Research paper

Intranasally administered polyethylenimine adjuvanted influenza M2 ectodomain induces partial protection against H9N2 influenza A virus infection in chickens

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ABSTRACT

This study aimed to investigate whether intranasally coadministered four tandem copies of extracellular domains of M2 (M2e) and polyethyleneimine (PEI), a mucosal adjuvant, can protect chickens against H9N2 influenza A virus infection. Groups of chickens were intranasally vaccinated with M2e plus PEI adjuvant, M2e alone or PEI adjuvant, and antibody (serum IgG and mucosal IgA) and cellular (CD4$^+$ T cells and IFN-γ levels) immune responses were measured post-vaccination. We demonstrated that the chickens vaccinated with M2e plus PEI adjuvant showed significantly ($p < 0.05$) higher M2e-specific systemic IgG and mucosal IgA responses compared to the chickens that received either M2e alone or PEI adjuvant. The IgA responses measured in lungs were almost comparable to that of the serum IgG levels. Upon restimulation of the vaccinated peripheral blood mononuclear cells (PBMCs) with M2e antigen, significantly ($p < 0.05$) higher IFN-γ levels were observed only in M2e plus PEI adjuvant vaccinated group. Lymphoproliferative and CD4$^+$ T cell responses, as measured by MTT-based assay and flow cytometry, respectively, were also observed significantly ($p < 0.05$) higher in M2e plus PEI adjuvant vaccinated chickens. On challenge with the H9N2 virus (10^4TCID$_{50}$) at 28th day post-vaccination, M2e plus PEI adjuvant vaccinated group exhibited lower lung inflammation and viral load compared to the chickens treated with either M2e alone or PEI adjuvant. In summary, we show that intranasally coadministered M2e and PEI adjuvant can elicit humoral and cell-mediated immune responses and can reduce viremia levels in chickens post H9N2 infection in chickens.

1. Introduction

Avian influenza viruses, defined as high pathogenic avain influenza (HPAI) or low pathogenic avain influenza (LAPI), cause significant economic losses in poultry industry worldwide (França and Brown, 2014; Röhm et al., 1995). H9N2, a LPAI virus, infection in poultry species is characterized by slight to moderate mortality with apparent clinical signs including depression, respiratory symptoms and a reduction in egg production (Lee et al., 2000). H9N2 virus is the most widespread LPAI subtype circulating endemically in poultry across Asia, the Middle East and Northern Africa (Lee et al., 2017; Thuy et al., 2016). In Korea, H9N2 virus was first documented in 1996 and since then, this influenza subtype has caused huge economic losses in the Korean poultry industry (Lee et al., 2007). Despite being primarily poultry pathogen, H9N2 virus has been reported to diversify the host range and conferring zoonotic transmission potential to H7N9 and H10N8 viruses, which are reported to cause deadly human infections

(Chen et al., 2014; Gu et al., 2014; Pu et al., 2015). Recently, Chrzastek et al. demonstrated a high degree of heterogeneity of glutamine and leucine residues at position 226 in the H9N2 hemagglutinin (HA) gene, which increases specificity to either avian or mammalian-type receptors (Chrzastek et al., 2018). Furthermore, H9N2 sporadic human cases have been reported in the past (Butt et al., 2005; Huang et al., 2015), suggesting that this influenza subtype has zoonotic potential as well. Therefore, the development of novel strategies and/or potent vaccines to confine this infection has immense public health importance. In South Korea, since 2007, H9N2 infection has been controlled mainly through the use of inactivated oil adjuvanted H9N2 LPAI vaccine (Choi et al., 2010). However, this oil adjuvanted H9N2 vaccine requires a large supply of specific-pathogen-free (SPF) embryonated eggs and a long timeline that could be threatened during epidemic and/or pandemic situations. Furthermore, because of vaccination and subsequent selective immune pressure, H9N2 virus has been continuously evolved through antigenic drift (Lee et al., 2007), thus, several studies reported

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that the current Korean H9N2 vaccine could provide only partial protection against the newly emerged LPAI H9N2 variants (Lee and Song, 2013; Park et al., 2011). Therefore, vaccination strategies targeting conserved epitopes that are known to provide universal protection should be devised to control LPAI viruses. Similar to hemagglutinin and neuraminidase, matrix protein 2 (M2) of influenza A viruses (IAVs) is an integral transmembrane protein and its ectodomain (M2e) is considered a promising candidate antigen to elicit heterologous protection (Fiers et al., 1999). Several studies have demonstrated the potential of M2e-based vaccines to elicit cross-protective immunity against various types of influenza A subtypes (Deng et al., 2015). While natural infections, live attenuated and inactivated vaccines generally induce very low M2e-specific antibody responses (Kim et al., 2013); however, presenting M2e on a suitable carrier or linking several copies of M2e in tandem greatly enhances its immunogenicity and cross-protective potential (Deng et al., 2015). Furthermore, M2e on its own is a very weak immunogen; therefore, potent adjuvants including flagellin (Huleatt et al., 2008), cholera toxin (Li et al., 2014), delivery via bacterial system (Hajam and Lee, 2017; Layton et al., 2009) etc., have been employed to induce efficient M2e-specific protective immune responses in various animal models. In the present study, we used polyethyleneimine (PEI) to augment immunogenicity of H9N2 M2e in chickens. PEI is a potent mucosal adjuvant for viral glycoprotein antigens (Sheppard et al., 2014) and several studies have demonstrated that coadministration of PEI with viral antigens induced efficient immune responses and subsequent protection against lethal infections (Diaz-Dinamarca et al., 2018; Song et al., 2017).

Herein, we report that intranasally co-administered four tandem copies of H9N2 M2e and PEI adjuvant induced M2e-specific humoral and cell-mediated immune (CMI) responses in chickens. Furthermore, we show that vaccination with M2e plus PEI adjuvant reduced viral shedding in cloacal swab samples and vaccinated birds exhibited lower lung inflammation than control groups.

2. Materials and methods

2.1. Cell line and virus

H9N2 influenza A virus (IAV) was purchased from Korean National Institute of Environmental Research (NIER) and propagated in the allantoic cavities of 9- to 10-day-old SPF embryonated chicken eggs as previously described (Hajam et al., 2018a). The tissue culture infective dose (TCID$_{50}$) of H9N2 IAV was calculated in Madin Darby Canine Kidney (MDCK) cell line as previously described (Kim et al., 2018).

2.2. Expression and purification of M2e tetramer

The four tandem repeats of conserved ectodomain of H9N2 M2 (M2e) gene, with amino acid sequence (MSLLTEVETPTRNGWECKCS-DSSD; Accession No.ACF08313.1), were cloned in frame into pET32a (+) prokaryotic expression vector as discussed and reported elsewhere (Hajam and Lee, 2017). Briefly, the M2e gene sequence was codon optimized for efficient gene expression in Escherichia coli, then synthesized (Bionee, Korea) and subsequently four tandem copies of M2e gene were physically linked to each other and built into the pET32a expression vector at EcoR1/HindIII site as previously described (Hajam and Lee, 2017). The pET32a-4M2e recombinant plasmid was transformed into E. coli BL21 (DE3) pLysS host strain (Novagen, San Diego, USA) for expression of M2e tetramer. The protein expression was induced by adding 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to transformed BL21 host strain (OD600 = 0.6) at 30 °C and the protein expression was allowed to 4 h. After 4 h, the bacterial cells were harvested and the M2e protein expression was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant protein was further confirmed by Western blot analysis using primary rabbit influenza A antiM2-specific polyclonal antibody

(catalog#, MBS9405612, MyBioSource) that was subsequently detected by the addition of HRP-conjugated goat anti-rabbit IgG secondary antibody (NB730-H, Novus Biologicals) as previously described (Hajam and Lee, 2017). The protein was purified by Ni-NTA chromatographic column under denaturation conditions and the purified protein was refolded and dialysed against PBS (pH 7.4) at 4 °C. The purified protein was quantified by a Bradford assay and stored at − 20 °C until further use.

2.3. Immunization and challenge studies

All animal experimentation work was approved by the Chonbuk National University Animal Ethics Committee (CBU 2014-1-0038) and the chicken experiments were carried out according to the guidelines of the Korean Council on Animal Care. One-day-old SPF female brown nick layer chickens (Corporation of Join hatchery, Republic of Korea) were maintained under standard conditions and provided antibiotic-free food and water ad-libitum. Four weeks later, the chickens were randomly divided into three groups (n = 10 in each group) and vaccinated intranasally with PEI adjuvant (100 μg/bird), M2e tetramer alone (25 μg/bird) or M2e tetramer (25 μg/bird) plus PEI adjuvant (100 μg/bird) in a volume of 100 μl (50 μl/nostril). The diluent used was PBS. Two weeks later, the chickens were boosted intranasally with the same dose. Blood was drawn from the jugular vein of five randomly vaccinated and control chickens on day 14 post-booster vaccination and serum and peripheral blood mononuclear cells (PBMCs) were collected as previously described (Kim et al., 2018). For analysis of mucosal M2e-specific IgA responses, four birds in each group were sacrificed by CO2 asphyxiation at 14th day post-booster vaccination and lung washings were aseptically collected in sterile PBS. Two weeks post-booster vaccination, the remaining vaccinated and control chickens (n = 6 in each group) were intranasally challenged with 10^4 TCID$_{50}$ of H9N2 virus and the cloacal swabs (n = 5) were collected post-challenge to determine the viral load by real time PCR (qRT-PCR) assay as described previously (Kim et al., 2018). Briefly, cloacal swab samples were transferred to 500 ml of sterile PBS containing antibiotics, vortexed and supernatant was processed for total RNA isolation by RNeasy Mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The cDNA was prepared using SuperScript™ III Reverse Transcriptase kit (Invitrogen, San Diego, California, USA) and the matrix gene primers recommended by World Health Organization (WHO) were used to determine the H9N2 viral RNA copy number in qRT-PCR assay. The qRT-PCR assay was performed with the ABI applied biosystems using Power SYBR Green Master Mix (#4367659, Applied Biosystems, USA) (Kim et al., 2017). For histopathological analysis, six chickens in each group were sacrificed on day 7 post-challenge. Histopathological analysis of lung tissues was performed as previously described (Kim et al., 2018). For comparison purposes, two naïve chickens of three weeks old were sacrificed and lung tissues were processed for histopathological analysis.

2.4. Systemic IgG and mucosal IgA specific antibody responses

The systemic and mucosal M2e-specific IgG and IgA responses were analysed in vaccinated sera and lung washings, respectively, by an indirect ELISA (Kim et al., 2018). Purified M2e (250 ng/well) was used as a coating antigen in an indirect ELISA.

2.5. CMI responses

Two weeks post-booster vaccination, in vitro proliferative capacity of vaccinated PBMCs (1 × 10^6, n = 5) in response to the recall M2e antigen (10 μg/ml) was determined by a MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide]-based assay (Kim et al., 2018). The proliferative capacity of vaccinated PBMCs in response to the recall M2e antigen was further analyzed by flow cytometry. To this end,

PBMCs isolated from vaccinated and control birds on 14th day post-booster vaccination were stimulated with M2e antigen (10 μg/ml) for 72 h and then M2e-specific CD4 + T cells were analysed by flow cytometry, following labelling of cells with FITC anti-CD3$^+$ (#Cat No 8200-02) and AF-700 anti-CD4$^+$ (#8210-27) antibodies (MiltenyiBiotec) as described previously (Hajam et al., 2018b). We further analysed IFN-γ cellular immune responses by qRT-PCR assay. In this regard, PBMCs (1 × 10^6, n = 5) isolated from vaccinated and control birds were stimulated with M2e antigen (10 μg/ml) for 24 h at 37 °C in 5% CO2. Subsequently, total RNA isolated from stimulated cells was analysed for IFN-γ mRNA transcription by qRT-PCR assay (Kim et al., 2017).

2.6. Statistical analysis

All the obtained data was analysed using GraphPad prism 6.00 program (San Diego, CA, USA). Statistical significance was determined by one-way ANOVA (with Tukey's multiple comparisons tests). The FACS data were analyzed using FlowJo software (Treestar, Inc., San Carlos, CA). P values of < 0.05 were considered statistically significant.

3. Results

3.1. M2-specific polyclonal antibody reacted specifically with E. coli expressed M2e

The four tandem copies of conserved M2e gene sequence were cloned in frame downstream to a Trx-His-S-enterokinase nucleotide sequence of pET32a expression vector at *EcoR1/HindIII* site. The pET32a-4M2e recombinant plasmid was then subsequently transformed into *E. coli* BL21 host strain for expression of fusion protein induced by IPTG. The expressed fusion protein was purified by Ni-NTA chromatography and confirmed by SDS-PAGE and Western blot analysis (Supplementary Fig. 1). SDS-PAGE analysis revealved that purity of the Ni-NTA purified recombinant fusion protein was > 95% as single band of approximately 31 kDa size was observed (Supplementary Fig. 1A). Our results indicated that M2e tetramer reacted specifically with anti-M2 specific polyclonal antibody giving a characteristic band of approximately 31 kDa, the expected size of our protein of interest, thus confirmed the authenticity of the expressed protein (Supplementary Fig. 1B).

3.2. Intranasally coadministered M2e and PEI adjuvant elicited antigen-specific systemic and mucosal antibody responses

To investigate the effect of vaccination on systemic and mucosal antibody responses, we vaccinated chickens with PEI adjuvant, M2e alone or PEI adjuvanted M2e and measured IgG in serum and mucosal IgA responses in lungs at 14th day post-booster vaccination (Fig. 1). Our

data demonstrated that vaccination with M2e plus PEI adjuvant induced significantly (p < 0.05) higher, but comparable M2e-specific IgG (Fig. 1A) and IgA responses (Fig. 1B) compared to the chickens that received either M2e alone or PEI adjuvant. Although IgG and IgA responses were detected in M2e alone vaccinated chickens, but the responses were nonsignificant compared to the PEI adjuvant group. These results clearly suggest that PEI adjuvant has significantly increased antigen-specific humoral immunity.

3.3. Vaccination with M2e plus PEI induced M2e-specific cellular immune responses

To assess the effect of vaccination on the M2e-specific cellular immunity, we measured capacity of vaccinated PBMCs to proliferate in response to the recall M2e antigen *in vitro* (Fig. 2). Flow cytometric analysis of vaccinated PBMCs revealed that CD3$^+$ and CD3$^+$CD4$^+$T cell responses were significantly (p < 0.05) higher (both 1.5 folds increase) in chickens vaccinated with M2e plus PEI adjuvant compared to the M2e alone vaccinated group (Fig. 2A). Although M2e alone vaccinated chickens showed some recall T cell responses, but the responses were non-significant compared to the PEI adjuvant control group. This finding was recapitulating with the lymphoproliferative responses measured by MTT-based assay. Our results showed that vaccination with M2e plus PEI adjuvant showed significantly (p < 0.05) higher (1.3 folds increase) proliferative responses compared to the chickens vaccinated with M2e alone (Fig. 2B). We further investigated IFN-γ responses in vaccinated and control PBMCs in response to the recall M2e antigen by qRT-PCR assay (Fig. 2C). Our results indicated that the chickens vaccinated with M2e plus PEI adjuvant showed significantly (p < 0.05) higher IFN-γ mRNA levels (3 and 4 folds increase, respectively) compared to the M2e alone and the PEI adjuvant chicken groups. All these results clearly indicate that PEI adjuvant has significantly augmented the immunogenicity of M2e antigen and intranasally administered M2e plus PEI adjuvant can elicit both humoral and CMI responses.

3.4. Intranasally administered PEI adjuvanted M2e tetramer reduced lung inflammation and viral shedding post-H9N2 challenge

To determine the protective efficacy of PEI-adjuvanted M2e vaccine, we intranasally challenged vaccinated and control chickens with 10^4 TCID$_{50}$ H9N2 virus. Subsequently, cloacal wash samples were collected post-challenge for the determination of viral RNA copy number by qRT-PCR assay (Fig. 3). The presence of viral RNA was found in both vaccinated and control chickens, albeit, chickens vaccinated with M2e plus PEI adjuvant showed lower viral copy number from day 1 to day 6 compared to the chickens that received either M2e alone or PEI adjuvant (Fig. 3). To further investigate the effect of vaccination on virus-
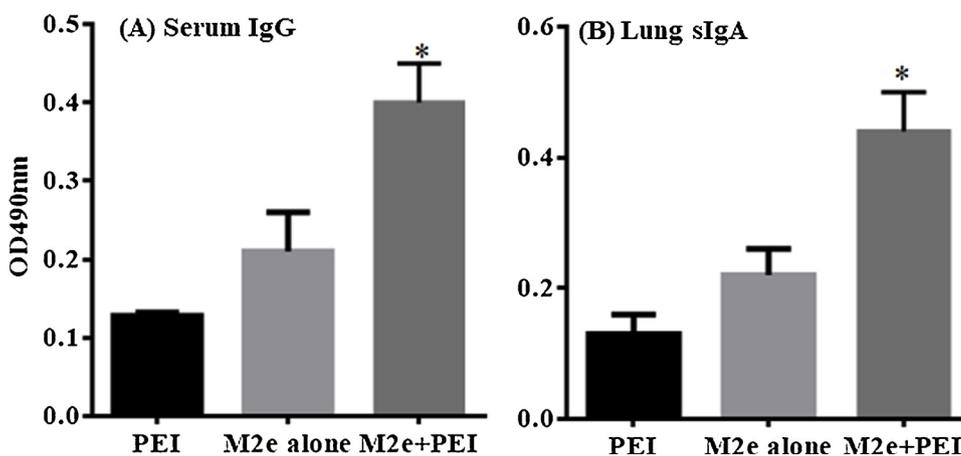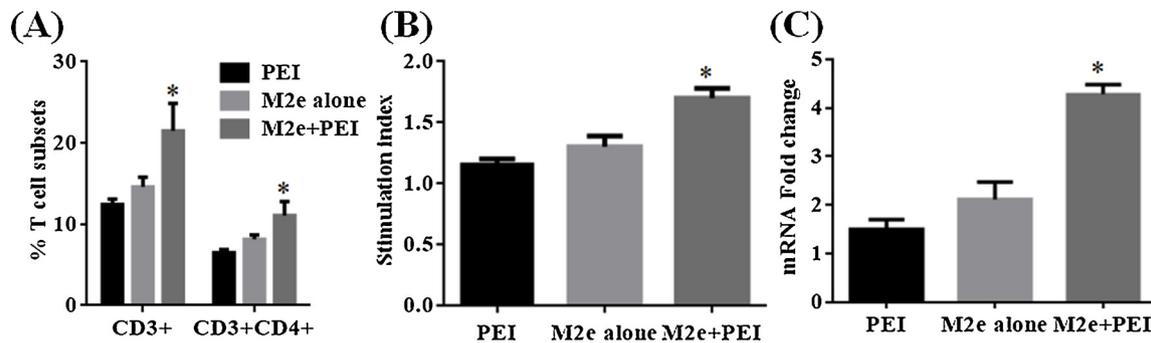

Fig. 1. Serum IgG and mucosal IgA antibody responses. Chickens (n = 10) were intranasally immunized with PEI adjuvant, M2e alone or M2e plus PEI adjuvant and two weeks later the vaccinated chickens were boosted intranasally with the same dose. (A) Serum samples (n = 5) were collected at 14th day post-booster vaccination for IgG analysis by an indirect ELISA. (B) Four chickens in each group were sacrificed 14 days post-booster vaccination and lung washings were collected for IgA analysis by an indirect ELISA. Each data points represent mean ± SD. *p < 0.05.

Fig. 2. Analysis of cellular immune response in M2e plus PEI vaccinated chickens. Chickens (n = 10) were intranasally immunized with PEI adjuvant, M2e alone or M2e plus PEI adjuvant, and 14 days post-booster vaccination PBMCs were restimulated with M2e antigen for either 24 h or 72 h for IFN-γ and lymphoproliferative responses, respectively. (A) The $CD3^+CD4^+$ T-cell subset in vaccinated and control chickens are expressed as a percentage of the gated cells. (B) Lymphocyte proliferative responses by MTT-based assay. (C) qRT-PCR analysis of IFN-γ mRNA transcription induction levels in vaccinated and control chickens and results are expressed as relative fold change in mRNA transcription levels of stimulated PBMCs from vaccinated chickens compared to the stimulated naïve PBMCs. Gene expressions were normalized to GAPDH and mRNA levels of 0 h were used as the calibrator. Data presented are mean ± SD of five chickens per group. *p < 0.05.
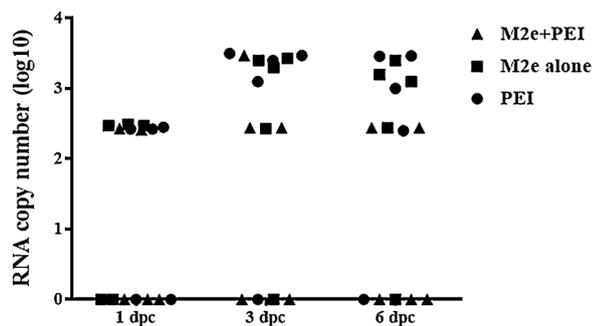

Fig. 3. Protective efficacy of M2e plus PEI vaccine against LPAI H9N2 challenge. Chickens (n = 10) were intranasally immunized with PEI adjuvant, M2e alone or M2e plus PEI adjuvant, and 14 days post-booster vaccination immunized chickens (n = 6) were challenged with 10^4 TCID50 H9N2 virus. The protective efficacy was determined by estimation of H9N2 viral RNA copy numbers in the cloacal swab samples of the vaccinated and control chickens (n = 5) after challenge with the virulent H9N2 virus. dpc; days post-challenge.

specific immune protection, histological studies were performed on lung tissues collected from birds on day 7 post-H9N2 challenge (Fig. 4). As expected, no lesion was found in the lung tissues of uninfected chickens (Fig. 4A); however, chickens treated with either PEI adjuvant or M2e alone had pulmonary lesions consisting of inflammation, congestion and hemorrhagic exudates in the lungs (Fig. 4B & C). In contrast, chickens vaccinated with M2e plus PEI adjuvant exhibited minimal inflammation at 7 day post-challenge (Fig. 4D). All these results clearly suggest that intranasally administered PEI adjuvanted M2e antigen is capable of reducing lung inflammation and viral loads in vaccinated birds.

4. Discussion

The present study was aimed to investigate the protective efficacy of PEI adjuvanted M2e-based vaccine against H9N2 challenge in chickens. H9N2 infection in Korean poultry industry has enormous economic importance, and since the introduction of oil-adjuvanted inactivated H9N2 vaccine, novel H9N2 variants have emerged, which have not only posed significant threat to the poultry industry, but also diversified the host range and zoonotic transmission potential of H7N9 and H5N1 IAVs (Ku et al., 2014; Thuy et al., 2016). A major obstacle in influenza vaccine development is the extent of genetic diversity among influenza subtypes, and, thus, immunity generated against one subtype provides little or no protection against the heterologous strain (Soema et al., 2015). M2e is an attractive and promising approach to induce broad spectrum of protection against many influenza subtypes as it is highly

conserved across IAVs (Fiers et al., 1999). Several studies have demonstrated that M2e alone is non-protective; however, M2e conjugated to various carrier molecules induces efficient immune responses and subsequent homologous and heterologous protection in various animal models (Deng et al., 2015). In this study, we show that intranasally coadministered M2e and PEI in chickens elicited humoral and CMI responses and vaccinated birds exhibited lower lung inflammation and viremia levels post-H9N2 viral challenge.

Intranasal administration of vaccines is a logical route to control infections caused by IAVs and has many advantages, especially for mass implementation of vaccination, thereby preventing the occurrences of cross-contamination of blood-borne pathogens as a result of parenteral injection and/or needle re-use. Furthermore, intranasal vaccination is needle-free, which seems to be a logistic approach in pandemic situations, when time and trained medical personnel could be the limiting factors. Several studies have explored intranasal administration of M2e-based vaccines and compared the effectiveness of this route with other routes of vaccination (Deng et al., 2015). Herve et al. showed that intranasal administration of recombinant nucleoprotein of respiratory syncytial virus substituted with three tandem copies of M2e elicited both M2e-specific systemic and mucosal antibody responses and efficient protection against PR8 challenge compared to the subcutaneous administration, which induced only IgG responses (Herve et al., 2014). The present study demonstrated that intranasally coadministered M2e and PEI adjuvant induced both systemic IgG and mucosal IgA responses. Induction of anti-M2e-specific IgG responses in peripheral blood circulation is the principal mode of protection mediated by M2e-based vaccines (Deng et al., 2015), while the protection mediated by anti-M2e IgA antibodies remains unclear. The present study showed that chickens vaccinated with M2e plus PEI adjuvant exhibited lower lung pathology and viral load compared to the M2e alone or the PEI adjuvant chicken groups. This finding was in agreement to the previously published report, which shows that prophylactic and therapeutic administration of M2e-specific Z3G1 monoclonal antibody resulted in significant protection in mice and alleviated clinical symptoms and lung pathology in monkeys following H1N1 infection (Song et al., 2014). In addition to antibody responses, T cell responses induced by M2e-based vaccines also play an important role in influenza protection. Pejoski et al. reported that immunization with Freund`s adjuvanted M2e2-16 peptide containing only B cell epitopes failed to induce antibody responses; however, M2e-specific antibodies are readily generated by including a chemically conjugated T helper epitopes derived from HA in the M2e conjugate (Pejoski et al., 2010). Further, Eliasson et al. shows that M2e-specific T cell responses are broadly protective against influenza infections and poor protection was observed in vaccinated mice that failed to develop CD4 + T cell responses following M2e based
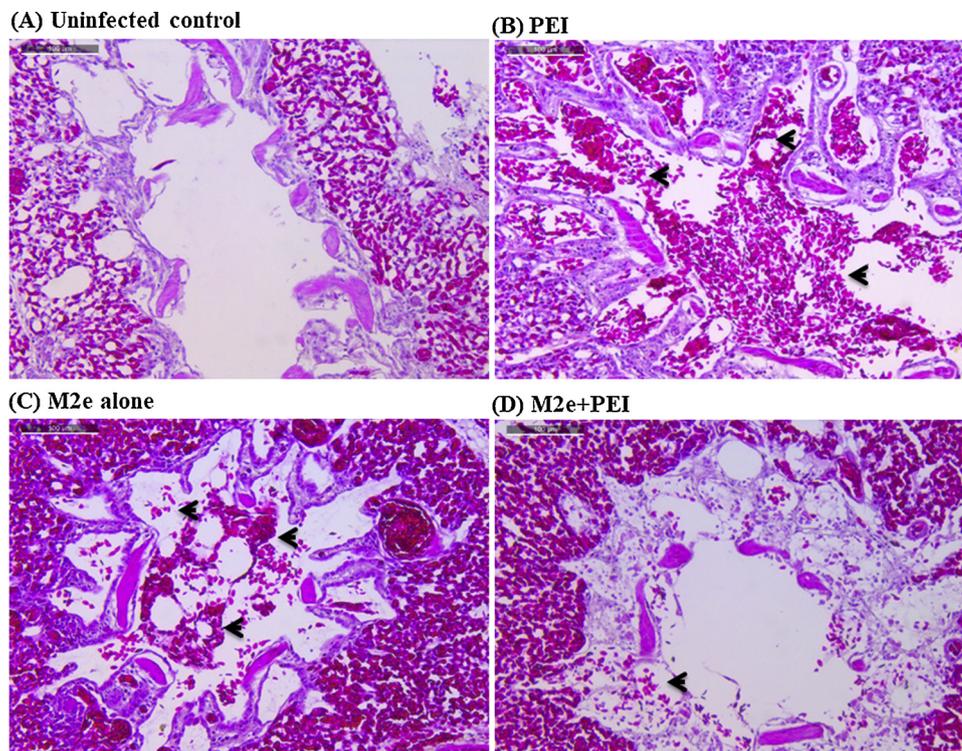
(A) Uninfected control

(B) PEI

(C) M2e alone

(D) M2e+PEI

Fig. 4. Photomicrographs of hematoxylin-and eosin-stained lung sections of chickens on 7th day post-H9N2 challenge. Chickens (n = 10) were intranasally immunized with PEI adjuvant, M2e alone or M2e plus PEI adjuvant, and 14 days post-booster vaccination immunized chickens (n = 6) were challenged with 10⁴TCID50 H9N2 virus. At 7th day post-challenge, six chickens were sacrificed and lung tissues were collected for histopathological analysis. Arrows indicate inflammation.

vaccination (Eliasson et al., 2018). The M2e-specific T cells drive B cells to accelerate and generate more potent M2e and hemagglutinating-specific IgG production (Eliasson et al., 2018). The present study used M2e1-24 peptide that contains two B cell epitopes and one T cell epitope and we showed that CD4$^+$ T cell responses were induced in M2e plus PEI vaccinated group. We also observed significantly higher IFN-γ responses, a surrogate measure of T cell responses, in chickens vaccinated with a co-mix of M2e plus PEI adjuvant. Consequently, the vaccinated chickens had shown lower lung pathology and viral load compared to the chickens that received either M2e alone or PEI adjuvant. These results clearly indicate that PEI adjuvant had significantly augmented the immunogenicity of M2e antigen in chickens. Our results are in agreement to the previously published reports, which show that PEI adjuvant is capable of eliciting both antigen-specific humoral and cell-mediated immunity (Song et al., 2017; Wegmann et al., 2012).

In conclusion, we show that intranasally coadministered M2e and PEI adjuvant can elicit significant amount of M2e-specific humoral and CMI responses in chickens. Other studies have shown that efficacious LPAI vaccines protect chickens against clinical signs and mortality, reduce virus shedding, and increase resistance to infection. Our results clearly demonstrate that vaccination with M2e plus PEI adjuvant reduced lung pathology and viral shedding in cloacal samples. Further studies are warranted to investigate the potential of M2e plus PEI-based vaccine to mediate protection against other influenza subtypes or, more reasonably, M2e antigen can be incorporated with currently available H9N2 vaccines to increase their effectiveness and cross-protective potential.

Conflicts of interests

The authors declare no conflict of interest.

Author contributions

The work was designed by JHL and IAH. Experiments and sampling were done by IAH and KJH. Manuscript draft preparation and statistical analysis was done by IAH. All authors have read and approved the final

manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.02.007.

References

Butt, K.M., Smith, G.J.D., Chen, H., Zhang, L.J., Leung, Y.H.C., Xu, K.M., Lim, W., Webster, R.G., Yuen, K.Y., Peiris, J.S.M., Guan, Y., 2005. Human infection with an avian H9N2 influenza a virus in Hong Kong in 2003. J. Clin. Microbiol. 43, 5760–5767. https://doi.org/10.1128/JCM.43.11.5760-5767.2005.

Chen, H., Yuan, H., Gao, R., Zhang, J., Wang, D., Xiong, Y., Fan, G., Yang, F., Li, X., Zhou, J., Zou, S., Yang, L., Chen, T., Dong, L., Bo, H., Zhao, X., Zhang, Y., Lan, Y., Bai, T., Dong, J., Li, Q., Wang, S., Zhang, Y., Li, H., Gong, T., Shi, Y., Ni, X., Li, J., Zhou, J., Fan, J., Wu, J., Zhou, X., Hu, M., Wan, J., Yang, W., Li, D., Wu, G., Feng, Z., Gao, G.F., Wang, Y., Jin, Q., Liu, M., Shu, Y., 2014. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. Lancet 383, 714–721. https://doi.org/10.1016/S0140-6736(14)60111-2.

Choi, J.G., Lee, Y.J., Kim, J.Y., Kim, Y.H., Paek, M.R., Yang, D.K., Son, S.W., Kim, J.H., 2010. Molecular identification of the vaccine strain from the inactivated oil emulsion H9N2 low pathogenic avian influenza vaccine. J. Vet. Sci. 11, 161–163.

Chrzastek, K., Lee, D., Gharaibeh, S., Zsak, A., Kapczynski, D.R., 2018. Characterization of H9N2 avian influenza viruses from the Middle East demonstrates heterogeneity at amino acid position 226 in the hemagglutinin and potential for transmission to mammals. Virology 518, 195–201. https://doi.org/10.1016/J.VIROL.2018.02.016.

Deng, L., Cho, K.J., Fiers, W., Saelens, X., 2015. M2e-based universal influenza a vaccines. Vaccines 3, 105–136. https://doi.org/10.3390/vaccines3010105.

Diaz-Dinamarca, D.A., Ibañez, F.J., Soto, D.A., Soto, J.A., Cespedes, P.F., Muena, N.A., Garate, D.S., Kalergis, A.M., Vasquez, A.E., 2018. Immunization with a mixture of nucleoprotein from human metapneumovirus and AbISCO-100 adjuvant reduces viral infection in mice model. Viral Immunol. 31, 306–314. https://doi.org/10.1089/vim.2017.0159.

Eliasson, D.G., Omokanye, A., Schön, K., Wenzel, U.A., Bernasconi, V., Bemark, M., Kolpe,

A., El Bakkouri, K., Ysenbaert, T., Deng, L., Fiers, W., Saelens, X., Lycke, N., 2018. M2e-tetramer-specific memory CD4 T cells are broadly protective against influenza infection. Mucosal Immunol. 11, 273–289. https://doi.org/10.1038/mi.2017.14.

Fiers, W., Neirynck, S., Deroo, T., Saelens, X., Vanlandschoot, P., Jou, W.M., 1999. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat. Med. 5, 1157–1163. https://doi.org/10.1038/13484.

França, M.S., Brown, J.D., 2014. Influenza pathobiology and pathogenesis in avian species. Curr. Top. Microbiol. Immunol. 385, 221–242. https://doi.org/10.1007/82_2014_385.

Gu, M., Chen, H., Li, Q., Huang, J., Zhao, M., Gu, X., Jiang, K., Wang, X., Peng, D., Liu, X., 2014. Enzootic genotype S of H9N2 avian influenza viruses donates internal genes to emerging zoonotic influenza viruses in China. Vet. Microbiol. 174, 309–315. https://doi.org/10.1016/j.vetmic.2014.09.029.

Hajam, I.A., Lee, J.H., 2017. An influenza HA and M2e based vaccine delivered by a novel attenuated Salmonella mutant protects mice against homologous H1N1 infection. Front. Microbiol. 8, 872. https://doi.org/10.3389/fmicb.2017.00872.

Hajam, I.A., Kim, J., Lee, J.H., 2018a. Salmonella Gallinarum delivering M2eCD40L in protein and DNA formats acts as a bivalent vaccine against fowl typhoid and H9N2 infection in chickens. Vet. Res. 49, 99. https://doi.org/10.1186/s13567-018-0593-z.

Hajam, I.A., Kim, J.H., Lee, J.H., 2018b. Incorporation of membrane-anchored fl agellin into Salmonella Gallinarum bacterial ghosts induces early immune responses and protection against fowl typhoid in young layer chickens. Vet. Immunol. Immunopathol. 199, 61–69. https://doi.org/10.1016/j.vetimm.2018.03.011.

Herve, P.-L., Raliou, M., Bourdieu, C., Dubuquoy, C., Petit-Camurdan, A., Bertho, N., Eleouet, J.-F., Chevalier, C., Riffault, S., 2014. A Novel Subnucleocapsid Nanoplatform for Mucosal Vaccination against Influenza Virus That Targets the Ectodomain of Matrix Protein 2. J. Virol. 88, 325–338. https://doi.org/10.1128/JVI.01141-13.

Huang, Y., Li, X., Zhang, H., Chen, B., Jiang, Y., Yang, L., Zhu, W., Hu, S., Zhou, S., Tang, Y., Xiang, X., Li, F., Li, W., Gao, L., 2015. Human infection with an avian influenza A (H9N2) virus in the middle region of China. J. Med. Virol. 87, 1641–1648. https://doi.org/10.1002/jmv.24231.

Huleatt, J.W., Nakaar, V., Desai, P., Huang, Y., Hewitt, D., Jacobs, A., Tang, J., McDonald, W., Song, L., Evans, R.K., Umlauf, S., Tussey, L., Powell, T.J., 2008. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine 26, 201–214. https://doi.org/10.1016/j.vaccine.2007.10.062.

Kim, E.-H., Lee, J.-H., Noriel, P., Pascua, Q., Song, M.-S., Baek, Y.-H., Kwon, H.-I., Park, S.-J., Lim, G.-J., Decano, A., Chowdhury, M.Y., Seo, S.-K., Song, M.K., Kim, C.-J., Choi, Y.-K., 2013. Prokaryote-expressed M2e protein improves H9N2 influenza vaccine efficacy and protection against lethal influenza a virus in mice. Virol. J. 10, 1. https://doi.org/10.1186/1743-422X-10-104.

Kim, J.H., Hajam, I.A., Lee, J.H., 2017. A consensus-hemagglutinin-based vaccine delivered by an attenuated Salmonella mutant protects chickens against heterologous H7N1 influenza virus. Oncotarget 8. https://doi.org/10.18632/oncotarget.16353.

Kim, J.H., Hajam, I.A., Lee, J.H., 2018. Oral immunization with a novel attenuated Salmonella typhimurium encoding influenza HA, M2e and NA antigens protects chickens against H7N9 infection. Vet. Res. 49, 12. https://doi.org/10.1186/s13567-018-0509-y.

Ku, K.B., Park, E.H., Yum, J., Kim, H.M., Kang, Y.M., Kim, J.C., Kim, J.A., Kim, H.S., Seo, S.H., 2014. Transmissibility of novel H7N9 and H9N2 avian influenza viruses between chickens and ferrets. Virology 450–451, 316–323. https://doi.org/10.1016/J.VIROL.2013.12.022.

Layton, S.L., Kapczynski, D.R., Higgins, S., Higgins, J., Wolfenden, A.D., Liljebjelke, K.A., Bottje, W.G., Swayne, D., Berghman, L.R., Kwon, Y.M., Hargis, B.M., Cole, K., 2009. Vaccination of chickens with recombinant Salmonella expressing M2e and CD154 epitopes increases protection and decreases viral shedding after low pathogenic avian influenza challenge. Poult. Sci. 88, 2244–2252. https://doi.org/10.3382/ps.2009-00251.

Lee, D.-H., Song, C.-S., 2013. H9N2 avian influenza virus in Korea: evolution and vaccination. Clin. Exp. Vaccine Res. 2, 26–33. https://doi.org/10.7774/cevr.2013.2.1.26.

Lee, C.W., Song, C.S., Lee, Y.J., Mo, I.P., Garcia, M., Suarez, D.L., Kim, S.J., 2000. Sequence analysis of the hemagglutinin gene of H9N2 Korean avian influenza viruses and assessment of the pathogenic potential of isolate MS96. Avian Dis. 44, 527–535.

Lee, Y.-J., Shin, J.-Y., Song, M.-S., Lee, Y.-M., Choi, J.-G., Lee, E.-K., Jeong, O.-M., Sung, H.-W., Kim, J.-H., Kwon, Y.-K., Kwon, J.-H., Kim, C.-J., Webby, R.J., Webster, R.G., Choi, Y.K., 2007. Continuing evolution of H9 influenza viruses in Korean poultry. Virology 359, 313–323. https://doi.org/10.1016/j.virol.2006.09.025.

Lee, E.-K., Kang, H.-M., Song, B.-M., Lee, Y.-N., Heo, G.-B., Lee, H.-S., Lee, Y.-J., Kim, J.-H., 2017. Surveillance of avian influenza viruses in South Korea between 2012 and 2014. Virol. J. 14, 54. https://doi.org/10.1186/s12985-017-0711-y.

Li, J., Arévalo, M.T., Chen, Y., Posadas, O., Smith, J.A., Zeng, M., 2014. Intranasal immunization with influenza antigens conjugated with cholera toxin subunit B stimulates broad spectrum immunity against influenza viruses. Hum. Vaccin. Immunother. 10, 1211. https://doi.org/10.4161/HV.28407.

Park, K.J., Kwon, H.-i., Song, M.-S., Pascua, P.N.Q., Baek, Y.H., Lee, J.H., Jang, H.-L., Lim, J.-Y., Mo, I.-P., Moon, H.-J., Kim, C.-J., Choi, Y.K., 2011. Rapid evolution of low-pathogenic H9N2 avian influenza viruses following poultry vaccination programmes. J. Gen. Virol. 92, 36–50. https://doi.org/10.1099/vir.0.024992-0.

Pejoski, D., Zeng, W., Rockman, S., Brown, L.E., Jackson, D.C., 2010. A lipopeptide based on the M2 and HA proteins of influenza A viruses induces protective antibody. Immunol. Cell Biol. 88, 605–611. https://doi.org/10.1038/icb.2010.15.

Pu, J., Wang, S., Yin, Y., Zhang, G., Carter, R.A., Wang, J., Xu, G., Sun, H., Wang, M., Wen, C., Wei, Y., Wang, D., Zhu, B., Lemmon, G., Jiao, Y., Duan, S., Wang, Q., Du, Q., Sun, M., Bao, J., Sun, Y., Zhao, J., Zhang, H., Wu, G., Liu, J., Webster, R.G., 2015. Evolution of the H9N2 influenza genotype that facilitated the genesis of the novel H7N9 virus. Proc. Natl. Acad. Sci. 112, 548–553. https://doi.org/10.1073/pnas.1422456112.

Röhm, C., Horimoto, T., Kawaoka, Y., Süss, J., Webster, R.G., 1995. Do hemagglutinin genes of highly pathogenic avian influenza viruses constitute unique phylogenetic lineages? Virology 209, 664–670. https://doi.org/10.1006/viro.1995.1301.

Sheppard, N.C., Brinckmann, S.A., Gartlan, K.H., Puthia, M., Svanborg, C., Krashias, G., Eisenbarth, S.C., Flavell, R.A., Sattentau, Q.J., Wegmann, F., 2014. Polyethyleneimine is a potent systemic adjuvant for glycoprotein antigens. Int. Immunol. 26, 531–538. https://doi.org/10.1093/intimm/dxu055.

Soema, P.C., Kompier, R., Amorij, J.-P., Kersten, G.F.A., 2015. Current and next generation influenza vaccines: formulation and production strategies. Eur. J. Pharm. Biopharm. 94, 251–263. https://doi.org/10.1016/j.ejpb.2015.05.023.

Song, A., Myojo, K., Laudenslager, J., Harada, D., Miura, T., Suzuki, K., Kuni-Kamochi, R., Soloff, R., Ohgami, K., Kanda, Y., 2014. Evaluation of a fully human monoclonal antibody against multiple influenza A viral strains in mice and a pandemic H1N1 strain in nonhuman primates. Antiviral Res. 111, 60–68. https://doi.org/10.1016/j.antiviral.2014.08.016.

Song, L., Xiong, D., Song, H., Wu, L., Zhang, M., Kang, X., Pan, Z., Jiao, X., 2017. Mucosal and Systemic Immune Responses to Influenza H7N9 Antigen HA1-2 Co-Delivered Intranasally with Flagellin or Polyethyleneimine in Mice and Chickens. Front. Immunol. 8, 326. https://doi.org/10.3389/fimmu.2017.00326.

Thuy, D.M., Peacock, T.P., Bich, V.T.N., Fabrizio, T., Hoang, D.N., Tho, N.D., Diep, N.T., Nguyen, M., Hoa, L.N.M., Trang, H.T.T., Choisy, M., Inui, K., Newman, S., Trung, N.V., van Doorn, R., To, T.L., Iqbal, M., Bryant, J.E., 2016. Prevalence and diversity of H9N2 avian influenza in chickens of Northern Vietnam, 2014. Infect. Genet. Evol. 44, 530–540. https://doi.org/10.1016/j.meegid.2016.06.038.

Wegmann, F., Gartlan, K.H., Harandi, A.M., Brinckmann, S.A., Coccia, M., Hillson, W.R., Kok, W.L., Cole, S., Ho, L.-P., Lambe, T., Puthia, M., Svanborg, C., Scherer, E.M., Krashias, G., Williams, A., Blattman, J.N., Greenberg, P.D., Flavell, R.A., Moghaddam, A.E., Sheppard, N.C., Sattentau, Q.J., 2012. Polyethyleneimine is a potent mucosal adjuvant for viral glycoprotein antigens. Nat. Biotechnol. 30, 883–888. https://doi.org/10.1038/nbt.2344.