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A randomized, double-blind, controlled clinical trial to evaluate the safety and immunogenicity of an intranasally administered trivalent inactivated influenza vaccine with adjuvant LTh(αK): A phase I study

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ABSTRACT

Background: A nasal influenza vaccine has been available only in a live attenuated form, which limits the range of recipients to immune-competent individuals. The present study evaluated a newly developed intranasal inactivated influenza vaccine with a novel adjuvant, heat-labile enterotoxin (LT) derived from *E. coli* (LTh(αK)).

Methods: The study was a randomized, double-blind, controlled phase I trial to evaluate the safety and immunogenicity of an intranasal vaccine containing the trivalent influenza HA antigen (7.5 μg each of A/California/7/09 (H1N1)-like virus, A/Victoria/210/2009 (H3N2) virus, and B/Brisbane/60/2008-like virus) in combination with 4 different doses of adjuvant LTh(αK) (7.5, 15, 30 or 45 μg) and 22.5 μg of influenza HA antigen alone (control vaccine). The vaccine was intranasally administered on Days 0 and 7. A safety evaluation commenced for 180 days, and hemagglutination inhibition (HI) antibody titers and nasal HA-specific IgA titers on Day 0 and Day 28 were assessed to determine whether an immunogenic response was elicited.

Results: From November 2012 to September 2013, a total of 36 subjects were enrolled. Twenty-four subjects received an adjuvanted vaccine, and 12 subjects received a control vaccine. The most common adverse event (AE) was mild nasal discomfort, and systemic AEs were mild fatigue and headache. Only two subjects discontinued the study because of an AE (one had grade 3 fever, and one had nodal arrhythmia). In the group with 45 μg of LTh(αK), the seroprotection rates were 100%, 100% and 80%, and the nasal IgA conversion factors were 7.90, 7.46 and 12.27 for the A/H3N2, A/H1N1 and split B strains, respectively. Adjuvant LTh(αK) vaccine showed a significant enhancement in mucosal immunity in split B -specific IgA.

Conclusion: The intranasal inactivated influenza vaccine is generally safe, and the LTh(αK)-adjuvanted vaccine is more immunogenic than non-adjuvanted control vaccine.

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1. Introduction

The World Health Organization (WHO) recognized influenza as a public health priority in 2012, since influenza contributes to the global disease burden with an annual attack rate of 20–30% among children and 5–10% among adults [1]. According to the most recent modeling study, the global influenza-associated respiratory mortality was as high as 290,000–650,000 annually, which is higher than previous estimates [2]. Thus, the WHO strategic advisory group of experts has recommended annual vaccination for pregnant women, healthcare personnel, children aged 6–59 months, elderly individuals and any individuals with certain risk factors [3]. A survey in the United States documented an increased influenza vaccine coverage rate in all age groups, except the group with

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individuals older than 65, when comparing the 2010–2011 to the 2005–2006 flu season [4]. However, there is still a substantial gap between the actual vaccination rate and the 90% target proposed by Healthy People 2020 [5].

Most conventional flu vaccinations are injected intramuscularly. Nevertheless, influenza vaccines can also act on the respiratory tract, the site of virus entry and the principal location of replication. Intranasal vaccines have the advantage of being simple to administer and could therefore facilitate delivery during mass vaccination campaigns. The first live attenuated influenza vaccine (LAIV) was licensed in Russia in the 1980s [6]. In 2006, the WHO recognized the potential advantage of LAIVs over inactivated influenza vaccines in pandemic situations and initiated a global action plan to manufacture vaccines in China, India, and Thailand [7]. However, the live attenuated nature of the vaccine has limited application, as it can be administered only to a healthy population; it cannot be used in immunocompromised individuals. There is also no consensus for the age group suitable for LAIVs; e.g., it is recommended for persons aged 2–49 years in the USA [8] and for persons aged 2–59 years old in Canada [9].

Thus, an intranasal inactivated vaccine is needed to combine the benefit of easy administration and administration to both immunocompetent and immunocompromised individuals. Different approaches have been tested, including virosome-formulated nasal vaccines, which revealed both HA-specific immunoglobulin and nasal IgA responses [10]. Different adjuvants have also been tested to increase the immune response [11,12]. The use of *E. coli* heat-labile enterotoxin as an adjuvant in animals has been extensively described for >30 years, with a wide variety of vaccine antigens [13,14]. Experiments in humans conducted 20 years ago demonstrated significantly higher antibody titers in humans who received intranasally administered heat-labile toxin-adjuvant influenza vaccines compared with those who received non-adjuvant formulations [15,16]. However, the native heat-labile enterotoxin (LT)-derived from *E. coli* causes side effects. The A1 portion is responsible for ADP-ribosylating activity, which results in increased intracellular cyclic AMP accumulation, causing diarrhea [17]. In addition, the association of Bell's palsy with the administration of NasalFlu (Berna Biotech), a nasal virosome-based vaccine adjuvanted with LT, was reported, which resulted in the withdrawal of NasalFlu [18]. Thus, several genetically modified mutants with reduced or completely absent ADP-ribosylating enzyme activity have been constructed, including LTK63, which has been tested in a phase I influenza vaccine trial [19], and there were no cases of Bell's palsy or diarrhea. However, two cases of transient Bell's palsy were also reported in studies of LTK63-adjuvanted human immunodeficiency virus and tuberculosis vaccines [20]. The mechanism of LTK63-related Bell's palsy has not been well identified, and Lewis et al. hypothesized it to be an accumulation of LTK63 molecules or inflammation following ganglioside binding. Although the two reported cases recovered without long-term sequelae, the phenomenon led to additional safety concerns regarding LTK63.

The Development Center for Biotechnology (DCB), Taiwan, developed a new detoxified version of LT, LTh(αK), with completely inhibited ADP-ribosylating enzyme activity. The preclinical biodistribution study was conducted in Institute of Cancer Research (ICR) male mice, with iodine −131 radiolabeled LThWT, labeled LTh(αK), and labeled LTh(αK) mixed trivalent influenza antigen. The results revealed that the majority of the tested article remained within the nasal passages. There was not a significant amount of the tested article observed in the olfactory bulb, brain, lungs, eyes, plasma, or other tissues [Supplement I]. Thus, we hypothesized that the risk for retrograde inflammation after nasal administration should be low. A preclinical animal study revealed a positive immune response in a ferret model after a 2-dose regimen, 7 days apart [Supplement II and Supplemental Tables S1–3]. To date, there are no reports of clinical experience in humans for the LTh(αK)-adjuvanted trivalent inactivated influenza virus antigen. In the present phase I study (NCT03293732), we aimed to assess the safety and immunogenicity of the intranasally administered trivalent influenza vaccine adjuvanted with LTh(αK) (DCB07030).

2. Materials and methods

2.1. Ethics approval

The study was approved by the Institutional Review Board of National Taiwan University Hospital (201112123 MSA).

2.2. Vaccine and trial design

The study vaccine (DCB07030) is a thimerosal free-split trivalent inactivated influenza virus antigen vaccine (22.5 μg total of influenza HA antigen, 7.5 μg of HA of each three strains) in combination with adjuvant LTh(αK) (developed by DCB and patent transferred to Advagene Biopharma Inc., Taipei, Taiwan) at doses of 7.5, 15, 30 or 45 μg. The inactivated influenza virus antigens included A/California/7/09 (H1N1)-like virus, A/Victoria/210/2009 (H3N2) virus, and B/Brisbane/60/2008-like virus, which are drug substances (DS) of AdimFlu-S trivalent influenza antigen, purchased from Adimmune Corp (Tai-Chun, Taiwan). Adimmune Corp is a seasonal influenza vaccine provider (AdimFlu-S trivalent influenza antigen (DOH-BM-000113)) for Taiwan and China. The DS was produced by monovalent pool harvest (MPH). The virus was produced in embryonated eggs. Following the inoculations, eggs were incubated, and the virus was harvested, pooled, centrifuged, filtered and ether-treated to split the virus. The ether-split MPH was formalin inactivated. The antigen of each MPH was analyzed by single radial immunodiffusion. The drug product used in this study contained 7.5 μg of split antigen from each strain combined with LTh(αK). The control vaccine is the same split trivalent inactivated influenza virus antigen vaccine (22.5 μg total of influenza HA antigen, including 7.5 μg of HA of the three strains identical to the study vaccine) without adjuvant.

This phase I trial was a single center, double-blind, randomized, dose-escalation design with sequential subject enrollment. The vaccine was intranasally administered on Day 0 and Day 7.

2.3. Participants

A total of 110 healthy Taiwanese volunteers aged 20–40 years old were screened, and 36 subjects were sequentially enrolled in this study. Participants with rhinitis or sinusitis and those who received any intranasal medication or nasal topical treatment within 7 days prior to enrollment were excluded. We also excluded subjects with laboratory-confirmed influenza and those who had been vaccinated against influenza within 6 months prior to enrollment. The additional inclusion and exclusion criteria are available in the supplementary documents (Supplemental Table S4).

2.4. Randomization and masking methods

Subjects who signed the informed consent and met the eligibility criteria were randomized within each cohort in a 2:1 ratio (blocking size of 3) to receive adjuvanted influenza vaccine or control vaccine on study Day 0 and Day 7. The blocked randomization was generated using PROC PLAN (SAS®). The randomization numbers were inspected and verified by an independent staff member prior to delivery to the project manager. Then, the program of randomization code generation was deleted, and only the coding seed

was saved. Subjects were assigned consecutive randomization numbers as they were entered into the study.

2.5. Administration

Two doses of the study vaccine or control vaccine were intranasally (100 μl in each nostril) administered on study Day 0 and Day 7 by a BD Accuspray device (Becton, Dickinson and Company, France.) An assigned ear, nose, and throat (ENT) doctor was in charge of administering the vaccine throughout the study.

The participants in the 4 cohorts were enrolled and treated sequentially with increasing doses of adjuvant. In each cohort, the proportion of the study vaccine groups to the control vaccine groups was 2:1 (Supplemental Table S5). Safety data were reviewed by a data and safety monitoring board (DSMB) after the completion of dosing in each cohort. At least a 7-day period passed between the last subject receiving the second dose in the lower cohort and the first subject receiving the first dose of the next higher adjuvant dose in the higher cohort to allow the DSMB to review the complete safety data for a cohort.

2.6. Concomitant and prohibited therapies

Concomitant medications deemed necessary for the safety of the subject were allowed, and their use is documented in the medical records and in the case record forms (CRFs). The following concomitant treatments were prohibited: Chinese medications and herbal medications, immunosuppressants, antiviral drugs, intranasal medication or nasal topical treatment, steroid, antibiotics and other investigational drugs.

2.7. Endpoint assessment

Safety assessment included vital signs, pre- and postvaccination physical examination findings, laboratory examination findings (including hematology, blood chemistry and urinalysis), electrocardiogram (ECG), cardiac echo, ophthalmoscopy, and chest X-ray. Diary cards were provided for participants to record the solicited events through Day 0 to Day 28. The unsolicited events and serious adverse events (SAEs) were collected from Day 0 to Day 180. The proportion of subjects in each treatment group with any indication of an adverse event (AE) was tabulated for each solicited and unsolicited event at intervals from Days 0–6, Days 7–13, Days 14–28 and Days 0–180.

2.8. Immunogenicity assessment

2.8.1. Measurement of antibodies by hemagglutination inhibition (HI) test

Details of the procedure for the HI test were described previously [21,22]. Briefly, all sera and nasal washes from swabs were stored at −80 °C until they were tested. Serum nonspecific inhibitors were treated with RED kits (Denka Seiken Co. Ltd. Tokyo, Japan) overnight at 4 °C, followed by inactivation at 56 °C for 30 min. Nonspecific agglutinator was removed by absorption with 50% turkey red blood cells. The antigens used as vaccine strains in the test were provided by AdimFlu-S MPH.

2.8.2. Measurement of antibodies by enzyme-linked immunosorbent assay (ELISA)

Measurement of antibodies by ELISA has been described previously [23]. In brief, the influenza antigens were diluted and coated onto a microtiter plate with coating buffer (KPL, Seracare, MA, USA). In the first incubation, diluted sample sera were incubated in influenza antigen-coated wells for two hours at 37 °C. After washing away the unbound substances, the horseradish peroxidase (HRP)-labeled antibody was added, followed by a two-hour incubation at 37 °C. Following another round of washing, the tetramethylbenzidine (TMB) substrate solution was added, followed by the addition of acid stop solution to terminate the reaction. The measurement was conducted by the optical density (OD) at selected wavelengths. The calibration curve for assessment of vaccine-specific IgG was established using a pool of serum or nasal wash samples with detectable ELISA signals.

2.8.3. Measurement of nasal IgA antibody response

The nasal specimens were collected by inserting the swab (NFS-SWAB APPLICATOR™, Noble Bio) into the nostril, gently rotating the swab inward until resistance was at the turbinates. The swab was then inserted into container with Universal Transport Medium. All samples were stored at −80 °C before testing [24].

Total nasal IgA was quantified by the Total Human IgA Assay Kit (Diagnostic Automation, Inc. Cat. No. 1802-09) following the manufacturer's instructions. Chromatographically purified human secretory IgA (Biorbyt, Cat. No. orb22122) was used as a reference sample. Before the total human IgA assay, the test samples were treated with receptor destroying enzyme (RDE) II as recommended by the manufacturer (Tunyen, 370013) and then diluted 40-fold with the IgA specimen diluent (KPL, Seracare, MA, USA). The reference sample was diluted with the IgA specimen diluent to generate six concentrations as a calibration curve. Total IgA concentrations of test samples were calculated against a calibration curve of the reference sample using a four-parameter logistic curve fitting technique.

The primary immunogenicity endpoint was the pre- to postvaccination change in the geometric mean of serum HI antibody titers. Geometric mean titers (GMTs) of HI were calculated by taking the anti-log of the mean log titer transformation. For calculation purposes, titers below the cut-off value were given an arbitrary value of half the cut-off value at different time points. Serum HI titers for each of the three vaccine components were performed prevaccination on Day 0 and Day 28 after the first vaccination.

The secondary immunogenicity endpoints included: (1) seroconversion rate, seroprotection rate and seroconversion factors of HI. The seroconversion rate is defined as the percentage of subjects who have a prevaccination HI titer <1:10 and postvaccination titer ≥1:40 or prevaccination titer ≥1:10 and at least a 4-fold increase in postvaccination titer. The seroprotection rate is defined as the percentage of subjects in each treatment group who developed seroprotective levels of antibody (HI titer >1:40). The seroconversion factor is defined as the fold increase in GMTs of HI after vaccination. All HI antibody titers were determined in blinded samples.

For adults aged 18–60 years, the seroconversion rate, seroprotective rate and seroconversion factor of a listed influenza vaccine should fulfill the requirements of 40%, 70% and 2.5, respectively, according to Committee for Proprietary Medicinal Products (CPMP) guidelines [25]; (2) anti-flu antigen IgA antibodies were assessed in deep nasal swab samples prevaccination on Day 0 and Day 28 post first vaccination; (3) GMTs of anti-LTh(αK) serum IgG and nasal IgA antibodies were assessed on Day 0 and Day 28 post first vaccination.

2.9. Statistics

Safety endpoints are listed and summarized as appropriate. Continuous variables were evaluated using Student's *t* test and are presented as the mean/median and standard deviation (SD) for normally distributed data. ANOVA was used for intergroup comparisons. For categorical data, the chi-squared test or Fisher's exact test was used to examine differences between groups, and the data are presented as the number and proportion. Summary

statistics, including two-sided 95% confidence intervals (CI), are presented for each immunogenicity parameter for each study group and the pooled control vaccine group. All the statistics were performed by SAS 9.3 (North Carolina, USA).

3. Results

A total of 110 healthy adult Asian volunteer subjects were screened, and 36 subjects were sequentially enrolled in the 4 study cohorts from November 28, 2012, to September 30, 2013. All 36 subjects who received at least one dose of study vaccine or control vaccine were included in the safety assessment for demographic and safety analyses. Among the 36 participants, nine (9/36, 25.0%) were male, and 27 (27/36, 75.0%) were female. The mean age was 29.1 years. There were no significant differences in age, sex, or BMI distribution among the four vaccination cohorts (Table 1).

A total of 34 subjects received the 2 scheduled doses of vaccination. Two subjects withdrew due to grade 3 AEs and did not receive the second vaccination (Fig. 1). We defined the subjects who received HA + 7.5 μg LTh(αK) vaccination as Group 1; HA + 15 μg LTh(αK) as Group 2; HA + 30 μg LTh(αK) as Group 3; HA + 45 μg LTh(αK) as Group 4; and HA alone as Group 5. The withdrawal rate was not significantly different among the five groups (P = 0.62).

3.1. Safety analysis

3.1.1. Solicited AE

After the first dose of vaccine, a total of 64 local AEs were reported by 22 subjects (61.1%), and 46 systemic solicited AEs reported by 19 subjects (52.8%) were recorded (Fig. 2). The most frequently reported local AE was mild nasal discomfort related to intranasal administration (including stuffy nose, runny nose, sneezing, and other nasal discomfort). Two participants showed changes in vision (subjectively and transiently, no related abnormality could be identified by the ophthalmologist). Most of the reported local AEs were of grade 1 severity (61/64, 95.3%).

The most frequently reported solicited systemic AEs were fatigue and headache. The intensity of most systemic AEs was mild. AEs possibly/probably related to the study vaccine accounted for 65.2% (30/46). One subject reported an SAE with fever (39.7 °C), runny nose and headache the day after the first vaccination (Group 4) and withdrew from the study. The event was evaluated by an investigator and diagnosed as tonsillitis, which was unlikely to be related to the study vaccine. Compared with the Group 5, only Group 1 had a significantly higher percentage of participants presenting with fatigue (83.3% in Group 1 and 16.7% in Group 5, P = 0.03), and Group 2 reported a higher percentage presenting with headache (50.0% in Group 2 and 0.0% in Group 5, P = 0.04). There were no significant differences in the percentage of other systemic AEs between each test group and the Group 5.

After the second dose of vaccination, 53 local and 30 systemic solicited AEs were reported by 19 (55.9%) and 15 (44.1%) subjects, respectively (Fig. 2). The most frequently reported local AE was mild nasal discomfort related to intranasal administration (including stuffy nose, runny nose, sneezing, and other nasal discomfort). No subject reported any changes in vision. Compared with the Group 5, only Group 1 had a higher proportion of participants who presented with a runny nose (66.7% in group 1 and 9.1% in group 5, P = 0.05). There were no significant differences in the percentage of local AEs between each test group and the Group 5.

The most frequently reported solicited systemic AEs were fatigue and headache (Fig. 2). No fever was reported. Most of the solicited systemic AEs were mild (27/30, 90.0%), and 73.3% (22/30) of the AEs were related to the study vaccine. No severe solicited systemic AEs were reported after the second vaccination.

The solicited local and systemic symptoms after the first and second doses of vaccine did not demonstrate a dose-response relationship with adjuvant LTh(αK).

3.1.2. Unsolicited AEs

Most hematology and blood chemistry results were within the normal range at the baseline and final visits. A total of 82 unsolicited AEs were reported by 28/36 subjects (77.8%). There was no statistical difference among the five vaccination groups. Most of the unsolicited AEs were mild (62/82, 75.6%) or moderate in severity (19/82, 23.2%) and unlikely/not related (56/82, 68.3%) to vaccination.

Asymptomatic hematuria was the most common AE (Supplemental Table S6). Among the 13 subjects with hematuria, most (11/13) had mild hematuria that appeared transiently (red blood cell (RBC) < 20/ high power field (HPF)), which resolved at the follow-up urinalysis 7–14 days later. For the two participants who had moderate hematuria, one event was noted on Day 7, which also resolved spontaneously at the follow-up urinalysis on Day 14. The other participant had hematuria on Day 28, which was related to her menstruation.

There was only one SAE during the study period. The participant developed nodal arrhythmia approximately 2 h after the first intranasal vaccine was administered, accompanied by mild dizziness but no other discomfort or hypotension. A close serial follow-up EKG found that nodal arrhythmia improved within the same day. However, nodal arrhythmia without any subjective symptoms persisted for several weeks, and no medical treatment was required. The investigator judged that prominent nodal arrhythmia on the

Table 1
Baseline characteristics of enrolled participants.

		Cohort 1		Cohort 2		Cohort 3		Cohort 4	
		HA	HA + 7.5 μg LTh(αK)	HA	HA + 15 μg LTh(αK)	HA	HA + 30 μg LTh(αK)	HA	HA + 45 μg LTh(αK)
n		3	6	3	6	3	6	3	6
Age (years)	Mean ± SD	24.9 ± 1.5	29.0 ± 4.4	30.8 ± 2.7	34.1 ± 4.5	31.7 ± 6.2	28.8 ± 3.9	25.8 ± 3.8	26.5 ± 2.4
P value		0.18		0.29		0.55		0.55	
Sex	Male	0	0	0	1	0	2	3	3
	Female	3	6	3	5	3	4	0	3
P value		N/A		1.00		0.50		0.46	
Weight (kg)	Mean ± SD	54.7 ± 2.5	52.2 ± 5.6	59.3 ± 7.0	57.8 ± 3.7	52.4 ± 7.8	55.4 ± 8.7	69.2 ± 9.7	59.4 ± 9.2
P value		0.49		0.22		0.59		0.37	
Height (cm)	Mean ± SD	158.3 ± 4.2	157.1 ± 4.9	161.0 ± 4.4	163.1 ± 3.6	157.7 ± 2.3	160.6 ± 7.3	172.0 ± 7.9	163.2 ± 9.7
P value		0.72		0.46		0.53		0.25	
BMI (Kg/m^2)	Mean ± SD	21.9 ± 2.1	21.2 ± 2.0	22.9 ± 2.9	20.6 ± 1.3	21.1 ± 1.7	21.5 ± 2.7	23.3 ± 1.4	22.9 ± 1.7
P value		0.64		0.12		0.94		0.75	

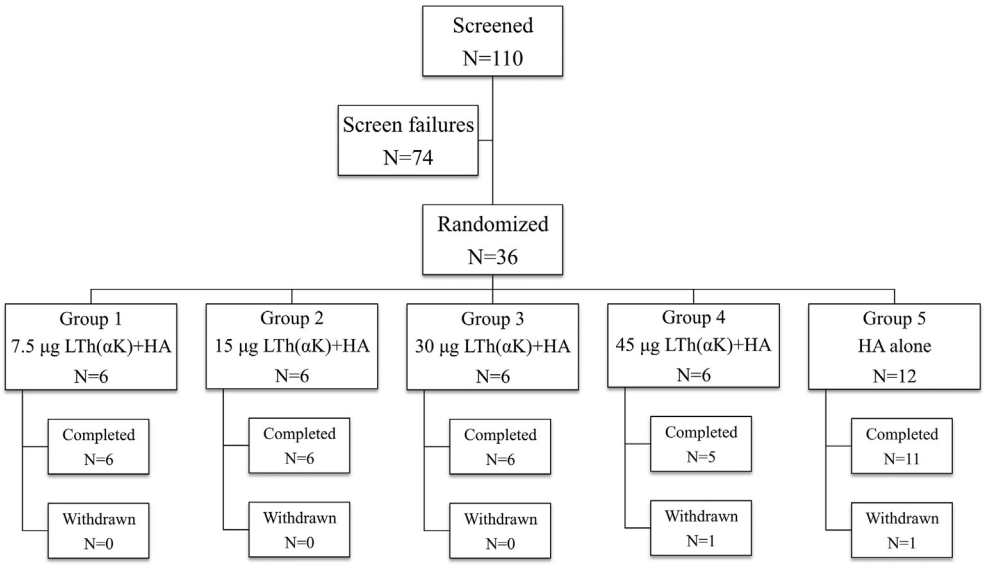

Fig. 1. Enrollment algorithm. One subject in the Group 4 withdrew because of grade 3 fever due to an unsolicited AE of tonsillitis. One subject had a grade 3 unsolicited AE of nodal arrhythmia possibly related to the study vaccine (Group 5). The withdrawal rate was not significantly different among the five groups (P = 0.62).

first day after vaccination was possibly related to the administration of the vaccine.

3.1.3. Withdrawal

Two subjects withdrew from the study and did not receive the second vaccination. One subject (Group 4) had grade 3 fever due to an unsolicited AE of tonsillitis. The subject showed high fever (39.6 °C) one day after receiving the first dose. After examination, the investigator made the diagnosis of acute tonsillitis, and antibiotics were prescribed. The fever and symptoms improved after antibiotic treatment, and the tonsillitis was fully resolved in two weeks. The event was judged as unlikely related to the vaccine by the investigator. One subject (Group 5) had a grade 3 unsolicited AE of nodal arrhythmia (as prescribed in unsolicited AEs) and judged as possibly related to the administered vaccine by the investigator.

3.2. Immunogenicity analysis

A total of 34 subjects completed the study and received the 2 scheduled doses of intranasal administration of vaccines. The 34 subjects were included in the analysis of immunogenicity.

3.3. Primary endpoint: GMT of serum HI

All GMTs increased on Day 28 in all groups (Fig. 3). The Group 4 had the highest HI titer against H3N2 (52.78) and H1N1 (69.64). The results showed that the highest GMT of HI on Day 28 to split B was present in the Group 1 (89.8). Compared with Day 0, the HI titer against H3N2 in the Group 4 and against split B in the Group 1 was significantly higher on Day 28 (P = 0.03 and <0.01, respectively).

In general, the Group 5 demonstrated lower HI titers than the four test vaccine groups on Day 28. Only the HI titer against H1N1 in the Group 2 was lower than that in the Group 5 (GMT 8.91 versus 13.70, P = 0.53), but the difference was not statistically significant. The HI titer against H1N1 in the Group 4 was significantly higher than that in the Group 1 (GMT 69.64 versus 13.70, P < 0.01).

3.4. Secondary endpoint

3.4.1. Seroconversion rates (SCR)

On Day 28, a high dose of adjuvant seemed to help achieve high seroconversion rates (SCR). The Group 4 had SCRs of 20.0% (1/5), 40.0% (2/5) and 60.0% (3/5) for the H1N1, H3N2, and split B strains, respectively (Fig. 4A). The SCRs in other groups were highly variable for the three influenza virus strains. None of the 4 study vaccine groups were significantly different from the Group 5.

3.4.2. Seroprotection rate (SPR)

The Group 4 achieved seroprotection rates (SPRs) for H1N1 (100%, 5/5), H3N2 (100.0%, 5/5) and split B (80.0%, 4/5). The Group 1 and Group 3 also achieved the required SPR for split B strains (100%, 6/6 and 83.3%, 5/6, respectively) but not for H3N2 (50.0%, 3/6 and 66.7%, 4/6, respectively) and H1N1 (16.7%, 1/6 and 16.7%, 1/6, respectively). The Group 2 and Group 5 achieved the lowest SPR (for H3N2, H1N1, and split B as 66.7, 16.7 and 33.3% and 45.5, 18.2, and 36.4%, respectively) (Fig. 4B). Among the 5 groups, Group 4 achieved an effective SPR. Compared with Group 5, Group 1 had a significantly higher SPR (P < 0.01) for H1N1.

3.4.3. Seroconversion factors

On Day 28, the seroconversion factors revealed a ≥2.5-fold increase in the GMT HI titers for H3N2 in Group 2 and split B strains in Groups 1 and 4 (Fig. 4C). The Group 3 and Group 4 showed elevated seroconversion factors with a ≥2-fold increase in GMT HI titers for H3N2 and split B strains. All groups did not elicit seroconversion factors ≥1.78 for H1N1. Compared with the Group 5, the four study vaccine groups had no significant differences in seroconversion factors for H1N1, H3N2, or split B.

3.4.4. Nasal mucosal IgA antibodies

On Day 0, the prevaccination IgA values were low in all groups. On Day 28, the GMT of the IgA increased against all three influenza virus strains in groups receiving adjuvanted vaccines (Fig. 5). Compared with Day 0, the GMT of nasal IgA in the Group 5 on Day 28 was nonsignificantly elevated for H3N2, H1N1, and split B (P = 0.75, 0.44, and 0.34, respectively). For split B, the adjuvanted vaccine groups revealed a significantly elevated GMT from Day 0

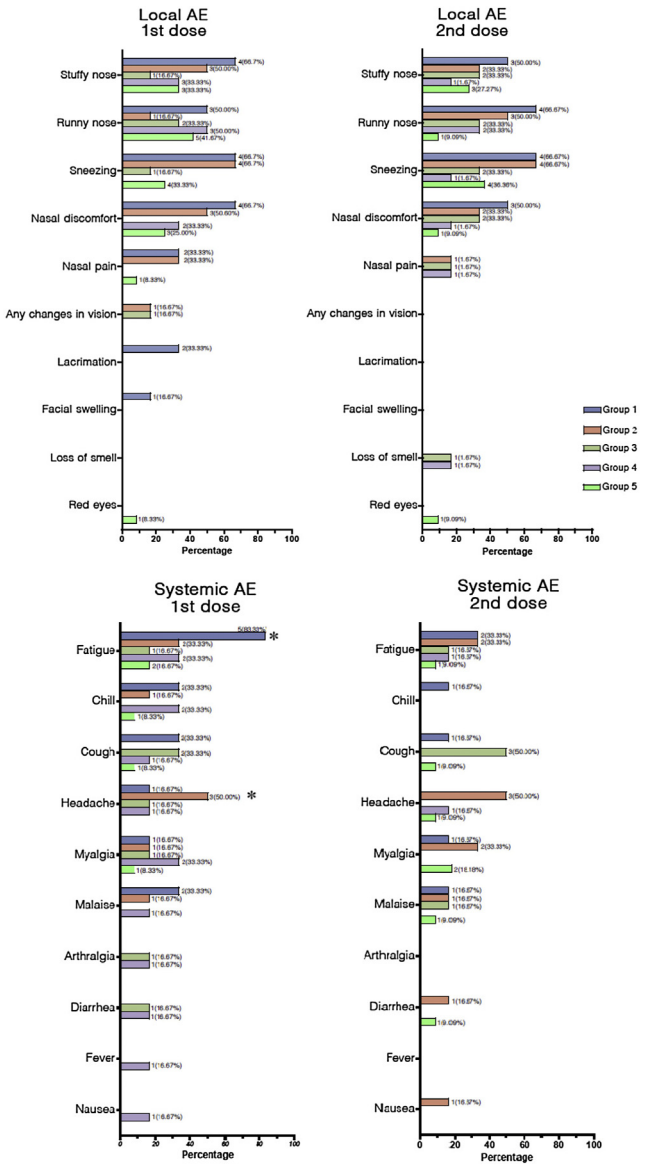

Fig. 2. Solicited adverse events after the first and second vaccinations. Group 1: HA + 7.5 μg LTh(αK); Group 2: HA + 15 μg LTh(αK); Group 3: HA + 30 μg LTh(αK); Group 4: HA + 45 μg LTh(αK); Group 5: HA alone. *In regard to systemic AEs, there was a statistically higher proportion of fatigue in Group 1 (P = 0.03) and headache in Group 2 (P = 0.04) than in Group 5 after the first dose, and no other groups had statistically significant differences compared with Group 5.

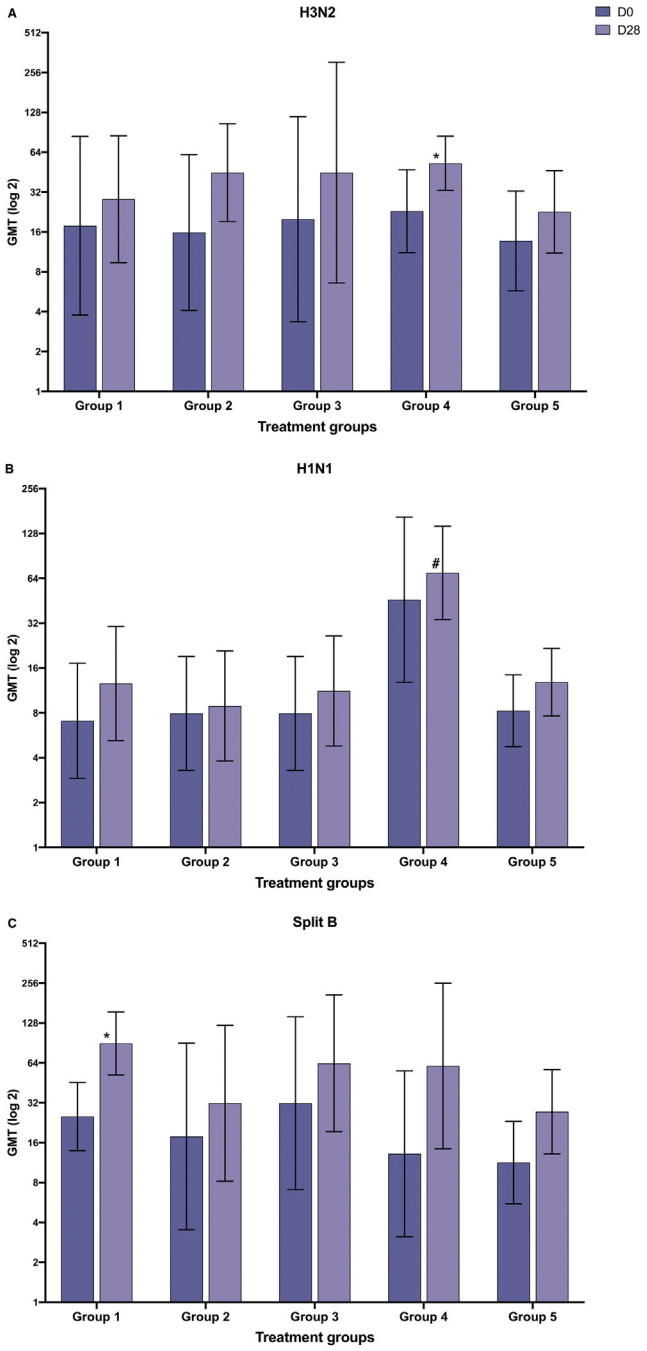

Fig. 3. Geometric mean titers (GMTs) of serum HI in the five vaccination groups. *Compared with Day 0, the HI titer against H3N2 in Group 4 and against split B in Group 1 was significantly higher on Day 28 (P = 0.03 and <0.01, respectively). #The HI titer against H1N1 in Group 4 was significantly higher than that in Group 5 on Day 28 (GMT 69.64 versus 13.70, P < 0.01).

to Day 28 (P = 0.05, 0.02, 0.03 and < 0.01 in Groups 1, 2, 3 and 4, respectively).

Unlike the seroconversion factor of serum HI titers, most adjuvanted vaccine groups showed increasing nasal IgA titers against HA antigens on Day 28 (Fig. 6). Group 5 did not elicit IgA reactivity (1.46- to 2.13-fold). The Group 4 had the highest fold increase in GMTs across the three strains, 7.46 (H1N1), 7.90 (H3N2) and 12.27 (split B), followed by the Group 1 (4.37–8.49). The geometric mean ratio (GMR) of nasal IgA titers for split B in the Group 4 was significantly higher than that in the Group 5 (P = 0.03).

3.5. Immune response of LTh(αK)

The seroconversion factors for LTh(αK) showed dosage escalation responses from 10.11 for the Group 1 to 32.63 for the Group 4 (Supplemental Fig. S1A). The fold increase in nasal IgA titers to LTh(αK) also showed dose-escalated responses from 4.45 for Group 1 to 16.09 for Group 4 (Supplemental Fig. S1B).

4. Discussion

The present study evaluated the safety, tolerability, and immunogenicity of intranasally administered trivalent influenza vaccine (DCB07030), which combines 22.5 μg of trivalent influenza HA antigen with 4 different doses of adjuvant LTh (αK) and influenza HA antigen alone. The DCB07030 vaccine was generally safe

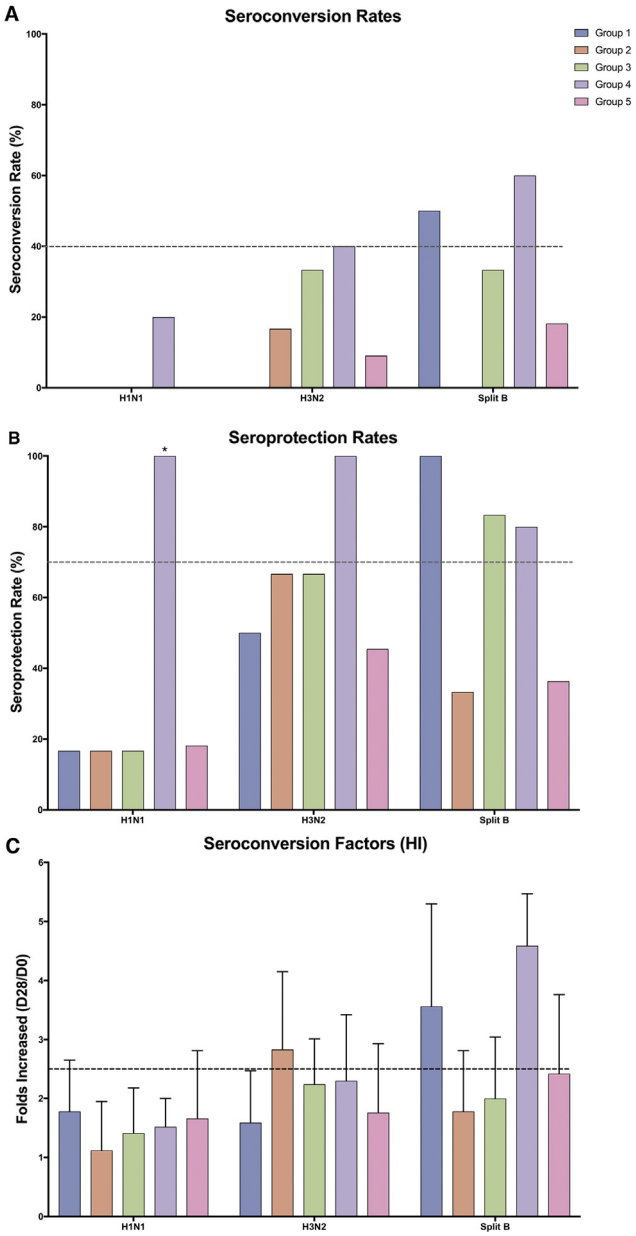

Fig. 4. (A) Seroconversion rate; (B) seroprotection rate; (C) seroconversion factors in the five vaccination groups. *Group 4 had a significantly higher seroprotection rate for H1N1 than did Group 5 ($P < 0.01$).

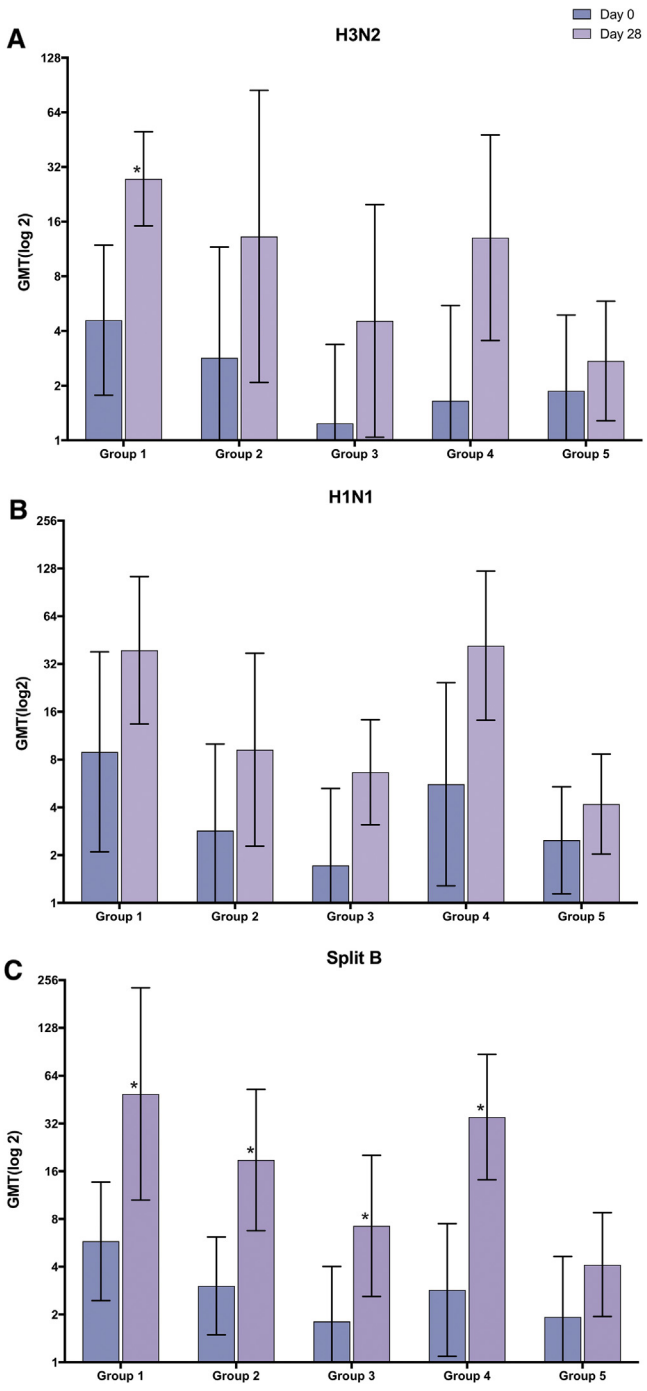

Fig. 5. Nasal mucosal IgA antibody response in the five vaccination groups. *The GMT of nasal IgA for split B increased significantly among all four study vaccine groups from Day 0 to Day 28 ($P = 0.05$, 0.02, 0.03 and <0.01 in Groups 1, 2, 3 and 4, respectively). The IgA for H3N2 also increased significantly in Group 1 ($P < 0.01$).

in 36 adult healthy Taiwanese volunteers and had acceptable immunogenicity. Solicited AEs related to vaccine were mostly mild. Most unsolicited AEs were also mild and may not be related to the vaccine. No participants developed Bell's palsy during follow-up.

The intranasal route of influenza vaccine has gained interest due to the advantage of eliminating injection-associated discomfort, cuts down on medical waste and may be more conveniently administered than injected vaccines [26]. More importantly, the intranasal influenza vaccine can induce a secretory IgA response in the nasal mucosa, where the infection occurs. IgA plays an important role in natural immunity against mucosal infection, and its polymeric form has been shown to prevent virally induced pathology in the upper respiratory tract of a murine model [27]. Muramatsu et al. demonstrated an improvement in the antiviral activity of polymeric IgA via cross-binding by cloning a monoclonal anti-HA IgG to an IgA backbone [28]. Furthermore, a clinical study revealed that the neutralizing capability of human IgA improved with increasing polymerization, indicating that polymeric IgA provides protection against both homologous and variant influenza viruses [29]. Currently available intranasal influenza vaccines are only available as LAIVs [30]. The LAIVs may boost IgA against influenza infection but have limited application in a healthy population [8,9], while immunocompromised and elderly populations are vulnerable to influenza-related complications.

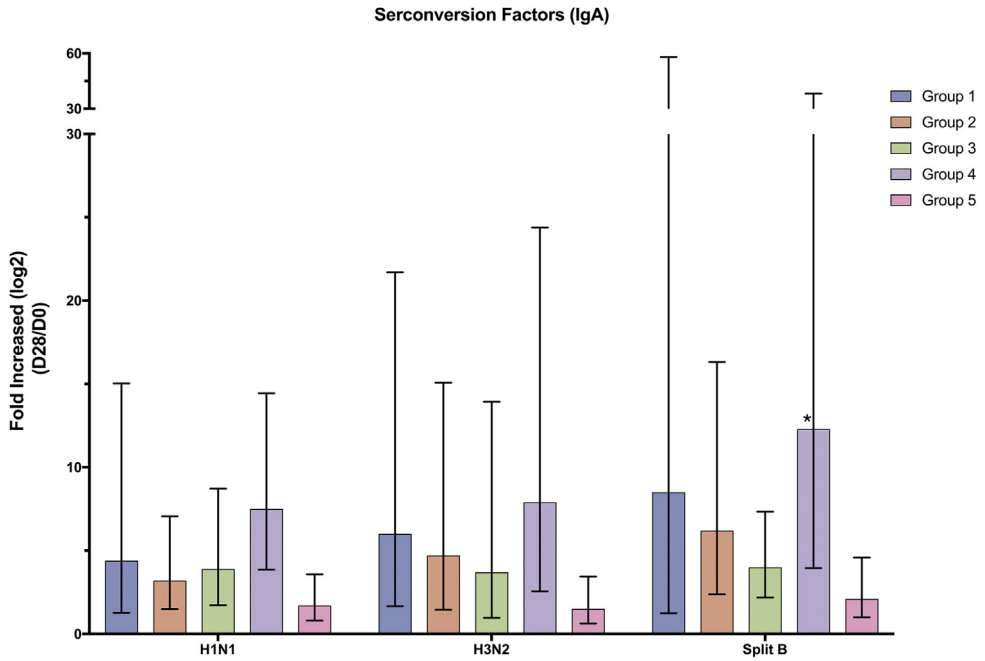

Fig. 6. Conversion factor of nasal IgA. *The folds increase of GMT of nasal IgA titers for split B in Group 4 was significantly higher than that in Group 1 (P = 0.03).

There have been different attempts to facilitate the immune response for intranasally administered influenza vaccines, and some of them had positive results in preclinical studies. These new attempts can be divided into two categories: (1) Delivery system: The prior virosome-based vaccine was withdrawn, possibly due to the side effect of Bell's palsy [18], and nanogel-based nasal vaccines were a new attempt. Current evidence has revealed effective immunity and no brain deposition after nasal administration in mice and nonhuman primates [31]. Mucoadhesive liposomes or intranasal immunization with an avian influenza virus vaccine have also revealed effective immunity in chicken experiments [32]. (2) Different adjuvants: Gram-positive enhancer matrix (GEM) derived from *Lactococcus lactis* was used as an adjuvant for intranasal influenza vaccine in mice and revealed a strongly enhanced immune response [33]. ISCOMATRIX (IMX)-adjuvanted intranasal influenza vaccine, which has particles composed of quillaia saponin, cholesterol, and phospholipids, was tested in mice and sheep and achieved a better serum HA titer and mucosal IgA response than non-adjuvanted vaccine [34,35]. In contrast to most other approaches, the available results were only from preclinical animal studies. Here, we presented a human phase I trial based on one intranasal influenza vaccine with a new adjuvant of LTh (αK).

In our study with the inactivated influenza vaccine, we noted that Group 4 was the most effective dose for IgA enhancement. The effect of heat-labile enterotoxin was previously demonstrated, as Tumpey et al. showed that mice receiving inactivated H3N2 vaccine with LT(R192G) via the intranasal route developed cross-immunity against lethal challenge from H5N1 [36]. In our phase I study, we further provided evidence of the IgA response in humans, and the intranasally inactivated nature showed the potential to achieve targeted local immunity in both immunocompetent and immunocompromised hosts.

Conventionally, the approval of influenza vaccines by either the European Medicines Agency (EMA) or US Food and Drug Administration (FDA) is based on the ability to induce serum IgG antibody responses. The FDA criteria for serum HI antibody responses in adults younger than 65 years are as follows: >40% (lower limit of 95% CI) of participants must seroconvert or show a significant increase in antibody titer; or >70% (lower limit of 95% CI) of participants must show a reciprocal titer of 40 after vaccination. The EMA criteria additionally suggest a geometric titer increase of >2.5-fold to each strain. The present results show that Group 4 fulfilled the FDA requirement by inducing seroprotection to all three strains. The lower seroconversion rate to H1N1 may be due to the sensitivity of the HI assay and/or low immunogenicity to a particular strain.

There was no clear dose response to the HI assay among the adjuvant groups, even though the highest elevated GMTs were reported from the Group 4. This finding may be due to the very small sample size enrolled in this phase I clinical trial. The other possible explanation is that the quantity of vaccination received by the route of intranasal administration may be different based on the administrator. To prevent this bias, we had a well-trained ENT doctor administrate the vaccine throughout the trial. In addition, the HI and IgA response to LTh(αK) both revealed positive dose responses to the adjuvant. Thus, the seroresponse to different influenza vaccine strains warrants further study.

The prevalence of local and systemic AEs after the first and second dose vaccination in the present study was 56–61% and 44–53%, respectively, which are similar to prior clinical trials of other intranasal inactivated influenza vaccinations [12]. Nasal discomfort and headache and fatigue were the most common local reactions and systemic AEs, which were similar to previous intranasal influenza vaccine studies by Glueck R et al. [11,12]. Some participants developed hematuria after vaccination, which was not LTh(αK) dose-dependent and could also be observed in the control vaccine group. The phenomenon was not reported in the phase I LTK63-adjuvanted intranasal vaccine [18]. However, prior studies reported that the inactivated influenza vaccine may be associated with hematuria, likely due to the inactivated virus causing inclusion bodies and fusion to cells within the epithelium of the kidneys that quickly revert to normal after vaccination. This reaction was also observed after influenza infection or influenza vaccination [37]. Although all subjects recovered spontaneously from hematuria in our present study, this phenomenon warrants further follow-up in future studies.

Two subjects withdrew from the present study. One subject withdrew due to tonsillitis, which was judged by an investigator as a nonvaccine-related AE. The other subject, who was in the Group 5, withdrew due to nodal arrhythmia. Thus, the adjuvanted intranasal influenza vaccine was generally well tolerated. Further studies on the impact of adjuvant doses on the occurrence of AEs and immunogenicity are needed.

The limitation of the study is the limited study size in the phase I design. The participants had different baseline seroresponses to H1N1 antigen. However, the phase I study was designed to explore the potential AEs of the novel adjuvant and the intranasal delivery route in titrated doses. The results revealed no clear dose response to the adjuvant in the local and systemic AEs, and most of these adverse events were mild. Thus, the effectiveness and preferred adjuvant dose should be further studied in future phase II trials.

5. Conclusion

The present study revealed that intranasally delivered, LTh(αK)-adjuvanted influenza vaccines are safe and capable of inducing enhanced immune responses compared with non-adjuvanted vaccines. Further phase II trials are warranted to identify the ideal immunogenic dose in future vaccine development.

Authorship

Pan SC and Hsieh SM prepared the manuscript. Pan SC, Hsieh SM, Lin CF, and Chang SC designed and conducted the study. Chang SC, Hsu YS and Chang M coordinated and organized the study. Pan SC, Hsieh SM, and Chang SC analyzed the data and interpreted the results independently of Advagene Biopharma Co., Ltd. and the Development Center for Biotechnology.

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Conflict of interest

The findings and conclusions of the present study are those of the authors and do not necessarily represent the official position of Advagene Biopharma, the patent owner of DCB07030.

The authors at National Taiwan University Hospital have no conflicts of interest and analyzed the data and interpreted the results independently of Advagene Biopharma Co., Ltd. and the Development Center for Biotechnology.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.02.006.

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