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Potent adjuvant effects of novel NKT stimulatory glycolipids on hemagglutinin based DNA vaccine for H5N1 influenza virus

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ABSTRACT

H5N1 influenza virus is a highly pathogenic virus, posing a pandemic threat. Previously, we showed that phenyl analogs of α-galactosylceramide (α-GalCer) displayed greater NKT stimulation than α-GalCer. Here, we examined the adjuvant effects of one of the most potent analogs, C34, on consensus hemagglutinin based DNA vaccine (pCHA5) for H5N1 virus. Upon intramuscular electroporation of mice with pCHA5 with/without various α-GalCer analogs, C34-adjuvanted group developed the highest titer against consensus H5 and more HA-specific IFN-γ secreting CD8 cells (203 ± 13.5) than pCHA5 alone (152.6 ± 13.7, $p < 0.05$). Upon lethal challenge of NIBRG-14 virus, C34-adjuvanted group (84.6%) displayed higher survival rate than pCHA5 only group (46.1%). In the presence of C34 as adjuvant, the antisera displayed broader and greater neutralizing activities against virions pseudotyped with HA of clade 1, and 2.2 than pCHA5 only group. Moreover, to simulate an emergency response to a sudden H5N1 outbreak, we injected mice intramuscularly with single dose of a new consensus H5 (pCHA5-II) based on 1192 full-length H5 sequences, with C34 as adjuvant. The latter not only enhanced the humoral immune response and protection against virus challenge, but also broadened the spectrum of neutralization against pseudotyped HA viruses. Our vaccine strategy can be easily implemented for any H5N1 virus outbreak by single IM injection of a consensus H5 DNA vaccine based on updated HA sequences using C34 as an adjuvant.

1. Introduction

Influenza A virus is a negative single-stranded RNA virus belonging to the *Orthomyxoviridae* family. Based on the antigenicity of its surface proteins, hemagglutinin (HA) and neuraminidase (NA), influenza A virus can be divided into many subtypes, among which H5N1 is the most virulent subtype identified so far (Chmielewski and Swayne, 2011). Although poultry is the major host of H5N1 influenza virus, it can occasionally be transmitted from chickens to human, causing serious infection with high mortality. Since 2003, 641 peoples have been infected by H5N1 worldwide, with 380 fatal cases, according to publication of World Health Organization (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/index.html). Fortunately, only small outbreaks of H5N1 virus were observed and no human-to-human transmission has been documented. However, continuing evolution of H5N1 influenza virus still poses concerns for public health (Amendola et al., 2011).

The most abundant surface glycoprotein on influenza A virus is HA, a homotrimer protein, which binds to sialic acid on host cells, followed by internalization of virus with subsequent release of viral RNA into cells (Rossman and Lamb, 2011). Thus, HA is an attractive target for H5N1 vaccine development (Dormitzer et al., 2011; Gerhard, 2001). However, in a sudden H5N1 outbreak, it is difficult to rapidly generate a large quantity of conventional virus particle based vaccine, due to its time consuming process of production and the high mutation frequency of HA. Yet another challenge is the safety concern for inactivating the highly pathogenic H5N1 viruses during vaccine preparation. Therefore, it is desirable and important to develop new strategy for H5N1 vaccine which can overcome the above challenges.

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The use of DNA based vaccine may circumvent shortcomings of conventional virus particle based influenza vaccine. DNA based vaccine has been shown to induce protective immune responses against infectious pathogens (Donnelly et al., 1995; Polack et al., 2000). Compared to virus particle based vaccine, manufacture of DNA vaccine is relatively simple and fast, making it relatively easy and more economical to generate a large quantity of vaccine in a short period of time. However, the drawback is the low expression levels of DNA encoded protein upon intramuscular injection. Many strategies have been used to improve the efficiency of DNA vaccine. These include codon optimization of DNA sequence to enhance the translation efficiency of DNA (Nagata et al., 1999; Uchijima et al., 1998), use of electroporation (EP) to facilitate delivery of DNA into cells by electric pulses (Andre and Mir, 2010; Mir, 2009) and generation of DNA vaccine with consensus DNA sequences to induce broad immune response against targets with subtle variations. Combining these three strategies, we have shown that DNA vaccine of consensus H5, pCHA5 delivered by intramuscular-electroporation (IM-EP) route, could induce both humoral and cellular immune responses with broad spectrum neutralizing antibodies (Chen et al., 2008). Another approach to improve vaccine potency is the addition of adjuvant such as aluminum phosphate, in DNA vaccine to enhance antibody production (Ulmer et al., 1999). Along this line, α-galactosylceramide (α-GalCer), which activates invariant natural killer T (iNKT) cells, was reported to exhibit adjuvant activities for protein and DNA vaccines (Courtney et al., 2009; Guillonneau et al., 2009; Huang et al., 2008; Ko et al., 2005; Yamaguchi et al., 1996). Recently, we have shown that α-GalCer analogs containing phenyl group in acyl tail displayed greater immune-enhancing activity than α-GalCer (Chang et al., 2007; Wu et al., 2011). In this study, we evaluated dose-sparing effects of α-GalCer and one of its analogs on the HA-specific cellular and humoral immune responses *in vivo* and spectrums of neutralizing antibodies elicited by HADNA vaccine.

2. Materials and methods

2.1. Vaccination procedure and virus challenge

Consensus HA DNA plasmids, pCHA5-I and pCHA5-II, were generated as described previously (Chen et al., 2008). α-GalCer and C34 were chemically synthesized as described (Wu et al., 2011) and dissolved in DMSO. Female BALB/c mice (10 mice/group, National Laboratory Animal Center, Taipei, Taiwan), 6–7 weeks old, were injected with HA plasmid with/without C34 (2 μg) at tibial cranial muscle via intramuscular electroporation using TriGrid Delivery System (Ichor Medical Systems, CA, USA). Two weeks after two vaccinations at 3-week interval, mice were intranasally challenged with NIBRG-14 virus (200 LD_{50}) (National Institute for Biological Standards and Control, UK). The subtype of NIBRG-14 is H5N1. The NIBRG-14 is a reassortant virus derived from A/PR/8/34 (H1N1) virus and A/Vietnam/1194/2004 (H5N1) virus (in which the polybasic HA cleavage site has been excised). Mouse studies were approved by the Institutional Animal Care and Use Committee of Academia Sinica.

2.2. HA-pseudotyped neutralization assay

Pseudotyped virus neutralization assay was done as described previously (Chen et al., 2008), using HA pseudotyped viruses carrying a luciferase reporter gene, including A/Anhui/1/2005 (RG5, clade 2.3.4), A/Turkey/1/2005 (TK, clade 2.2), A/Indonesia/5/2005 (ID5, clade 2.1) and A/Viet Nam/1194/2004 (VN1194, clade 1). HA-pseudotyped virus was mixed with diluted sera for 30 min at 37 °C, and then infected MDCK cell for 4 h. After washing with 200 μL of PBS, cells were cultured for 2 days at 37 °C with complete DMEM medium (10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM Glutamax). Cells were washed with 200 μL of PBS and lysed with 50 μL of Bright-Glolysis buffer (Promega, WI, USA) for 2 h at room temperature. Luciferase assay regent (Promega, WI, USA) were added and incubated for 1 min at room temperature, and then read with TopCount Liquid Scintillation Counter (Packard, CT, USA). Data were reported as serum dilutions giving 50% of HA-pseudotyped virus neutralization (ID_{50}).

2.3. ELISA and ELISpot

Anti-HA titer was determined by ELISA using plates coated with HA-Fc protein and HA-specific mouse splenocytes producing IFN-γ was detected by mouse IFN-γ ELISpot kit (BD Pharmingen, CA, USA) as described previously (Chen et al., 2008). For ELISA, Maxisorp plates (Nunc, Roskilde, Denmark) were coated with HA-Fc protein (0.1 μg/well) in bicarbonate buffer (pH 9.6) overnight at 4 °C. After coating, blocking buffer (50 mmol/L Tris, 0.14 mol/L NaCl, 1% BSA, pH 8.0) was added and incubated for 1 h at RT. Sera were serially diluted, transferred into each coated well, incubated for 1 h at RT, followed by 10000×-diluted goat anti-mouse IgG-HRP antibody (Promega, Madison, WI, USA) for 1 h incubation. TMB solution (BD Pharmingen, CA, USA) were added and incubated for 30 min. The reaction was stopped by adding 2 N H_2SO_4 (J.T. Baker, NJ, USA) and read at OD450 with SpectraMax M2 (Molecular Device, CA, USA). Antibody titers were those dilutions that gave an optical density (OD) 2.5-fold higher than the OD obtained in pre-immune sera. For ELISpot, briefly, capture antibodies were coated into each well. After washing, splenocytes (5×10^5/well) were mixed with HA-specific peptide (9mers) and incubated for 24 h at 37 °C. Detection antibody was added and incubated for 2 h at RT, followed by streptavidin-HRP for 1 h. AEC substrate solution (BD Pharmingen, CA, USA) was added to reveal signal, and reaction was stopped with deionized water. The plate was dried overnight, and spots were counted by ELISpot reader (Autoimmune Diagnostika GMBH, Straßberg, Germany).

2.4. Cytokines/chemokines

The Bio-Plex mouse cytokine 23-plex kit (Bio-Rad, Hercules, CA, USA) were used as described previously (Chang et al., 2007).

2.5. Statistical analysis

Statistical significance was calculated using Prism 5 (GraphPad, USA), with One-way analysis of variance (ANOVA) and Tukey's multiple comparison post hoc test for antibody titers, log-rank test for mouse survival, and curve fit with nonlinear regression for neutralization assay. p Values < 0.05 were considered significant.

3. Results

3.1. Adjuvant effects of α-GalCer and its analogs on pCHA5 vaccine

Previously, we showed that immunization of mice with DNA vaccine of consensus H5, pCHA5 (30 μg) delivered by intramuscular electroporation (IM-EP) was sufficient to provide protection against lethal challenges by various clades of H5N1 viruses (Chen et al., 2008). To develop this DNA-based vaccine for large-scale application, it will be desirable to minimize dosage of pCHA5 by combining with optimal adjuvant. Hence, dose-sparing effects of α-GalCer and its analogs on the pCHA5 vaccine were evaluated. BALB/c mice were injected twice three weeks apart through IM-EP route with pVAX (vector only as control, 30 μg) or pCHA5

at dosages of 5, 15, 30, and 45 μg in combination of α-GalCer (2 μg). Two weeks after the second vaccination, sera were collected to determine the titers of HA-specific antibody by ELISA. A dose dependent response was observed from 5 to 15 μg of pCHA5, but it plateaued at ⩾15 μg (Supplementary Fig. 1), and no detectable anti-HA antibody response was noted in mice injected with pVAX. No obvious adjuvant effects of α-GalCer were observed at these dosages of pCHA5 (Supplementary Fig. 1). We then lowered the dosages of pCHA5 to 0.2, 0.5 and 2 μg to evaluate the adjuvant effect of α-GalCer. For pCHA5 alone, there was little antibody response to pCHA5 at ⩽2 μg, until the dose reached 5 μg ($p < 0.0001$ by one-way ANOVA). The addition of α-GalCer increased production of HA-specific IgG by 3.36, 4.63, and 3.18-folds at 0.2, 0.5, and 2 μg of pCHA5, respectively, when compared to the pCHA5 only groups (Fig. 1A). To evaluate optimal modes of delivering pCHA5 and C34, we tested three different modalities of administering pCHA5 (0.2 μg) and α-GalCer (2 μg). Group M1 received injection of mixtures of pCHA5 and C1 at single site in thigh, group M2 were injected with pCHA5 and C1 separately at different thighs, and group M3 received pCHA5 and C1 separately at adjacent sites next to each other. Administration of α-GalCer and pCHA5 as a mixture induced significantly greater production of anti-HA specific antibody (GMT 1108, 95% CI 610.1–2011) when compared with pCHA5 only group (GMT 382.2, 95% CI 198.7–735.2) (Fig. 1B). No adjuvant effects of α-GalCer were evident for either group M2 (GMT 167.8, 95% CI 76.4–368.4) or group M3 (GMT 153.9, 95% CI 47.2–501.4). Based on these findings, adjuvant effects of α-GalCer analogs were examined by injecting the mixture of suboptimal dose (0.2 μg) of pCHA5 with various α-GalCer analogs (2 μg). Antibody titers of C34-adjuvanted group were higher than C1 by 1.4-fold (Fig. 1C). The remaining five analogs, including C13 (0.8-fold), C14 (0.9-fold), C16 (0.9-fold), C17 (1-fold) and C26 (0.8-fold), had slightly lower or equivalent adjuvant effects as C1. These results suggested that α-GalCer could enhance antibody production at the suboptimal dose of pCHA5 and the adjuvant effect of C34 was at least comparable or better than α-GalCer.

3.2. Neutralizing capability of antisera generated by pCHA5/C34 against HA-pseudotyped viruses

Two weeks after second immunization with pCHA5/C34, sera were collected for determination of neutralizing activities against four HA-pseudotyped viruses, which encoded luciferase gene and HA genes of H5N1 influenza viruses, including VN1194 (clade 1), ID05 (clade 2.1), TK05 (clade 2.2) and Anhui05 (2.3.4). Antisera were incubated with HA-pseudotyped viruses and neutralizing titers were measured by luciferase activity. Data were reported as reciprocal of serum dilutions giving 50% of neutralization (IC_{50}) to HA-pseudotyped virus. The pCHA5 only group produced more neutralizing antibodies against VN1194 ($IC_{50} = 170.6$) and TK05 ($IC_{50} = 168$) HA-pseudotyped viruses than pVAX group ($IC_{50} = 36.1$ and 37.4, respectively, $p < 0.0001$ for both) (Fig. 2). Moreover, neutralization ability of C34 adjuvanted group against VN1194 ($IC_{50} = 245.1$) and TK ($IC_{50} = 327.4$) was significantly greater when compared with pVAX or pCHA5 groups ($p < 0.0001$ for both). On the other hand, there was little neutralization activity against ID05 and Anhui05 HA-pseudotyped viruses in sera obtained from all groups. These results suggest that pCHA5 vaccine could induce antisera with cross neutralization activity for selected clades and addition of C34 as an adjuvant significantly boosted neutralization activity.

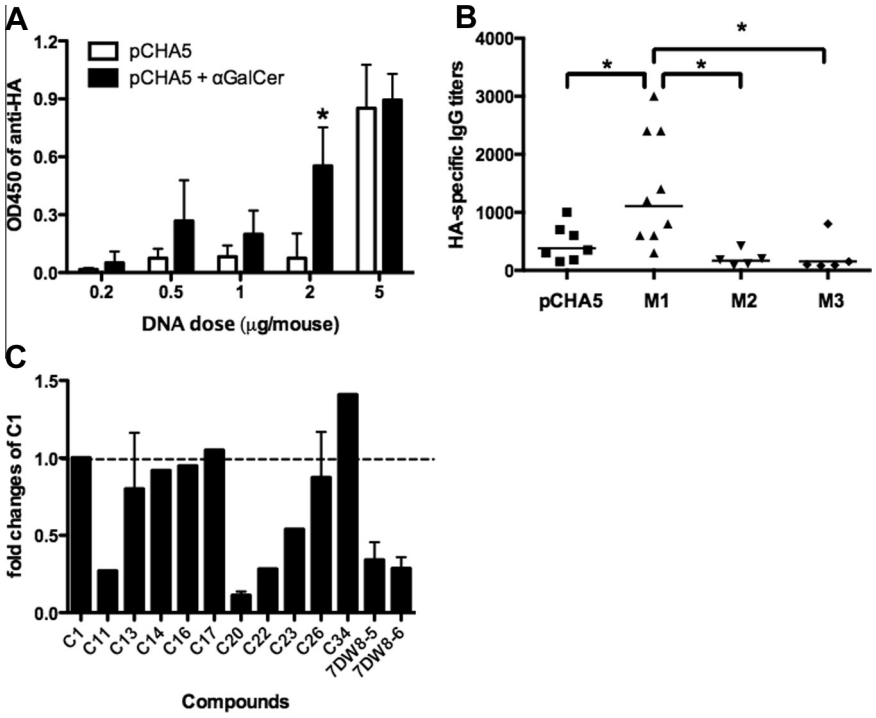

Fig. 1. Adjuvant effect of α-GalCer analogs on pCHA5 vaccine. (A) Female BALB/c mice ($n = 5$ per group) were vaccinated with the indicated dose of pCHA5 mixed with α-GalCer (C1) or DMSO vehicle control by intramuscular electroporation (IM-EP) at week 0 and 3. Two weeks later, sera were collected and diluted 1000-fold to determine HA-specific IgG by ELISA. The production of HA-specific IgG was presented as mean ± SD. $^*p < 0.05$ by one-way ANOVA and Tukey's multiple comparison test. (B) The mice were immunized with pCHA5 (0.2 μg/mouse, $n = 7$) according to the above described time schedule. The mice were divided into three groups. In group M1 ($n = 9$), pCHA5 and C1 were mixed together and injected as a mixture by IM-EP. For group M2 ($n = 5$), pCHA5 was injected on left thigh and C1 on right thigh. For group M3 ($n = 5$), pCHA5 and C1 were injected separately at adjacent sites near each other on left thigh. Titers of HA-specific IgG for each mouse were presented in dot plot. Horizontal lines represent the geometric mean titers for each group. $^*p < 0.05$ by one way ANOVA with Tukey's multiple comparison test. (C) The adjuvant effect of α-GalCer analogs in pCHA5 (0.2 μg) vaccine was compared *in vivo* by using the same immunization schedule as described above and GMT of each glycolipid was used to normalize with GMT of C1 and presented as fold changes.

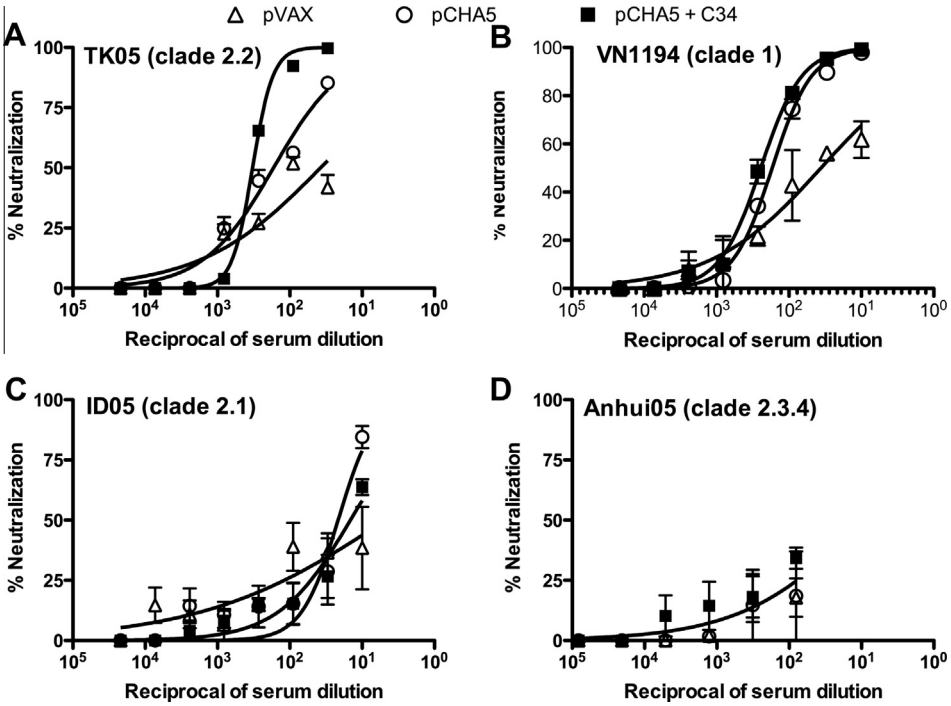

Fig. 2. Infection of MDCK cell by HA-pseudotyped viruses was inhibited by pCHA5 + C34 vaccine induced antisera. Female BALB/c mice (n = 13 per group) were vaccinated with pVAX (0.2 μg), pCHA5 (0.2 μg), or pCAH5 (0.2 μg) + C34 (2 μg) by IM-EP at week 0 and 3. Two weeks later, sera were collected for determination of their neutralizing activities against HA-pseudotyped virus. Four HA-pseudotyped viruses, including (A) TK, (B) VN1194, (C) ID05, and (D) Anhui05, were used in the assays and a dose–response curve fit with nonlinear regression analysis was shown.

3.3. Induction of cytokines/chemokines by vaccination with pCHA5/C34 in mice

Sera were collected at 0 h (before) and 20 h after the second vaccination with pVAX or pCHA5 ± C34 through IM-EP route for determination of the production of cytokines/chemokines by luminex. Production of cytokines/chemokines from pCHA5/C34 group was significantly increased at 20 h than 0 h for IFN-γ, G-CSF, IL-5, IL-17, KC, MIP-1β and RANTES (Fig. 3). Furthermore, production of following cytokines/chemokines at 20 h was substantially higher in pCHA5/C34 group than in pCHA5 only group: IL-2, IL-5, IL-12p40, IL-13, IL-17, IFN-γ, MIP-1α, MIP-1β, KC, RANTES and G-CSF. Curiously, levels of IL-1β decreased in pCHA5/C34 group when compared with pCHA5 only group. There were little to undetectable levels of IL-1α, IL-3, IL-4, IL-6, IL-10, IL-12p70, IL-15 and TNF-α in both pCHA5 only or pCHA5/C34 groups. Taken together, these data suggested that increased production of cytokines/chemokines, which are involved in cell proliferation and chemotaxis, might contribute to the adjuvant effect of C34.

3.4. Protective effects of pCHA5/C34 vaccine against reassortant H5N1 virus challenge in mice

Protective effect of pCHA5/C34 against virus challenge was evaluated. Female BALB/c mice received two vaccinations with pVAX (0.2 μg), pCHA5 (0.2 μg), or pCHA5 (0.2 μg)/C34 (2 μg) through IM-EP at 3 weeks interval. Two weeks after last immunization, protective effect of pCHA5/C34 was evaluated by challenge with reassortant NIBRG-14 virus. The survival of pCHA5/C34 group was significantly higher (84.6% with median survival >14 days) than pCHA5 only group (46.1% with median survival 8 days, $p < 0.05$) (Fig. 4C). No mice survived in pVAX group (0% with median survival 6 days). Moreover, humoral immune response as reflected by anti-HA IgG showed GMT of 8520 in C34 adjuvanted group (95% CI 6153–11799) which was significantly higher than in pCHA5 only group (GMT 1594, 95% CI 532.6–4773; $p < 0.0001$) (Fig. 4A). To assess cellular immune response, the number of IFN-γ-producing $CD8^+$ splenocytes upon stimulation with HA-specific peptide was determined by ELISpot. The numbers of IFN-γ secreting $CD8^+$ cells were significantly greater in pCHA5/C34 group (203 ± 13.5) than in pCHA5 only group (152.7 ± 13.7; $p = 0.001$) (Fig. 4B). Overall, use of C34 as adjuvant has dose sparing effects for suboptimal dosage of pCHA5 vaccine by increasing the titers of HA-specific antibody, raising the number of IFN-γ producing cells, and improving the survival after challenge with H5N1 reassortant virus.

3.5. Protective effects of single intramuscular injection of pCHA5-II/C34 against reassortant H5N1 challenge

Since DNA sequences of pCHA5 were deduced from 467 full-length H5 sequences available as of May 2006, it was no surprise that pCHA5 conferred little protection against clade 2.3 which emerged subsequently. Hence, a new consensus H5 plasmid, pCHA5-II which contain 5 mutated residues (Chen et al., 2011), was generated based on 1192 full-length H5 sequences known by 2007. As expected, the pCHA5-II induced broader protection profile than pCHA5. Moreover, in case of a sudden H5N1 outbreak, there may not be sufficient time for large-scale administration of two vaccinations by IM-EP route. We therefore evaluated the efficacy of single high dose vaccine given by IM instead of IM-EP. Mice were immunized with pCHA5-II at 50 and 75 μg ± C34. Three weeks later, sera were collected for determination of the titers of anti-HA antibodies by ELISA. Low titers of anti-HA antibodies were detected in pCHA5-II only groups, and no dose-dependent effects were observed (Fig. 5A). In contrast, the titers of anti-HA antibodies increased by 8–9-folds at each indicated dose of pCHA5-II in C34 adjuvanted groups ($p < 0.01$). We further assessed HA-specific cellular responses to pCHA5-II ± C34 by determining the secretion of IFN-γ with ELISpot assay. The IFN-γ producing cells increased

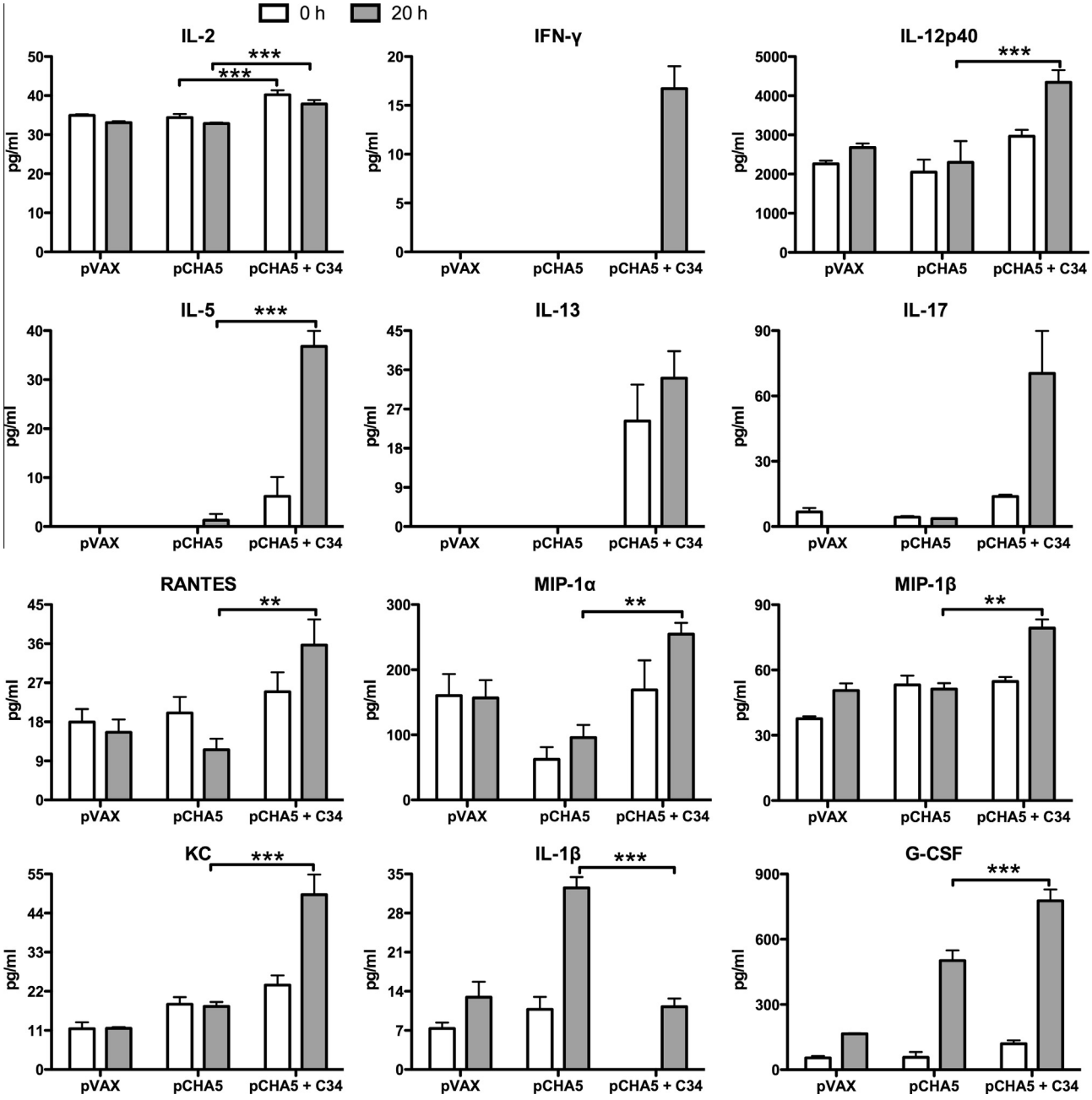

Fig. 3. Profiles of cytokines/chemokines in pCHA5 ± C34 vaccinated mice. Female BALB/c mice ($n = 6$ per group) were vaccinated with pVAX (0.2 μg), pCHA5 (0.2 μg) or pCHA5 (0.2 μg) + C34 (2 μg) by IM-EP at week 0 and 3. Sera were collected at 0 h (before) and 20 h after second vaccination and concentrations of cytokines/chemokines were assayed by Bio-Plex mouse cytokine 23-plex kit. $^{**}p < 0.01$ and $^{***}p < 0.001$ using one-way ANOVA followed by Tukey's multiple comparison test.

to 187.1 ± 9.9 in 50 μg ($p < 0.0001$) or 155.5 ± 8.4 in 75 μg ($p < 0.0001$) of pCHA5-II/C34 groups, when compared with pCHA5-II only groups (50 μg: 50 ± 1.6 and 75 μg: 40 ± 4.5, Fig. 5B). Upon intranasal challenge with lethal dose of NIBRG-14, the survivals of mice were 10% for both pVAX group (median survival 6 days) and 50 μg pCHA5-II group (median survival 6.5 days, vs. pVAX, $p = 0.16$ by log-rank test). The survival increased to 40% for 75 μg pCHA5-II group (median survival 7.5 days, vs. pVAX, $p = 0.23$), although not significantly different from pVAX group (Fig. 5C). On the other hand, in C34 adjuvanted groups, survivals of mice significantly increased from 10% to 100% ($p < 0.0001$) and from 40% to 100% ($p = 0.006$) for 50 μg PCHA5-II group (median survival > 14 days) and 75 μg group (median survival > 14 days), respectively. Our technology platform provided an easily implemented method to update the HA sequence upon any H5N1 virus outbreak, which can confer protective immunity with single IM injection using C34 as an adjuvant.

3.6. Neutralization activity of antiserum after single dose of pCHA5-II/C34 vaccine

Furthermore, we tested cross-neutralization capability of antisera from mice immunized with 50 μg of pCHA5-II ± C34. Three weeks after vaccination, serum was assessed with HA-pseudotyped viruses as described above. The antisera of pCHA5-II only group showed greater neutralizing ability against TK05 HA-pseudotyped virus than pVAX group ($IC_{50} = 420$ vs. 96.7, $p < 0.0001$), but not against ID05 (48.5 vs. 14.6, $p = 0.13$), Anhui05 (3.8 vs. 0, $p = 0.08$), and VN1194 (6.9 vs. 18.6, $p = 0.2$) (Fig. 6). Importantly, in C34 adjuvanted group the neutralization efficacy

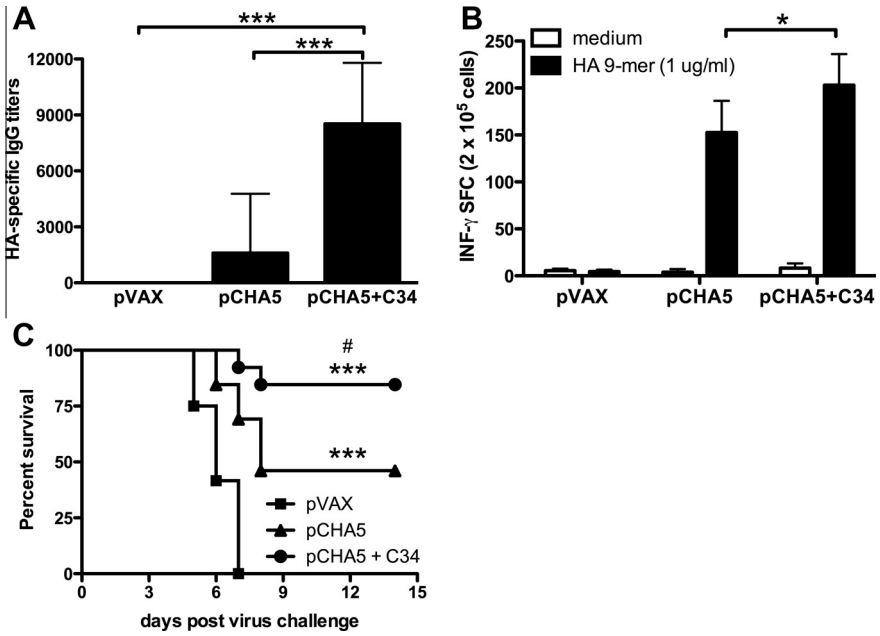

Fig. 4. Induction of HA-specific humoral and cellular immune responses by pCHA5 + C34 vaccine in mice. Female BALB/c mice were vaccinated with pVAX (0.2 μg), pCHA5 (0.2 μg), or pCAH5 (0.2 μg) + C34 (2 μg) by IM-EP at week 0 and 3. Two weeks later, sera were collected and mice were sacrificed. (A) Titers of HA-specific IgG antibody were determined by ELISA and GMT ± 95% CI were presented. $^{***}p < 0.0001$ by one way ANOVA with Tukey's multiple comparison test. (B) Splenocytes from immunized mice ($n = 3$ per group) were stimulated with HA specific peptide. HA-specific IFN-γ producing cells were determined by ELISpot and mean of spot forming colonies (SFC) ± SD were shown. $^{*}p = 0.01$ by one way ANOVA and Tukey's multiple comparison post hoc test. (C) These vaccinated mice ($n = 10$ per group) were challenged with 200 LD_{50} of NIBRG-14 virus and monitored for survival as shown by Kaplan–Meir curve. $^{***}p < 0.0001$ (vs. pVAX) and $^{\#}p < 0.05$ (vs. pCHA5) by log-rank test for survival.

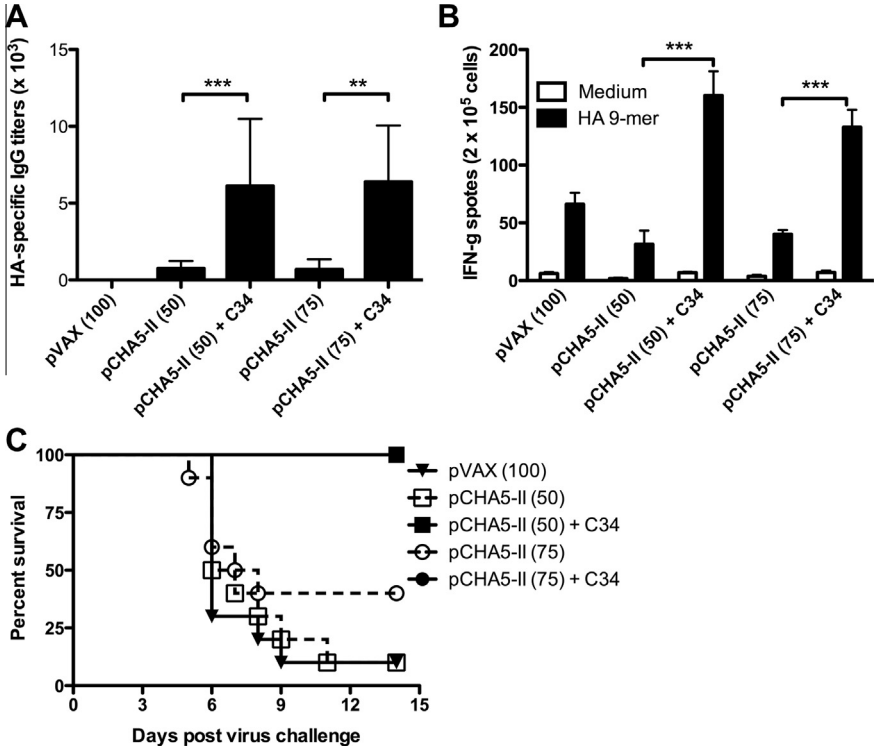

Fig. 5. Single injection of pCHA5-II + C34 intramuscularly could induce protection against NIBRG-14 virus challenge. Mice were injected IM with indicated doses of pCHA5-II (50 and 75 μg) in the presence/absence of C34 (2 μg) as adjuvant. Three weeks after vaccination, sera were collected and tested. (A) Titers of HA-specific IgG antibody were determined by ELISA and GMT ± 95% CI were presented. (B) Splenocytes from vaccinated mice ($n = 2$) were stimulated with HA specific peptide (HA 9-mer, 1 μg/mL) and the HA-specific IFN-γ producing cells were assessed by ELISpot assay. Mean of spot forming colonies ± SD were shown. $^{**}p < 0.01$ and $^{***}p < 0.001$ using one-way ANOVA followed by Tukey's multiple comparison test. (C) These vaccinated mice ($n = 10$) were challenged with 200 LD_{50} of NIBRG-14 virus and monitored for survival as shown by Kaplan–Meir curve.

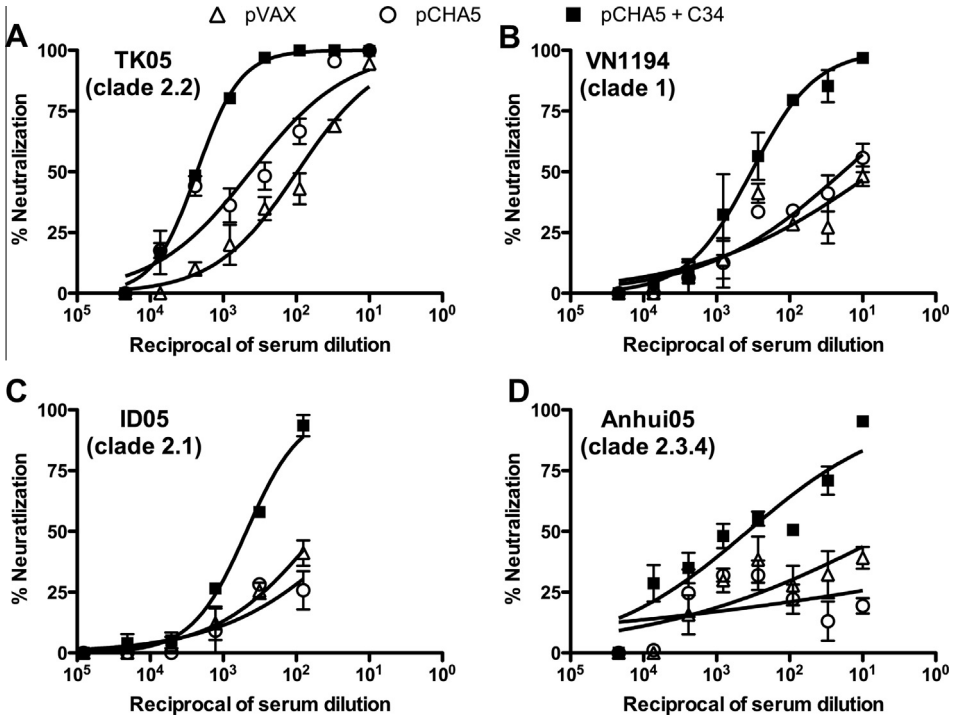

Fig. 6. Serum from mice immunized with single dose of pCHA5-II + C34 inhibited the infection of MDCK cells by HA-pseudotyped viruses. Female BALB/c mice (n = 10 per group) were vaccinated IM with pVAX (50 μg), pCHA5-II (50 μg), and pCAH5-II (50 μg) + C34 (2 μg). Three weeks later, sera were collected and examined for the neutralizing activity against HA-pseudotyped virus. Four HA-pseudotyped viruses, including (A) TK, (B) VN1194, (C) ID05, and (D) Anhui05, were used in the assay and a dose–response curve fit with nonlinear regression analysis was shown.

was significantly greater than pCHA5-II and pVAX groups against VN1194 (IC_{50} = 333.1), TK05 (IC_{50} = 2289), Anhui05 (IC_{50} = 374.9), and ID05 (IC_{50} = 480.6) ($p < 0.0001$ for all). Taken together, these results indicate that C34 adjuvant not only increase the potency of neutralization but also broaden the spectrum against different clades of H5N1 virus.

4. Discussion

In this study we showed that C34 could enhance the efficacy of DNA vaccine administrated by IM or IM-EP routes by increasing titers of HA-specific antibody, broadening spectrum of HA neutralizing antibody, raising the number of HA-specific IFN-γ producing CD8 T cells, and providing better protection against virus challenge. The results of adjuvant effects of α-GalCer (C1) on antibody production and IFN-γ secretion were consistent with those observed in HIV DNA vaccine reported by Huang et al. (2008). On the other hand, production of cross-protecting neutralizing antibody was reported by Kamijuku with H1N1 HA protein vaccine + C1 through intranasal administration (Kamijuku et al., 2008). Here, we showed that adding C34 as adjuvant for HA DNA vaccine induced higher anti-HA titers than C1, broadened the spectrum of neutralizing antibody, and also enhanced $CD8^+$ T cell responses.

Adjuvant activity of C1 was observed only when C1 and pCHA5 were delivered as a mixture but not when they were injected separately. It had been reported that plasmid DNA could induce inflammatory responses and cell infiltration at the site of injection (McMahon et al., 1998). In this study, we showed significant increases of MIP-1α and RANTES in C34 adjuvanted group, which was consistent with our previous report of their induction (Wu et al., 2011) by i.v. injection of C34. Thus, migration of immune cells to the injection site might be attributed to MIP-1α that recruits dendritic cells and B cells to the injected site and lymph nodes (Zhao et al., 2010). Besides, RANTES is a potent chemoattractant for monocytes, NK cells (Loetscher et al., 1996), memory T cells (Schall et al., 1990), eosinophils (Rot et al., 1992) and DCs (Dieu et al., 1998). It also plays an important role to regulate CD8 T cells responses during chronic viral infection. It is thus possible that when pCHA5 was given with glycolipid as a mixture, plasmid-induced inflammatory responses attracted T cells and glycolipid-induced MIP-1α and RANTES chemokine further recruited DC and T cells to the site of injection and draining lymph node leading to enhanced immune responses. Collectively, adjuvant effects of C34 might be attributed to both NKT cell stimulation and APC recruitment mediated by these chemokines.

C34 differs from C1 in having a shorter acyl chain (11 carbons) than C1 (26 carbons) with a 4-phenyl-4-fluorophenoxyl group at the end of acyl chain. In this study, the adjuvant effect of C34 was at least comparable or better than C1. This is in line with our recent report that although C1 induced anergy upon repeated administration, C34 does not (Huang et al., 2014). C1 also elicits liver toxicities (Fujii et al., 2005; Nakagawa et al., 2001), but no such toxicities were observed in our preclinical animal toxicity study of C34 (unpublished data). Furthermore, C34 displayed higher binding affinity and stability in CD1d-glycolipid complex to TCR of NKT cells, as we reported (Wu et al., 2011). Moreover, C1 analogs with phenyl group at acyl chain have been shown to induce Th1-biased response with higher ratio of IFN-γ/IL-4 (Chang et al., 2007; Fujio et al., 2006), which could activate CD8 T cells against viral infection. In line with this, we also observed higher ratio of IFN-γ/IL-4 production in C34 adjuvanted group, which may contribute to greater antiviral activities.

DMSO has been widely used as cryoprotectant, for research purposes and clinical applications. It has been shown that DMSO concentration >10% could damage cell through pore formation of plasma membrane (de Menorval et al., 2012). However, there are case reports of using DMSO for the treatment of amyloidosis (Iwasaki et al., 1994; McCammon et al., 1998). In addition, FDA has approved the use of Rimso-50 (50% DMSO) in patients with

bladder inflammation known as interstitial cystitis (FDA important alert 62–06), suggesting that DMSO is well-tolerated at concentrations up to 50%. In this study, C34 and alpha-GalCer were dissolved in DMSO and diluted to a working concentration of 1% of DMSO in PBS before injection. Thus, the toxicity of such a low concentration of DMSO would be negligible. Nonetheless, we are in the process of developing a new formulation of C34 without DMSO for clinical trials.

Although many DNA vaccines are undergoing clinical evaluation, none has yet to be approved by FDA, largely because DNA vaccines have elicited only modest immune responses in humans. We had proven that C34 as an adjuvant for DNA vaccine delivered by IM-EP or IM could enhance protective immunity against H5N1 infection. In addition, C34 is a potent adjuvant not only for DNA vaccine but also for the poorly immunogenic carbohydrate antigen, as demonstrated in our recent study showing that C34 could induce the production of IgG against target carbohydrate (Huang et al., 2013). Moreover, in view of low toxicity profiles of C1 up to 4800 $\mu g/m^2$ in a phase I study (Giaccone et al., 2002), the use of C34 as an adjuvant might be well tolerated in human. Development of C34 as a vaccine adjuvant is warranted.

5. Conclusion

In this study, we provided an easily implemented technology platform to update the HA sequence upon any H5N1 virus outbreak, which can confer broad-spectrum protective immunity when using C34 as an adjuvant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.04.007.

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