Analysis of the immunogenicity and bioactivities of a split influenza A/H7N9 vaccine mixed with MF59 adjuvant in BALB/c mice

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ABSTRACT

The H7N9 influenza virus caused significant mortality and morbidity in humans during an outbreak in China in 2013. A recombinant H7N9 influenza seed with hemagglutinin (HA) and neuraminidase (NA) gene segments from A/Zhejiang/DTID-ZJU01/2013(H7N9) and six internal protein gene segments from A/Puerto Rico/8/34(H1N1; PR8) were generated using reverse genetics. We sought to determine the immunogenic, protective properties, and mechanisms of a split avian influenza A/H7N9 vaccine mixed with MF59 adjuvant in comparison to vaccines that included other adjuvant. BALB/c mice were vaccinated with two doses of different amounts and combinations of this novel A/ZJU01/PR8/2013 split vaccine with adjuvant. Mice were subsequently challenged with A/Zhejiang/DTID-ZJU01/2013(H7N9) by intranasal inoculation. We verified that MF59 enhanced the HI, MN, and IgG antibody titers to influenza antigens. Compared with alum, MF59 could more potentially induce humoral immune responses and Th2 cytokine production after virus infection, while both MF59 and alum can slightly increase NK cell activity. This split H7N9 influenza vaccine with MF59 adjuvant could effectively induce antibody production and protect mice from H7N9 virus challenge. We have selected this vaccine for manufacture and future clinical studies to protect humans from H7N9 virus infection.

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1. Introduction

The threat of a novel influenza H7N9 virus, A/Shanghai/1/2013 (H7N9), and related isolates were highlighted by outbreaks in China in March 2013 [1]. Subsequently, a second wave of infections occurred in the winter of 2013–2014 [2]. A total of 571 cases that caused 212 deaths (37.1%) had been reported to the World Health Organization by February 23, 2015 [3].

A more feasible approach to reducing the impact of a pandemic would require the development of safe and effective vaccines against H7N9 [4]. One recent advance in influenza vaccine production is the utilization of plasmid-based reverse genetics systems [5], which can provide advantages in the development of seed strains, such as decontaminate to remove pathogenic traits at the plasmid stage [6]. Reverse genetics technology has been applied to construct candidate viruses that harbor HA and NA glycoproteins of A/Zhejiang/DTID-ZJU01/2013(H7N9), while all other internal genes were derived from A/Puerto Rico/8/34(H1N1; PR8). Subunit vaccines, such as split or subvirion vaccines, are thought to be safer alternatives. HA is the mainstay of conventional influenza vaccine approaches [7], efforts to develop vaccines against H7-containing subtypes have been hampered because these HAs are poor vaccine antigens [8–10], although antibody responses could be improved substantially with adjuvants. The oil-in-water adjuvant MF59 has been reported to improve the antibody response, permit dose sparing, enhance efficacy, and lower the antigen dose required to induce protection. It also has an acceptable safety profile when paired with inactivated influenza vaccines [11,12]. Clinical trial also verified the MF59 adjuvant even boost the 3.75 μg split H7N9 to achieve the HI titer of 40 or greater [13]. Alum is a conventional adjuvant that has been used for over 80 years, and is the only adjuvant that is currently widely licensed for human use [14]. To determine whether
MF59 or alum was a better adjuvant for this H7N9 influenza split vaccine, we compared the adjuvanticity of MF59 and other adjuvanted split vaccines.

Several H7N9 seed strains, which were mostly generated using HA and NA sequences of the A/Shanghai/2/2013 and A/Anhui/1/2013 strains, form the basis of H7N9 candidate vaccines [15]. Importantly, this split vaccine is the first to use the A/Zhejiang/1/2013 strain. Herein, we carried out these preclinical experiments in a relatively comprehensive and systematic manner to evaluate the immunogenicity and mechanisms of this split avian influenza A/H7N9 vaccine mixed with MF59 adjuvant in mice.

2. Materials and methods

Male BALB/c mice that were 6–7 weeks old were purchased from Joint Ventures SIPPER-BK Experimental Animal Co. (Shanghai, China). All animal studies were performed in accord with the Guide for the Care and Use of Laboratory Animals of Zhejiang Province and were approved by the local Ethics Committee. Zhejiang Tianyuan Bio-Pharmaceutical Co., Ltd. (which was affiliated with Novartis Vaccine Inc.) provided the MF59™ adjuvant (MF59 is a trade mark of Novartis AG and Affiliate Companies) and the Yuganning seasonal flu vaccine, which contains 15 μg HA of each of the following substances: A/California/7/2009 (H1N1) pdm09-like virus, A/Texas/50/2012 (H3N2) virus, and B/Massachusetts/2/2012-like virus. Madin-Darby canine kidney cell line (MDCK) and Yac-1 were obtained from ATCC (Rockville, MD, USA).

2.1. Viruses and vaccines

The A/Zhejiang/DTID-ZJU01/2013 (H7N9) virus was isolated from a patient in Zhejiang Province, China in 2013, by transfecting 8 individual pHW2000 plasmids into Vero E6 cells [5]. We created a A/ZJU01/PR8/2013 vaccine seed strain which harbored HA and NA genes from the aforementioned virus and six internal genes from the PR8 virus, then the seed virus was grown to a high titer in eggs and the virions were purified by centrifugation, inactivated with formalin, and filtered to remove bacteria. The H7N9 split vaccines were produced under Good Manufacturing Practices.

2.2. Animal immunization

Groups (n = 12) of male 6 to 7-week-old BALB/c mice that weighed 22 ± 2 g were immunized twice with 100 μl with 100 μl of either split vaccine alone, MF59 alone, MF59-adjuvant vaccine, alum-adjuvant vaccine, seasonal flu vaccine (Yuganning), or PBS by intramuscular injection in the hind legs (half dose per injection site) at a 2-week interval (Table 1). All animals were bled via the tail vein on the day before each immunization and virus inoculation and again 1 week after virus inoculation. Lack of pre-existing immunity against influenza virus was ascertained in all mice, which was defined as hemagglutinin inhibition (HI) activity ≤10.

2.3. Virus inoculation

At 2 weeks after the second immunization, mice in each group (n = 6) were inoculated intranasally with 50 μl 10^6 TCID50 wild type H7N9 virus A/Zhejiang/DTID-ZJU01/2013(H7N9) diluted in PBS. The TCID50 assay was performed according to the recommended protocol of the World Health Organization [16,17]. Mice were observed for illness, weight loss, and death for 7 days after infection. Then, mice were sacrificed and lungs were removed—parts of the lungs were fixed in 10% buffered formalin or were harvested and used to determine the virus titers using TCID50 assay.

2.4. HI test

All serum samples were treated with receptor destroying enzyme [18]. Prior to HI tests, a two-fold serial dilution series of serum was mixed 1:1 with four hemagglutinating units of virus (wild type H7N9 virus A/Zhejiang/DTID-ZJU01/2013(H7N9)) and incubated at 37°C for 1 h. Subsequently, 50 μl 1% chicken erythrocytes were added, mixed, and incubated for 1 h at 4°C; all agglutination patterns were read within 10 min.

2.5. Micro-neutralization (MN) assay

MDCK cells were seeded at 2 × 10^4 cells/well in 96-well plates and cultured to 80–90% confluency at 37°C. Heat-inactivated serum samples were diluted 1:10 with DMEM, then were serially diluted two-fold and mixed with 50 μl of 100 TCID50 A/Zhejiang/DTID-ZJU01/2013(H7N9) virus for 1 h at 37°C. Cells were incubated in the presence of TPCK-treated trypsin at 37°C for 72 h post-infection. Cell supernatants were harvested and transferred to V-bottom 96-wells plates. The presence of virus was detected using a hemagglutination assay.

2.6. Immunoglobulin G enzyme-linked immunosorbent assay (IgG-ELISA)

96-wells of polystyrene plate was coated with anti-H7N9 antibody (Sigma–Aldrich, USA) and incubated for 2 h after three washes. Next, wells were washed and 2-fold serial dilutions of serum were inoculated in 100 μl volumes for 1 h. Plates were washed and coated with 1% bovine serum albumin (Sigma–Aldrich, USA) and incubated for 1 h. After another wash,
100 μL/well substrate TMB (Sigma) was added and then the reaction was stopped. Absorbance was measured at 450 nm. ELISA titers were expressed as reciprocal dilutions and yielded an OD that was higher than the average OD of the blanks plus 3 times the SD.

2.7. Splenocyte proliferation assays

Spleens were aseptically removed from mice 2 weeks after the final immunization and were gently dissociated through a stainless steel sieve into RPMI medium supplemented with 5% FBS. Splenocytes were collected by pelleting at 1200 rpm in a Beckman GPKR centrifuge. Erythrocytes were lysed for 1 min in 155 mM NH4Cl, 17 mM Tris–HCl (pH 7.2).

50 μL triplicates of splenocyte suspensions (5 × 10^6 cells/ml) in complete RPMI-1640 medium with 5% FBS in 96-well plates were stimulated with 10 μg/50 μL HA for 72 h. After inoculation, 100 μL freshly prepared reconstituted Cell Titer-Glo reagent (Promega) was added to all wells, which were mixed for 2 min and then incubated for 10 min. Luminescence intensity was measured using the GloMax®-Multi Detection System (Promega).

2.8. Determination of NK cell activity and cytotoxic T lymphocyte (CTL) activity

The NK activities of splenocytes were measured: 96-well plates containing various amounts of splenocytes and 10^4 Yac-1 cells per well in 200 μL RPMI-1640 supplemented with 5% FBS were incubated for 72 h. Then the percentage of specific lysis was determined using a CytoTox96 non-radioactive cytotoxicity assay (Promega), and samples (50 μl) from each well were transferred to fresh 96-well flat-bottom plates. Reconstituted substrate mixtures (50 μl) were added to each well, which were incubated for 30 min at room temperature. Stop solution (50 μl) was added before absorbance of samples was measured at 490 nm on an automated plate reader.

The CTL activity of splenocytes was measured: target cells were prepared using 10^5 Yac-1 cells co-cultured with 10 μg/ml HA. Then, 10^4 target cells and various amounts of effector splenocytes were added to each well of the 24-well plates then incubated for 4 h. The percentage of specific lysis was determined as described above for NK cell activity.

2.9. Immunoassay–Luminex measurement

Splenocytes cultured at 5 × 10^6/well were seeded into 24-well plates in 5% FBS RPMI-1640 medium. Cells were cultured either in the presence or absence of 10 μg/ml HA antigen, and then supernatants were collected after a 72 h activation period.

Serum samples and supernatants of stimulated splenocytes were measured using multiplex bead assays (MCYT MAG-70K-PX32, Millipore; USA) incorporated into MILLIPLEX MAP panels that we ran on a Luminex 200 instrument. Samples were first diluted as recommended, and bead–antibody complexes were vortexed. A total of 25 μL assay buffer was added to either 25 μL controls or samples followed by the addition of 25 μL bead–antibody complexes. Plates were incubated at 4 °C for 16 h with constant agitation. Plates were washed three times and then were incubated for 1 h with 25 μL biotinylated detection antibody. Next, 25 μL streptavidin–PE was added to detection antibody for another 30 min. Finally, plates were washed, and beads were suspended in 100 μL sheath fluid. For analysis, the 96-well plate was placed in a BioPlex reader and data were collected, analyzed, and quality controlled as previously described by Lovestone [19].

2.10. Virus titers and Immunohistochemistry of lung tissues

Viral lung titers were determined using 10-fold serial dilutions of tissue extracts, and were tested for infectivity of MDCK cells in 96-well plates after 48 h incubation. Virus titers were estimated based on the method of Reed and Muench.

Paraffin sections of lungs were dewaxed then subjected to Ag retrieval by heat treatment, and endogenous peroxidase activity was quenched with 0.3% H₂O₂ in methanol. Sections were blocked for 1 h with 3% BSA in PBS and incubated sequentially overnight at 4 °C with 1:400 dilution of polyclonal rabbit anti-H7N9 Ab; antibody binding was detected using EnVision System reagents (DAKO, Denmark). All slides were counterstained with hematoxylin.

2.11. Statistical analysis

Statistical analyses of data for HI titers, antibody titers, splenocyte proliferation, NK cell activity, CTL activity, and virus titers were performed using SPSS software (SPSS Inc., USA) using a two-way ANOVA test with the Turkey post hoc assessment. Values represent means ± SEM for indicated sample sizes. A threshold of p < 0.05 was used to denote statistical significance.

3. Results

3.1. Evaluation of antibody responses

Antibody responses in each of the 24 groups are shown in Fig. 1. After the first vaccination, the GMTs of HI titers in the groups immunized with HA antigen (H7N9 vaccine with adjuvant or not and Yuganning) all were within the range of 10–80, while all MN titers were below the limit of detection (1:20).

After second boost, titers of HI, MN, and IgG of groups immunized with HA antigen increased substantially. We observed that antibody titers declined in correlation with the reduction in the amount of HA antigen. Reducing the HA antigen dose to 0.024 μg per mouse with or without adjuvant (MF59 or Alum) resulted in a significant reduction in antibody and MN titers to less than 40. By comparing the same amount HA antigen with or without MF59, we observed that MF59 adjuvant significantly increased the immune response (for the HI titer: p = 0.004, MN titer: p = 0.014, IgG titer p = 0.128). There were also significant differences between groups of mice administered MF59 adjuvant vs. Alum adjuvant (HI titer p = 0.016, MN titer p = 0.033, IgG titer p = 0.017).

3.2. Cellular immune responses in each experimental group

The splenocyte proliferation levels (Fig. 2A) in mice immunized with H7N9 antigen with or without adjuvant were higher than those of animals vaccinated with PBS, Yuganning, or MF59 (p < 0.001). The difference between group vaccinated with H7N9 (group 1) and H7N9 + MF59 (group 5) was also significant (p = 0.03), while the difference between H7N9 + MF59 group (group 5) vs. H7N9 + alum (group 20) was not (p = 0.2). MF59 and alum adjuvants stimulate the innate immune response of splenocytes 2 weeks after the second immunization. Using a standard 4h NK cell assay against YAC-1 targets (Fig. 2B), we revealed that the NK activity in the adjuvant groups (MF59 and alum) was slightly higher than that of the PBS groups, while there was no significant difference between antigens administered with MF59 (group 5) or alum (group 20) adjuvants (p = 0.4). We found that the adjuvants alum and MF59 were poor inducers of CTL activities (Fig. 2C) and that there was no significant difference between antigens administered with either MF59 or alum adjuvant (p = 0.5).

Fig. 3A shows systemic levels of cytokines in the serum. The cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 are mainly produced by
Th2 cells. Immunization resulted in low, but detectable levels of IL-5, IL-6, and IL-10 in serum, whereas IL-4 was not detectable; levels of all four of these cytokines increased after virus inoculation. By contrast, levels of IL-13 were relatively high after immunization, but decreased significantly after virus inoculation. Levels of IL-6 and IL-13 expression significantly increased in the H7N9 + MF59 group after the second immunization, while levels IL-4, IL-5, IL-6, IL-10, IL-13 were greatest in the H7N9 + MF59 group among all experimental groups after virus inoculation (IL-4, IL-5, IL-6, and IL-10, all \( p < 0.001; \) IL-13, \( p = 0.07 \)). IL-17 is mainly produced by Th17 cells. Detectable levels of IL-17 were not induced in the serum by a single or second injection of any vaccine/adjuvant combination that we tested. The group of mice that received MF59 + H7N9 exhibited the highest levels of IL-17 after virus inoculation. The cytokines IFN-γ and IL-2 are mainly produced by Th1 cells. IFN-γ and IL-2 levels varied throughout the period following immunization, and the addition of MF59 adjuvants to the vaccine resulted in remarkable changes IL-2 levels after virus inoculation (\( p = 0.018 \)), but also resulted in lower levels of IFN-γ during both the immunization and virus inoculation periods (2 weeks, \( p = 0.95 \); 4 weeks, \( p = 0.65 \);
Most of the Th1 and Th2 cytokines increased when cocultured with H7N9 antigen (Fig. 3B). The addition of MF59 or alum adjuvant to the H7N9 split vaccine induced strong Th2 cytokine responses, including IL-4, IL-5, IL-6, IL-10, and IL-13, as well as the Th17 cytokine IL-17. It has been observed that MF59 boosts levels of IL-4, IL-5, IL-10, and IL-17 more highly compared with alum (for each, p < 0.05), while levels of IL-6 and IL-13 levels elicited by alum were higher than those elicited by MF59 (for both, p < 0.05). The levels of IFN-γ secreted by splenocytes were inconsistent with the levels detected in the serum. IL-2 and IL-13 levels peaked in that group received H7N9 + MF59 (both p < 0.05), whereas the cytokines IL-12 and IL-7 were not detectable in any of the groups.

### 3.3. Protective effects of each of the immune formulations

For most of groups, all antibody titers were significantly increased after virus inoculation (Fig. 4), as the antibody response increased progressively with MF59 or Alum adjuvant, although the MF59 adjuvant showed a slight advantage in stimulating humoral responses. Groups 6, 7, 8, and 13, 14, and 15 were compared separately to evaluate the effects of different amounts of MF59 adjuvant

![Graphs showing cytokine expression](image)
(group 6 vs. group 13: HI titer, \( p = 0.783 \); MN titer, \( p = 0.9 \); IgG titer, \( p = 0.8 \); group 7 vs. group 14: HI titer, \( p = 0.9 \); MN titer, \( p = 0.5 \); IgG titer, \( p = 0.04 \); group 8 vs. group 15: HI titer, \( p = 0.045 \); MN titer, \( p = 0.036 \); IgG titer, \( p < 0.001 \)), while for relatively large doses of HA antigen there was little impact of a five-fold increase or decrease of MF59 adjuvant concentration on the antibody response. The comparison of Group 4 vs. Group 5 showed that even 0.05 ml MF59 could enhance the antibody response to the smallest amount HA antigen tested, which was 0.024 \( \mu \)g (HI titer, \( p = 0.016 \); MN titer, \( p < 0.001 \); IgG titer, \( p < 0.001 \)).

We observed lethargy, rough hair and loss of appetite in the immunized mice after wide type H7N9 challenge but no deaths. There were varying degrees of weight loss in all 24 experimental groups (Fig. 5A). In a comparison of mice vaccinated with the non-adjuvanted H7N9 vaccine, both MF59 and alum were effective in delaying weight loss. Weight loss in the Yunganning group suggested that any cross-protection effect was negligible. Fig. 5B shows an analysis of whether MF59 or alum could significantly reduce viral titers compared with groups treated with H7N9 split vaccine alone (\( p < 0.001 \)), and indicates that H7N9 antigen was more effective when paired MF59 adjuvant (\( p = 0.11 \)).

The extent and characteristics of the lesions varied among these groups 1 week after virus inoculation (Fig. 6). The H7N9 vaccine + MF59 treated mice (group 5) had minimal lesions. Microscopic lung lesions included severe bronchiolitis and alveolitis and damage that included lymphocytic infiltration around peribronchiolar and perivascular areas in the PBS group (group 24). By immunohistochemical analysis, viral antigens could be detected in the lung, especially in the bronchiolar epithelium (Fig. 6C). Based on these gross and microscopic lesions, the pathogenicity of H7N9 viruses could be ranked in the following order, from greatest to least: H7N9 + MF59 (group 5), H7N9 + alum (group 20), H7N9 (group 1), and finally MF59 (group 9), Yunganning (group 16), and PBS group (group 24); no obvious differences were observed between the final three groups.

4. Discussion

Reverse transcription technology can aid the development of seed strains especially for the highly pathogenic H5 or H7 strains in which the major molecular features that confer this high virulence can be removed [20,21]. The H7N9/PR8 virus has many characteristics that are desirable in an H7N9 vaccine candidate. Unfortunately, because of the relatively poor immunogenicity of split vaccines, at least two doses of vaccine must be provided [9] along with an adjuvant [22]. MF59 seasonal influenza vaccines were first licensed for elderly individuals and previous large-scale analysis supported its good safety profile [23]. Although vaccines must undergo strict preclinical safety evaluation before licensing. However, cases of developing serious adverse events following influenza vaccinations with adjuvant had been reported [24,25], among them the implications of adjuvants in the development of autoimmune diseases continues to receive considerable attention at recent years [25], although such adverse reaction was rare and in most cases related to myotoxic drugs use [26,27]. Some researchers even claim influenza vaccine does not produce myopathy in patients taking...
Fig. 4. Antibody responses, including (A) HI antibodies, (B) MN antibodies, and (C) IgG titers, for groups of mice 1 week after virus inoculation. Each dot represents the geometric mean titer obtained from groups of 6 mice. Mouse were intramuscular injected twice with various dosage of following immune formulations: Groups 1, 2, 3 and 4 (H7N9), Groups 5, 6, 7 and 8 (H7N9 + MF59, dosage of MF59 decreased synchronize with dosage of H7N9 in these four groups), Groups 9, 10, 11 and 12 (MF59), Groups 13, 14, and 15 (H7N9 + MF59, dosage of MF59 maintained the highest dosage of 0.05 ml/mouse); Groups 16, 17, 18 and 19 (Yuganning); Groups 20, 21, 22 and 23 (H7N9 + alum); Groups 24 was PBS control group.

Fig. 5. (A) Weight loss after virus inoculation. The Y-axis shows weight loss that was calculated as follows: (weight at 1 week after virus inoculation – weight before virus inoculation)/weight before virus inoculation) × 100. (B) Virus titers of lungs 1 week after H7N9 virus inoculation. Replications of H7N9-WT challenged viruses 1 week after virus inoculation in lungs of immunized BALB/c mice were determined using the TCID_{50} method in MDCK cells. Virus titers in lungs are expressed as means ± SE of the log_{10} TCID_{50} per g tissue. Mouse were intramuscular injected twice with various dosage of following immune formulations: Groups 1, 2, 3 and 4 (H7N9), Groups 5, 6, 7 and 8 (H7N9 + MF59, dosage of MF59 decreased synchronize with dosage of H7N9 in these four groups), Groups 9, 10, 11 and 12 (MF59), Groups 13, 14, and 15 (H7N9 + MF59, dosage of MF59 maintained the highest dosage of 0.05 ml/mouse); Groups 16, 17, 18 and 19 (Yuganning); Groups 20, 21, 22 and 23 (H7N9 + alum); Groups 24 was PBS control group.

statins [28]. Unfortunately, cases of developing rhabdomyolysis following influenza vaccinations are not accidental and the cause is unclear, careful attention should be paid to adjuvant safety especially to concomitant medication inquiry when administrating the influenza vaccine. It has been reported that MF59 can reduce the antigen requirements of H5N1 vaccines by at least six-fold and can induce a protective response after only a single dose [10,29]. By contrast, the addition of the classic immunoadjuvant alum to split or subunit influenza vaccines has only been reported to induce marginal improvements [30,31]. In this present study, we note that adjuvanting H7N9 antigen with MF59 or alum resulted in comparatively higher levels of HI and MN antibodies and IgG titers, and ultimately increased protection against H7N9 influenza virus. Our findings suggest that MF59 has more advantages in improving immune responses of split influenza vaccine compared with alum, while neither MF59 nor alum could induce MN antibody production after single dose immunization. In our present study, the HI, MN, and IgG titers in serum after a first immunization were obviously low and were considered to be insufficient to provide adequate protection.

The induction of cellular immune responses is an attractive goal for influenza vaccine research because such immunity targets the
most conserved viral proteins [32] and tends to be more cross-reactive against other virus strains [33,34]. The cellular immune response to influenza virus was determined using a splenocyte proliferation assay, along with measurements of Th1 and Th2 cytokines in serum and supernatants of HA-stimulated cultured splenocytes. The MF59-adjuvanted H7N9 split vaccine induced slightly higher proliferative responses against homologous antigen compared with the PBS- or H7N9 alone-treated groups, but showed a response that was similar that of the alum + H7N9-treated group. Cytokines play a critical role in mediating immune responses, and it is likely that many of the activities induced by adjuvants are mediated by cytokines. Notably, more Th-2 cytokines, including IL-4 and IL-10, were detected in serum from mice immunized with H7N9 + MF59, especially after virus inoculation of from the supernatants of splenocytes isolated from mice immunized with H7N9 + MF59 that were restimulated with HA antigen ex vivo. Th2-type responses have been typically associated with B cell stimulation and antibody production. By contrast, the release of Th1-type cytokines including, IFN-γ and of IL-2, have typically been associated with cellular immunity, which is responsible for the stimulation of CTLs [32]. We verified that the adjuvants MF59 and alum are poor inducers of Th1 responses and CTL activities, which might be related to the high concentrations of IFN-γ and can be generally immunosuppressive. Indeed, most Th1 clones are directly cytoxic for activated B cells [35]. Recent advances have suggested a critical role for IL-17 in vaccine-induced protective cellular responses and in generating effective long-lived vaccine-induced antiviral immunity [36]. Thus, we verified that MF59 can robustly stimulate Th17 responses.

In summary, a monovalent inactivated split vaccine that we prepared from the H7N9/PR8 virus and mixed with MF59 was immunogenic and protected mice from subsequent Zhejiang virus challenge and we believe this split H7N9 vaccine is able to protect against other H7N9 virus without major HA antigenic drift. Both MF59 and alum could enhance the priming of Ab levels, exert a Th2 bias, while MF59 exerted more robust responses. Further studies that include the challenge of vaccinated mice at a later time point, antibody isotype analysis, duration of immunity, and identification of protective epitopes and most importantly, human testing and risk evaluations will be necessary.

**Contributors and authorship**

Conceived and designed the experiments: H. Yao, L. Li, N. Wu. Performed the experiments: H. Ou, X. Wu, K. Chen. Analyzed the data: L. Cheng. Wrote the first draft of the manuscript: H. Ou, H. Yao. Provided H7N9 vaccine and MF59 adjuvant: W. Yao, H. Chen. All authors read and approved the final manuscript.

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**Conflict of interest statement:** Two authors have declared that the following interests are relevant to the submitted work. Wei Yao and Chengcong Han are employees of Zhejiang Tianyuan Bio-Pharmaceutical Co., Ltd. (which was affiliated with Novartis Vaccine Inc.) They participated in the production of H7N9 vaccine and MF59 adjuvant and had nothing to do with the investigation of the immunogenicity and bioactivities of this vaccine. The other authors declare no competing interests.
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