Interleukin-2 shows high adjuvanticity for an inactivated vaccine against duck Tembusu virus disease

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ABSTRACT Currently, the widely used vaccine against duck Tembusu virus (**DTMUV**) disease is inactivated vaccine which, however, facing the limits of large inoculation dose, short immunization period, and incomplete effectiveness. Access to efficient adjuvants aiding for DTMUV inactivated vaccine seems to be of critical importance. Interleukin-2 (**IL-2**) was reported to induce a persistent expansion of effector T cells and could be a promising molecular adjuvant for many kinds of vaccines. In this study, the efficacy of duck interleukin (**dIL**)-2 as an adjuvant for a DTMUV inactivated vaccine was evaluated. Fifty-five Pekin ducks were divided into 5 groups and intramuscularly administered with 5 batches of vaccines at 42 D (A: DTUMV + dIL-2; B: 1/2DTUMV + dIL-2; C: DTUMV; D: 1/2DTUMV and E: PBS), respectively, and received the second vaccination 2 wk later. Fifty-six days after immunization, 6 ducks from each group were randomly selected to conduct a challenge protection test. Antibody titers and cytokine responses were detected to assess humoral and cellular immune responses in serum of inoculated ducks by hemagglutination inhibition and ELISA, respectively; virus isolation and RT-PCR method were used in immunity protective test. Our results showed that dIL-2 exerted an enhanced effect on the vaccine while reducing the dose of inoculated antigen highlighting high adjuvanticity of IL-2. The vaccines supplemented with IL-2 induced a higher level of antibodies and higher percentage of inhibition values than inactivated vaccines without IL-2 to a significant extent. The production level of IFN-α, IFN-γ, and IL-6 genes were elevated, enhancing both humoral and cellular responses. Furthermore, it provided higher protection after virus challenge. Therefore, IL-2 can be considered as a potential adjuvant for inactivated vaccine against DTMUV disease.

Key words: duck Tembusu virus, dIL-2, adjuvant, inactivated vaccine, dose

2020 Poultry Science 99:6454–6461
https://doi.org/10.1016/j.psj.2020.08.022

INTRODUCTION

Ducks Tembusu virus disease, characterized by a clinical symptom with severe egg drop in duck, first outbroke in the coastal provinces of southeast China in April 2010 (Yan et al., 2011) and has been spread to Zhejiang, Jiangsu, Shanghai, Fujian, Shandong and other provinces so far (Su et al., 2011). The causative agent of the disease was isolated by several research groups and named as duck Tembusu virus (**DTMUV**), which belongs to the Genus *Flavivirus*, Family *Flaviviridae* (Yan et al., 2011; Tang et al., 2012; Ninvilai et al., 2018). Duck Tembusu virus has a relatively wide host range. It could infect not only almost all species of ducks such as Pekin ducks, Cherry Valley ducks (Tang et al., 2013a), and Muscovy ducks (Su et al., 2011; Tang et al., 2015) but also other poultry and wild birds such as chickens (Chen et al., 2014), geese (Ti et al., 2015), penguins (He et al., 2019), and sparrows (Tang et al., 2013a). Even more alarming, a recent report demonstrated that DTMUV could also infect humans (Tang et al., 2013b). As with traditional diseases such as avian flu, duck plague, and duck viral hepatitis, DTMUV disease caused huge economic loss and has already became one of the major diseases severely affecting the healthy development of the Chinese duck industry (Wang et al., 2011). The prevention and control of this disease meets urgent need of development on duck industry in China.

Vaccine is the most cost-effective way to prevent and control infectious diseases. To date, inactivated vaccine against DTMUV disease is still the most widely used in

Received February 14, 2020.
Accepted August 15, 2020.

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China because of its characteristics of safety, stability, and efficacy (Lin et al., 2015). However, inactivated vaccines typically require large doses of vaccination and have limitations in antibody production, antibody titers, and immune duration, which may result in incomplete or ineffective immune protection (Lü, 2018). Administration in cooperation with proper adjuvants to enhance the immune response presents a conventional optimization strategy to generate more efficient vaccine (Cox and Coulter, 1997).

Interleukin-2 (**IL-2**), which has extensively up-regulative effect on the proliferation and differentiation of effector T cells and on B lymphocytes in the process of immune activation and regulation (Taniguchi, 1992; Caligiuri et al., 1993) has been reported as a promising adjuvant for various vaccines against diseases of human (Baek et al., 2015), rabbits (Deng et al., 2019), and swine (Rompato et al., 2006). However, few studies applied IL-2 to vaccines against the diseases of duck, and the effect of IL-2 as a molecular adjuvant on the quantity and quality of humoral and cellular immune responses induced by DTMUV inactivated vaccine remains unknown.

In this study, the effects of recombinant duck IL-2 (**dIL-2**) as an adjuvant for the DTMUV-HB inactivated vaccine were comprehensively evaluated. The results were assessed against several immune parameters including the titer of hemagglutination inhibition (**HI**) antibodies in serum, percentage of inhibition (**PI**) of anti-DTMUV neutralizing antibodies, steady-state protein levels of immune response genes postimmunization, and protection efficacy postchallenge.

MATERIALS AND METHODS

Expression and Purification of dIL-2 Protein in E. Coli System

The dIL-2 gene with a full-length of 434 bp (GenBank: JX239765.1) was synthesized by GenScript Biotech Co., Ltd. (Nanjing, China) and then cloned into the pET-28a expression vector. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells and then was induced by 1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG, Solarbio, Beijing, China) at 37°C for 6 h. The protein containing His-tag was purified using a high-affinity Ni-NTA column (GenScript USA Inc., Nanjing, China) and carried out with endotoxin removal twice. The presence of expressed recombinant proteins was evaluated by 12% SDS-PAGE then recognized by Western blotting. Purified dIL-2 protein was determined by bioactivity assay of recombinant human interleukin-2 in Veterinary Pharmacopoeia of the People's Republic of China. The PET protein (1 mg/mL, the tag protein of the pET-28a vector was expressed and purified in our lab, with endotoxin at a concentration of less than 10 EU/mg) was used as control for dIL-2.

Cells, Virus Preparation, and Animals

Cytotoxic T-cell line-2 cells were cultured in Roswell Park Memorial Institute 1,640 media with 10% fetal bovine serum, 1% streptomycin/penicillin, and 400∼800 IU/mL IL-2 at 37°C, 5% CO_2. The Tembusu-HB viral strain, SPF duck eggs, 6-day-old SPF chicken embryos used in this study were the same as we used in our previous research (Ren et al., 2020).

Vaccine Formulation

The vaccines were prepared according to the production standard of the Ringpu biological pharmaceutical Co., Ltd. (Baoding, China). As shown in Figure 1, the allantoic fluid containing inactivated DTMUV-HB (Final concentration: $10^{6.5}$ ELD_{50}/0.5 mL), 1% tween 80 coupled with or without dIL-2 (Final concentration: 120 IU/mL) were prepared as aqueous phase, and the sterilized mixture consisting of 94% (v/v) Marcol 52, 6% (v/v) span-80 and 2% (w/v) aluminum stearate was used as oil phase. In the same way, a batch of control vaccine was prepared with the allantoic fluid obtained from PBS-inoculated duck embryos.

Immunization and Sample Collection

As Figure 1 shows, totally 55 healthy Pekin ducks were selected and randomly divided into 5 groups and then inoculated through intramuscular injection at 42-day-old with 0.5 mL of 5 batches of vaccines (A: DTUMV + dIL-2; B: 1/2DTUMV + dIL-2; C: DTUMV; D: 1/2DTUMV and E: PBS), respectively. The vaccine groups with full dose antigen (DTMUV) and half dose antigen (1/2 DTMUV) were added to explore the effect of dIL-2 adjuvant on the dosage of antigen in the vaccines. Because the ovaries of these ducks were not dissected and observed, there was no special requirement for sex. Blood samples were collected at day 14, day 24, day 35, and day 56 after primary immunization.

Hemagglutination Inhibition Assay to Determine Serum Antibody Titers

Postvaccination, sera were isolated from blood samples to measure the antibody levels by a modified HI assay (Ren et al., 2020). Positive titer was interpreted as inhibition of hemagglutination at a serum dilution of 1:20 or higher. The positive rate of antibody and the geometric mean titer (**GMT**) for each group were further determined. The GMT was quantified using the formula, $GMT = lg^{-1}[(N1 \times lgX1 + N2 \times lgX2 + \ldots + Nn \times lgXn)/(N1 + N2 + \ldots + Nn)]$ (N: number of ducks; X: the value of HI titer; $lg^{-1}(X) = 10^{x}$).

Detection of Positive Rate of Anti-DTMUV Antibodies

Blocking ELISA assay was carried out using DTMUV ELISA kit provided by Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences to calculate the positive rate of anti-DTMUV antibodies (Li et al., 2012). Each sample were tested in triplicate,

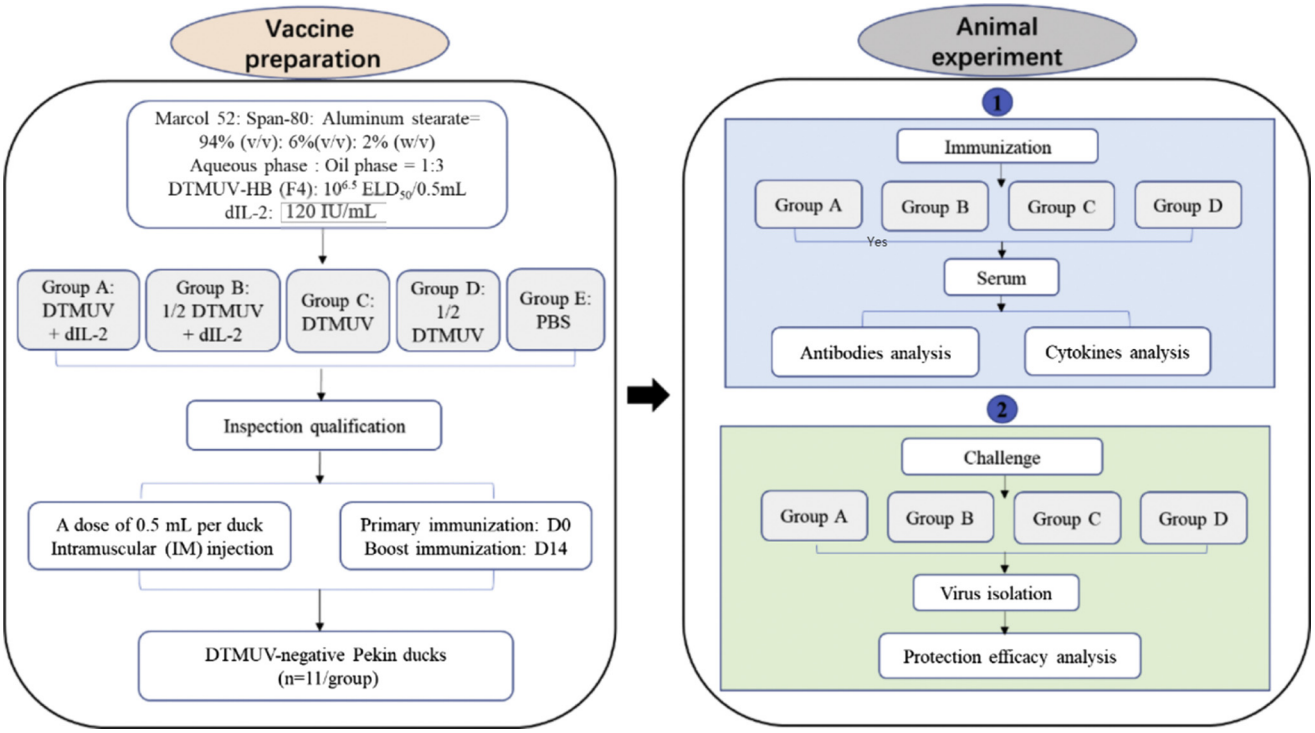

Figure 1. The workflow of the study. Abbreviations: DTMUV, duck Tembusu virus; dIL-2, duck interleukin-2.

and PI value higher than 18.4% was considered as DTMUV antibody positive, whereas lower than 12.6% was considered as DTMUV antibody negative. If the PI value is between 12.6 and 18.4%, the test should be repeated. If it is still between 12.6 and 18.4%, it is judged to be negative.

Determination of Cytokine Levels in Serum

To evaluate the cytokine responses at the protein level, the concentrations of IFN-α, IFN-γ, and IL-6 in serum of group A, B, C, D, and E at different time points after vaccination were measured using Duck interferon α, IFN-α ELISA kit, Duck interferon γ, IFN-γ ELISA kit, and Duck interleukin 6, IL-6 ELISA kit, respectively. (Meimian, Jiangsu, China). In short, diluted standards and serum samples (1:4) were added to micropores coated with corresponding antibodies. Horseradish peroxidase-conjugated second antibody was used to detect bound cytokines, and finally, the OD value was measured at 450 nm. The concentrations of each cytokine were calculated by respective standard curve.

Immune Protection Experiment, Virus Isolation, and RT-PCR

Six ducks randomly selected from each group were challenged with 100 DID_{50} (50% duck infection dose) of the DTMUV in a volume of 0.5 mL on day 42 after primary immunization. Six-day-old chicken embryos, highly susceptible to the virus, was inoculated with serum separated from day 2 postchallenged ducks for virus isolation. Since death of embryos typically at 60 h after virus inoculation, embryo death was monitored and recorded at 24 to 72 h after virus inoculation. The PD_{50} (50% protection dose) of each vaccine batch was determined by using the Spearman–Kärber method.

Positive results of virus isolation were further confirmed by a flavivirus-specific RT-PCR (Tang et al., 2018; Ren et al., 2020). Finally, gel electrophoresis was employed to visualize the specific gene product with the expected length of 998 bp.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA). Differences among the treatments at each time point were analyzed by two-way analysis of variance. Data were shown graphically as the mean ± SD. A P-value of <0.05 was considered significant.

RESULTS

dIL-2 Was Expressed and Determined

In this study, full-length dIL-2 gene was cloned into pET-28a vector to express the IL-2 protein. The recombinant plasmids were expressed in *E. coli*, and the protein was purified by high-affinity chromatography followed by gel filtration chromatography. The purified protein band at approximately 22 kDa was visualized by SDS-PAGE and Western blot (data not shown, Supplementary Figure 1). Purified dIL-2 protein was determined as 3,000,0 IU/mL, with endotoxin at a concentration less than 10 EU/mg.

Table 1. Summary of HI/ELISA and virus challenge results.

					HI/ELISA assay																Virus challenge		
					14 dpi					24 dpi				35 dpi				56 dpi			Virus isolation		
					HI			ELISA		HI			ELISA	HI			ELISA	HI					
Groups	Animals no.	Content	Ino culum dose (mL)	Chip no.	HI antibody titer	Positive rate	GMT	PI value	Positive rate	HI anti body titer	Positive rate	GMT	PI value	Positive rate	HI anti body titer	Positive rate	GMT	PI value	Positive rate	HI anti body titer	Positive rate	GMT	No.of dead embryos/ total	RT-PCR	Protective rate
A	1	DTMUV	0.5	413910	1:20	54.5%	1:11.1	18.95%	54.5%	1:80	100%	1:160.0	45.25%	100%	1:80	100%	1:85.2	52.76%	100%	/	100%	1:50.4	/	/	83.3% (5/6)
	2	+ IL-2		413325	1:20	(6/11)		19.23%	(6/11)	1:320	(11/11)		51.38%	(11/11)	1:160	(11/11)		69.74%	(11/11)	1:40	(6/6)		0/5	/	
	3			413421	1:40			28.86%		1:640			69.24%		1:320			75.29%		1:80			0/5	/	
	4			413397	<1:5 (-)			−6.19%		1:80			38.61%		1:40			41.82%		1:20			1/5	P	
	5			413494	1:5			14.28%		1:160			42.10%		1:80			66.09%		/			/	/	
	6			413321	1:20			27.36%		1:320			60.37%		1:320			80.28%		1:160			0/5	/	
	7			413367	1:20			20.01%		1:320			40.49%		1:40			45.23%		/			0/5	/	
	8			413372	1:10			4.59%		1:80			54.54%		1:80			63.03%		1:40			/	/	
	9			431363	1:20			19.16%		1:80			40.87%		1:40			49.88%		/			/	/	
	10			413439	1:5			5.26%		1:80			35.68%		1:40			54.34%		/			/	/	
	11			413500	1:10			12.34%		1:160			31.22%		1:80			58.66%		1:40			0/5	/	
B	12	1/2	0.5	324910	1:5	54.5%	1:12.6	4.65%	54.5%	1:80	100%	1:219.2	55.54%	100%	1:80	100%	1:102.9	62.40%	100%	1:40	100%	1:63.5	1/5	P	83.3% (5/6)
	13	DTMUV		325124	1:40	(6/11)		28.18%	(6/11)	1:640	(11/11)		70.17%	(11/11)	1:320	(11/11)		79.91%	(11/11)	/	(6/6)		/	/	
	14	+ IL-2		421839	1:20			22.18%		1:640			57.96%		1:320			66.55%		1:160			0/5	/	
	15			324697	<1:5 (-)			−1.12%		1:80			50.41%		1:40			76.98%		1:20			0/5	/	
	16			324904	1:20			19.49%		1:320			54.13%		1:80			66.09%		/			/	/	
	17			325153	1:10			8.48%		1:320			75.72%		1:320			85.32%		1:160			0/5	/	
	18			433677	1:10			17.78%		1:320			47.42%		1:80			75.46%		1:80			0/5	/	
	19			433732	1:20			19.25%		1:80			54.54%		1:40			53.97%		/			/	/	
	20			433631	1:10			14.16%		1:80			49.51%		1:80			49.35%		/			/	/	
	21			433649	1:40			30.17%		1:640			53.12%		1:80			74.34%		/			/	/	
	22			433700	1:20			20.62%		1:160			51.58%		1:80			50.86%		1:40			0/5	/	
C	23	DTMUV	0.5	325152	<1:5 (-)	18.2%	1:2.6	−2.79%	18.2%	1:5	63.6%	1:26.9	56.80%	72.7%	1:5	54.5%	1:24.2	18.01%	63.6%	/	66.7%	1:17.8	/	/	66.7% (4/6)
	24			324909	<1:5 (-)	(2/11)		−3.21%	(2/11)	1:40	(7/11)		24.83%	(8/11)	1:10	(6/11)		13.69%	(7/11)	/	(4/6)		/	/	
	25			324725	<1:5 (-)			4.10%		1:40			44.10%		1:80			66.26%		1:20			5/5	P	
	26			325204	1:10			−1.53%		1:40			23.11%		1:20			43.87%		1:20			0/5	/	
	27			325202	<1:5 (-)			11.26%		1:10			73.95%		1:10			19.69%		1:10			0/5	/	
	28			325190	<1:5 (-)			−12.62%		<1:5			10.20%		1:5			17.43%		1:10			3/5	P	
	29			421837	1:5			−0.28%		1:40			32.49%		1:40			51.80%		/			/	/	
	30			316497	1:40			20.74%		1:640			50.62%		1:320			80.73%		1:40			0/5	/	
	31			325201	1:20			23.52%		1:160			−2.20%		1:80			62.05%		1:20			0/5	/	
	32			324898	<1:5 (-)			2.02%		1:5			12.10%		1:5			14.75%		/			/	/	
	33			325057	<1:5			−2.04%		1:80			32.65%		1:80			54.13%		/			/	/	
D	34	1/2 DTMUV	0.5	324918	<1:5	0%	1:2	6.20%	0%	1:80	54.5%	1:16.9	16.22%	54.5%	1:80	63.6%	1:14.9	55.27%	63.6%	1:20	66.7%	1:15.8	3/5	P	50% (3/6)
	35			435718	<1:5	(0/11)		−4.30%	(0/11)	1:10	(6/11)		17.46%	(6/11)	1:10	(7/11)		19.35%	(7/11)	/	(4/6)		/	/	
	36			433585	<1:5			4.75%		1:40			49.99%		1:20			35.40%		1:10			5/5	P	
	37			421836	<1:5 (-)			2.39%		1:10			21.13%		1:40			25.18%		1:10			1/5	P	
	38			433607	<1:5 (-)			3.77%		1:320			9.41%		1:80			58.73%		1:20			0/5	/	
	39			433593	<1:5 (-)			−1.34%		1:10			16.60%		1:5			13.61%		/			/	/	
	40			433592	<1:5 (-)			−1.36%		1:40			35.07%		1:20			22.20%		1:20			0/5	/	
	41			421846	01:10			−0.51%		1:40			76.22%		1:40			53.44%		/			/	/	
	42			433609	<1:5 (-)			3.07%		<1:5 (-)			54.90%		<1:5 (-)			16.62%		/			/	/	
	43			433588	<1:5 (-)			9.16%		<1:5			14.21%		<1:5 (-)			8.67%		/			/	/	
	44			433703	<1:5 (-)			−4.31%		1:20			32.42%		1:40			38.73%		1:20			0/5	/	

(*continued on next page*)

Table 1. (*continued*)

Groups	Animals no.	Content	Ino culum dose (mL)	HI/ELISA assay														Virus challenge		
				14 dpi					24 dpi				35 dpi			56 dpi		Virus isolation		
				HI		ELISA			HI		ELISA		HI		ELISA	HI				
				Chip no.	HI antibody titer	Positive rate	GMT	PI value	Positive rate	HI anti body titer	PI value	Positive rate	HI anti body titer	Positive rate	GMT	PI value	Positive rate	HI anti body titer	Positive rate	GMT	No.of dead embryos/ total	RT-PCR	Protective rate
E	45	PBS	/	ctl1	<1:5 (-)	0%	/	−0.54%	0%	<1:5 (-)	0%	/	<1:5 (-)	0%	/	0.38%	0%	<1:5 (-)	0%	/	5/5	P	0% (0/6)
	46			ctl2	<1:5 (-)	(0/11)		3.02%	(0/11)	<1:5 (-)	(0/11)		<1:5 (-)	(0/11)		−2.33%	(0/11)	/	(0/6)		/	/	
	47			ctl3	<1:5 (-)			−6.56%		<1:5 (-)			<1:5 (-)			−1.35%		/			/	/	
	48			ctl4	<1:5 (-)			0.31%		<1:5 (-)			<1:5 (-)			2.69%		<1:5			5/5	P	
	49			ctl5	<1:5 (-)			0.65%		<1:5 (-)			<1:5 (-)			4.05%		<1:5 (-)			5/5	P	
	50			ctl6	<1:5 (-)			2.86%		<1:5 (-)			<1:5 (-)			0.71%		/			/	/	
	51			ctl7	<1:5 (-)			8.42%		<1:5 (-)			<1:5 (-)			0.48%		/			/	/	
	52			ctl8	<1:5 (-)			−4.11%		<1:5 (-)			<1:5 (-)			6.09%		<1:5 (-)			5/5	P	
	53			ctl9	<1:5 (-)			−5.53%		<1:5 (-)			<1:5 (-)			1.38%		<1:5			5/5	P	
	54			ctl10	<1:5 (-)			4.45%		<1:5 (-)			<1:5 (-)			−2.40%		<1:5 (-)			/	/	
	55			ctl11	<1:5 (-)			−5.76%		<1:5 (-)			<1:5 (-)			1.82%		<1:5 (-)			5/5	P	

Abbreviations: DTMUV, duck Tembusu virus; GMT, geometrical mean titer; HI, hemagglutination inhibition; PI, percentage of inhibition.

dIL-2 Enhanced the Specific-Antigen Immune Response

Collected sera were subjected to HI and ELISA assays for antibody detection, and the results were summarized in Table 1. Antigen-specific antibody responses detected by HI and ELISA assays showed consistency, and the data of HI antibody response was selected for further analysis.

No antibody response in sera from control group was detected in the course of experiment. Meanwhile, significant difference of the HI antibody titer was observed neither among 4 experimental groups at 14 and 56 dpi (day postimmunization) nor between group C and D at any time points. Notably, HI antibody titers in group B were significantly higher than those in group D at 24 dpi and 35 dpi ($P < 0.001$; $P < 0.01$), whereas HI antibody titer in group A was significantly higher than that in group C at 24 dpi ($P < 0.05$; Figure 2A). The HI titer of each vaccine group reached a peak at 24 dpi, and the HI titer of the groups containing dIL-2 increased significantly during 14 to 24 dpi, but there was no significant difference in the conventional vaccine groups during this period. The PET protein included in dIL-2 had no significant effect on the vaccine immune efficiency (Supplementary Figure 2).

The positive antibody response rate and GMT were calculated and displayed in Table 1 and Figures 2B and 2C. The positive rates of the 4 groups showed similar trends during the experiment, increased significantly from 14 dpi to 24 dpi, and maintained at the high level during the subsequent period. The positive rates of group A and B were far more than those of group C and D at all time points. Furthermore, the positive rates of group A and B were almost the same, whereas the positive rates of group C were higher than that of group D during the period except at 35 dpi. The GMT trend of all groups was inverted V-shaped with a peak at 24 dpi. The relationship of GMT among 4 groups was B > A > C > D, and GMT of group A and B was far higher than that of group C and D at all time points except at 14dpi.

dIL-2 Enhanced Both Th1-type and Th2-type Cytokine Response in Protein Level

As shown in Figure 3, cytokines responses to different vaccines were examined using ELISA. Compared with conventional vaccine group (group C and D), upregulated protein expression in dIL-2-added vaccine group (groups A and B) were detected for all 3 cytokines (IFN-α, IFN-γ, and IL-6). Furthermore, the levels of IFN-α ($P < 0.05$) and IL-6 ($P < 0.001$) in group B were significantly higher than those in group D at 24 dpi, whereas no significant difference was observed in pair-comparison of group A and C for those 2 cytokines at that time point.

dIL-2 Enhanced Protection Efficacy

To evaluate the protection efficacy of the vaccines, 6 ducks per group were challenged with DTMUV, and

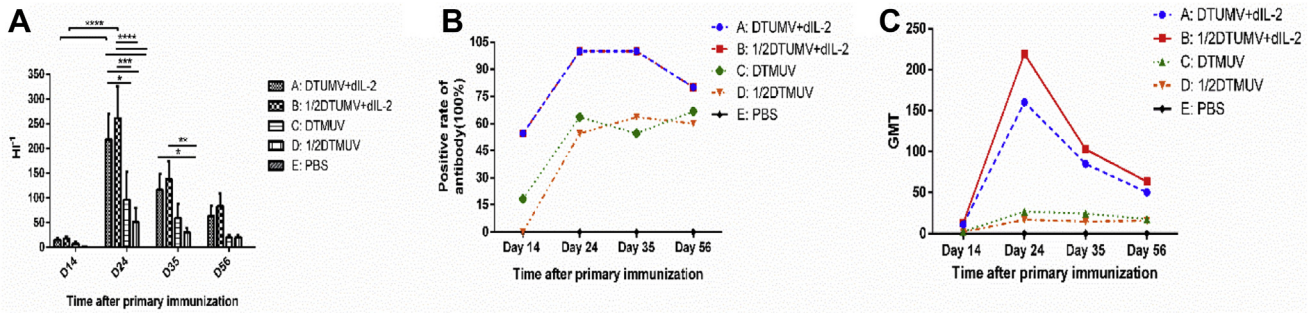

Figure 2. Serum hemagglutination inhibition (HI) results in vaccinated ducks. (A) HI titer, (B) positive rate of antibodies and (C) geometrical mean titer in each group after immunization. Serum samples were collected at different time point after immunization for HI antibody level. Antibody titers were determined using the HI assay with 4 HA units of the DTMUV-HB. The HI titer is expressed as the reciprocal form. Positive titers were interpreted as inhibition of hemagglutination at a serum dilution of 1:20 or greater. Data are expressed as means ± SEM. The significance between HI titers in pair-wise comparisons was analyzed by two-way ANOVA. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). Abbreviations: DTMUV, duck Tembusu virus; dIL-2, duck interleukin-2.

sera were collected postchallenge for virus isolation. A series of clinical symptoms were observed in control group and partly in the other vaccine groups, including green watery diarrhea, reduced feed intake, and depression. The RT-PCR data were consistent with the virus isolation results.

The detailed results of virus isolation in each duck, protective rate in each group were exhibited in Table 1. Approximately, 83.3% of ducks receiving dIL-2-added vaccines were protected against virus challenge as shown by the absence of virus in the sera. About 66.7 and 50% of ducks in conventional vaccine groups (group C and D) were, respectively, protected based on virus isolation results.

DISCUSSION

Duck Tembusu virus disease, an acute infectious disease caused by DTMUV, brought huge economic loss to the poultry industry and posed a threat to public health (Yan et al., 2011), highlighting the urgency of preventing and controlling the disease. Development of an effective vaccine would be the most cost-effective strategy.

Currently, scientists have started to develop subunit vaccine (Ma et al., 2016), DNA vaccine (Tang et al., 2018), inactivated vaccine (Lin et al., 2015), and live attenuated vaccine (Li et al., 2014; He et al., 2019) against the disease, of which some live attenuated and inactivated vaccine candidates against DTMUV have been evaluated and proven to be effective and used in some areas (Li et al., 2014; Lin et al., 2015; He et al., 2019). Application of live-attenuated vaccine gives rise to safety and differentiating infected from vaccinated animals issues and should be wary. Administration of inactivated vaccine in conjunction with appropriate adjuvants to enhance the immune response presents a novel optimization strategy to generate more efficient vaccine.

Mineral oil, with depot effect as its major adjuvant action, is widely used in the veterinary field (Potter and Boyce, 1962). In this study, the adjuvanticity of IL-2 was comprehensively evaluated while retaining the initial mineral-based oil emulsion formulation widely used to prepare the inactivated DTMUV vaccine. Our results showed that the HI or anti-DTMUV antibody titers, their positive rates, GMT, and protective rate in dIL-2-added vaccine group (group A and B) were all higher than those in core vaccine groups (group C and

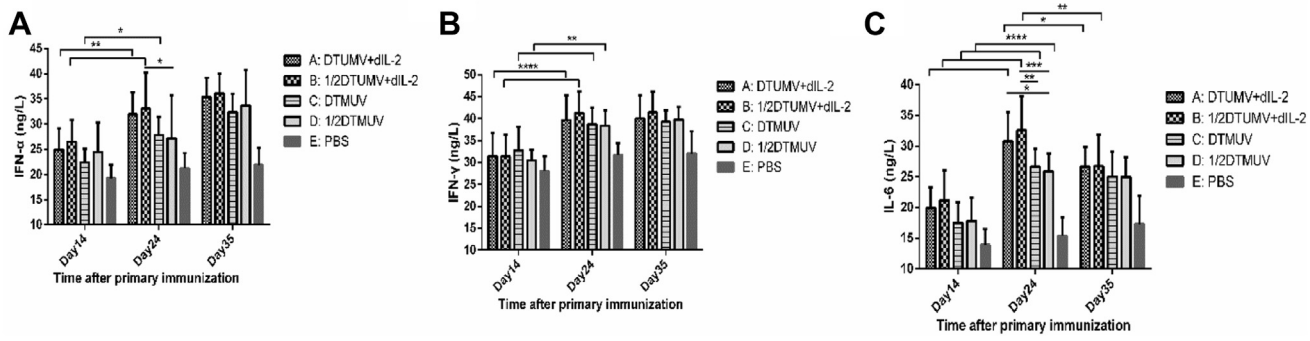

Figure 3. The protein levels of cytokines in vaccinated ducks. Ducks were immunized I.M. twice (D0 and D14) with vaccines formulated with dIL-2 or not and ducks in the control groups were immunized with saline. The protein expression of (A) IFN-α, (B) IFN-γ, and (C) IL-6 was detected by ELISA. Data are presented as the mean ± SD of ducks in the same treatment. The significance between cytokine levels in pair-wise comparisons was analyzed by two-way ANOVA. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Abbreviations: DTMUV, duck Tembusu virus; dIL-2, duck interleukin-2.

D) at all time points (Table 1, Figure 2), indicating IL-2 augment the antigen-specific immune response. In pair-comparison of core vaccine groups, full antigen dose vaccine (group C) generated higher values of antibody-related parameters than half antigen dose vaccine (group D) at almost all time points and offered higher protection rate (66.7%) in virus challenge than half dose vaccine (50%), suggesting that inactivated DTMUV core vaccines were incapacity of eliciting enough immune response on the one hand and that the generation of anti-DTMUV antibody may be antigen dose-dependent within a certain range, on the other hand. Interestingly, when IL-2 was added to those 2 core vaccines, group B showed higher values of antibody-related parameters than those of group A. This discrepancy was supported by the observation that HI antibody titers triggered by dIL-2-added half antigen-dose vaccine (group B) at 24 and 45 dpi were all significantly higher than those triggered by half antigen-dose vaccine (group D), whereas the significant different of HI antibody titer between group A and C was observed only at 24 dpi. The possible reason is that in the case of IL-2 addition, both the efficiency of antigen utilization and the level of immune response are all enhanced, and excessive antigen means more non-structural proteins, which may cause side-effect.

Many inactivated vaccines or antigens have a weak ability to induce innate immune responses (Petrovsky and Aguilar, 2004; Pellegrino et al., 2015), which was followed by our cytokine assays showing no significant different of cytokines (IFN-α, IFN-γ, and IL-6) protein responses between core vaccine groups (group C and D) and control group. The expression of IFN-γ all significantly improved in all vaccine groups during at 14 to 24 dpi, because it is generally accepted that IFN-γ dependent Th1 cell responses are necessary for the prevention of *Flavivirus* infection (Singla et al., 2016). And the expression of group A and B were higher than those of group C and D, but the differences were not significant. Furthermore, in pair-comparisons of group A vs., C and B vs. D, the significant different of IFN-α and IL-6 protein responses were observed only between group B and D at 24 dpi (Figure 3), indicating IL-2 can synergistically induce a certain innate immune response in the middle of immunization in the case of an appropriate amount of antigen.

There are several limitations in this study. First, maybe the group inoculated only with IL-2 should be added to make the experimental design more complete, and a group without booster vaccination may be added to explore the possibility of IL-2 in simplifying immunization procedure of the vaccine. Second, the dose of IL-2 used in this study was not optimized but only referred to the conclusion of previous research in our lab, which has not been published yet. However, we had to temporarily ignore these 2 issues because of insufficient funding and limited isolators supporting more grouping. Third, previous publication has showed that the alum plays its adjuvant action through Syk−NFAT−IL-2 pathway in dendritic cells (Khameneh et al., 2016), indicating IL-2 is an effector or a mediator. So, is the adjuvanticity of IL-2 observed in our study mainly regulated by this pathway? What is mechanism about the downstream of this pathway? Those queries should be addressed in further research.

In summary, our findings support that IL-2 enable antigen dose sparing and increase antigen-specific and innate immune responses. According to the standard of adjuvant selection (e.g., increasing vaccine's immunogenicity, lower antigen dose, the route of administration) (Byars and Allison, 1990; Lindblad et al., 2004), IL-2 has high adjuvanticity, thus can be recommended as a potential adjuvant for inactivated vaccine against DTMUV.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (No. 2017YFD0500806) and the National Natural Sciences Foundation of China (No. 31670156 and 31872430).

Ethics Statement: The animal care and use protocol was approved by the Shandong Agricultural University Animal care and use Committee (SDAUA-2016-002). All animals used in this study were cared for and maintained throughout of the experiments strictly following the ethics and biosecurity guidelines approved by the Institutional Animal Care and Use Committee of Shandong Agricultural University.

Conflict of Interest Statement: The authors did not provide a conflict of interest statement.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at http://doi.org/10.1016/j.psj.2020.08.022.

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