Biologicals 70 (2021) 28–37

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Research paper

Evaluation of adjuvant activity of Astragaloside VII and its combination with different immunostimulating agents in Newcastle Disease vaccine

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ARTICLE INFO

Keywords:
Vaccine adjuvant
Natural products
Triterpenoid saponin
Astragaloside VII
Astragalus polysaccharide
Newcastle disease virus

ABSTRACT

Astragaloside VII (AST-VII), a major cycloartane saponin isolated from Turkish *Astragalus species*, turned out to be one of the most active metabolites demonstrating Th1/Th2 balanced immune response. As *Quillaja* saponins are extensively used in adjuvant systems, this study made an attempt to improve AST-VII based adjuvant systems by using different immunostimulatory/delivery agents (monophosphoryllipid A (MPL), *Astragalus* polysaccharide (APS) and squalene) and to induce cellular and humoral immune response against a viral vaccine. For this purpose, Newcastle Disease vaccine (NDV) was chosen as a model vaccine. Swiss albino mice were immunized subcutaneously with LaSota vaccines in the presence/absence of AST-VII or developed adjuvant systems. AST-VII administration both in live/inactivated LaSota vaccines induced neutralizing and NDV specific IgG, IgG1 and IgG2b antibodies response as well as IL-2 and IL-4 production. APS based delivery systems enhanced the production of neutralizing antibody and the minor augmentation of IFN-γ and IL-2 levels. Squalene emulsion (SE) alone or combined with AST-VII were effective in NDV restimulated splenocyte proliferation. As a conclusion, AST-VII and AST-VII containing adjuvant systems demonstrated Th1/Th2 balanced antibody and cellular immune responses in NDV vaccines. Thus, these systems could be developed as vaccine adjuvants in viral vaccines as alternative to saponin-based adjuvants.

1. Introduction

Astragalus membranaceus is one of the most popular herbal medicines in China. It has been used for the treatment of cold, diarrhea, fatigue and cardiovascular diseases in traditional medicine. In addition, *Radix Astragali* and their secondary metabolites are well known for its pharmacological properties such as immunomodulatory, antioxidant, antitumor, antidiabetic, antiviral, anti-inflammatory etc. [1]. In the flora of Turkey, *Astragalus* genus is represented by 224 endemic species in a total of 447 species [2]. In the Southeast Turkey, the water extracts of *Astragalus* roots have been used to cure leukemia and for its wound healing properties in traditional medicine. Preliminary cytotoxicity panels revealed that saponins isolated from Turkish *Astragalus* species had no cytotoxic activity. Therefore, we further focused on immunomodulatory properties of these compounds, which showed promising results for their development as immunotherapeutic agents and vaccine adjuvants *in vitro* and *in vivo* [3–7].

Vaccination is the most successful method to prevent the infectious diseases in farm animals and human. Conventional veterinary vaccines mainly consisted of live-attenuated pathogens, whole inactivated organisms or inactivated bacterial toxins [8]. Attenuated vaccines and bacterial toxins are superior because of high level of neutralizing antibody responses and ability of being phagocytosed by antigen presenting cells [9]. The utilization of attenuated vaccines is limited because of their side effects, reversion to virulence of the attenuated pathogen etc. On the other hand, inactivated vaccines have low immunogenicity and are not effective in the induction of cellular immune response [10]. Therefore, they require adjuvants to increase the immunogenicity of

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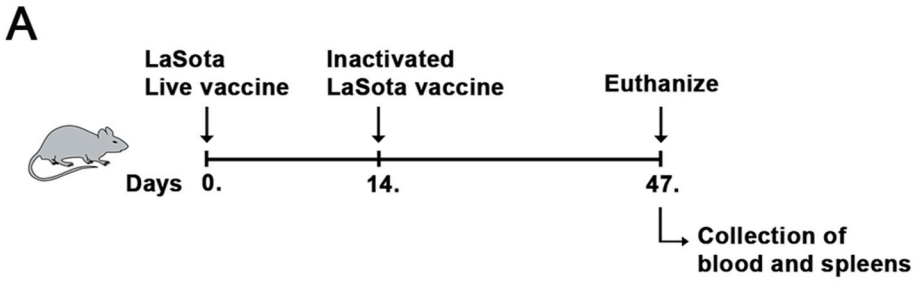
https://doi.org/10.1016/j.biologicals.2021.01.005
Received 25 September 2020; Received in revised form 26 January 2021; Accepted 31 January 2021
Available online 16 February 2021

antigen to give an appropriate immune response, provide dose-sparing and reduce side effects [11]. Alum, widely used adjuvant in veterinary and human vaccines, generally demonstrated antibody and Th2 mediated immune responses. Incapability of Th1 mediated immune response and several side effects induced by alum led researchers to investigate new adjuvants [12].

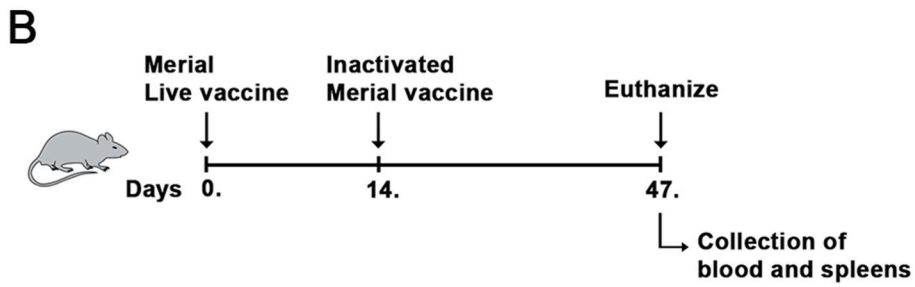
Newcastle disease (ND) is an acute viral disease in domestic poultry and other bird species, which causes substantial mortality and morbidity worldwide [13]. Vaccination programs for ND contain the administration of lentogenic live-virus or inactivated vaccines to stimulate protective immunity [14]. Inactivated ND vaccines are generally poor inducer of mucosal and cell mediated immune response, thus, an adjuvant is needed to increase its immunogenicity. Oil emulsion-based ND vaccines are widely used for a long time because they produce high antibody levels [15], but destabilization problems of vaccine emulsion formulations and induction of no or weak cellular immune response shows that necessity of novel stable adjuvants to enhance strong humoral and cellular immune responses.

Saponins are natural triterpene or steroidal glycosides that are widely distributed in plants and marine organisms. They have exhibited diverse biological activities such as anti-inflammatory, adjuvant, antioxidant, hemolytic, neuroprotective etc. [16,17]. *Quillaja* saponins have gain attention since 1950 due to their strong immunomodulatory properties. Quil-A, an aqueous extract of *Quillaja saponaria*, induced both humoral and cellular immune responses and was used as a vaccine adjuvant in veterinary medicine for many years, especially for Foot and Mouth Disease. QS-21, purified fraction of Quil-A, has been used as golden standard for saponin based adjuvants. In mice, QS-21 showed IgG1/IgG2a balanced antigen specific immune response, IFN-γ and IL-2 production and effective $CD8^+$ T cell response [18].

Besides strong adjuvant properties, toxicity and undesirable hemolytic activity of QS-21 limited its utilization in human vaccines. To overcome these problems, synthetic [19] and semi-synthetic analogs of QS-21 [20,21] have been synthesized, QS-21 based formulation studies with different immunostimulating agents [22] carried out and, their immunomodulatory activities are extensively studied with different types of antigens in pre-clinically and clinically [23]. One of the saponins isolated from *Astragalus* species, Astragaloside VII (AST-VII), induced humoral and Th1/Th2/Th17 balanced cellular immune responses [4–7,24]. High solubility in water, slight hemolytic activity at high concentrations, high stability, and appropriateness to lyophilization make AST-VII a good candidate as vaccine adjuvant. A substantial amount of polysaccharides (*Astragalus* polysaccharide-APS) is present in the roots of *Astragalus* plants together with saponin glycosides. These polysaccharides are also known as Gum Tragacanth, which has been used in the food and pharmaceutical industries as emulsifying and stabilizing agents for years. Moreover, APS demonstrated immunomodulatory activities such as boosting the production of IL-2, IFN-γ, TNF-α,



Groups	0. Day	14. Day
Control	Saline	Saline
LaSota	LaSota Live Vaccine	Inactivated LaSota Vaccine
AST-VII/ AST-VII	LaSota Live Vaccine + AST-VII	Inactivated LaSota Vaccine + AST-VII
AST-VII	LaSota Live Vaccine	Inactivated LaSota Vaccine + AST-VII
AST-VII+MPL	LaSota Live Vaccine	Inactivated LaSota Vaccine + AST-VII+MPL
ASE (AST-VII+SE)	LaSota Live Vaccine	Inactivated LaSota Vaccine + ASE



Groups	0. Day	14. Day
CEVAC	Merial Live Vaccine	CEVAC
AST-VII/ AST-VII	Merial Live Vaccine + AST-VII	Inactivated Merial Vaccine + AST-VII
MPL	Merial Live Vaccine	Inactivated Merial Vaccine + MPL
SE	Merial Live Vaccine	Inactivated Merial Vaccine + SE
AST-VII+MPL	Merial Live Vaccine	Inactivated Merial Vaccine + AST-VII+MPL
ASE (AST-VII+SE)	Merial Live Vaccine	Inactivated Merial Vaccine + ASE
AST-VII/ ASE	Merial Live Vaccine + AST-VII	Inactivated Merial Vaccine + ASE
APNS (APS)	Merial Live Vaccine	Inactivated Merial Vaccine + APNS
ANS (APS+AST-VII)	Merial Live Vaccine	Inactivated Merial Vaccine + ANS

Fig. 1. Immunization schedules of NDV vaccines and vaccination groups indicated for **A)** LaSota and **B)** commercial LaSota (Merial) vaccines.

and activating mouse macrophages, B cells and dendritic cells [25]. The effects of APS on NDV vaccines were investigated and the results showed that APS augmented NDV specific antibody response and splenocyte proliferation in chicken [26].

In previous studies, we reported novel adjuvant carrier system combining *Astragalus* polysaccharide and AST-VII (ANS), and evaluated its immunomodulatory potential in inactivated influenza vaccine (H3N2) in mice. This adjuvant system demonstrated humoral and cellular immune response [7]. In the light of these findings, an attempt was made to develop AST-VII based adjuvant systems utilizing TLR-4 (Toll like receptor-4) agonist MPL, squalene and APS, and evaluate their vaccine adjuvant properties in NDV vaccines.

2. Materials and methods

2.1. Materials

The RPMI-1640 medium (Biochrom, Berlin, Germany), was supplemented with 10 μg/mL gentamycin (Biochrom, Berlin, Germany), 2 μmol/mL L-glutamine (Biochrom, Berlin, Germany), 50 μmol/L 2-mercaptoethanol (Sigma, Missouri, USA) and 10% fetal bovine serum (Gibco, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), polyoxyethylene sorbitan monooleate (Tween 80) and sorbitan trioleate (Span 85) were purchased from Sigma Chemical Co., Missouri, USA. Goat polyclonal anti-mouse IgG, IgG1 and IgG2b peroxidase conjugate were purchased from Southern Biotech. Assoc., Birmingham, USA. Mouse IL-2, IFN-γ and IL-4 ELISA kits were purchased from ebioscience (San Diego, USA). Newcastle Disease Virus (B1 type, LaSota strain, Live Virus) and CEVAC Broiler NDK were obtained from Merial Select, Inc., (GA, USA) and CEVAC (Sheffield, England). AST-VII, APS and squalene were donated by Bionorm Natural Products (Izmir, Turkey).

2.2. Production, isolation and inactivation of NDV (LaSota strain) vaccines

NDV vaccine (LaSota strain) was propagated in 9–10 days-old specific pathogen free (SPF) chicken eggs as described by Yakuboğulları et al. [7]. LaSota virus titer was calculated as $10^{6.0}$ EID_{50}/mL according to Spearman-Karber method. NDV vaccines were inactivated with 3–5% of BEI (binary ethylenimine).

2.3. Preparations of adjuvanted LaSota vaccines

The oil in water (o/w) squalene emulsions (SE) were prepared using the following components: 5% squalene, 0.5% polyoxyethylene sorbitan monooleate (Tween 80) and 0.5% sorbitan trioleate (Span 85) [27]. Oil phase, 0.5 mL squalene was mixed with 0.05 mL of tween 80 and 0.05 mL of span 85. Then, ultrapure water was slowly added into the oil phase by adjusting final volume to 10 mL under the agitation of a homogenizer (Yellowline DI 25 basic). To obtain an adjuvant system combining AST-VII and SE, stated as ASE, same procedure for the preparation of SE was followed by adding 10 mg AST-VII in water phase. The particle size and surface zeta potential of SE was measured by Malvern Nanosizer/Zetasizer Nano-ZS ZEN 3600 (Malvern Instruments, USA).

For AST-VII + MPL combination, each component was dissolved in physiological saline, and simple admixture to administrate 120 μg AST-VII [4] and 10 μg MPL [28] per mice.

Astragalus polysaccharide (APS) based nanocarrier system (APNS) and adjuvant nanocarrier system (ANS) composed of AST-VII and APS were prepared according to the following methodology by Yakuboğulları et al. [7]. All formulations were filtered through 0.22 μm sterile filter before mixing with LaSota or Merial vaccines in 1:1 volume ratio.

2.4. Experimental animals

Male-female Swiss albino mice (4–6 weeks old) weighing 18–22 g were purchased from Bornova Veterinary Control Institute (Bornova, Izmir, Turkey). Mice were maintained in the groups of 6, under standard conditions of temperature 22 ± 1 °C with regular 12 h light:12 h dark cycle and were allowed free access to standard laboratory food and water. The experimental protocol was approved by the Local Ethics Review Committee for Animal Experimentation of Uludağ University (2014-14/04).

2.5. Immunizations

The immunomodulatory effects of adjuvants/adjuvant systems were evaluated on two different NDV (LaSota strain) vaccines, expressed as LaSota vaccine and commercially available one, Merial® vaccine. Immunization schedule and vaccination groups are shown in detail in Fig. 1. At day 0, four to six weeks old Swiss albino mice were subcutaneously vaccinated with live $10^{6.0}$ EID_{50}/mL LaSota virus or Merial with/without AST-VII (120 μg) [29]. Fourteen days after the first immunization, boosting immunization was done subcutaneously with inactivated LaSota or inactivated Merial vaccines adjuvanted with following groups: AST-VII (120 μg), MPL (10 μg), AST-VII (120 μg) + MPL (10 μg), SE, ASE, APNS, ANS and CEVAC (commercially available oil-adjuvanted LaSota vaccine). Control group was only saline treatment without NDV vaccines. Thirty-three days after the second immunization, mice were euthanized, and blood samples were collected by cardiac puncture methodology using 1 mL syringe [30]. To obtain mouse sera, the blood samples were centrifuged at 500×*g* for 5 min, and upper yellow phase was collected and stored −20 °C for HI assay, antibody and cytokine measurements by ELISA. Moreover, the spleens were collected for splenocyte proliferation assay.

2.6. Determination of neutralizing antibody response by hemagglutination (HA) and hemagglutination inhibition (HI) assays

HA and HI assays were carried out following the methodology by Yakuboğulları et al., [7]. The HI assay was performed using BEI inactivated LaSota (4 HA virus/antigen).

2.7. Measurement of NDV-specific antibody response

NDV-specific IgG, IgG1 and IgG2b antibody titers in mouse sera were measured by commercial ELISA kits (IDEXX, Portland, Maine, USA) with modified methodology of Nalbantsoy et al. [4]. The diluted serum samples (1:500) were added into NDV pre-coated 96-well microtiter plates and incubated for 1 h at 37 °C. Following serial washing steps, 1:8000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1 or IgG2b were added and incubated for 1 h at 37 °C. The enzymatic reaction was developed with the addition of H_2O_2 (Merck, Darmstadt, Germany) and O-phenylenediamine (Sigma, St. Louis, USA). Data was expressed as the mean OD value of the samples minus the mean OD value of the control. The results were expressed as log2 titers.

2.8. Measurement of IL-2, IFN-γ and IL-4

IL-2, IFN-γ and IL-4 titers in mouse sera were determined by commercial ELISA kits (eBioscience, USA) according to manufacturer's instructions.

2.9. Splenocyte proliferation assay

Splenocyte proliferation assay was performed based on the methodology described by Nalbantsoy et al. [4]. Thirty-three days after last immunization, the mouse spleens were collected to obtain single cell suspension. These suspensions were treated with Concavalin A (Con

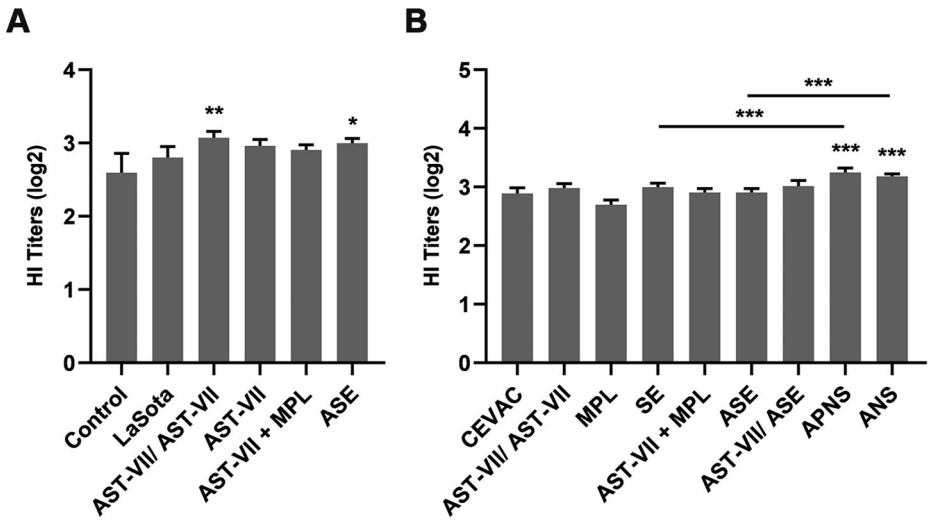


Fig. 2. Hemagglutination Inhibition (HI) analysis for **A)** LaSota and **B)** Merial vaccines. Swiss albino mice were immunized subcutaneously with AST-VII (120 μg) adjuvanted/unadjuvanted live LaSota and Merial vaccines at day 0. Boosting immunization was performed with inactivated LaSota and Merial vaccines alone or combined with following adjuvants: AST-VII (120 μg), MPL (10 μg), AST-VII (120 μg) + MPL (10 μg), SE, ASE, APNS and ANS. AST-VII/AST-VII or AST-VII/ASE indicated the vaccine group immunized with AST-VII adjuvanted live NDV vaccine/AST-VII or ASE adjuvanted-inactivated NDV vaccine. Control group ($n = 3$) indicates only saline treatment without NDV vaccine. Thirty-three days after last immunization, mouse sera ($n = 6$) were collected and analyzed for HI by using 1% mouse RBCs (red blood cells). Data was expressed as log 2 titers. Statistically significant differences of adjuvanted groups versus LaSota or CEVAC are presented. $^{*}p < 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

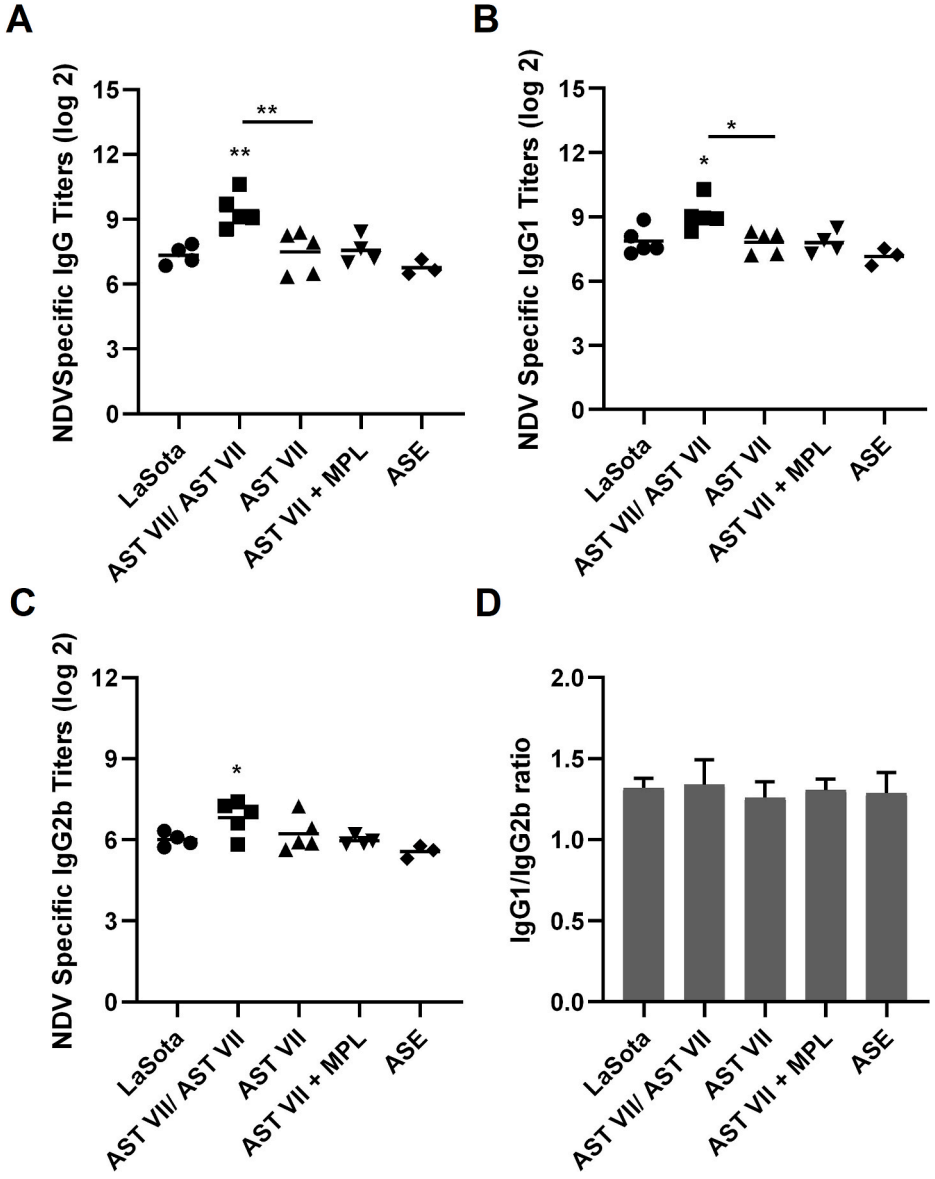


Fig. 3. Effects of adjuvanted/unadjuvanted LaSota vaccine on the production of NDV specific **A)** IgG, **B)** IgG1, **C)** IgG2b, **D)** IgG1/IgG2b ratio. Swiss albino mice were immunized subcutaneously with live LaSota alone or combined with AST-VII (120 μg) at day 0. Boosting immunization was performed with inactivated LaSota vaccines alone or combined with following adjuvants: AST-VII (120 μg), AST-VII (120 μg) + MPL (10 μg), ASE. AST-VII/AST-VII or AST-VII/ASE indicated the vaccine group immunized with AST-VII adjuvanted live NDV vaccine/AST-VII or ASE adjuvanted-inactivated NDV vaccine. Thirty-three days after last immunization, mouse sera ($n = 5$) were collected and analyzed for NDV-specific IgG, IgG1 and IgG2b antibodies by ELISA. Data was expressed as log 2 titers. Statistically significant differences of adjuvanted groups versus LaSota are presented. $^{*}p < 0.05$, $^{**}p \leq 0.01$.

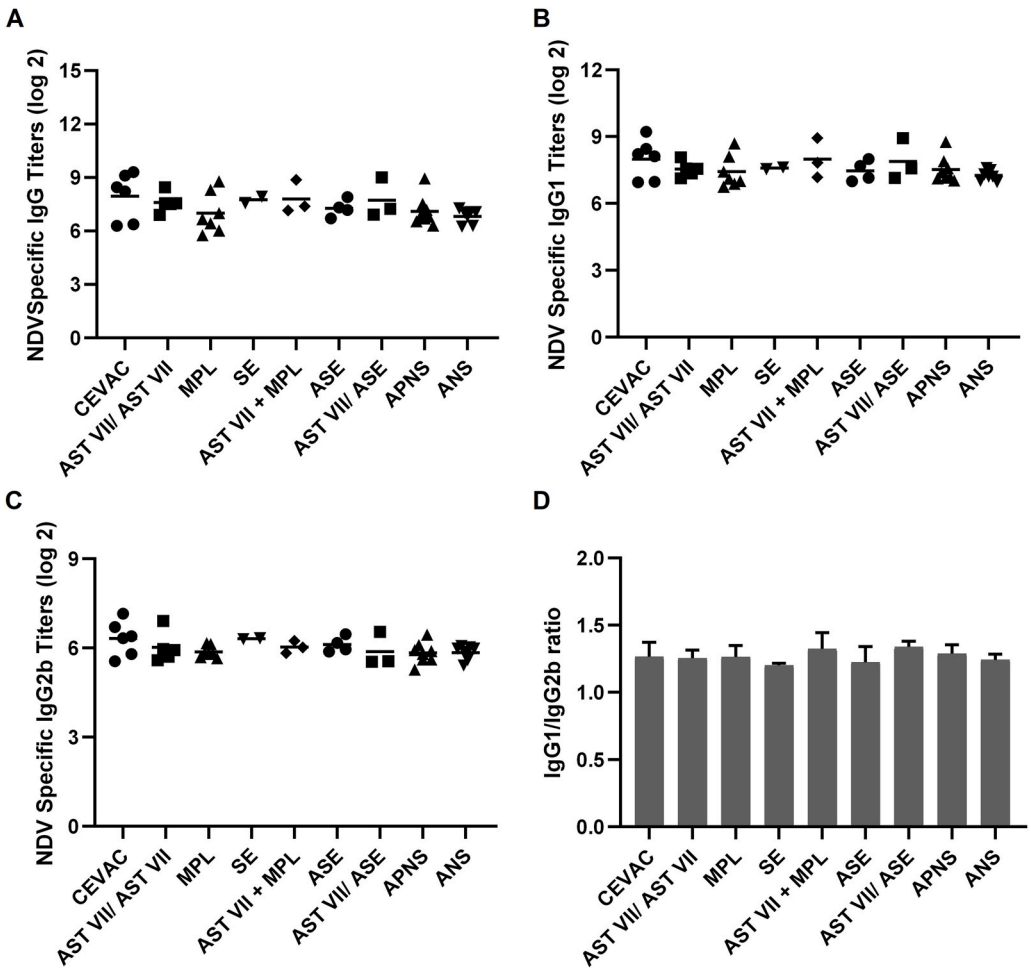


Fig. 4. Effects of adjuvanted/unadjuvanted Merial vaccine on the production of NDV specific **A)** IgG, **B)** IgG1, **C)** IgG2b, **D)** IgG1/IgG2b ratio. Swiss albino mice were immunized subcutaneously with live Merial vaccine alone or combined with AST-VII (120 μg) at day 0. Boosting immunization was done with inactived Merial alone or with following adjuvants: AST-VII (120 μg), MPL (10 μg), AST-VII (120 μg) + MPL (10 μg), SE, ASE, APNS and ANS. AST-VII/AST-VII or AST-VII/ASE indicated the vaccine group immunized with AST-VII adjuvanted live NDV vaccine/AST-VII or ASE adjuvanted-inactivated NDV vaccine. Thirty-three days after last immunization, mouse sera ($n = 6$) were collected and analyzed for NDV-specific IgG, IgG1 and IgG2b antibodies by ELISA. Data was expressed as log 2 titers.

A-final concentration 5 μg/mL) and 4 HA LaSota and incubated for 72 h at 37 °C. LPS (final concentration 10 μg/mL) restimulation was done only for some vaccination groups. Stimulation index (SI) was calculated according to the following formula: SI=The absorbance value for mitogen-stimulated cultures/The absorbance value for non-stimulated cultures.

2.10. Statistical analysis

The data was expressed as mean ± SD. Statistically significance of differences was examined by using One-way ANOVA, Student-*t* test, and Dunnett's test for post hoc test (GraphPad Prim 6.0). *P*-values of less than 0.05*, 0.01** and 0.001*** were considered as statistically significant.

3. Results

3.1. Characterization of squalene-based adjuvant systems

Particle size distribution and surface zeta potential of SEs were measured through DLS (Dynamic Light Scattering) methodology. The hydrodynamic particle size of SE and ASE was measured as 155.1 and 183.2 nm in diameter. Moreover, SE and ASE demonstrated negative surface potential as −38.1 and −33.7 mV, respectively. The load of AST-VII into SE increased the particle size and reduced the surface zeta potential. All SE formulated vaccine groups showed a smaller polydispersity index than 0.5, indicating the homogeneous size distribution (Supplemental Fig. 1). The stability of SEs was examined in terms of pH variations and phase inversion. SEs at pH 7 were stable at room temperature for up to two weeks and there was no phase inversion.

3.2. Evaluation of hemagglutination inhibition assay

HI activity of AST-VII and AST-VII based adjuvant systems were evaluated in LaSota and Merial vaccines. Administration of AST-VII both in live and inactivated LaSota vaccine provided an increase in HI titers compared to LaSota group ($p \leq 0.01$) (Fig. 2A). Administration of AST-VII only in inactivated LaSota did not make a statistically significant augmentation in HI titers compared to unadjuvanted LaSota group. Moreover, ASE adjuvanted group was prominent in HI titers in contrast to unadjuvanted LaSota ($p < 0.05$). In Merial case, all adjuvant combinations except MPL demonstrated similar HI titers as oil-based adjuvanted LaSota vaccine, CEVAC. APNS and ANS adjuvanted Merial revealed higher HI titers compared to CEVAC ($p \leq 0.001$). When the effect of delivery systems on neutralizing antibody induction (HI) was analyzed, APNS or ANS systems composed of APS demonstrated statistically significant HI titers than SE or ASE ($p \leq 0.001$) (Fig. 2B).

3.3. Assessment of NDV-specific antibody response

Unadjuvanted LaSota or Merial vaccines, and their combinations with different adjuvants were investigated in terms of induction of IgG, IgG1 and IgG2b antibody responses. No significant induction was observed in the antibody response between adjuvanted LaSota vaccine and unadjuvanted form. Only administration of AST-VII (120 μg) with live and inactivated LaSota meaningfully augmented IgG, IgG1, IgG2b antibody levels in mouse sera compared to unadjuvanted LaSota ($p \leq 0.01$, $p < 0.05$, $p < 0.05$, respectively) (Fig. 3A–C). Adjuvanted and

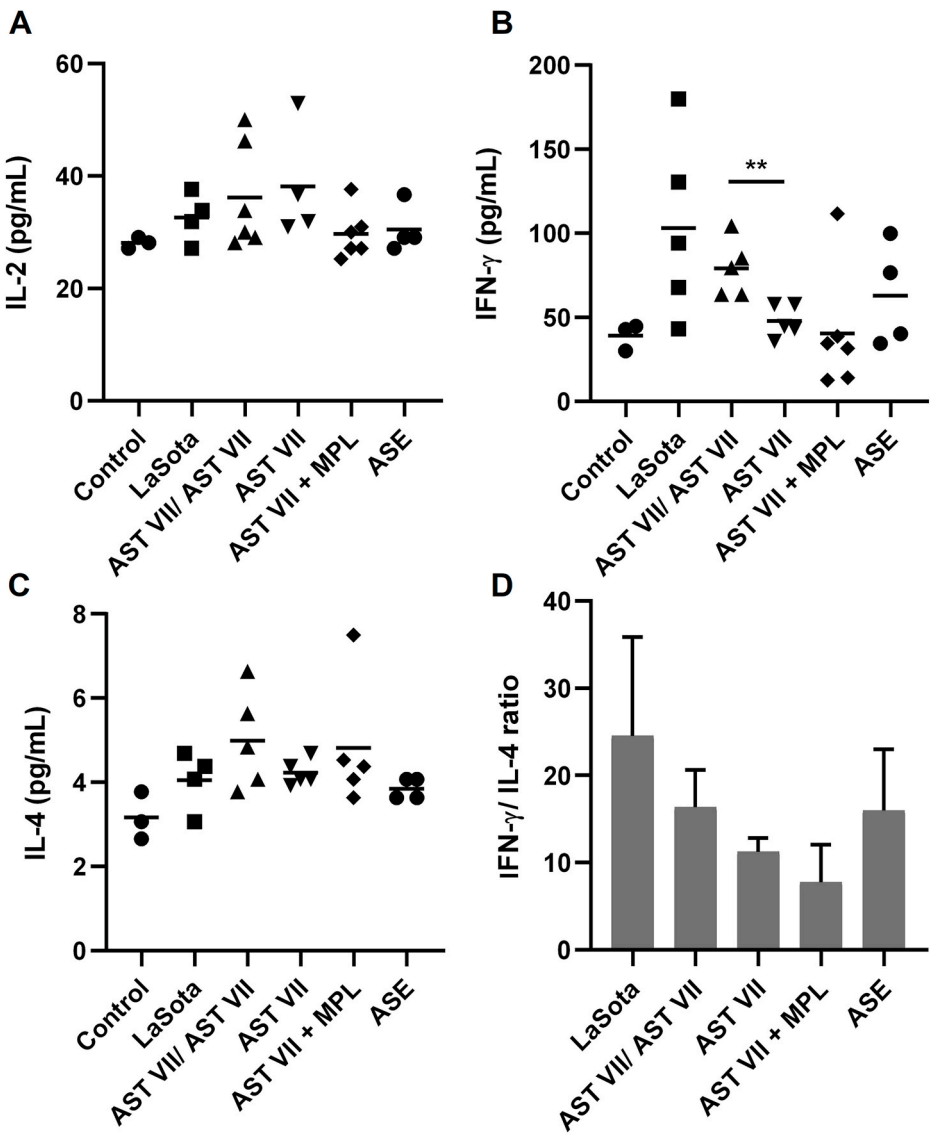


Fig. 5. Effects of adjuvanted/unadjuvanted LaSota vaccines on the production of **A)** IFN-γ, **B)** IL-2 and **C)** IL-4, **D)** IFN-γ/IL-4 ratio. Swiss albino mice were immunized subcutaneously with live LaSota vaccine alone or combined with AST-VII (120 μg) at day 0. Boosting immunization was performed with inactivated LaSota vaccine alone or combined with following adjuvants: AST-VII (120 μg), AST-VII (120 μg) + MPL (10 μg) and ASE. AST-VII/AST-VII or AST-VII/ASE indicated the vaccine group immunized with AST-VII adjuvanted live NDV vaccine/AST-VII or ASE adjuvanted-inactivated NDV vaccine. Control group ($n = 3$) indicates only saline treatment without NDV vaccine. Thirty-three days after last immunization, mouse sera ($n = 6$) were collected and analyzed for IFN-γ, IL-2 and IL-4 by ELISA. Statistically significant differences of adjuvanted groups versus LaSota are presented. $^{**}p \leq 0.01$.

unadjuvanted LaSota vaccines demonstrated a similar IgG/IgG2b ratio, indicating balanced Th1/Th2 immune responses (Fig. 3D).

In regards to Merial vaccine, there was no substantial improvement between adjuvanted vaccine and CEVAC. In general, SE, ASE and AST-VII + MPL provided similar antibody titers with CEVAC (Fig. 4A–C). There was no alteration in IgG1/IgG2b ratio of adjuvanted vaccines, demonstrating prominent Th2 mediated immune response (Fig. 4D).

3.4. Effects of AST-VII and AST-VII based adjuvant systems on the production of IFN-γ, IL-2 and IL-4

Cytokine response induced by adjuvanted/unadjuvanted vaccines was investigated and the results were indicated in Figs. 5 and 6. In LaSota vaccinated groups, AST-VII adjuvanted vaccines slightly increased IL-2 and IL-4 titers compared to unadjuvanted LaSota (Fig. 5A and C). IFN-γ levels decreased in all adjuvanted groups. Administration of AST-VII both in live and inactivated LaSota augmented IFN-γ levels in mouse sera compared to AST-VII administration only in inactivated LaSota ($p \leq 0.01$) (Fig. 5B). The highest IFN-γ/IL-4 ratios were seen in the groups immunized with unadjuvanted LaSota, AST-VII administration in live and inactivated vaccine and ASE adjuvanted LaSota vaccine (Fig. 5D).

In the case of Merial vaccinations, there is no statistically significant increase in IFN-γ, IL-2 and IL-4 titers between adjuvanted groups and CEVAC ($p > 0.05$). However, IFN-γ titer was slightly enhanced by the immunization of AST-VII + MPL, APNS and ANS compared to CEVAC (Fig. 6A). IL-2 levels were not shown statistical difference between tested groups but prepared adjuvant systems, especially AST-VII + MPL and APNS, demonstrated a similar IL-2 response as widely used oil adjuvanted LaSota vaccine (CEVAC) in poultry (Fig. 6B). IL-4 titers in adjuvanted vaccine groups slightly reduced compared to CEVAC (Fig. 6C). The highest IFN-γ/IL-4 ratios were seen in the groups immunized with AST-VII administration in live and inactivated vaccines, SE, AST-VII + MPL, APNS and ANS adjuvanted LaSota vaccine (Fig. 6D).

3.5. Evaluation of splenocyte proliferation capacity of adjuvanted/unadjuvanted LaSota vaccines

Effects of AST-VII and its combination with SE, MPL and APNS on LaSota and Con A stimulated splenocytes were shown in Figs. 7 and 8. In LaSota vaccinated groups, administration of AST-VII both in live/inactivated vaccine ($p < 0.05$; $p \leq 0.01$), AST-VII ($p \leq 0.01$), AST-VII + MPL ($p < 0.05$; $p \leq 0.01$), ASE ($p \leq 0.01$; $p \leq 0.01$) induced splenocyte proliferation in Con A and NDV restimulated splenocytes, respectively (Fig. 7).

In Merial vaccinated groups, only SE adjuvanted Merial vaccine

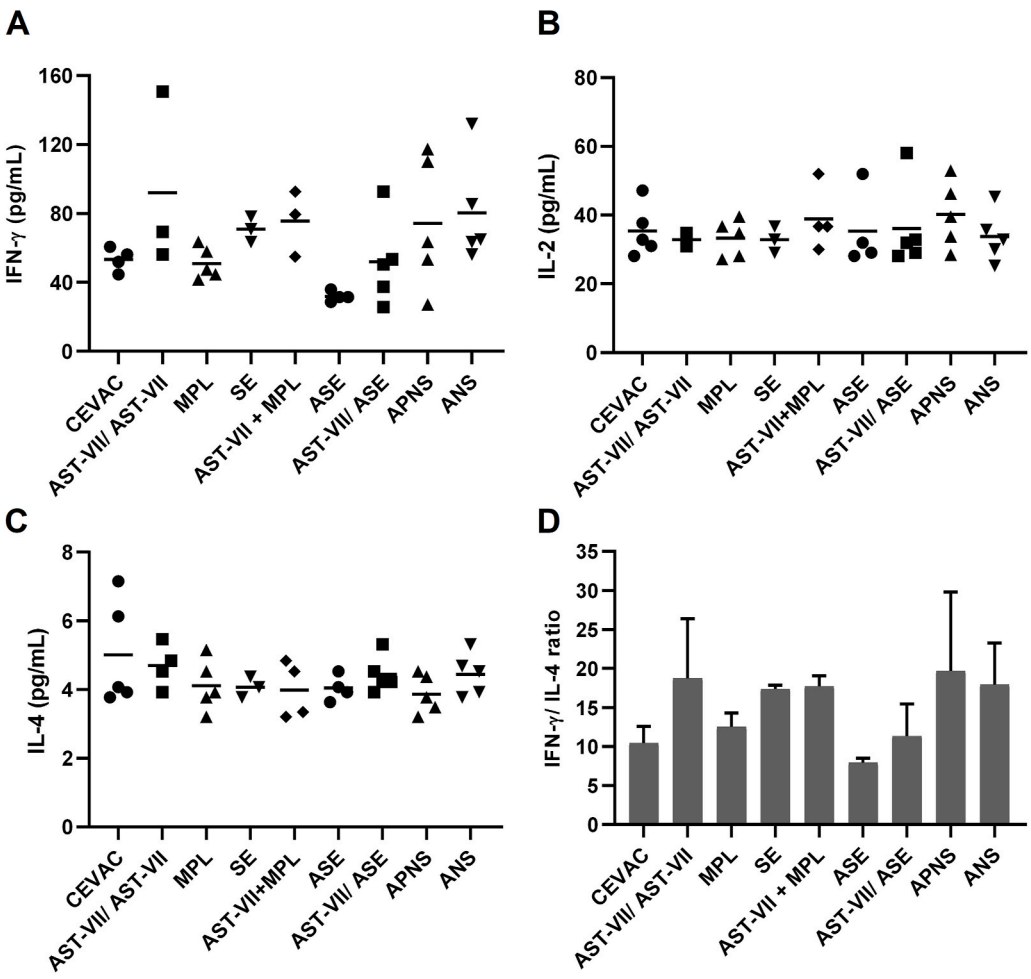


Fig. 6. Effects of adjuvanted/unadjuvanted Merial vaccine on the production of **A)** IFN-γ, **B)** IL-2 and **C)** IL-4, **D)** IFN-γ/IL-4 ratio. Swiss albino mice were immunized subcutaneously with live Merial vaccine alone or combined with AST-VII (120 μg) at day 0. Boosting immunization was performed with inactivated Merial vaccine alone or combined with following adjuvants: AST-VII (120 μg), MPL (10 μg), AST-VII (120 μg) + MPL (10 μg), SE, ASE, APNS and ANS. AST-VII/AST-VII or AST-VII/ASE indicated the vaccine group immunized with AST-VII adjuvanted live NDV vaccine/AST-VII or ASE adjuvanted-inactivated NDV vaccine. Thirty-three days after last immunization, mouse sera ($n = 5$) were collected and analyzed for IFN-γ, IL-2 and IL-4 by ELISA.

induced splenocyte proliferation in Con A stimulated splenocytes ($p < 0.05$). In the case of NDV restimulation, the following adjuvants combined with Merial were able to enhance splenocyte proliferation: AST-VII/AST-VII, SE ($p \leq 0.01$), AST-VII + MPL ($p \leq 0.01$), ASE ($p \leq 0.01$) and AST-VII/ASE ($p \leq 0.01$). In the vaccination groups where AST-VII combined with different immunostimulating agents, SE was more effective than MPL in NDV stimulated splenocyte proliferation ($p \leq 0.001$). There was no enhancement in the APNS or ANS adjuvanted vaccinations compared to CEVAC (Fig. 8).

The single cell suspension of splenocytes obtained from adjuvanted LaSota and Merial vaccine restimulated with LPS. The lowest stimulation was observed in APNS, whereas AST-VII + MPL, ASE and AST-VII/ASE groups demonstrated higher HI titers compared to other groups (Supplemental Table 1).

4. Discussion

Vaccination elicits strong and long-term immunity against infectious agents. In live NDV vaccinations, reversion to virulence of pathogen is a major problem, thus inactivated or subunit NDV vaccines are used. However, the problems related to poor immunogenicity are come up and bring the use of adjuvants [31]. Oil-based emulsion adjuvants have been widely used for NDV vaccinations in poultry. Stability issues and lack of cell-mediated immune response by administrating oil-emulsion adjuvants led researchers to investigate stable, efficacious adjuvants and cost-effective delivery systems that can induce humoral and cellular immune response for NDV [29].

In this study, we tried to develop safe AST-VII based adjuvant systems by formulating AST-VII with an immunostimulatory agent such as MPL, squalene and APS to induce humoral and cellular immune response. Immunomodulatory activities of these adjuvant systems are investigated on two different NDV vaccines stated as LaSota and Merial in terms of neutralizing antibody, NDV specific antibody, cytokine responses along with a capacity to stimulate splenocytes.

All adjuvanted NDV vaccines (except MPL) demonstrated slightly increase in neutralizing antibody titers compared to LaSota and Merial. In LaSota vaccine, AST-VII/AST-VII and ASE revealed significant HI titers compared to unadjuvanted LaSota. The results obtained from Merial vaccine showed that AST-VII containing formulations provided a similar neutralizing antibody responses with oil-emulsion adjuvants. Interestingly, APNS and ANS consist of the polymeric delivery system (APS) augmented significantly higher HI titers over CEVAC ($p \leq 0.001$). Rehmani et al. show that saponin-based adjuvants, Quil-A and ISCOMs formulation with live NDV vaccine (V4 strain) did not enhance neutralizing antibody response [32]. However, Homhuan et al. revealed that NDV (Clone-30) containing ISCOMs increased HI titers and induced >80% protection in NDV challenged chickens [33]. Chen et al. revealed that APS with inactivated LaSota vaccine augmented HI titers in chicken in contrast to inactivated LaSota [26]. Polysaccharide extracted from *Ganoderma lucidum* (GLP-25 mg/kg) was orally administrated with LaSota vaccine into chickens and enhanced HI antibodies compared to LaSota vaccine alone [34]. These results are consistent with the data obtained from LaSota and Merial vaccines. According to HI titers obtained from adjuvanted NDV vaccines, *Astragalus* saponins and polysaccharides could be an alternative to oil-based adjuvants.

As antibody response is important in virus neutralization by complement activation or opsonization, NDV specific antibody response was evaluated along with HI [31]. Antibody titers could not be measured in

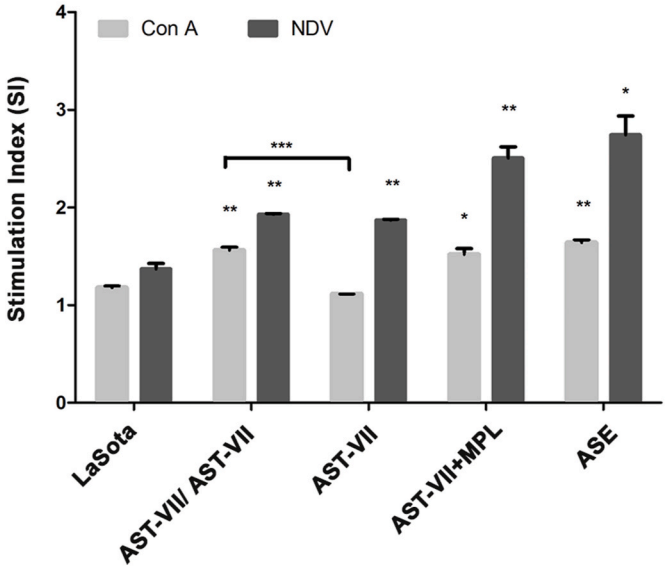


Fig. 7. Stimulation index (SI) of Con A and NDV re-stimulated splenocytes obtained from mice immunized with adjuvanted/unadjuvanted LaSota vaccine. Swiss albino mice were ($n = 6$) immunized subcutaneously with live LaSota vaccine alone or combined with AST-VII (120 μg) at day 0. Boosting immunization was performed with inactivated LaSota vaccine alone or combined with following adjuvants: AST-VII (120 μg), AST-VII (120 μg) + MPL (10 μg) and ASE. AST-VII/AST-VII or AST-VII/ASE indicated the vaccine group immunized with AST-VII adjuvanted live NDV vaccine/AST-VII or ASE adjuvanted-inactivated NDV vaccine. Thirty-three days after last immunization, mouse spleens were collected, minced and passed through a sterile mesh to obtain single splenocyte suspension. These cells were re-stimulated with Con A and NDV, incubated for 72 h and analyzed for stimulation index (SI). Statistically significant differences of adjuvanted groups versus LaSota are presented. *$p <$ 0.05, **$p \leq 0.01$ and ***$p \leq 0.001$.

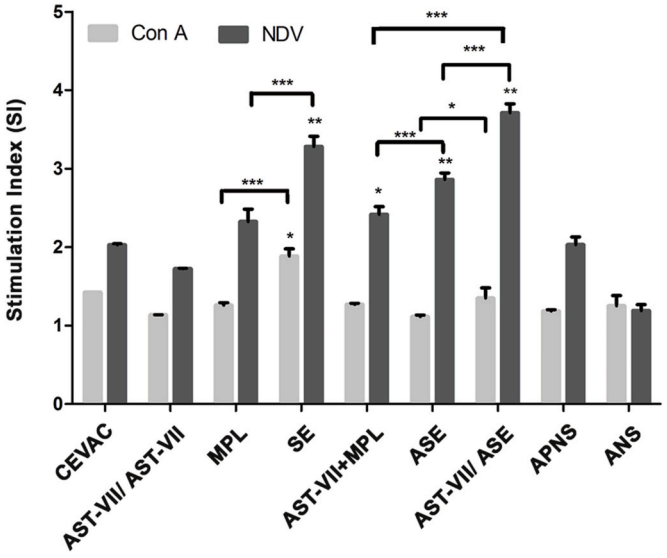


Fig. 8. Stimulation indexes (SI) of Con A and NDV re-stimulated splenocytes obtained from mice immunized with adjuvanted/unadjuvanted Merial vaccine. Swiss albino mice were ($n = 6$) immunized subcutaneously with live Merial vaccine alone or combined with AST-VII (120 μg) at day 0. Boosting immunization was performed with inactivated Merial vaccine alone or combined with following adjuvants: AST-VII (120 μg), MPL (10 μg), SE, AST-VII (120 μg) + MPL (10 μg), ASE, APNS and ANS. AST-VII/AST-VII or AST-VII/ASE indicated the vaccine group immunized with AST-VII adjuvanted live NDV vaccine/AST-VII or ASE adjuvanted-inactivated NDV vaccine. Thirty-three days after last immunization, mouse spleens were collected, minced and passed through a sterile mesh to obtain single splenocyte suspension. These cells were re-stimulated with Con A and NDV, incubated for 72 h and analyzed for stimulation index (SI). Statistically significant differences of adjuvanted groups versus CEVAC are presented. *$p < 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

mouse sera for a particular mice groups as well as cytokine titers. This limitation could be resulted from an insufficient amount of mice sera collected from mice during cardiac puncture methodology. However, in measured antibody response, AST-VII administration both in live and inactivated LaSota vaccine statistically increased NDV specific IgG ($p \leq 0.01$), IgG1 ($p < 0.05$) and IgG2b ($p < 0.05$). Administration of AST-VII in live and inactivated LaSota vaccine revealed higher antibody response (IgG, IgG1 and IgG2b) over AST-VII administration only in inactivated LaSota vaccine ($p < 0.01$, $p < 0.05$, respectively). In Merial vaccinated groups, there was no statistically significant increment over CEVAC. However, similar IgG, IgG1 and IgG2b antibody responses were obtained by adjuvanted vaccines as CEVAC. Sun et al. isolated seven saponins from the roots of *Platycodon grandiflorum* (PD, PD2, PD3, PA, PE, DPE and PGD) and immunized these compounds with rL-H5 antigen (Newcastle disease virus-based recombinant influenza vaccine) into mice. PD and PD2 significantly increased IgG, IgG1, IgG2a and IgG2b antibody responses whereas PA and PGD induced IgG and IgG1 responses [35]. These results were consistent with our antibody data. A liposomal system containing TLR-4 agonist (LPS) was administrated into the chicken with NDV vaccine, and induced IgA and IgG antibody response as well as $CD4^+$ and $CD8^+$ T cell response [36]. In Merial vaccine, AST-VII and MPL (TLR-4 agonist) combination also produced IgG, IgG1 and IgG2b response. As IgG1 antibody response corresponds Th2 mediated immune response, IgG2b antibody indicates Th1 bias immune response. It can be concluded that saponin-based adjuvants demonstrated Th1/Th2 balanced NDV specific antibody response.

Antibody response prevents the spreading and binding of the virus to the cell membrane, but it did not avoid transduction and transcription of viral genes, and viral replication using newly produced nucleocapsid [37]. Hence, an active cell-mediated immune response is required in NDV vaccines as well as humoral immune response. To investigate the role of cytokines in adjuvanted NDV vaccines, IFN-γ, IL-2 and IL-4 cytokine responses were evaluated. IFN-γ is one of the crucial cytokines in anti-viral immunity due to its immunoregulatory properties such as activation of cytotoxic T lymphocytes, natural killer cells and recruitment of macrophages to the injection site [38]. The production of IFN-γ was decreased with adjuvanted LaSota vaccines compared to unadjuvanted LaSota. In adjuvanted Merial vaccine, there was no statistical augmentation on IFN-γ, IL-2 and IL-4. However, AST-VII in live/inactivated Merial, AST-VII + MPL, APNS and ANS provided higher mean IFN-γ titers than CEVAC. AST-VII integration into SE did not enhance the production of IFN-γ, whereas AST-VII combination with APS (ANS) slightly increased the levels of IFN-γ.

IL-2 is a Th1 type cytokine that is responsible for the proliferation of $CD4^+$, $CD8^+$ T cells and NK cells, the stimulation of cytokines in the microenvironment and induction of antibody response [39]. In LaSota vaccines, AST-VII formulations slightly increased IL-2, whereas AST-VII adjuvanted live/inactivated Merial vaccine did not alter IL-2 response over CEVAC. The most increment in the mean IL-2 titers was obtained from APNS adjuvanted Merial vaccine due to APS itself. Sun et al. demonstrated that NDV strain I formulated with APS + LMS (levamisole)+Se (Selenoprotein) enhanced the production of IL-2 and IL-6 cytokines, IgG antibody response as well as T cell proliferation [40]. Moreover, AST-VII combination with MPL enhanced the production of IFN-γ ($p < 0.05$) and IL-2 compared to MPL adjuvanted Merial vaccine.

IL-4 is a Th2 type cytokine, which ensures B cell differentiation and stimulation, promotes Th2 cell differentiation while inhibiting Th1 cell differentiation [5]. AST-VII both in live/inactivated LaSota vaccine increased the mean IL-4 titers compared to unadjuvanted LaSota. In Merial, IL-4 production approximately showed similar titers between vaccinated groups. Integration of AST-VII into APS slightly affected the

production of IL-4. According to cytokine secretion profiles, AST-VII administration in live/inactivated NDV vaccine, AST-VII + MPL, APNS and ANS demonstrated augmentation on the mean titers of Th1/Th2 cytokines, indicating Th1 mediated immune response.

To elucidate recall response, splenocytes isolated from adjuvanted mice re-stimulated with Con A, NDV antigen. In all AST-VII adjuvanted LaSota vaccinations, stimulation index was statistically significant ($p < 0.05$; $p \leq 0.01$) compared to control group. It is expected because AST-VII stimulated the Con-A and antigen retreated splenocytes in previous studies [4,5]. Moreover, AST-VII administration in live/inactivated LaSota vaccine demonstrated higher splenocyte proliferation than AST-VII adjuvanted-inactivated LaSota vaccine in Con A treatment ($p \leq 0.001$). In Merial vaccinations, all SE formulations were able to stimulate splenocytes. The highest stimulation index (SI) was determined by AST-VII injection in live vaccine and ASE administration in inactivated Merial vaccine (AST-VII/ASE) in NDV re-stimulation. ASE formulation enhanced the proliferation of splenocytes more effectively than AST-VII + MPL in NDV treated splenocytes. Moreover, AST-VII + MPL and AST-VII/ASE vaccination groups demonstrated the highest SI in LPS stimulated splenocytes. As APNS or ANS adjuvanted vaccines were the prominent vaccination groups in terms of the induction of neutralizing antibody responses, they did not make an augmentation on splenocyte proliferation. These results are contradicting the literature regarding the administration of APS with NDV inducing splenocyte proliferation in chicken [26]. Our previous study carried out on H3N2 vaccine adjuvanted with APNS or ANS in mice revealed the potency of APNS as a splenocyte stimulating agent [7]. However, APS based delivery systems did not make an effect on NDV vaccine. Although AST-VII in live/-inactivated vaccine increased the relevant NDV specific antibody and cytokines titers, its effect on splenocyte proliferation was not quite high as SE based vaccinations.

In conclusion, AST-VII administration both in live/inactivated LaSota vaccine induced the mixed Th1/Th2 and neutralizing antibody responses along with a slight increase in IL-2 and IL-4. AST-VII/ASE vaccinations also demonstrated the highest splenocyte proliferation in NDV re-stimulation, which is one of the indicators of the cell-mediated immune response. These two combinations revealed the importance of adjuvant utilization in a live vaccine. Other promising adjuvant combinations, APNS or ANS demonstrated higher neutralizing antibody response along with the production of IFN-γ, IL-2 and IL-4, implying the potential of APS as delivery and immunostimulatory agent in NDV vaccine. Overall, AST-VII and AST-VII containing adjuvant systems are promising to be used to potentiate a viral antigen in veterinary/human vaccines as demonstrating humoral and cellular immune response.

Author's contributions

NY, FOC, NC and AN carried out all experiments. FC helped with the production of LaSota vaccines and, HI and HA assays. NC helped with animal studies. RC was consultant for the production of APS based systems. AN also involved in all steps in this study, writing, review and editing. EB managed all steps in this study, writing, review and editing.

Funding

This study partially supported by The Scientific and Technological Research Council of Turkey (TUBITAK) under Grant 1139B411402292.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

We would like to extend our thanks to AREL (Research and Education Laboratory, Ege University School of Medicine) and Institute for Nuclear Science (Ege University) for their laboratory facilities to perform *in vitro* and characterization studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biologicals.2021.01.005.

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