UK) antibodies (data not shown).

The recombinant fusion protein 4xM2e.HSP70c (carrying four copies of M2e) retained its immunogenicity and displayed the

Fig. 1. Schematic representation of a construct encoding four copies of M2e fused to *HSP*70c gene as a one open reading frame (~1100 bp). The fragments, M2e and HSP70c genes, amplified separately by specific primers containing determined restriction enzymes were gel-purified using high pure PCR product purification kit produced by Roche Co., according to manufacturer's protocol. The products were then single digested with *Bam*HI restriction enzyme separately. The digested products were again gel-purified and ligated by *T4* DNA ligase produced by Roche Co., to form 4xM2e.HSP70c gene as a single ORF in a pQE-60 ecpression vector. To minimize interference between adjacent proteins, each protein was separated from its neighboring one by a glycine and a serine codon.

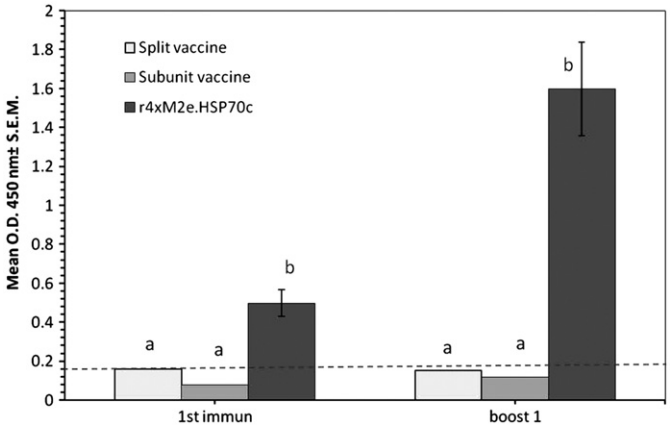

Fig. 2. M2e specific antibodies (1:400 diluted in PBS) were determined by ELISA. 96-well plates were coated with peptide M2e. Anti-sera were collected 13 days after the first immunization (1st immun) and the second immunization (boost 1), respectively, from mice immunized with 4xM2e.HSP70c administrated in F105 buffer. The bound antibodies were detected by horseradish peroxidase-linked rabbit-anti-mouse antibodies and TMB solution. Mean value ± S.E.M of A450 in each group were presented. The titers of antibodies were expressed as the highest dilution of a serum which has twice average value of A450 in control group. Bars show mean value ± standard error of the mean (S.E.M) of five mice/group per time point. The dashed line shows the limit of detection. Mean with different letters between bars are statistically different at the same time point ($p < 0.05$).

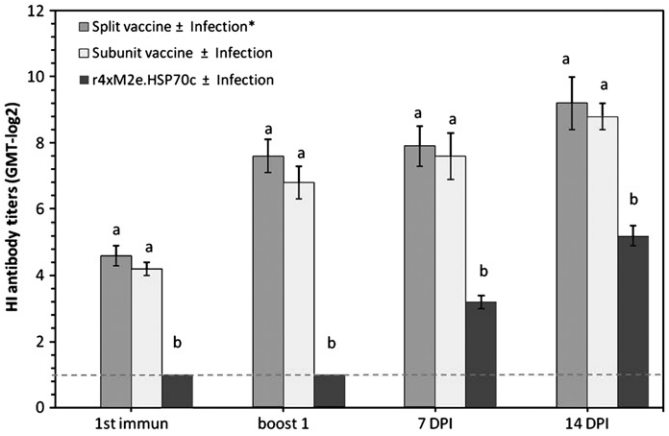

Fig. 3. Mean hemagglutinin-inhibition (HI) antibody titers from mice immunized with split- or subunit- conventional vaccines and 4xM2e.HSP70c recombinant fusion protein 13 days after the first immunization (1st immun) and the second immunization (boost 1), respectively. β-propiolactone (BPL)-treated Iran/H1N1 antigen was used in HI assay for analysis of anti HA-antibody responses induced by conventional vaccines. The mean titers of antibodies enhancement with HA glycoprotein were detected in mice vaccinated with 4xM2e.HSP70c fusion protein on 7 and 14 day post infections (7DPI and 14DPI) by using homologous BPL-treated Iran/H3N2, Iran/H1N1, or Iran/H9N2 antigens. Bars show log 2 geometric mean ± standard error of the mean (S.E.M) of five mice/group per time point. The dashed line shows the limit of detection. Mean with different letters between bars are statistically different at the same time point ($p < 0.05$). * (± Infection) indicates as before (−) or after (+) infection.

protective epitope of M2e by ELISA assay using M2e synthetic peptide, sera from immunized and unimmunized mice, and commercial anti-mouse HRP conjugated antibody (Fig. 2), according to the methods described by our previous studies (Ebrahimi and Tebianian, 2010). In addition, the results of FITC assay also confirmed the correct exposure of protective epitope of M2e in which only anti-M2e protein IgG Abs taken from immunized mice could bind M2 on cells infected with H1N1, H3N2, or H9N2 influenza A isolates; however, the absence of fluorescence emission in uninfected cells showed the specificity of the experiment (data not shown). This allows two conclusions. First, Abs induced against M2e-based vaccine, 4xM2e.HSP70c, are cross-reactive with a protein expressed on either of virus-infected cells, apparently native M2 protein. Second, Abs can act as an effective role in clearance of infected cells in hosts by binding to the surface of infected cells (Jegerlehner et al., 2004). Thus, these data suggest that the antibody induced against 4xM2e.HSP70c could protect the host against virus challenge through antibody dependent cell-mediated cytotoxicity (ADCC) and Ab-dependent cell-mediated phagoctosis as recently reported by Bakkouri et al. (2011).

Serologic response to immunization

Polyclonal serum from mice immunized with M2e-based vaccines or commercial vaccines were analyzed by ELISA and HI assays to identify IgG antibodies directed against M2e peptide or HA glycoprotein of influenza A isolates, respectively.

No anti-M2e or anti-HA antibody was detected in mice of different groups prior to vaccination. All mice initially immunized and subsequently boosted with M2e-based vaccine or commercial vaccines containing HA had an anti-M2e (Fig. 2) and anti-HA (Fig. 3) antibody responses as measured by ELISA and HI assays, respectively.

All mice initially immunized and subsequently boosted with commercial vaccines containing HA had an anti-HA antibody response as measured by HI assay (Fig. 3). No anti-M2e antibody was detected in mice immunized with commercial vaccines. There was no statistically significant differences between groups of mice immunized with split or subunit commercial vaccine (Fig. 3; $P > 0.05$). However, mice immunized with split vaccine showed relatively higher titer of anti-HA antibody than observed with subunit vaccine.

No anti-HA antibody was produced in mice immunized with 4xM2e.HSP70c as measured by HI assay, prior to viral challenge. However, the mean titers of antibodies enhancement with HA glycoprotein were detected in mice vaccinated with 4xM2e.HSP70c fusion protein on days 7 and 14 after challenges by using homologous BPL-treated viruses antigen (Fig. 3). A lower mean titer of anti-HA antibody was induced by virus infections in the mice immunized with 4xM2e.HSP70c than observed with conventional vaccines containing HA (Fig. 3; $P < 0.05$), indicating that the challenge viruses in the 4xM2e.HSP70c immunized groups of mice were not sufficient to induce an antibody boost.

Moreover, there was no significant difference in induced anti-HA antibodies among 4xM2e.HSP70c immunized groups of mice on day 14 after challenge with either of mentioned viral isolates (data not shown; $P > 0.05$).

The significant increase on anti-HA antibody titers on day 14 after viral challenges in both conventional and M2e-based vaccinated mice further indicated a replicative infection of the challenge viruses in these mice (Fig. 3). These data confirm that inactivated conventional vaccines could not protect against clinical signs and mortality in mice. In contrast, 4xM2e.HSP70c fusion protein as a vaccine candidate significantly decreased clinical signs, lungs viral load, and fully protected mice against hetero-or homosubtypic virus challenges, but this did not fully prevent infection.

Vaccination with M2e-based vaccines protected mice against influenza virus challenge

To evaluate the potency of the 4xM2e.HSP70c fusion protein against lethal influenza virus challenges, Balb/C mice were immunized twice as described above and challenged intranasally with a 90% lethal dose (LD90) of mouse adapted H3N2, H1N1, or H9N2 influenza A isolates on day 14 after the last immunization.

The immune response in mice was evaluated by monitoring clinical signs and percent survival.

The mice were monitored for adverse reactions to the vaccination procedure including redness, swelling, and the formation of granulomas at the site of injection. No macroscopic signs of adverse reactions to the 4xM2e.HSP70c fusion protein were observed.

Following the challenge, animals were monitored for weight change (as a measure of illness) and mortality. As expected, the LD90 challenge of mouse-adapted H1N1, H3N2, or H9N2 influenza virus isolates resulted in 100% mortality in the non-vaccinated groups (Fig. 4A–C, Tables 2–4). In contrast, 100% of mice immunized with 4xM2e.HSP70c survived against H3N2, H1N1, or H9N2 viral challenges, respectively (Fig. 4A–C, Tables 2–4), and began to recover on day 8 post-challenge. Of mice vaccinated with egg-derived subunit vaccine 33.34% (7 of 21 mice), 14.29% (3 of 21 mice), and 23.81% (5 of 21 mice) survived challenge with H3N2, H1N1, or H9N2, respectively (Fig. 4A–C, Tables 2–4), and began to recover on day 9–10 post-challenge. Of mice vaccinated with egg-derived split vaccine 28.58% (6 of 21 mice), 19.05% (4 of 21 mice) and 28.58% (6 of 21 mice) survived challenge with H3N2, H1N1, or H9N2 influenza isolates, respectively (Fig. 4A–C, Tables 2–4), and began to recover on day 9–10 post-challenge. Thus, in contrast to commercial vaccines (split or subunit), M2e-based vaccines protected against challenge viruses in which M2e sequences were similar to or divergent from those of the vaccine. In general, the differences in survival of 4xM2e.HSP70c immunized groups of mice following challenges with compared to other groups of infected mice were highly significant (Fig. 4A–C, Tables 2–4; $P<0.05$).

Immunized mice with M2e-based vaccines showed significant weight loss following the viral challenges; however, this was less severe than that observed for the challenged non-vaccinated mice and mice vaccinated by split or subunit commercial vaccines. The differences in body weight loss of 4xM2e.HSP70c immunized groups of mice following viral challenges with compared to other groups of challenged mice were also highly significant (Fig. 4D–F; $P<0.05$).

In all experiments, morbidity following challenges was monitored by measuring body weight and observation of clinical symptoms. There was usually a weight loss after challenges, even in the fully protected groups (Fig. 4D–F). It seems that the incomplete prevention of morbidity may be explained by the severity of the viral challenge. A highly lethal dose for challenge ensures that all, or nearly all, control mice will die as a consequence, and unambiguously confirms the protective efficacy of the vaccine. These results suggest that this strategy for development of M2e-based vaccine could induce fully protection against homo -and heterosubtypic influenza A viral challenges in mice.

Clinical signs, histopathologic examination and lung viral load

Following the inoculation of mice against a lethal dose of H3N2, H1N1, or H9N2 influenza virus isolates; the vaccinated mice were further evaluated in terms of cross-protective ability by daily observation of the clinical symptoms, viral shedding rate and then histopathological examination following removal of lung tissues compared to the unchallenged control group of mice.

Unchallenged mice in the negative control group (Table 1; group 1) remained negative for virus shedding and clinical sings throughout the study period (data not shown).

From day 3 after viral challenges, the challenged and non-vaccinated mice developed obvious clinical signs including: ruffled fur, hunched posture, rapid breathing, inactivity and paralysis of posterior limb. These clinical signs were delayed for 2–3 days in the mice vaccinated with the 4xM2e.HSP70c fusion protein, in which they exhibited mild clinical signs for 3–4 days

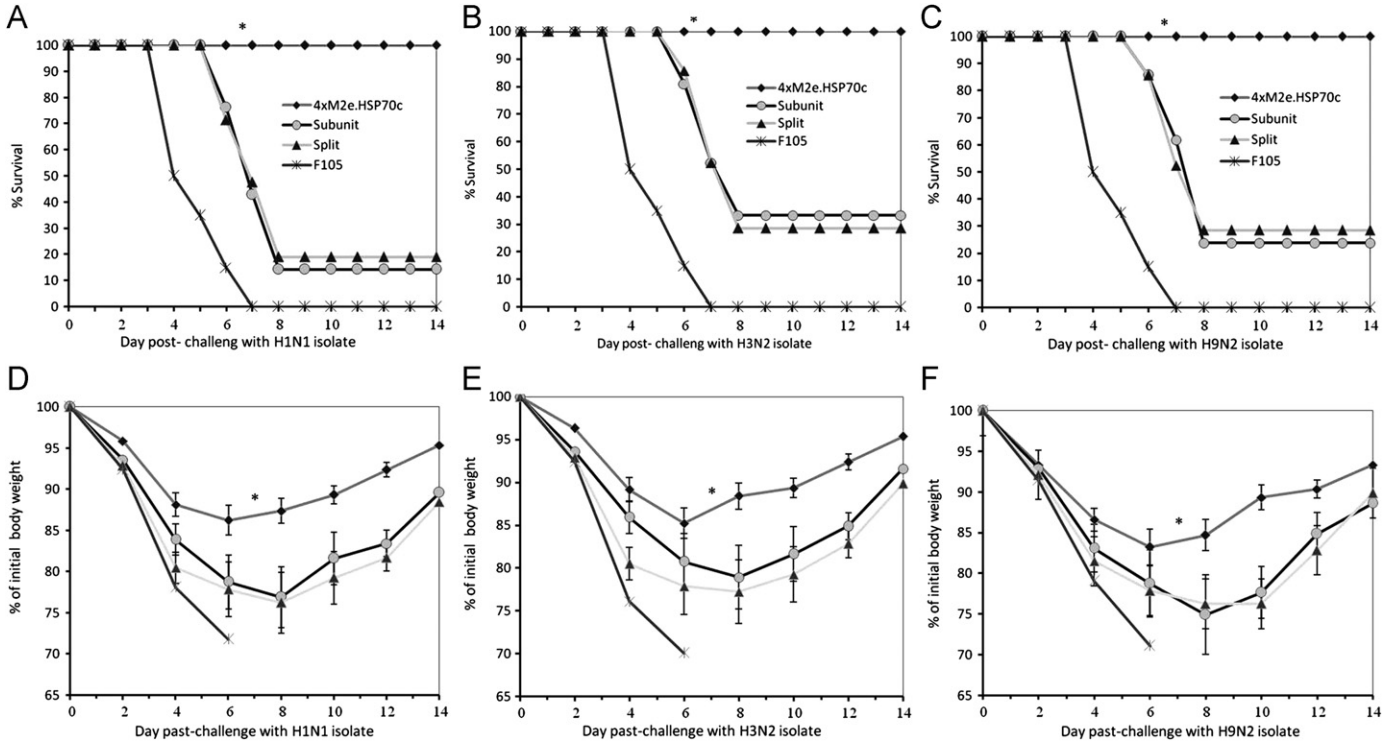

Fig. 4. Results of prime-boost immunization with 4xM2e.HSP70c recombinant fusion protein administrated in F105 buffer and conventional egg-derived subunit-or split vaccines in mice (21 mice/group) and challenges with lethal dose (MLD90) of mouse-adapted H1N1, H3N2, or H9N2 influenza A isolates. Mice immunized with F105 buffer were challenges as negative controls. Survival rate (A–C; panels are based on viral challenge isolate) were monitored on a daily basis and percent of initial body weight (D–F; panels are based on viral challenge isolate with standard error bars) were monitored at two-day intervals for 2 weeks after viral challenges. * Indicates statistically difference between 4xM2e.HSP70c immunized mice with other groups ($P<0.05$).

Table 1
Experimental design vaccination status, Influenza virus infection status and number of mice per group.

Groups	Vaccination	Challenge virus	Total number of mice
1	F105 buffer	No	30
2	4xM2e.HSP70c	H1N1	30
3	Subunit vaccine	H1N1	30
4	Split vaccine	H1N1	30
5	F105 buffer	H1N1	30
6	4xM2e.HSP70c	H3N2	30
7	Subunit vaccine	H3N2	30
8	Split vaccine	H3N2	30
9	F105 buffer	H3N2	30
10	4xM2e.HSP70c	H9N2	30
11	Subunit vaccine	H9N2	30
12	Split vaccine	H9N2	30
13	F105 buffer	H9N2	30

Table 2
Pulmonary viral replication of either immunized or unimmunized mice following challenges with mouse adapted (m.a.) H1N1 isolate of influenza virus circulating in Iran on 3, 6, and 9 days post infection (dpi). Numbers show log 10 geometric mean TCID50/ml ± standard error of the mean (S.E.M) of three mice/group per time point. Mean with different letters within columns (same dpi) are statistically different ($p < 0.05$).

Mean viral titers ($\log_{10}$ TCID50/ml) ± S.E.M				
Groups	3 dpi	6 dpi	9 dpi	Mortality (no.of dead/no. of challenged)
4xM2e.HSP70c	3.0 ± 0.18^{a}	2.1 ± 0.22^{a}	$< 0.9^{B}$	$0/21^{a}$
Subunit vaccine	5.9 ± 0.44^{b}	4.5 ± 0.25^{b}	≤ 1.8	$18/21^{b}$
Split vaccine	6.1 ± 0.33^{b}	4.9 ± 0.50^{b}	≤ 1.9	$17/21^{b}$
F105 buffer	7.9 ± 0.55^{c}	6.8 ± 0.49^{c}	≤ 2.7	$21/21^{c}$

[B]Mean viral titers ($\log_{10}$ TCID50/ml) ± S.E.M on 9 dpi was determined below the level of detection (< 0.9).

Table 3
Pulmonary viral replication of either immunized or unimmunized mice following challenges with mouse adapted (m.a.) H3N2 isolate of influenza virus circulating in Iran on 3, 6, and 9 day post infection (dpi). Numbers show log10 geometric mean TCID50/ml ± standard error of the mean (S.E.M) of three mice/group per time point. Mean with different letters within columns (same dpi) are statistically different ($p < 0.05$).

Mean viral titers ($\log_{10}$ TCID50/ml) ± S.E.M				
Groups	3 dpi	6 dpi	9 dpi	Mortality (no. of dead/no. of challenged)
4xM2e.HSP70c	3.2 ± 0.12^{a}	2.3 ± 0.14^{a}	$< 0.9^{B}$	$0/21^{a}$
Subunit vaccine	4.8 ± 0.15^{b}	3.9 ± 0.22^{b}	≤ 1.2	$14/21^{b}$
Split vaccine	5.5 ± 0.24^{b}	4.1 ± 0.12^{b}	≤ 1.6	$15/21^{b}$
F105 buffer	7.3 ± 0.31^{c}	6.1 ± 0.22^{c}	≤ 2.5	$21/21^{c}$

[B]Mean viral titers ($\log_{10}$ TCID50/ml) ± S.E.M on 9 dpi was determined below the level of detection (< 0.9).

Table 4
Pulmonary viral replication of either immunized or unimmunized mice following challenges with mouse adapted (m.a.) H9N2 isolate of avian influenza circulating in Iran on 3, 6, and 9 days post infection (dpi). Numbers show log 10 geometric mean TCID50/ml ± standard error of the mean (S.E.M) of three mice/group per time point. Mean with different letters within columns (same dpi) are statistically different ($p < 0.05$).

Mean viral titers ($\log_{10}$ TCID50/ml) ± S.E.M				
Groups	3 dpi	6 dpi	9 dpi	Mortality (no. of dead/no. of challenged)
4xM2e.HSP70c	3.5 ± 0.18^{a}	2.6 ± 0.15^{a}	$< 0.9^{b}$	$0/21^{a}$
Subunit vaccine	5.6 ± 0.34^{B}	4.9 ± 0.25^{b}	≤ 2.1	$16/21^{b}$
Split vaccine	5.3 ± 0.30^{b}	4.5 ± 0.17^{b}	≤ 1.9	15/21
F105 buffer	6.8 ± 0.39^{c}	5.5 ± 0.18^{c}	≤ 2.3	$21/21^{c}$

[B]Mean viral titers ($\log_{10}$ TCID50/ml) ± S.E.M on 9 dpi was determined below the level of detection (< 0.9).

and then began to recover. Subunit- or split-vaccinated mice after challenge with the heterosubtypic influenza virus, H9N2, demonstrated mildly increased clinical signs compared to those challenged with the homosubtypic viruses, H1N1 or H3N2, but the difference in the mortality rate between them was not significant.

Moreover, compared to the challenged and non-vaccinated groups of mice (Table 1; groups 5, 9 and 13), the subunit- or split-vaccinated mice that did not survive the challenges were observed to have delayed the onset of illness and longer survival days.

Grass pathology of lungs in all challenged control mice and subunit- or split-vaccinated mice on day 6 after infection showed multiple foci of hemorrhage visible on the surface. Lungs of mice vaccinated with 4xM2e.HSP70c fusion protein showed a few focal areas of hemorrhage on the surface on day 6 after infection (data not shown).

Light microscopy observations, including damage of bronchial epithelium with necrosis, congestion, lymphocytic infiltration, and edema were consistent with the gross examinations among challenged groups (data not shown).

To further determine the effect of 4xM2e.HSP70c fusion protein as a universal vaccine candidate on viral replication in the respiratory system of mice, we evaluated the lung viral load from all challenged mice on days 3, 6, and 9 after viral challenges (Tables 2–4).

No virus was isolated from the lungs of the unchallenged control mice (Table 1; group 1). All challenged mice showed pulmonary viral replication at days 3 and 6 after challenges with H3N2, H1N1, or H9N2 influenza virus isolates. Mice immunized with 4xM2e.HSP70c fusion protein had overall significantly lower lung viral load than did the challenged control groups or challenged subunit-or split-vaccinated mice (Tables 2–4; $p < 0.05$).

At day 9 after challenge, no viruses were detected in the mice immunized with 4xM2e.HSP70c fusion protein; whereas, viruses were detected in the challenged control mice and subunit-or split-vaccinated mice (Tables 2–4). With regard to periods of viral shedding, the mice that received 4xM2e.HSP70c fusion protein had lower periods of virus replication and this suggest this recombinant fusion protein as a universal vaccine candidate can induce protective immunity against heterosubtypic virus, H9N2, and homosubtypic viruses, H1N1 or H3N2, in vaccinated mice.

Discussion

There is a continuing need for improved influenza A vaccines to provide a broader and longer lasting immunity. The development of a truly universal vaccine would present the potential opportunity for both reducing the threat of a pandemic and the impact of seasonal influenza. M2e protein-based vaccine approaches represent one such vaccine as its N-terminal epitope SLLTEVET (residues 2–9) found to be conserved at a rate of over 99.3% among all subtypes of influenza A viruses and 100% among isolates from human (Fiers et al., 2004; Liu et al., 2005).

Previous studies have shown that vaccine-induced antibody responses against M2e protein could protect animal models (mostly mice) against lethal challenge of influenza A viruses.

However, different results reported from them may be due to the difference of carrier proteins, adjuvant, dose of viral challenge, or route of vaccine administration (Fan et al., 2004; Fan et al., 2007; Frace et al., 1999; Liu and Chen, 2005; Mozdzanowska et al., 2003; Neirynck et al., 1999; Okuda et al., 2001; Slepushkin et al., 1995).

Laboratory mouse has proven a useful tool for the study of influenza viruses due to its utility in measuring infectivity, pathogenesis, and subsequent application for the evaluation of vaccine and antiviral candidates (Haga and Horimoto, 2010; Lu et al., 1999).

Hence, in the present study for the first time we used the C-terminal fragment of *mycobacterial* HSP70 as a potent carrier to develop an effective M2e-based recombinant vaccine in order to stimulate $CD8^+$ T cell responses that are crucial to immune responses against viruses.

Considering the successful results reported from preclinical study with 4xM2e.Flagellin fusion protein vaccine candidate in mice by Huleatt et al. (2008) and the recently published report of relevant phase I/II clinical trials in human with promising results include its good safety and potency (Turley et al., 2011). We implemented a similar approach of M2e-based vaccine development, but the adjuvant fragment of *Mycobacterial* HSP70 (mHSP70) as a potential approach to increase the potency of M2e was used instead of flagellin. The reason of linking M2e to adjuvant portion of mHSP70 is that the targeting of antigen to APCs through HSP70 receptors such as CD91 is a useful strategy to induce CD8+ T cell and antibody responses by alternate MHC-I Ag processing (cross-processing) mechanisms, resulting in cross-presentation of the exogenous antigens to CD8+T cells that are crucial to immune responses against viruses (Arnold et al., 1995; Blachere et al., 1997; Castellino, 2000a, b; Tobian, 2004). Moreover, Hsp70 can also activate the innate immunity through CD40 and Toll-like receptor-2 (TLR-2) and TLR-4 to induce cytokine secretion (Takakura et al., 2007; Takemoto et al., 2005).

Hence, based on our previous studies (Ebrahimi and Tebianian, 2010), we sought to construct a fusion plasmid harboring four tandems repeats of the extracellular domain of the influenza A M2 protein (M2e), which was fused to the N-terminus of the truncated HSP70 ($HSP70_{359-610}$) molecule. This chimeric sequence was heterologously expressed in *E.coli* (M15 strain), purified and further analyzed as a 4xM2e.HSP70c recombinant fusion protein. The purified protein was formulated in F105 buffer and then tested for its ability to stimulate immune responses and level of its protectivity against viral challenges in comparison with conventional influenza vaccines in mice.

The purified protein formulated in F105 buffer (in the absence of other adjuvants) was able to induce strong and effective M2e-specific immune responses in Balb/C mice and completely retained its structural conformation so that the induced antibodies against this recombinant protein was able to recognize and bind to the native M2 antigen on the surface of infected cells by either of H3N2, H1N1 or H9N2 influenza isolates.

We have also shown that genetically fusing an M2e to adjuvant fragment of mHSP70 significantly increased the immunogenicity with the retained native epitopes in the absence of other adjuvants is in consistent with previous studies in which the adjuvancity role of the mHSP70c were reported (Cho et al., 2000; Li et al., 2006; Suzue and Young, 1996).

To determine the optimal dosing schedule and route of administration, mice were vaccinated with different doses of 4xM2e.HSP70c formulated in F105 buffer and then challenged with influenza virus (PR8/H1N1) and monitored for survival. The results from these studies established that a 9 μg dose of this fusion protein by intramuscular route of injection was sufficient to stimulate immune responses and provide protection against viral challenge. This dose level and route of injection is comparable to that observed for conventional haemagglutinin (H) vaccines preparation.

In contrast to the egg-derived split or subunit vaccines, which is currently in use to provide immune protection against influenza viruses in Iran, immune response stimulated by the 4xM2e.HSP70c fusion protein fully protected the mice against lethal dose of mouse-adapted H3N2, H1N1, or H9N2 isolates circulating in Iran. The pronounced level of protection generated by the 4xM2e.HSP70c fusion protein is probably related to the interaction of the mHSP70c, which can activate the innate and adaptive immunity through its specific receptors and the multiple presentation of the M2e antigen.

From this data, several important observations can be made. First, the four tandem repeats of influenza M2e antigen linked genetically with mHSP70c as fusion protein induced the immune responses to this antigen well. Second, in contrast to conventional vaccines, the 4xM2e.HSP70c fusion protein as a vaccine candidate provided a full level of protection against hetro-or homosubtypice of influenza A virus challenges. Since the M2e sequences chosen in this construct are homologous to most of the H1N1 and H3N2 strains infecting humans circulating in Iran.

Third, Vaccine to M2e is predicted to limit the severity of influenza A disease by its inhibitory role on the uncoating of viral particles in endosomes and preventing viral replication; consequently, delaying the onset of morbidity in mice for 2–3 days rather than other control groups can allow the host immune response to develop adaptive immunity to the dominant neutralizing influenza antigen, HA. Therefore, humeral immune responses against HA provoked by infection could help to reduce the lung viral replication and restricted histopathological damage in lung tissues. Histopathologic analysis of lungs from such infected mice immunized with 4xM2e.HSP70c fusion protein revealed markedly reduced tissue injury, mononuclear cell accumulation, hemorrhage, and pulmonary edema.

This explanation can be considered by the results of the present study in which we detected influenza virus-induced antibody by day 7 after challenge (Fig. 3) and previous studies in which the early influenza virus-induced antibodies detected systemically in the serum by day 5 to 6 after challenge; in other words, 2–3 days after symptom onset (Fabrice et al., 2008; Lincoln et al., 2010; Sealy et al., 2003), could have a significant impact in controlling viral replication, and thus contributes to a less severe disease outcome (Baumgarth et al., 1994; Doherty et al., 1997).

In contrast, mice immunized with split or subunit conventional vaccines did not result to decreased level of lung viral load, morbidity, mortality, and histopathological damage in lung tissues against lethal dose of viral challenges compared to mice immunized with 4xM2e.HSP70c vaccine candidate. Whereas, both of conventional vaccines induced high level of HA-antibody in mice, but these derived antibodies could not reduce the level and duration of viral replication in mice. This failure effects of conventional vaccines in mice against H1N1 and H3N2 isolates circulating in Iran can be expected and occur frequently as a result of frequent point mutation (antigenic drift) of haemagglutinin (HA) (Seo et al., 2002). This vaccine failure for H9N2 avian influenza was also expected because of HA-mismatched among those heterosubtypic strains. In addition, it seems the extreme pathogenicity of the mentioned isolates of mouse adapted viruses are directly linked to the high viral replication rate and the consequent production of peak steady state viral titers in the lungs within 48 h after infection so that the immune responses could not restrict viral replication.

In the present study, we focused more on potential protectivity of the 4xM2e.HSP70c fusion protein as a universal influenza A vaccine candidate in comparison with conventional vaccines which have been using in Iran for more than one decade. This

report was limited by the lack of isotype analysis and cytokine data, but assessment of antibody isotype and cytokine responses have been completed and will be reported in detail in future.

The successful use of the adjuvant fragment of *mycobacterial* HSP70, as an adjuvant for this vaccine candidate, is an innovative approach to inducing immune response and protecting mice against lethal challenge of influenza infections in the absence of exogenous adjuvant. The use of mHSP70c as carriers seems to be safe for the development of vaccine strategies, since most humans have been in contact with mHSPs through BCG which is currently used as a vaccine against tuberculosis, and a large number of people are sensitized to mycobacteria or other parasites through natural contacts.

Conclusion

Based on evidence to our finding, M2e fusion to HSP70 is intrinsically very immunogenic and being able to protect mice against lethal challenge of homo-or heterosubtypic influenza A isolates, and 4xM2e.HSP70c recombinant fusion protein as a universal influenza A vaccine candidate can be easily produced in efficient prokaryotic-expression systems that require minimal containment. For these reasons, they are promising candidates for further clinical evaluation to study its immunogenicity and safety in people. Now, the results of preclinical studies on the recombinant 4xM2e.HSP70c fusion protein have convinced the Iranian health officials to prepare conditions for beginning of Phase I clinical trial as soon as possible, in which the safety and immunogenicity of this recombinant fusion protein as a universal influenza A vaccine will be tested.

Materials and methods

Expression of recombinant 4xM2e.HSP70c fusion proteins

According to the study by Huleatt et al. (2008), a construct encoding a single copy of M2e derived from the consensus sequence of the human influenza A virus (H1N1, H2N1, H3N2; amino acid sequence: SLLTEVETPIRNEWGCRCNDSSD) was chemically synthesized in pGH cloning vector, designated as pGHM2e (Generay Biotech Co., Shanghai, China). In this synthetic gene the two cysteine residues at sites 17 and 19 of M2e were modified to serine (SLLTEVETPIRNEWG**S**R**S**NDSSD; the modified amino acids are determined by bold and underlined letters), to prevent disulfide bond formation that would be incompatible with *Eschrichia coli* expression since these cysteines could result in formation of intramolecular disulfide bonds and aggregation of recombinant proteins under oxidative conditions. Indeed, Huleatt et al. (2008) and Mozdzanowska et al. (2003) also used the same strategy when they produced synthetic multiple antigenic peptides containing four copies of M2e without affecting on its antigenecity. The plasmid DNA, pGH-M2e, served as a template to generate the 4xM2e fusion gene according to the molecular strategy of our previous study (Ebrahimi and Tebianian, 2010). The PCR product was digested and then ligated to the 5′ end of the C-terminal domain of *mycobacterium tuberculosis* (H37Rv) HSP70 (mHSP70c) in a prokaryotic expression vector containing a mHSP70c, pQE-60/HSP70c, constructed from our previous study (Ebrahimi and Tebianian, 2010). The schematic diagram of this fusion strategy is demonstrated in Fig. 1.

The construct, pQE-60/4xM2e.HSP70c, was confirmed by DNA sequencing (MWG Biotech Co.,Germany).

The fusion protein was expressed in *E. coli* M15 cells, transformed with the expression vectors pQE-60 (Qiagen, USA) bearing 4xM2e.HSP70c sequence exactly according to our previous study (33). Four hours after induction the bacteria was harvested and the lysate was analyzed by SDS–PAGE.

Protein purification and endotoxin measurement

Histidine (His)-tagged recombinant 4xM2e.HSP70c was purified from an *E. coli* expression system as described previously (Ebrahimi and Tebianian, 2010), using nickel affinity chromatography (Ni-NTA agarose, Qiagen, USA) under denaturing condition by following the manufacturer's instructions.

To refold the purified denatured fusion protein, dialysis was additionally performed by slowly diluting them in freshly made 50x Tris-HCl (pH 7.4) for 15 h at 4 °C, and Triton X-114 was then used to remove its LPS, according to the method described in Aida et al.(1990). Fusion protein was tested for the presence of endotoxin by E-TOXATE^{TM} (limulus amebocyte lysate) test kit (Sigma, USA). Endotoxin-levels were below 20 endotoxin units/mg (<0.02 EU/μg).

After concentration through millipore ultrafiltration-15 ml (UF) system having a membrane with a molecular weight cut-off of 10 kDa (Sartorius, Germany) and sterile filtration through 0.22 μm, the purified fusion protein was stored in F105 buffer which contains 10 mM Tris, 10 mM histidine, 5%(w/v) sucrose,75 mM NaCl, 0.1 mM EDTA, 0.5% (v/v) ethanol and 0.02%(w/v) polysorbate-80 (pH 7.2) at 4 °C until use.

Purified fusion protein was characterized by Coomassie blue staining and Western blotting analysis, and their concentrations were determined using Micro BCA protein assay kit (Thermo scientific, USA), according to the manufacturer's instructions.

Experimental design

Three hundred and ninety 6-week-old female Balb/C mice were purchased from the Animal Rearing Department of Pasteur Institute, Iran. All of the mice at the age of 6-week-old were randomly divided into thirteen 30-mouse groups (Table 1), and kept two weeks for acclimatization before vaccination. They were fed by standard grower diet throughout our experiment according to the National Research Council Requirements (1995). Throughout the study, challenged mice (Table 1; groups 2–13) were housed in identical isolation rooms based on the same viral isolate as the challenge virus and non-challenged mice (Table 1; group 1) were housed in separate room.

Mice were reared, before and after vaccination, in four different cages in each room with similar environmental conditions, same availability of feeder and drinker conditions and offered feed and drinking water ad libitum at the animal research unit of Baqiyatallah University of Medical Sciences, Tehran, Iran. Before the experiment, rooms and cages were vigorously washed and fumigated using formaldehyde.

Virus preparation and titration

Iranian isolates of influenza viruses H3N2 and H1N1were obtained as passaged in Mardin–Darby Canine Kidney (MDCK) cells (a kindly gift from Dr. Mokhtari, Virus Research Dept., Tehran University of Medical Science, Tehran, Iran), and avian influenza virus, H9N2, was obtained as passaged in allantoic fluid of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (a kindly gift from Marand branch of Iran's Razi Vaccine and Serum research Institute). Viral lung-to-lung passages (10 serial passages) in Balb/C mice and one passage in embryonated chicken eggs was also used to obtain a mouse-adapted strains for influenza A/Tehran/631/2007 (H3N2), A/Tehran/5963/2010 (H1N1), and avian influenza A/chicken/Iran/101/1998 (H9N2), according to previously described method (Neirynck et al., 1999), and then stored in −80 °C. Infectious virus

was quantitated by 50% tissue culture infective dose (TCID50) assay using MDCK cells and expressed as TCID50 units/ml.

Ninety percent mouse lethal doses (MLD90) of the influenza A isolates H3N2, H1N1, or H9N2 were determined separately by inoculating groups of 6- to 8-week-old female Balb/C mice (n= 5/group) (Animal rearing Dept., Baqiyatallah University of Medical Sciences, Tehran, Iran) by intranasal route during light anesthesia with Ketamin/xylasine solution, with 30 μl of serial 10-fold dilutions of above mentioned isolates. The titer of the propagated virus in cells and mice were then calculated by the Reed and Muench (1938) method.

Mice immunization

Eight-week-old mice in the appropriate groups as shown in Table 1 were each immunized twice (days 0 and 14) by the intramuscular (IM) route with the purified 4xM2e.HSP70c fusion protein administrated in F105 buffer (without conventional adjuvants) at a dose of 0.1 ml containing 9 μg/mouse, with injection of both quadriceps in 50 μl volume per site without anesthesia. Conventional inactivated vaccines: egg-derived subunit vaccine (influvac®, Solvay, Netherlands) and egg-derived split vaccine (Split Virion, BP, Sanofi Pasteur MSD,UK) of the trivalent influenza vaccine formulations of vaccination season 2009/2010, consisting of inactivated virus strains A/Brisbane/59/2007, A/Brisbane/10/2007 and B/Brisbane/60/2008 at a dose of 0.1 ml containing 9 μg/mouse were also administered to each mice of the other groups twice (days 0 and 14) by IM injection of both quadriceps in 50 μl volume per site without anesthesia, separately, as shown in Table 1.

Mice of groups 1, 5, 9, and 13 (Table 1) were kept as unimmunized controls and were injected intramuscularly with equal volume of F105 buffer.

The mice were monitored for adverse reactions to vaccination including redness, swelling, or the formation of granulomas at the IM injection site. Mice were weighed daily for one week post vaccination and weekly between the prime and the boost.

Serum samples analysis

Serum samples were obtained from each mouse by retro-orbital bleeding in the time points of 1 day prior to the second immunization (13 days after the first immunization) and 2 days prior to the viral challenges (13 days after the second immunization) for the study of M2e- or HA-specific antibody responses. Serum samples were also collected in the surviving mice on days 7 and 14 after viral challenges for detection of HA specific antibody responses.

Serum samples from all groups and all time points were heat inactivated at 56 °C for 30 min before being analyzed in ELISA or hemagglutinin inhibition (HI) assays for analysis of anti-M2e or HA-antibody responses, respectively.

The HI assay was carried out according to our previous study (Ebrahimi et al., 2011) using β-propiolactone (BPL)-treated Iran/H3N2, Iran/H1N1, or Iran/H9N2 antigens and 1% v/v suspension of chicken red blood cells (RBCs). All samples were analyzed on the same day, and HI titers were scored as the reciprocal of the highest serum dilution producing 50% inhibition of hemagglutination.

The ELISA assay was performed as described previously (Zhao et al., 2010) with some modifications. Briefly, 96 well plates (Maxisorb,Nunc, Denmark) were coated either with 1 μg/ml of synthetic M2e-peptide (23 amino acids, 2 to 24, SLLTEVETPIRNEWGCRCNDSSD, synthesized by solid phase technology at the GL Biochem, China), in 50 mM sodium bicarbonate buffer, pH 9.6, and incubated overnight at 4 °C. After blocking, different serum samples were loaded on peptide coated plates. The plates were incubated with the different serum samples and then incubated with 1:10000 goat anti-mouse IgG-HRP conjugates (Sigma-Aldrich, USA), for 1 h at 37 °C. The color reaction was developed with 3, 3′, 5, 5′-tetramethylbenzidine, TMB, (Pishtazteb, Tehran, Iran), at OD 450 nm. For comparison between groups, the averages of A450 values of different sera were analyzed. The antibody titer is defined as the reciprocal of the highest dilution that yields an OD 450 nm value above 2 times the mean of negative control wells.

Surface staining of virus-infected MDCK cells with anti-M2e

Sera were tested for reactivity with influenza A infected MDCK cells as previously described (Ebrahimi and Tebianian, 2010). Briefly, Six-well plates were seeded with 10^7 Madin–Darby canine kidney (MDCK) cells per well and until cells were near confluence they were infected (or left uninfected as a control) with 10 multiplicity of infection with H1N1, H3N2, or H9N2 influenza isolates. Infected cells were incubated for 8 h at 37 °C. After washing steps with PBS, the influenza A virus matrix protein (M2) on the surface of infected MDCK cells were detected by sera from mice immunized with 4xM2e.HSP70c (1:400 diluted in PBS). The samples were stained with an FITC-labeled anti-mouse IgG Ab (sigma, USA) and fluorescence was visualized under an inverted fluorescence microscope (Olympus IX70). Here, the uninfected cells were applied as negative control.

Viral challenge and follow-up

To assess efficacy, mice immunized on days 0 and 14 as described above were challenged in parallel on day 28 by intranasal (i.n.) administration of an LD90 (dose lethal to 90% of mice) of mouse adapted H3N2, H1N, or H9N2 influenza isolates under light anesthesia with Ketamin/xylasine solution. Group 1 in Table 1 containing non-vaccinated and non-challenged mice were also kept as negative control, separately. Three mice in each group were randomly selected as representatives for lung viral titration on days 3, 6, and 9 post challenge and 21 mice in each challenged group were observed by monitoring mortality on a daily basis for 2 weeks. Morbidity was also followed at two-day intervals by monitoring body weight and by measuring body weight and observation of clinical symptoms. Mice that lost more than 25% body weight were humanely euthanized.

Viral titration and histopathologic examination

To assess viral titer of infected mice with H3N2, H1N1, or H9N2 influenza challenge isolates. Three mice from each infected group were killed on days 3, 6, and 9 post challenge by cervical dislocation under deep Ketamin/xylasine anesthesia, and their lungs were harvested and then homogenized for evaluation of viral load. Individual lungs were then titrated by inoculation of decimal dilutions (10^{-2} to 10^{-8}) of lung (lung homogenized at 10^{-1} [wt./vol.] dilution) in Madin–Darby canine kidney cells (MDCK) cell cultures as described previously (Rimmelzwaan et al., 1998).

Viral titer in lung homogenate was expressed as $\log_{10}$ geometric mean titer of TCID50/ml ± SEM. The infectious titers were calculated according to the method of Reed and Muench (1938) using BPL-treated Iran/H3N2, Iran/H1N1, or Iran/H9N2 antigens. All negative samples for the virus titration test were set at 0.9 $\log_{10}$ TCID50/ml.

Lungs from infected mice were also taken and fixed in 10% neutral formalin solution for histopathologic analysis on day 6 after challenge in order to be examined microscopically for evidence of cellular inflammation and necrosis. The paraffin

sections were stained with hematoxylin and eosin (H&E) for examination.

Statistical analysis

Data were analyzed using the SPSS 13.0 software (SPSS Inc., Chicago, USA).

Data were analyzed for significance ($P < 0.05$) by the one-way analyses of variance when variance between groups was homogeneous and distribution of the data was normal or a nonparametric test (Kruskal–Wallis) when normality test or homogeneity of variance test failed.

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