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Evaluation of aqueous extracts of *Cistanche deserticola* as a polysaccharide adjuvant for seasonal influenza vaccine in young adult mice

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ABSTRACT

Adjuvants can improve the efficacy of influenza vaccines and are still a hot spot in the study and development of influenza vaccines. In this report, the effects of aqueous extracts of *Cistanche deserticola* (AECD) as a polysaccharide adjuvant on seasonal influenza vaccines (IVV) were explored. The experimental data of anti-IVV IgG_1 and IgG as well as hemagglutinin inhibition (HI) titers in young adult mice indicated that IVV intramuscularly co-injected with AECD was significantly more immunogenic than alum-adjuvanted or non-adjuvanted IVV. AECD-adjuvanted vaccine could rapidly initiate specific IgG response. Similarly, IVV with AECD augmented significantly lymphocyte proliferation and increased the positive rates of $CD4^+$, $CD8^+$ and $CD44^+$ T cells from draining lymph nodes and spleens. Importantly, IVV with AECD could induce the Th1 immune response, as indicated by higher IgG_{2a} levels accompanied by the induction of IFN-γ in $CD4^+$ and $CD8^+$ T cells. Additionally, IVV with AECD activated dendritic cells (DCs) and decreased the expression of Treg cells. There were no noticeable side effects after the vaccination. In brief, the addition of AECD enhanced immunogenicity to seasonal influenza vaccine by the induction of HI antibody generation, more rapid humoral immune responses, and a balanced Th1-/Th2-type response, effective T-cell responses, which may be important for seasonal influenza vaccines with broad and long lasting immunity.

1. Introduction

Vaccination of influenza vaccines is still the most effective strategy to prevent the infection of seasonal and pandemic influenza virus. However, conventional seasonal influenza vaccines have some defects, such as antigenic drift. Therefore, it is necessary to develop alternative approaches to influenza vaccines, such as “universal” influenza vaccines with higher effectiveness, longer duration, and broader protection. In addition, H1N1 pandemic and sporadic human infections of avian H5N1 and H7N9 further highlight the demand for more efficacious universal vaccines. In particular, more balanced Th1/Th2 responses may be beneficial for improving such vaccines. Adjuvants can improve the vaccine potency and efficacious adjuvants may largely promote the development of universal influenza vaccines [1]. At present, new adjuvants are still a key way to improve the immune response to vaccines for humans and animals.

However, commercially available adjuvants are limited. AS03, MF59, and alum adjuvants are currently licensed for pandemic influenza vaccines. AS03 can enhance Th2/Th1 responses. Alum mainly activates Th2 responses [1–3]. A rational selection of adjuvants can induce the appropriate type of immune response (Th1- or Th2-type response) and achieve the optimal protection against different infections. Proper adjuvants should promote appropriate cellular and/or humoral immune responses and show high safety, tolerance, stability, biodegradability and biocompatibility. Therefore, it is necessary to develop a safer, more potent and economically viable influenza vaccine adjuvant.

Unlike conventional adjuvants, polysaccharides-based adjuvants are promising candidates widely used in human vaccine development due to their high biocompatibility and low toxicity. Polysaccharides are important in the stimulation and regulation of immune responses. Therefore, polysaccharides-based adjuvants are widely concerned [4,5]. Numerous polysaccharides from plants have been identified to possess immunological activities. Several compounds have been used as vaccine adjuvants in clinical trials, whereas the majority of natural polysaccharides are not used as adjuvants for human vaccines. So far, a

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variety of plant-derived polysaccharides have been discovered and investigated as novel adjuvants, including Astragalus polysaccharides, Lycium barbarum polysaccharides, and Ginseng polysaccharides [6–8]. Advax™adjuvant, a polysaccharide adjuvant derived from delta inulin, has been identified as a more stable and potent adjuvant in human and veterinary against infectious diseases including HIV, influenza, and hepatitis B virus. Current results suggested that Advax™ was a suitable substitute for alum adjuvant in the next-generation human vaccines [9–11].

Cistanche deserticola (CD, in Xinjiang) is used in human foods due to its good tonic activity. Polysaccharides are one of the main components in the aqueous extracts of *Cistanche deserticola* (AECD) plants and have immunoregulatory activities [12–14]. In recent studies, our groups reported that AECD as a water-soluble polysaccharide adjuvant exhibited good adjuvanticity to OVA antigen in mice and might contribute to the improved effective humoral immunity and cellular immunity, DCs activation, B and T cell responses with a Th1/Th2 balance [15]. In this study, we explored the immunostimulatory effect of AECD on seasonal influenza vaccine. We provided the evidence for the induction of humoral and cellular responses which were the rapid onset of immune responses in young adult mice after the intramuscular injection with AECD-adjuvanted vaccine. These observations provide the important basis for the development of an improved influenza vaccine adjuvant against seasonal or pandemic influenza.

2. Materials and methods

2.1. Animals and vaccination

In the experiment, female ICR mice (Xinjiang, China) were used. The mice were maintained under pathogen-free conditions. The animal experiments were approved by the Committee on the Ethics of Animal Experiments of Xinjang Key Laboratory of Biological Resources and Genetic Engineering (BRGE-AE001) in Xinjiang University.

The young adult mice (2–3 months old) were randomized into six groups with eight mice per group and injected intramuscularly (i.m.) once or twice at a two-week interval with a total volume of 100 μL of final vaccine formulation per mouse (50 μL/hind limb) with different concentrations of AECD prepared in our lab [15] or Imject Alum Adjuvant (Thermo Scientific Pierce, USA) mixed with IVV (The trivalent split influenza vaccine antigens containing A/Michigan/45/2015(H1N1)-like strain, A/HongKong/4801/2014(H3N2)-like strain, and B/Brisbane/60/2008, 0.1 μg of each subtype HA/mouse, Shenzhen Sanofi Pasteur, China). The mice immunized with 0.9% NaCl were used as blank controls and the mice immunized with alum adjuvant were used as positive controls. In addition to the blank controls, the 5 groups of mice were respectively injected with the following agents: 0.1 μg IVV alone (IVV group), 0.1 μg IVV plus 100 μg AECD (AECD-L group), 0.1 μg IVV plus 400 μg AECD (AECD-M group), 0.1 μg IVV plus 800 μg AECD (AECD-H group), and 0.1 μg IVV plus alum adjuvant (alum group). After the vaccination, the blood was obtained via the retro-orbital sinus. These groups of mice were immunized with the same immunization strategy and then HI titers, splenocyte proliferation, and T-cell response were detected.

2.2. Hemagglutination inhibition

Animals were bled individually in three weeks after single vaccination for HI measurements with routine methods. All samples in the same experimental group were pooled because the amount of serum from a mouse was insufficient for individual examination. The pooled sera were examined three times. Briefly, 4 haemagglutinin (HA) units were prepared with guinea pig red blood and then two-fold serially diluted sera of mice were incubated with 4 HA units for 60 min at room temperature. Subsequently, guinea pig red cell suspensions were added and incubated for additional 60 min, followed by the visual inspection and HI measurements. HI titers represent the reciprocal of the last dilution concentration of serum which fully inhibited haemagglutination of guinea pig red cells [16].

2.3. Enzyme-linked immunosorbent assay (ELISA)

Mouse serum samples were analyzed for detecting influenza-specific total IgG, IgG_1 and IgG_{2a} (Southern Biotech, USA) by ELISA. Briefly, ELISA plates were coated with vaccine antigen (25 ng/well) overnight in carbonate-bicarbonate buffer at 4 °C. Then serum dilutions (IgG at 7d: 1:100, IgG, IgG1 or IgG_{2a} at 21d: 1:1000) were sequentially incubated with horseradish peroxidase goat anti-mouse IgG, IgG_1 or IgG_{2a} antibodies (Southern Biotech, USA). The stop reagent (Sangon Biotech, Shanghai, China) was added and the spectral intensity at 450 nm/655 nm was measured in a microtiter plate reader (Bio-Rad, USA).

2.4. Splenocyte proliferation and IFN-γ production

In splenocyte proliferation studies, the spleens from mice were aseptically collected in seven days after booster vaccination. Single-cell suspensions were obtained in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS) (Hyclone), followed by the lysis of erythrocytes. Splenocyte suspensions in triplicates were stimulated with 0.5 μg/well ConA (Sigma) or 0.25 μg/well LPS (Sigma) in 96-well plates for 48 h. After inoculation, MTT (Sigma) was added to each well and stimulated for 4 h. After stopping the color development, the colorimetric reaction was measured at 570 nm/655 nm by a microtiter plate reader. Stimulation index (SI) was calculated as: SI = (OD of stimulated cells - medium OD)/(OD of unstimulated cells-medium OD).

In IFN-γ expression assays, intracellular cytokine staining was performed with IFN-γ-PE antibody (BD Biosciences). Single-cell suspensions were prepared as described above. Cells were firstly stimulated in the presence of 0.5 μg/ml total HA of influenza vaccine for 4 h or in the medium, followed by the addition of Golgistop (BD Bioscience) for 12-h treatment. Cells were washed twice with perm/washing buffer and then treated with Mouse BD FcBlock (BD Bioscience) before pre-staining with specific monoclonal antibodies for cell surface markers (including CD8-FITC or CD4-APC) (BD Bioscience) for 30 min at 4 °C. For the detection of intracellular IFN-γ, cells were fixed and permeabilized with Cytofix/Cytoperm buffer. Then, the appropriate fluorescently labeled IFN-γ-PE was added for 30-min treatment at 4 °C. Finally, cells were analyzed on FACSCalibur (BD Bioscience). Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2.5. T cell subsets and DC surface markers

Individual spleen or lymph node cell suspensions from mice in seven days after booster vaccination were subjected to surface marker staining. T cell subsets, including $CD4^+$ naive T helper cells, $CD8^+$ cytotoxic T cells, $CD4^+CD44^+$ and $CD8^+CD44^+$ effector T cells, were identified through double or triple labeling with different antibodies. Briefly, single cells from the spleen or lymph nodes were labeled with CD3-PE, CD8-FITC, CD4-APC, and CD44-PE antibodies (BD Bioscience) for 30 min, respectively. The cells in each sample were analyzed by FACS. Splenocyte suspensions were also used to test the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells with a mouse regulatory T cell staining kit (eBioscience, San Diego, CA) according to manufacturer's instructions. Data analysis was performed with FlowJo software.

For DC cell surface co-stimulatory molecules staining, single-cell suspensions from the spleens were collected after booster vaccination. After rinsing once with PBS-FBS, cells were double-stained with CD40-FITC, CD11c-PE, C86-APC, CD80-APC, and MHC II-FITC (BD Bioscience). Cells were measured by FACS and data were analyzed by FlowJo software.

2.6. Safety evaluation of AECD in mice

The safety of influenza vaccine containing AECD was tested in preclinical studies. The mouse weight was weekly monitored and the site of injection in mice was checked for local reaction.

2.7. Statistical analysis

The differences between experimental and control groups were analyzed by one-way ANOVA through Turkey's post-test. Experimental data are expressed as mean ± SD. Data analysis and drawing was carried out in GraphPad Prism 5. Differences with $p < 0.05$ were considered to be significant.

3. Results

3.1. Effects of AECD on HI titers and Th1/Th2 responses

In seven days after booster vaccination, HI serum antibody levels were determined in standard HI assays. The co-immunization of AECD-L with IVV increased the HI titers by 2~3 times. Compared to the influenza vaccine alone, AECD-L only increased HI antibodies subtly. HI titers of the mice in the alum group were the highest (Fig. 1A), but HI titers were not detected in the sera of the mice in the control group.

To assess whether rapidly antibody responses were elicited and the balance between humoral and cellular immune responses were enhanced by AECD, anti-influenza IgG responses and antibody subtypes in all serum samples were determined by ELISA. Even seven days after single vaccination, AECD-induced IgG was clearly detectable compared to that in alum group (Fig. 1B). In seven days after booster vaccination, the addition of AECD induced the significantly higher level of vaccine-specific total IgG antibodies than the group without adjuvant. AECD-M was found to be the optimal dose (Fig. 1C). In particular, AECD also significantly enhanced IgG_1 and IgG_{2a} levels than the alum group (Fig. 1D and E).

3.2. Effects of AECD on splenocyte proliferation and T cell responses

The above data revealed that AECD induced a mixed Th1/Th2 response in mice. To explore whether AECD could elicit cellular immunity, the effects of AECD on splenocyte proliferation were assessed by MTT assays in seven days after booster vaccination. After the exposure to ConA, the splenocyte proliferation index in the mice immunized with AECD-M was significantly higher than that of the mice immunized with IVV alone, but the difference between alum group and IVV group was not significant (Fig. 2A). Similar results were detected in the proliferation response after the exposure to LPS (Fig. 2B).

To assess whether AECD-adjuvanted influenza vaccine increased T-cell responses, lymphocytes from lymph nodes and spleens were obtained and determined by FACS. AECD elicited higher rates of $CD4^+$ and $CD8^+$ T cells from spleens than the control group or IVV group and the significant difference was observed between AECD group and alum group (Fig. 2C and D). The levels of $CD4^+$ T cells from lymph nodes were also slightly increased, but no significant difference was detected between AECD group and IVV group (Fig. 2E). The levels of $CD8^+$ T cells from lymph nodes were slightly increased in the AECD-M group and AECD-L group (Fig. 2F). Lymphocytes from spleens and lymph nodes of the mice immunized with AECD-adjuvanted influenza vaccine had the significantly higher $CD4^+CD44^+$ and $CD8^+CD44^+$ T-cell proliferation than that of the mice immunized IVV alone (Fig. 2G-J). The results revealed that AECD was an inducer of T-cell activation. AECD-adjuvanted vaccine significantly increased positive rates of $CD4^+CD44^+$ and $CD8^+CD44^+$ T lymphocytes from spleens and lymph nodes compared to influenza vaccine group and alum group

3.3. Effects of AECD on Th1-type cytokines IFN-γ

$CD8^+$ and $CD4^+$ T cells can significantly affect the viruses from infected cells by secreting antiviral cytokine, IFN-γ. In seven days after booster vaccination, the levels of IFN-γ from $CD8^+$ and $CD4^+$ T cells in spleen cells were analyzed by FACS. Notably, only AECD-L group showed the significantly enhanced activation of antigen-specific T cells and the differences between AECD-L group and alum group were significant (Fig. 3A-D). AECD is a potent adjuvant for inducing Type-1 cellular immune responses to influenza vaccine.

3.4. Effects of AECD on DCs maturation and Treg frequency

To better understand the role of AECD adjuvant and verify whether innate immunity could enhance antigen-specific antibody and T cell responses, the changes in DCs from spleens were evaluated by FACS. In three days after single vaccination, the co-stimulation markers of DC, including CD11c, CD40, CD80, CD86 and MHCII, were detected. The addition of AECD resulted in the higher expressions of CD40, CD80, CD86, and MHC II (Fig. 4A-H) on the DC surface than the immunization with IVV alone and the activated phenotype of DC was not detected in alum group.

Next, the Treg frequency was determined with a mouse regulatory T cell staining kit. In seven days after booster immunization, splenocyte was obtained and the frequency of Treg cells was measured (Fig. 4I-J). The frequencies of Treg cells in the AECD-L group and AECD-M group were lower than that in the mice treated with IVV alone. The decreased number of Treg cells was not detected in the alum group.

3.5. AECD-adjuvanted vaccine showed no adverse effect in mice

In order to explore adverse effects of AECD adjuvant in immunized mice, feeding behaviors and growth of mice as well as injection site reactions were monitored. The mice receiving AECD-adjuvanted vaccine showed no injection site lesion and displayed normal weight gain and development compared to the control mice (Table 1). The control groups and treated groups showed the similar weight gain during the experimental period. The weights of the mice vaccinated with influenza vaccine containing AECD adjuvant were not decreased, indicating the safety of vaccine and adjuvant. Adverse effects of AECD adjuvant were not observed in our previous OVA immunization study [11].

4. Discussion

Adjuvants can make influenza vaccines more potent [17–19]. Although alum adjuvants have been licensed for human use as adjuvants in influenza vaccines, alum adjuvants is not a potent adjuvant for the induction of Th1 cellular immune responses. Previously, we reported that AECD possessed the higher immunostimulatory activity in cellular immune response compared to alum adjuvants [15]. Thus, it is necessary to experimentally assess their potential as influenza vaccine adjuvants. In the study, the experimental data of young adult mice administered intramuscularly twice indicated the potential of AECD as influenza vaccine adjuvant. The effects of AECD were mainly assessed from four aspects: multifunctional T cells, serum haemagglutination inhibition titers, IFN-γ expression in $CD4^+$ and $CD8^+$ T cells, and specific IgG_1 and IgG_{2a} serum antibodies. Our studies showed that the addition of AECD significantly enhanced influenza vaccine efficacy, especially a Th1/Th2 response, and showed high IgG1/IgG2a, IFN-γ and T-cell response compared to alum adjuvants.

An appropriate combination of vaccine and adjuvant could significantly stimulate the increased immunogenicity. To determine the optimal dose of AECD, the mice were immunized with serially diluted AECD doses (low, medium and high) with a certain IVV dose. The immunity to influenza was determined in terms of the levels of HI antibodies, which were the generally accepted and well-defined surrogate

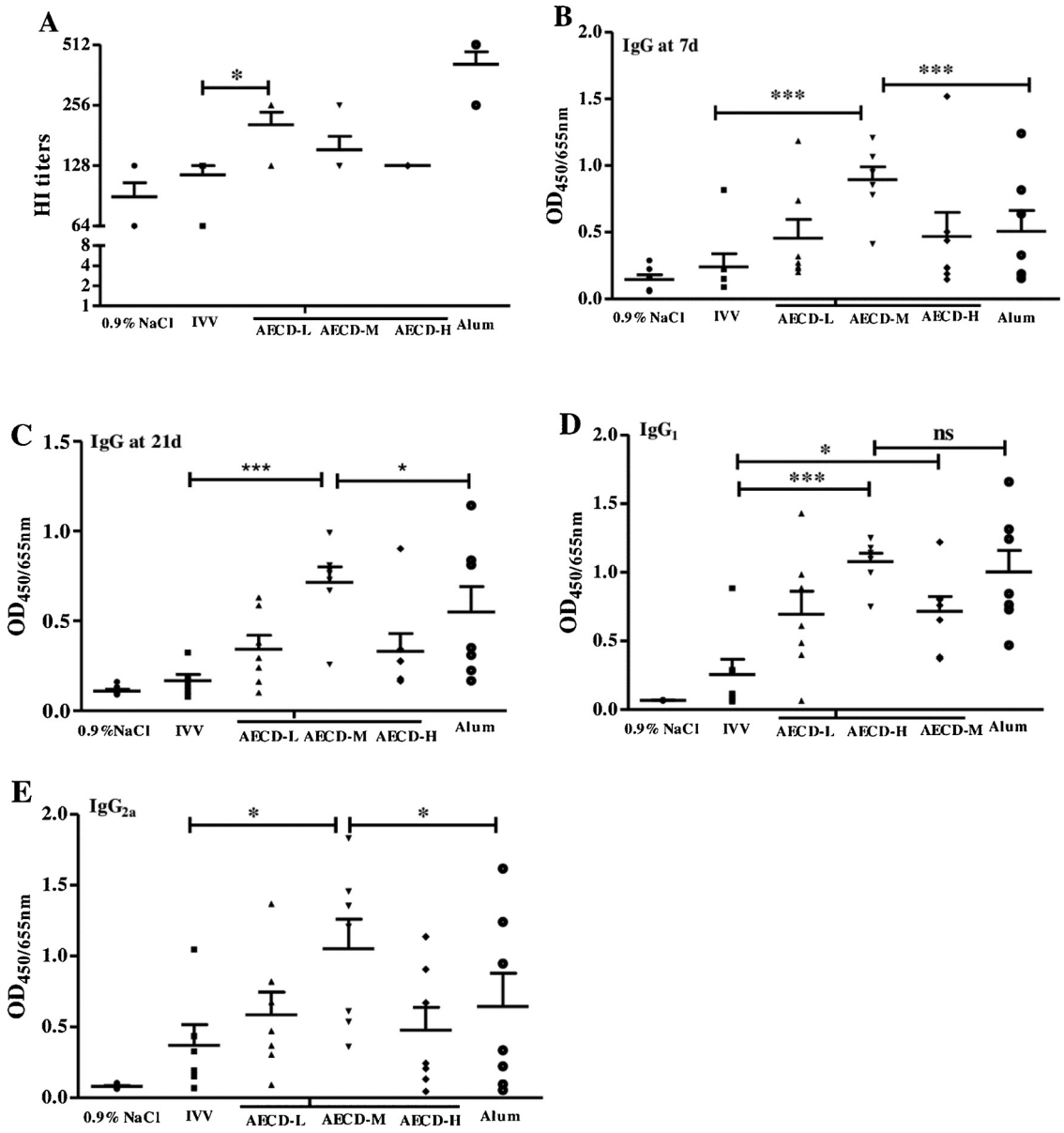

Fig. 1. Co-administration of AECD with IVV improved HI titers and Th1/Th2 responses. (A) The HI titers in 21 days are expressed as the log2 titers plus SD ($n = 5$). Three experiments were performed. ICR mice (n = 7) were immunized intramuscularly with IVV alone or with different doses of AECD. (B) IgG in 7 days. (C) Total IgG response in 21 days. (D–E) IgG_1 and IgG_{2a} response in 21 days. The data are expressed as mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{ns}p > 0.05$.

markers of protection against influenza in human beings and laboratory animals [20,21]. In the study, we observed that AECD-L could significantly enhance HI titers compared to IVV alone. HI titers in alum group were the highest, but there was no significant difference between AECD-L and alum groups. The results suggested that AECD slightly boosted HI responses. The dose of HA in vaccine was important in the induction of protective immunity against influenza. Therefore, the optimal vaccine HA doses in mice might improve the potency of influenza vaccine. We further examined the appropriate combination of the optimal vaccine HA dose with a certain AECD dose in terms of immune response.

After demonstrating increased HI titers, the changes in IgG and IgG subclasses were investigated. Our data also showed that AECD group was superior to alum group in terms of IgG responses in 7 or 21 days after single vaccination. The addition of AECD into influenza vaccines induced Th1-/Th2-type immune responses, but alum only induced IgG1 other than IgG2a, indicating that alum could induce only Th2-type immune response. It has been proposed that the humoral response is well known to be a key protection factor in subsequent challenges. Especially, IgG_1 and IgG_{2a} serum antibodies are involved in virus neutralization in mice and all antibodies which are completely protective in vivo belong to the IgG_{2a} subclass [22].

Although the antibody responses, including HI titer and IgG responses, have been thought to be associated with the protection against influenza, cellular immunity is also important for cross-protection and immune memory. The immunity to influenza is also dependent on effective T cell-mediated immune responses [23]. An appropriate $CD4^+$ helper T cell response is believed to be a prerequisite for an adequate humoral response. In addition, T cell responses may contribute to the extended longevity of humoral immunity. The $CD8^+$ T cell response is mainly responsible for influenza virus removal. The effective immunization with vaccines also requires the induction of effector T cells in response to vaccines [24,25]. In the study, the addition of AECD at an appropriate dose induced $CD4^+$ and $CD8^+$ T cell responses and an effective $CD44^+$ T cell response compared to alum adjuvants from spleens and draining lymph nodes. The results were consistent with

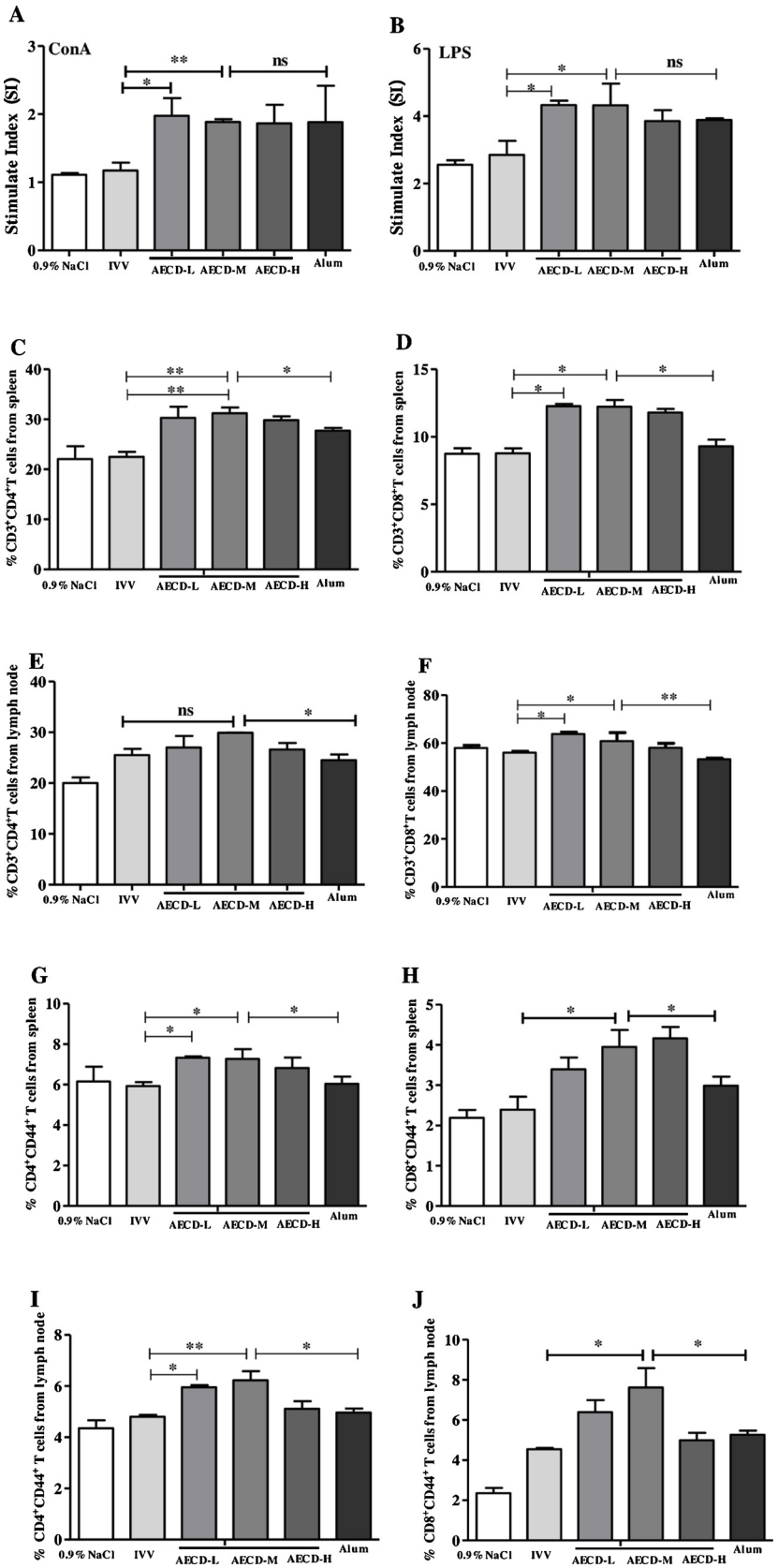

Fig. 2. Co-administration of AECD with IVV enhanced T cell–mediated immunity. (A–B) Splenocytes stimulated in vivo with ConA or LPS and analysis results by MTT. Cell proliferation of each sample is shown as the stimulation index (mean ± SD) ($n = 3$). (C–J) Splenocytes from spleens and lymph nodes of immunized mice were detected by FACS and the percentages of $CD4^+$(C),$CD8^+$(D), $CD4^+CD44^+$ (G), $CD8^+CD44^+$(H) T cells from spleen and $CD4^+$ (E), $CD8^+$ (F), $CD4^+CD44^+$ (I), $CD8^+CD44^+$ and (J) T cells from lymph nodes are expressed as mean ± SD ($n = 3$). $^*p < 0.05$, $^{**}p < 0.05$, $^{ns}p > 0.05$.

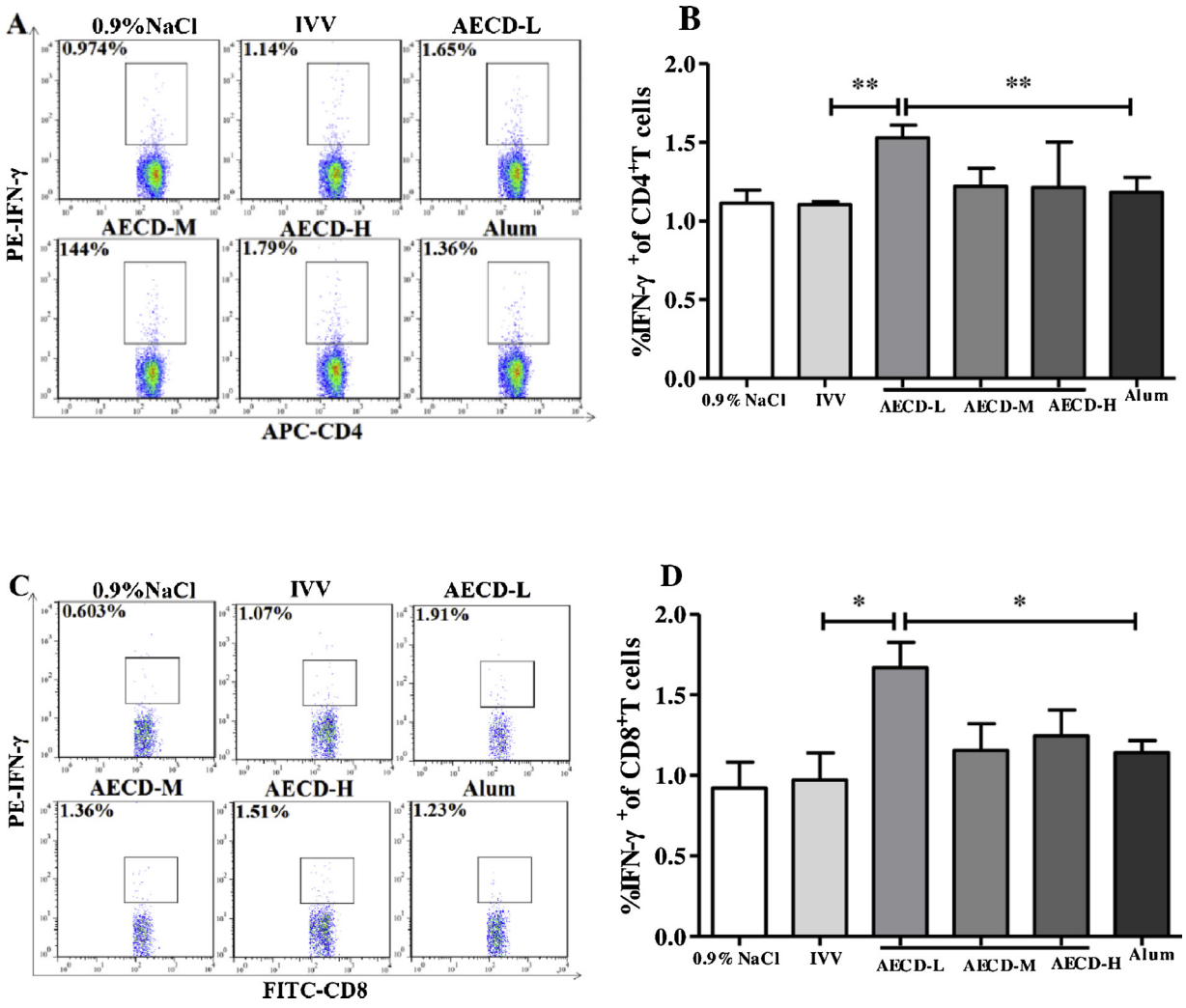

Fig. 3. Co-administration of AECD with IVV induced Th1 cytokine secretion. Splenocytes from mice were re-stimulated with influenza vaccine and the IFN-γ production was measured by FACS. (A–D) Influenza vaccine-specific IFN-γ production from $CD4^+$ and $CD8^+$ T cells is expressed as mean ± SD ($n = 3$). *$p < 0.05$, **$p < 0.05$.

typical humoral and cellular responses evoked by AECD-adjuvanted vaccine with the enhancement of IgG_1 and IgG_{2a} serum antibodies. Traditional influenza vaccines generally have the poor induction of Th1 cellular immune responses. Moreover, T-cell (CD8 and CD4)-derived cytokines, especially IFN-γ, are important for microbial clearance in animals and human beings [26,27]. In the study, IFN-γ expression from splenocytes was investigated in the mice. FACS data showed that the mice immunized with AECD and IVV elicited a higher percentage of vaccine antigen-specific IFN-γ secretions than the mice immunized with alum and IVV. Therefore, the addition of AECD elicited a more potent Th1 cellular immune response, which was characterized by lymphocyte proliferation and the induction of a strongly enhanced IFN-γ response from $CD4^+$ and $CD8^+$ T cells in splenocytes from the immunized mice.

In vivo results clearly showed that AECD promoted antibody responses and T cell responses. Since the mechanism underlying this discrepancy is unknown, we aimed to address it in vitro. DC is important in $CD8^+$ T cell responses in the early infection phase of an influenza virus. Activated T cells can also differentiate into regulatory T cells. Treg cells are important in maintaining the balance between immune tolerance and activation [28–30]. Our results revealed that the co-administration of AECD with IVV resulted in an increase of DC maturation which facilitated T cell differentiation and development. The frequency of Treg cells were also decreased by an appropriate dose of AECD due to the induction of more T cells and the enhanced antibody response.

To develop new formulations of adjuvants, more attention should be paid to side effects resulting from the interaction in new complex formulation adjuvants and the proper combination of different adjuvants. The toxic effects of AECD had not been observed in our previous toxicity experiments [15]. Under the same conditions, clinical signs in mice (such as ruffled hair, inappetence, inactivity and cluster) were not observed and body weight also gradually increased in the immunization period. Side effects were not observed in any immunized mice after vaccination. The results revealed a safety profile of the AECD-adjuvanted influenza vaccine.

In summary, AECD-adjuvanted influenza vaccine rapidly initiated specific IgG response and HI titers and increased the frequency of multifunctional $CD4^+$ and $CD8^+$ T cells in spleen and draining lymph nodes, specific $CD44^+$ cells, IFN-γ sections, and a balanced Th1/Th2 response. The improvement of seasonal influenza vaccine adjuvanted with AECD indicates the potential of AECD in novel vaccine formulations. We will further explore the optimal vaccine Ag dose, dose-sparing ability, duration of immunity and risk evaluations. However, the correlation between AECD and virus clearance is not clear. It is necessary to clarify the mechanisms that AECD promotes immune responses after immunization with influenza vaccines. The study may be helpful to understand the effects of AECD as polysaccharide adjuvant on seasonal influenza vaccines in genetically heterogeneous human beings.

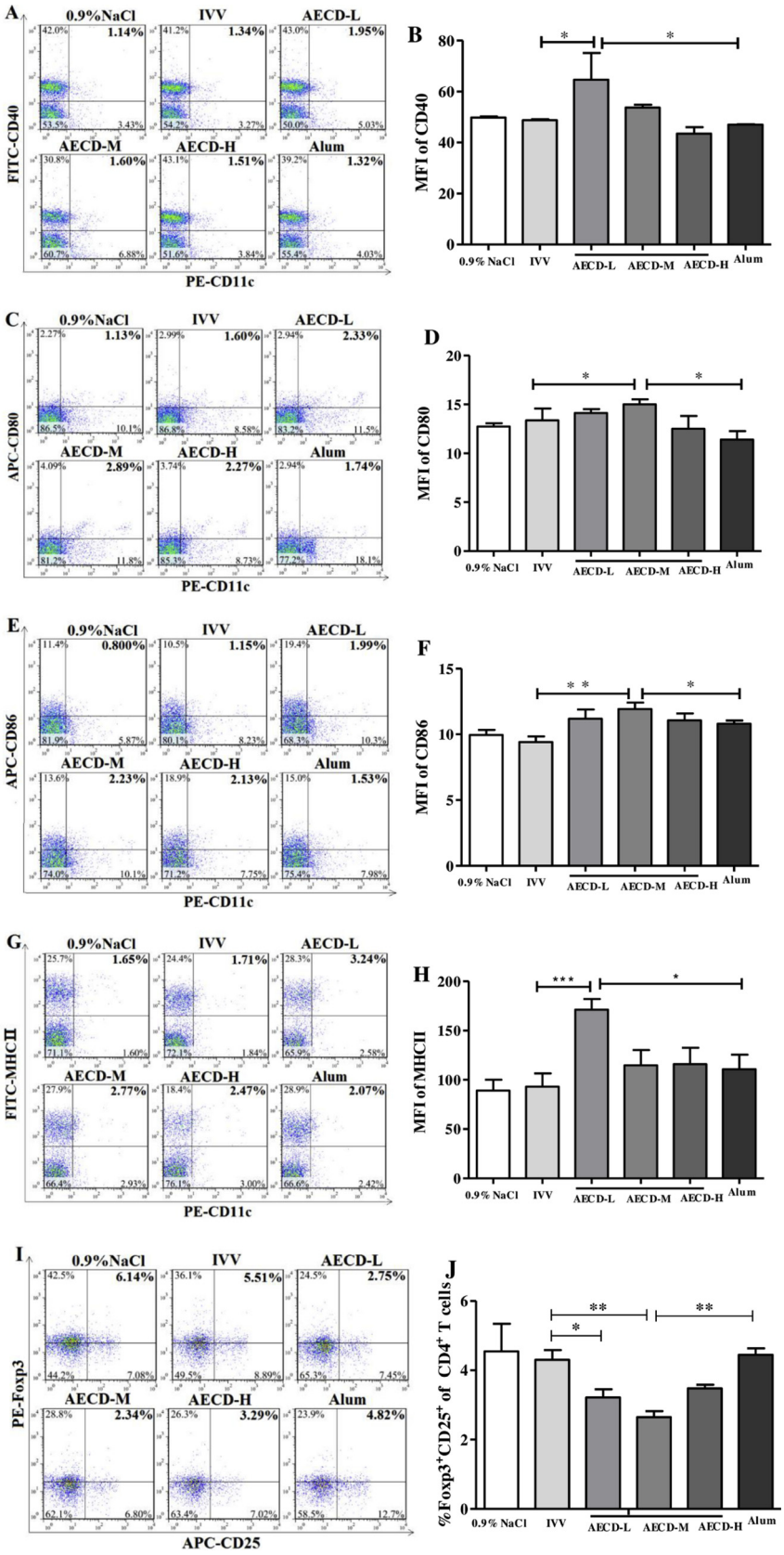

Fig. 4. Co-administration of AECD with IVV increased DC maturation and decreased Treg frequency. (A–D)In three days after single vaccination, the changes in DCs from spleens of immunized mice were examined by FACS. The activation of DCs was assayed via the expression of (A–B) $CD11c^+CD40^+$ cells (%), (C–D) $CD11c^+CD80^+$ cells (%), (E–F) $CD11c^+CD86^+$ cells (%), and (G–H) $CD11c^+$ MHC II cells (%). (I–J) In seven days after booster vaccination, the Treg frequency was determined with a mouse regulatory T cell staining kit and expressed as the percentage of $Foxp3^+CD25^+$ (%). The percentage of cells is expressed as mean ± SD ($n = 3$). *$p <$ 0.05, **$p <$ 0.05, ***$p <$ 0.001.

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Table 1
Influences of subcutaneous vaccination of AEAR on the mean body weight.

Groups	0 d	7 d	14 d	21 d
0.9%NaCl	23.43 ± 2.22	26.06 ± 1.49	28.74 ± 1.35	29.79 ± 1.17
IVV	21.8 ± 1.28	23.4 ± 1.95	25.11 ± 2.35	27.1 ± 1.50
AECD -L	22.51 ± 3.68	23.59 ± 2.56	27.29 ± 2.57	29.17 ± 1.98
AECD-M	20.4 ± 2.44	24.69 ± 1.51	27.29 ± 2.32	28.66 ± 2.21
AECD -H	21.7 ± 1.37	24.24 ± 1.43	26.36 ± 2.02	27.77 ± 1.75
Alum	21.81 ± 0.81	25.44 ± 1.03	27.44 ± 1.41	28.73 ± 1.40

Values are mean ± SD ($n = 8$). There was no significant difference in the body weight between the groups administered with the adjuvanted or non-adjuvanted seasonal influenza vaccine ($p > 0.05$).

Ethical approval

The animal experiments were approved by the Committee on the Ethics of Animal Experiments of Xinjang Key Laboratory of Biological Resources and Genetic Engineering (BRGE-AE001) of Xinjiang University.

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