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Advax, as a Co-adjuvant, in Combination with Poly(I:C) Elicits Enhanced Th1 Immune Responses and Parasite Growth-Inhibitory Antibodies Against *Plasmodium falciparum* Merozoite Surface Protein-1 ($PfMSP\text{-}1_{42}$) in BALB/c Mice

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ABSTRACT

Background: One of the main challenges in protein-based vaccines is the poor immunogenicity of antigens, which can be solved by the use of adjuvants. Advax is a novel microparticle polysaccharide adjuvant that in combination with antigens can induce both cellular and humoral immunity based on the intrinsic features of the antigen. It has been shown that poly(I:C) can be a suitable adjuvant for the $PfMSP\text{-}1_{42}$-based malaria vaccine. Advax is a suitable co-adjuvant for poly(I:C) to increase its half-life and reduce dose-dependent toxicity.

Objectives: To investigate whether advax alone or advax /poly(I:C) combination can enhance the immunogenicity with increased parasite inhibitory anti-$PfMSP\text{-}1_{42}$ antibodies in comparison to poly(I:C).

Methods: Mice groups were inoculated with $rPfMSP\text{-}1_{42}$ alone or formulated in poly(I:C), poly(I:C)/advax, or advax. Then, humoral and cellular immune responses, the ratio of Th1/Th2 and growth inhibitory activity of induced antibodies were analyzed.

Results: Poly(I:C)/advax formulated $PfMSP\text{-}1_{42}$ induced higher levels of anti-$PfMSP\text{-}1_{42}$ IgG, IgG2a, and IgG2b antibodies relative to poly(I:C)-formulated $PfMSP\text{-}1_{42}$. The maximum ratio of IFN-γ/IL-4 (50.13) and IgG2a/IgG1 (2.65), was induced in mice received advax-formulated $PfMSP\text{-}1_{42}$. Besides, poly(I:C)/advax formulated $PfMSP\text{-}1_{42}$ induced a higher ratio of IFN-γ/IL-4 (25.33) and IgG2a/IgG1 (1.89) when compared with poly(I:C) alone. Strong growth inhibitory activity was observed in antibodies induced in mice received poly(I:C)/advax-formulated $PfMSP\text{-}1_{42}$.

Conclusion: These findings indicate that advax is a favorable adjuvant to be combined with poly(I:C), and this combination of adjuvants could induce Th1 immune responses and growth inhibitory antibodies against $rPfMSP\text{-}1_{42}$.

Keywords: Advax, Malaria vaccine, Th1 responses, MSP-1, Poly(I:C)

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INTRODUCTION

Despite a major reduction in clinical cases and mortality due to malaria in recent years, malaria remained the main infectious disease in the world. Since 2007, elimination and eradication of malaria have been the primary goal of the World Health Organization (WHO); nonetheless, anti-malarial drugs and insecticides are insufficient to achieve this goal due to drug-resistant parasites (1) and insecticide-resistant mosquitoes (2). Therefore, new strategies such as malaria vaccine development are needed to prevent malaria.

The first approach in malaria vaccine development was the use of the whole sporozoite as a more effective strategy than others (3, 4). However, to achieve the eradicated world of malaria, the whole parasite vaccine is not applicable for the vaccination of half the world's population. One of the basic approaches in malaria vaccine development is using recombinant antigens, which are safe and affordable vaccines with easy production and administration. However, the main challenge of this type of vaccines is the weak immunogenicity, which is required to be considered in vaccine development (5).

The application of adjuvants could be an avenue to overcome the poor immunogenicity of antigen. One of the novel and potent adjuvants is a delta inulin polysaccharide microparticle (6). Delta inulin consists of spherulite-like discoid particles in variable sizes ranging from 1-2 μm (7). The Good Manufacture Practice (GMP) process of delta inulin has led to the production of advax^{TM} discoid particles with equal sizes, acting as an immunological adjuvant (8). Preclinical and clinical studies have revealed that this adjuvant is safe and well-tolerated in vaccines for infectious diseases (8-12) and, thus, can serve in this kind of human vaccines. In addition, advax in combination with antigens could induce both cellular and humoral immunity. For instance, its combination with the HBS antigen elevates IgG1- and IgG2a/2c-specific antibodies (13). This adjuvant also increases both CD4+ and CD8+ T-cell responses as well as elicits various cytokines, including IFN-γ, TNF-α, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, and GM-CSF (13). Advax has been used as an adjuvant for infectious diseases in several vaccine experiments, and the results have shown an improvement in the immunogenicity of these vaccine formulations (13-21). Interestingly, different types of immune responses (Th1 or Th2) induced by advax in combination with various antigens have been attributed to the intrinsic properties of antigens (22). However, this adjuvant has not yet been studied with Plasmodium antigens.

Among malaria vaccine candidate antigens, the 42-kDa fragment of merozoite surface protein 1 (MSP-1_{42}) is a promising blood-stage vaccine candidate for *P. falciparum* (23, 24). In clinical trials, the use of PfMSP-1_{42} antigen formulated in adjuvants that can be used in human vaccines, including alum (25), AS02 (26), alum/CpG (27), AS01 (28), and ISA720 (29) has shown disappointing results. Recently, the efficiency of poly(I:C)-formulated PfMSP-1_{42} revealed that poly(I:C) is a proper adjuvant for the induction of Th1 immune responses and also parasite inhibitory anti-PfMSP-1_{42} IgG antibodies in BALB/c mice (30). There are, nonetheless, still challenges, including short half-life in body fluids (31-33) and dose-dependent toxicity of poly(I:C) in intravenous or intraperitoneal injections (34), in using this adjuvant in vaccine formulations. To overcome these challenges, it is rational to suggest advax as a safe adjuvant alone or in co-administration with poly(I:C) in the PfMSP-1_{42}-based vaccine.

The current study was designed to investigate whether advax alone or in co-administration with poly(I:C), in comparison to the single use of poly(I:C), could elicit the higher immune responses with increased parasite inhibitory anti-PfMSP-1_{42} antibodies. To this end, the cellular and humoral immune responses and growth inhibitory of induced antibodies were measured in immunized mouse groups with poly(I:C)-, poly(I:C)/advax-, or advax-formulated rPfMSP-1_{42}

or antigen alone without any adjuvant. The results obtained from this study would be helpful in the formulation of rPfMSP-1_{42} with suitable adjuvants to have desired immune responses with the most safety.

MATERIALS AND METHODS

Antigen Preparation and Mice Immunization

The *pfmsp-1_{42}* gene (K1 strain, accession no. X03371) was cloned in pQE30 plasmid, then was expressed and purified in *E. coli* M15, as described previously (30). For mice immunization, in a primary experiment, the optimal dose of rPfMSP-1_{42} and poly(I:C) was determined. For the main experiment, 80 female BALB/c mice (6-8 weeks old) in eight groups were used for immunization with purified rPfMSP-1_{42} (10 µg in prime and 5 µg in boosts) on days 0, 14, and 28, subcutaneously in the base of tail at different formulations including antigen alone (group 1), formulated with 10 µg poly(I:C) (0.5 mg/kg body weight, InvivoGen, USA; group 2), poly(I:C)/advax (Vaxine Pty Ltd., Adelaide, Australia, group 3) combination or 1mg Advax (group 4). The control mouse groups were inoculated with PBS 1× (pH 7:2) (group 5), poly(I:C) (group 6), poly(I:C)/advax (group 7), or advax (group 8). Antibody responses were evaluated on days 10, 24, and 38 and the elicited cytokines were measured on day 38.

Antibody Responses Elicited in Response to rPfMSP-1_{42} in Immunized Mice with Different Vaccine Formulations

The elicited antibody responses to rPfMSP-1_{42} (total IgG, IgG1, IgG2a, IgG2b, and IgG3) were analyzed, as described previously (30). Besides, the titration endpoint of anti-PfMSP-1_{42} antibodies was measured for each mouse group using twofold serial dilutions (1:200 to 1:409,600) of pooled sera in ELISA. The last dilution of serum that had an $OD_{450nm} \geq$ cut-off was determined as the titration endpoint. In addition, the avidity of IgG antibodies and IgG subclasses elicited against PfMSP-1_{42} were measured for the sera of immunized mice by ELISA using 8 M urea as dissociation buffer, as described previously (30, 35).

Measurement of Lymphocyte Proliferation

This assay was performed 10 days after second boost on day 38. In this experiment, 4 mice from each group were euthanized by cervical dislocation in sterile conditions. Preparation of single spleen cells suspension and stimulation of the splenocytes in the presence of target antigen was carried out as described previously (30). After 72h, the cell proliferation was measured by using MTT [3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl-blue] dye assay as described previously (30).

Measurement of Released Cytokines from Splenocytes

The released cytokines from splenocytes of examined mice stimulated with rPfMSP-1_{42} (5 µg/mL) were measured using murine cytokine immunoassay kits (R&D system, Minneapolis, USA). For this experiment, the supernatants were collected at 48h and 72h for IL-4 and IFN-γ analysis, respectively. Besides, the supernatants of cells in the presence of Con A and culture medium alone were collected as positive and negative controls, respectively,

Continuous Culture of P. falciparum and Growth Inhibitory Assay (GIA)

Laboratory adapted *P. falciparum* (K1 strain) was continuously cultured based on the previous method (36), with some modifications. The parasites were cultivated in human blood group O+ erythrocytes (blood transfusion organization, Tehran, Iran) at 10% hematocrit, in the presence of RPMI 1640 medium (Gibco, Invitrogen, Scotland, UK), 25 mM $NaHCO_3$, 60 µg/mL gentamycin, 1 mM HEPES (Sigma, USA), 1.96 gr/L Glucose (Sigma, USA), 0.2% Albumax I (Gibco, UK), and pooled AB^+ blood group human sera (12%). A mixture of 3% O2, 6% CO2, and 91% N2 gasses were used for parasite culture, and the flasks were maintained at 37°C, as

previously described (30). Synchronization of parasites in the culture was performed by using 5% D-sorbitol, two times in 4 days intervals (Sigma, St Louis, MO).

On day 38 after primary immunization, IgG antibodies were purified from pooled mouse sera and then, growth inhibitory assay was performed as described previously (30). Then, the growth inhibitory activity of antibodies was measured by using Lactate Dehydrogenase (LDH), as described previously (37, 38). The OD_{650nm} values from infected RBCs treated with pre-immune sera were considered as 100% growth and the proportion of growth inhibitory (GI) in examined sera was calculated with the below formula.

$$GI = \left(1 - \frac{(OD_{650}\ \text{of infected RBCs with test IgG} - OD_{650}\ \text{of normal RBCs only})}{(OD_{650}\ \text{of infected RBCs with preimmune sera} - OD_{650}\ \text{of normal RBCs only})}\right) \times 100$$

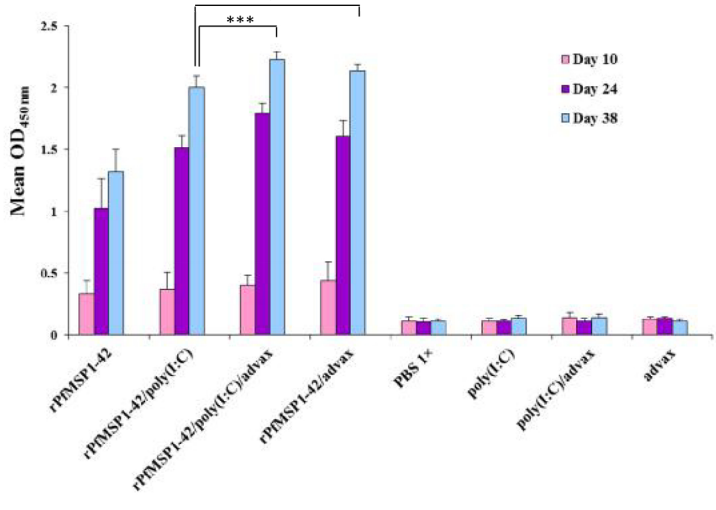
Statistical Analysis

SPSS software, Version 25.0 was employed for statistical analysis. One-way ANOVA test was used for comparing antibody levels, avidity, and cellular responses between mouse groups; and Tukey's honestly significant difference [HSD] post hoc test was used for multiple comparison analysis between each pair of groups. In addition, paired sample *t*-test was used for comparing the level of IgG antibodies in each group in 2-time points. P values<0.05 were considered statistically significant.

RESULTS

IgG Antibody Responses to rPfMSP-1_{42} in Different Mouse Groups

After the primary immunization (day 10), a significant increase in the level of anti-rPfMSP-1_{42} IgG antibody was identified in all vaccinated mouse groups relative to control groups (P<0.05; Figure 1). After the first and second boosts (on days 24 and 38,



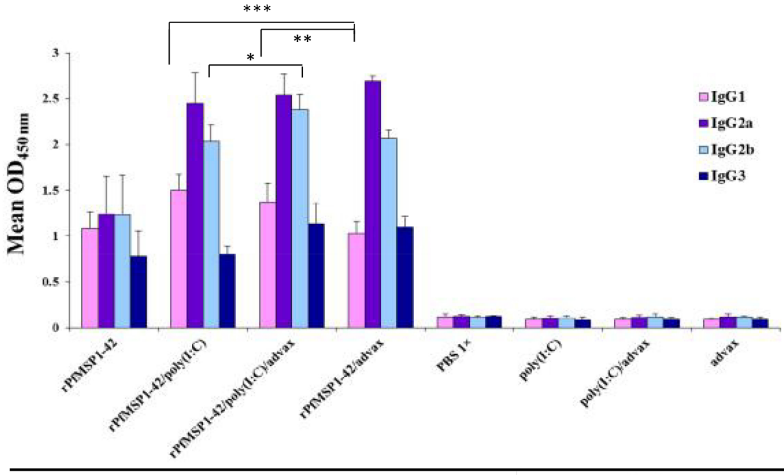
Compared mouse groups (Total IgG)		*P* values		
		D10	D24	D38
rPfMSP-1_{42}	rPfMSP-1_{42}/poly(I:C)	0.926	**<0.0001**	**<0.0001**
	rPfMSP-1_{42}/ poly(I:C)/advax	0.607	**<0.0001**	**<0.0001**
	rPfMSP-1_{42}/advax	0.276	**<0.0001**	**<0.0001**
rPfMSP-1_{42}/poly(I:C)	rPfMSP-1_{42}/ poly(I:C)/advax	0.925	**0.002**	**<0.0001**
	rPfMSP-1_{42}/advax	0.622	0.565	**0.031**
rPfMSP-1_{42}/ poly(I:C)/advax	rPfMSP-1_{42}/advax	0.923	0.050	0.253

Figure 1. Evaluation of the level of anti-PfMSP-1_{42} IgG antibodies in immunized mouse groups at different time points. Comparison of anti-PfMSP-1_{42} IgG antibody levels on days 10, 24, and 38 were performed using the paired-sample *t*-test and revealed a statistical difference between varied time points of the immunization in vaccine groups. The bars show the mean OD values and the error bars display standard deviations (SD) for 10 individual mice in each mouse group. A statistically significant difference was detected in the level of IgG between mice receiving poly(I:C)-formulated antigen and mice receiving advax- or poly(I:C)/advax-formulated antigen. *P<0.05, ***P<0.0001. The table indicates multiple comparison analyses of mean ODs for anti-PfMSP-1_{42} IgG antibodies among vaccine groups using Tukey's HSD post hoc test on days 10, 24, and 38 after the first immunization.

respectively), a significant elevation in the level of anti-rPfMSP-1_{42} IgG antibodies was observed in vaccine groups compared to day 10 (P<0.0001). After third immunization (on day 38), the highest and lowest levels of anti-rPfMSP-1_{42} IgG antibodies were found in mouse groups 3 and 1 receiving poly(I:C)/advax-formulated rPfMSP-1_{42} (mean OD_{450}: 2.221±0.051) and rPfMSP-1_{42} antigen alone (mean OD_{450}: 1.318±0.182), respectively (P<0.05; Figure 1). Among adjuvant vaccine groups, the higher levels of anti-rPfMSP-1_{42} IgG antibodies were detected when rPfMSP-1_{42} was delivered in poly(I:C)/advax relative to poly(I:C) (P<0.05, Figure 1). No detectable anti-rPfMSP-1_{42} IgG antibodies were observed in control mouse groups 5-8 that received PBS 1×, poly(I:C), poly(I:C)/advax, or advax adjuvants (Figure 1).

Profiles of Anti-rPfMSP-1_{42} IgG Subclasses in Immunized Mouse Groups

The anti-rPfMSP-1_{42} IgG subclasses were determined on day 38 after the second boost. The obtained results revealed an increase in the level of anti-rPfMSP-1_{42} IgG1 antibodies in mice received rPfMSP-1_{42} formulated with poly(I:C) or poly(I:C)/advax (mean OD_{450}: 1.5 and 1.372, respectively) compared to mice receiving rPfMSP-1_{42} alone (mean OD_{450}: 1.08; P<0.05, Figure 2). It was notable that the administration of advax-formulated rPfMSP-1_{42} could not enhance the level of anti-rPfMSP-1_{42} IgG1 antibodies, compared to immunized mice with rPfMSP-1_{42} antigen alone, (P>0.05, Figure 2). Regarding IgG2a response, adjuvant vaccine groups revealed a significant increase in anti-rPfMSP-1_{42} IgG2a antibodies when compared to mice receiving antigen alone (P<0.05, Figure 2). Remarkably, antigen delivery in poly(I:C)/advax (mean OD_{450}: 2.54) in compared to poly(I:C) alone (mean OD_{450}: 2.45) could boost anti-rPfMSP-1_{42} IgG2a antibodies. Besides, all the adjuvant vaccine groups showed an increase in the level of IgG2b



Compared mouse groups (D38)		*P* values			
		IgG1	IgG2a	IgG2b	IgG3
rPfMSP-1_{42}	rPfMSP-1_{42}/poly(I:C)	**<0.0001**	**<0.0001**	**<0.0001**	0.997
	rPfMSP-1_{42}/ poly(I:C)/advax	**0.004**	**<0.0001**	**<0.0001**	**0.001**
	rPfMSP-1_{42}/advax	0.919	**<0.0001**	**<0.0001**	**0.004**
rPfMSP-1_{42}/poly(I:C)	rPfMSP-1_{42}/ poly(I:C)/advax	0.340	0.896	**0.020**	**0.002**
	rPfMSP-1_{42}/advax	**<0.0001**	0.256	0.993	**0.007**
rPfMSP-1_{42}/ poly(I:C)/advax	rPfMSP-1_{42}/advax	**0.001**	0.646	0.039	0.971

Figure 2. Estimation of anti-PfMSP-1_{42} IgG subclass profiles on day 38. The bars show the mean OD values and the error bars display standard deviations (SD) for 10 individual mice in each mouse group. Statistical analysis showed higher levels of anti-PfMSP-1_{42} IgG1 antibodies in mice receiving poly(I:C)- or poly(I:C)/advax-formulated rPfMSP-1_{42} antigen relative to mice receiving this antigen formulated in advax. In addition, the level of IgG2a was statistically different in mice that received poly(I:C)- and poly(I:C)/advax-formulated rPfMSP-1_{42} antigen. *P<0.05, **P<0.001, ***P<0.0001. The table displays multiple comparison analyses of means for anti-PfMSP-1_{42} IgG subclasses antibodies among vaccine groups using Tukey's HSD post hoc test on day 38.

relative to mice receiving rPfMSP-1_{42} alone. It was remarkable that the higher level of anti-rPfMSP-1_{42} IgG2b antibodies was elicited by PfMSP-1_{42}/poly(I:C)/advax formulation (mean OD_{450}: 2.34) in comparison to poly(I:C) (mean OD_{450}: 2.04) or advax formulated PfMSP-1_{42} (mean OD_{450}: 2.06; $P<0.05$, Figure 2). The comparable and high level of IgG3 antibody was identified in mice receiving rPfMSP-1_{42} formulated with poly(I:C)/advax or advax adjuvants ($P>0.05$, Figure 2).

Antibody Titers and Avidity in Immunized Mice

The titers of IgG and IgG subclasses including IgG1, IgG2a, IgG2b, and IgG3 antibodies against PfMSP-1_{42} were calculated in mice collected sera on day 38. The highest IgG end-point titer (204,800) was detected in mice receiving advax- or poly(I:C)/advax-formulated rPfMSP-1_{42} antigen, that it was higher than IgG titer in mice receiving poly(I:C)-formulated rPfMSP-1_{42} antigen (102,400) or alone antigen (12,800). Administration of all adjuvants in combination with rPfMSP-1_{42} antigen increased the titer of IgG1 (51,200) relative to the administration of antigen alone (12,800). The highest IgG2a antibody titer was detected in mice receiving PfMSP-1_{42}/advax formulation (204,800), followed by mouse groups immunized with antigen formulated in poly(I:C)/advax, poly(I:C), or alone (102,400, 51,200, 12,800, respectively; Figure 3). In the mouse group

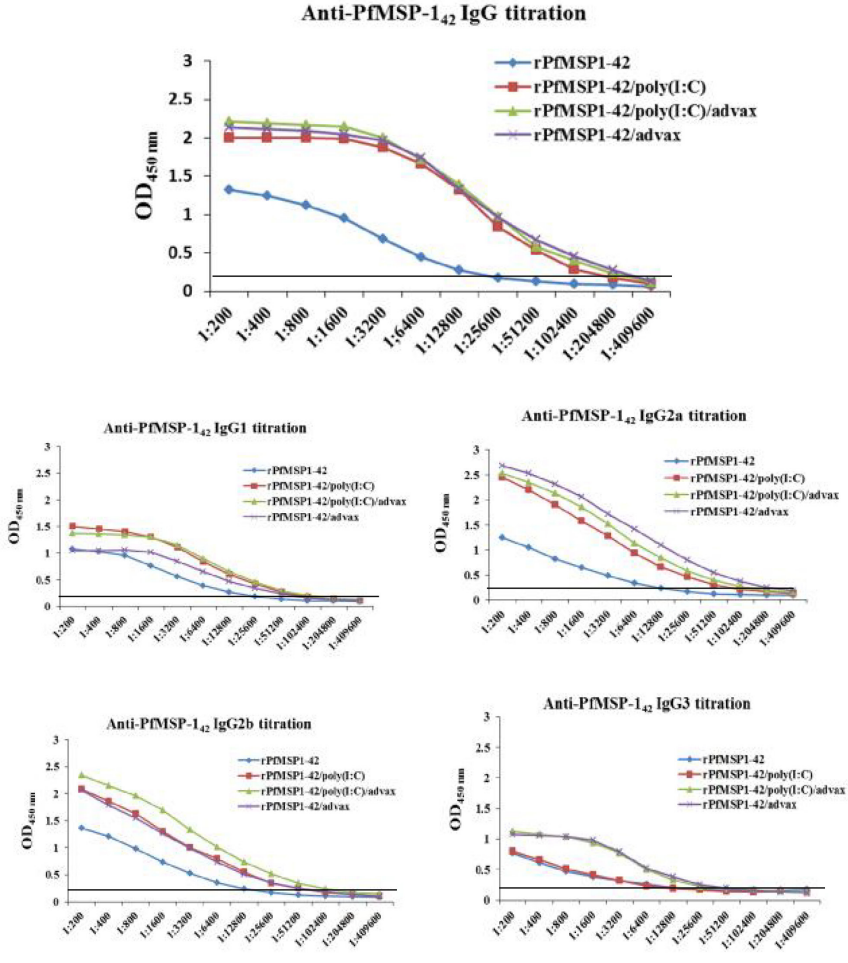


Figure 3. Titration analysis of anti-PfMSP-1_{42} IgG and IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3 antibodies) in mouse groups. Serial dilutions of mouse sera (1:200 – 1:409600) were used for titration. Different titrations of antibodies were observed among vaccinated mice. The horizontal lines indicate cut-offs. To calculate ELISA cut-offs, the mean OD of 30 pre-immune sera plus 3SD were measured. The cut-off OD_{450nm} values for IgG, IgG1, IgG2a, IgG2b, and IgG3 antibodies were 0.191, 0.158, 0.19, 0.178, and 0.164, respectively.

receiving poly(I:C)/advax-formulated PfMSP-1_{42}, IgG2b end-point titer (102,400) was greater than the mice receiving PfMSP-1_{42} alone (12,800) or formulated with poly(I:C) (51,200) or advax (51,200) adjuvants (Figure 3). The highest titer of IgG3 was detected in mice receiving poly(I:C)/advax- or advax-formulated antigen (Figure 3).

All mice receiving antigen plus any of adjuvants indicated IgG antibodies with high-avidity feature (mean AI: 54.4%-64.8%, Figure 4). Regarding AI of IgG subclasses, administration of poly(I:C)/advax-formulated PfMSP-1_{42} elicited high-avidity IgG2a (mean AI: 72.9) and IgG2b (mean AI: 70.6) but intermediate-avidity IgG1 (mean AI: 41.8) and IgG3 (mean AI: 30, Figure 4).

Cellular Immune Responses in Different Mouse Groups

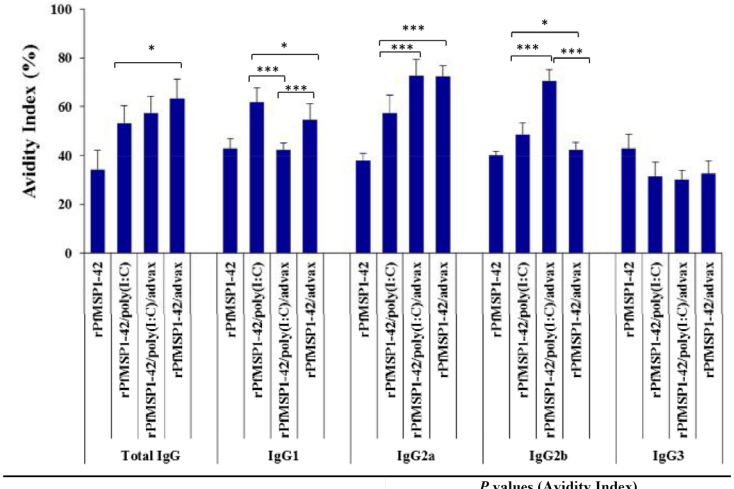
Significant proliferation of splenocytes was obtained in all mice receiving antigen alone or formulated with adjuvants when compared to the control mice (P<0.05, one-way ANOVA, data not shown).

Cytokine release measurement revealed that significant levels of IFN-γ (as Th1 type response) were elicited in vaccine groups receiving rPfMSP-1_{42} alone or in combination with adjuvants as compared to control mice (P<0.05, Figure 5a). Antigen delivery in poly(I:C)/advax (1866 pg/ml) could raise the level of IFN-γ relative to antigen delivery with poly(I:C) alone (1162 pg/ml, *P*<0.05, Figure 5). However, the highest (2485 pg/ml) and lowest (505 pg/ml) levels of IFN-γ were induced in mice receiving the antigen plus advax or alone, respectively (Figure 5).

Multiple comparison analyses of IL-4 secretion in immunized mouse groups revealed decreasing IL-4 in each of adjuvant vaccine groups (50-74 pg/ml) relative to mice receiving antigen alone (122 pg/ml).

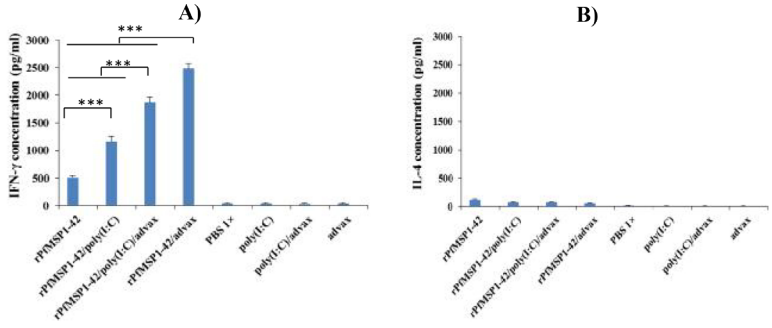
Th1/Th2 Ratio of Immune Responses in Immunized Mice

Mouse group that received rPfMSP-1_{42} formulated with advax (group 4) showed the highest IgG2a/IgG1 ratio (2.65; P<0.05) on day 38, followed by mouse groups receiving poly(I:C)/advax- or poly(I:C)-formulated rPfMSP-1_{42} (1.89 and 1.65, respectively; Figure 6).



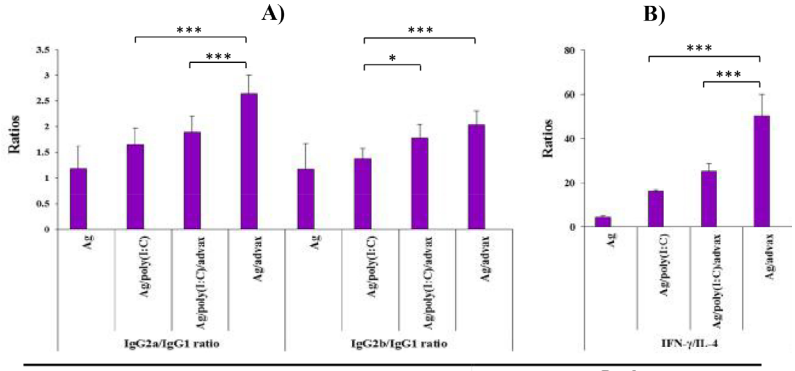
Compared mouse groups (D38)		*P* values (Avidity Index)				
		IgG	IgG1	IgG2a	IgG2b	IgG3
rPfMSP-1_{42}	rPfMSP-1_{42}/poly(I:C)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.001**
	rPfMSP-1_{42}/ poly(I:C)/advax	**<0.0001**	0.999	**<0.0001**	**<0.0001**	**<0.0001**
	rPfMSP-1_{42}/advax	**<0.0001**	**<0.0001**	**<0.0001**	0.504	**0.003**
rPfMSP-1_{42}/poly(I:C)	rPfMSP-1_{42}/ poly(I:C)/advax	0.576	**<0.0001**	**<0.0001**	**<0.0001**	0.906
	rPfMSP-1_{42}/advax	**0.022**	**0.014**	**<0.0001**	**0.005**	0.968
rPfMSP-1_{42}/ poly(I:C)/advax	rPfMSP-1_{42}/advax	0.317	**<0.0001**	1.000	**<0.0001**	0.674

Figure 4. Avidity indices of anti-PfMSP-1_{42} IgG, IgG1, IgG2a, IgG2b, and IgG3 antibodies were measured by an avidity ELISA. The bars and error bars display the mean AIs and SD, respectively. Significant differences are shown in the bar chart by the star. *P<0.05, ***P<0.0001. The comparisons of AIs between vaccinated mouse groups were performed by one-way ANOVA test, followed by Tukey's HSD post hoc test. Multiple comparison analysis and related *P* values are indicated in the table, and significant differences are presented in bold. Ag: rPfMSP-1_{42}



Compared mouse groups		*P* values	
		IFN-γ	IL-4
rPfMSP-1$_{42}$	rPfMSP-1$_{42}$/poly(I:C)	**<0.0001**	**<0.0001**
	rPfMSP-1$_{42}$/ poly(I:C)/advax	**<0.0001**	**<0.0001**
	rPfMSP-1$_{42}$/advax	**<0.0001**	**<0.0001**
rPfMSP-1$_{42}$/poly(I:C)	rPfMSP-1$_{42}$/ poly(I:C)/advax	**<0.0001**	0.997
	rPfMSP-1$_{42}$/advax	**<0.0001**	**0.049**
rPfMSP-1$_{42}$/ poly(I:C)/advax	rPfMSP-1$_{42}$/advax	**<0.0001**	**0.034**

Figure 5. Measurement of IFN-γ and IL-4 cytokines production in response to rPfMSP-1$_{42}$ among examined mouse groups (on day 38). **A**) IFN-γ production and, **B**) IL-4 production. The bars and error bars indicate the mean concentration of the cytokine and standard deviations (SD) in each mouse group, respectively. Significant differences are shown in the bar chart by the star. ***P<0.0001. Multiple comparisons of IFN-γ and IL-4 among different vaccine groups are displayed in the table. P values<0.05 are shown in bold in the table. Ag: rPfMSP-1$_{42}$

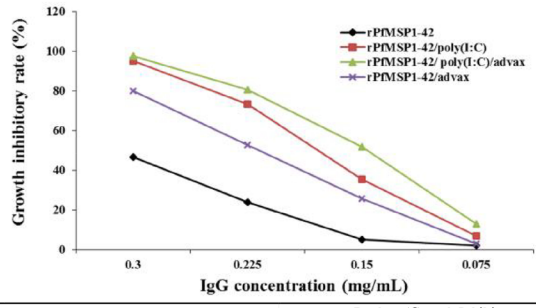


Compared mouse groups (D38)		*P* values		
		IgG2a/IgG1	IgG2b/IgG1	IFN-γ/IL-4
rPfMSP-1$_{42}$	rPfMSP-1$_{42}$/poly(I:C)	**0.028**	0.540	**0.036**
	rPfMSP-1$_{42}$/ poly(I:C)/advax	**0.001**	**0.001**	**0.001**
	rPfMSP-1$_{42}$/advax	**<0.0001**	**<0.0001**	**<0.0001**
rPfMSP-1$_{42}$/poly(I:C)	rPfMSP-1$_{42}$/ poly(I:C)/advax	0.472	**0.048**	0.113
	rPfMSP-1$_{42}$/advax	**<0.0001**	**<0.0001**	**<0.0001**
rPfMSP-1$_{42}$/ poly(I:C)/advax	rPfMSP-1$_{42}$/advax	**<0.0001**	0.324	**<0.0001**

Figure 6. Evaluation of Th1/Th2 ratios. Th1/Th2 ratio was measured on day 38 among vaccine groups. Anti-PfMSP-1$_{42}$ IgG2a and IgG2b antibodies and cytokine IFN-γ were considered as Th1 immune response and anti-PfMSP-1$_{42}$ IgG1 antibodies and the cytokine IL-4 were considered as Th2 responses. **A**) Ratio of IgG2a/IgG1 and IgG2b/IgG1 among vaccine mouse groups. **B)** Ratio of IFN-γ/IL-4 in vaccine mouse groups. Significant differences are shown in the bar chart by the star. *P<0.05, ***P<0.0001. The table illustrates multiple comparisons of IgG2a/IgG1, IgG2b/IgG1, and IFN-γ/IL-4 ratios between vaccine groups. Statistical differences are displayed in bold numbers. Ag: rPfMSP-1$_{42}$

Multiple comparison analyses disclosed that mouse groups receiving rPfMSP-1$_{42}$ formulated with advax or poly(I:C)/advax indicated a higher ratio of IgG2b/IgG1 in comparison to the mice receiving this antigen formulated with poly(I:C) or alone (P<0.05, Figure 6).

Regarding the IFN-γ/IL-4 ratio, as another indicator of Th1/Th2 immune response, the highest ratio was identified in mice receiving advax-formulated rPfMSP-1$_{42}$ on day 38 (50.13; P<0.05) after the primary immunization. Moreover, poly(I:C)/advax-formulated PfMSP-1$_{42}$ increased the IFN-γ/IL-4 ratio in comparison to poly(I:C)-formulated antigen (P>0.05, Figure 6).



		P values (Growth Inhibitory activity)			
Compared mouse groups		**0.3 mg/mL**	**0.225 mg/mL**	**0.15 mg/mL**	**0.075 mg/mL**
rPfMSP-1_{42}	**rPfMSP-1_{42}/poly(I:C)**	**<0.0001**	**<0.0001**	**<0.0001**	**0.003**
	rPfMSP-1_{42}/ poly(I:C)/advax	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	rPfMSP-1_{42}/advax	**<0.0001**	**<0.0001**	**<0.0001**	0.814
rPfMSP-1_{42}/poly(I:C)	**rPfMSP-1_{42}/ poly(I:C)/advax**	0.843	0.07	**<0.0001**	**0.011**
	rPfMSP-1_{42}/advax	**0.002**	**<0.0001**	**0.007**	**0.012**
rPfMSP-1_{42}/ poly(I:C)/advax	**rPfMSP-1_{42}/advax**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

Figure 7. Growth inhibitory activity of the purified IgG antibodies elicited among vaccine mouse groups on *P. falciparum* K1 parasites. A growth-inhibitory assay was carried out with purified IgGs from vaccine mouse groups in the final concentrations of 0.3, 0.225, 0.15, and 0.075 mg/mL by using the LDH assay. The table shows multiple comparison analyses for the growth inhibitory activity of anti-PfMSP-1_{42} IgG antibodies among vaccine groups (adjuvanted and non-adjuvanted). P values<0.05 are shown in bold.

Growth Inhibition Activity of Elicited Anti-PfMSP-1_{42} IgG Antibodies

The result of the growth inhibitory assay from different concentrations of purified IgG antibodies in immunized mouse groups is shown in Figure 7. The IgGs from mice receiving poly(I:C)/advax-formulated rPfMSP-1_{42} showed the highest growth inhibitory activity (mean GI: 97.5%, and 80.5% in 0.3 and 0.225 mg/mL of antibodies, respectively). The second most potent sera for the growth inhibitory activity was obtained from the mouse group receiving poly(I:C)-formulated rPfMSP-1_{42} antigen (mean GI: 95% and 73.1% in the final concentrations of 0.3 and 0.225 mg/mL of IgGs, respectively). Sera from mice receiving poly(I:C)- or poly(I:C)/advax-formulated rPfMSP-1_{42} antigen showed no significant difference in the growth inhibitory activity in the final concentration of 0.3 and 0.225 mg/mL (P>0.05). However, in the final concentrations of 0.15 mg/mL of purified IgG antibodies, sera obtained from mice receiving poly(I:C)/advax-formulated rPfMSP-1_{42} antigen displayed higher growth inhibitory activity than each of the other vaccine groups (P<0.05, Figure 7). Besides, no growth inhibitory activity was detected for antibodies in different concentrations in the control mice. In addition, the growth inhibitory activity of antibodies was checked using a microscopy experiment (data not shown).

DISCUSSION

Applying adjuvants is an important approach to improve the weak immunogenicity of recombinant protein-based malaria vaccines. However, a few adjuvants, such as alum and MF59, licensed for human use (39) are not suitable for malaria vaccines. Some non-licensed adjuvants, such as poly(I:C) (40) are allowed to be used in clinical trials with a specific dose. Besides, poly(I:C) has been introduced as a potent adjuvant for eliciting Th1 immune responses and growth-inhibitory antibodies against rPfMSP-1_{42} (30). However, poly(I:C) could be administered with another adjuvant to diminish its concentration and, consequently, its toxicity as well as to increase the raised immune responses against the rPfMSP-1_{42}/poly(I:C). Therefore, to achieve a safe adjuvant formulation for PfMSP-1_{42}-based vaccine, poly(I:C) was combined with advax, as a safe nanoparticle adjuvant, and the eliciting immune responses against rPfMSP-1_{42} were evaluated in examined formulations.

The highest and lowest growth inhibitory activity of anti-PfMSP-1_{42} IgG antibodies

was observed in mice receiving rPfMSP-1_{42} formulated in poly(I:C)/advax and advax adjuvants, respectively. This difference in the growth inhibitory efficiency of antibodies can be attributed to the activity of adjuvants since some adjuvants have more ability to produce antibodies against weak inhibitory epitopes (30, 41). Another possible explanation for this result could be the expansion of a limited number of the B-cell clones that are specific for the growth inhibitory epitope(s) in this formulation of the vaccine. From these explanations, it can be concluded that among the examined adjuvants, poly(I:C)/advax is the most appropriate adjuvant combination for the induction of antibodies against inhibitory epitopes.

Earlier studies have disclosed that Th1 immune responses, IFN-γ (42, 43) and IgG2a (42-44), are associated with protection against malaria. Similar to IgG2a, IgG2b can bind to activating FcγR receptors (45), leading to promote complement fixation (46). All the adjuvant-vaccinated mouse groups directed the immune responses to Th1 type responses. Particularly, PfMSP-1_{42}/advax could induce potent Th1 immune responses (IgG2a/IgG1: 2.65, IgG2b/IgG1: 2.03, and IFN-γ/IL-4: 50.13). In addition, combining advax with poly(I:C) could increase the Th1 immune responses (IgG2a/IgG1: 1.89, IgG2b/IgG1: 1.776, and IFN-γ/IL-4: 25.33) against rPfMSP-1_{42} relative to poly(I:C) alone. Therefore, it could be postulated that rPfMSP-1_{42}/advax or the addition of advax to the rPfMSP-1_{42}/poly(I:C) formulation can enhance Th1 immune responses and may elevate the protection.

In malaria infections, high-avidity antibodies play an essential role in the prevention of a severe disease (47). In this regard, the highest anti-PfMSP-1_{42} IgG avidity was induced in mice receiving the rPfMSP-1_{42}/advax (63.35%), while immunized mice with the rPfMSP-1_{42}/poly(I:C)/advax had the highest avidity for IgG2a (72.87) and IgG2b (70.65), suggestive of a strong Th1 immune response. Once more, these results suggested that advax in combination with poly(I:C) could improve the quality of Th1 antibody responses that could help protection.

Previous studies have connected protection against PfMSP-1_{42} vaccine with parasite growth inhibitory activity of anti-PfMSP-1_{42} antibodies (25, 48, 49). One of the effective factors in the induction of growth-inhibitory antibodies is using adjuvant formulation (41, 50). The important point to consider is that in the addition of an adjuvant, the used concentration of adjuvant and antigen is critical for inducing the growth inhibitory antibodies. In this regard, the growth inhibitory activity of 25 μg of the rPfMSP-1_{42}/50 μg of poly(I:C) reduced from 94.6% and 82.3% (30) to 73.1% and 35.5% growth inhibitory activity in 0.225 and 0.15 mg/mL of purified IgG, respectively, when vaccine dose decreased to 10 μg of rPfMSP-1_{42}/10 μg of poly(I:C). However, this reduction in growth inhibitory activity of induced antibodies was somewhat compensated by the administration of advax in this formulation [rPfMSP-1_{42}/poly(I:C)], denoting that advax could be a suitable adjuvant for combining.

Based on our knowledge, the current investigation is the first study to evaluate advax, as an adjuvant, for a malaria vaccine. The efforts to find the mechanism of action of advax have exhibited that this adjuvant could maximize the immune responses based on the intrinsic features of the used antigen in vaccine formulation (22). The obtained results from the current study also suggested that rPfMSP-1_{42} alone induces Th1/Th2 ratios >1, indicating a Th1 type immune response. Administration of advax with rPfMSP-1_{42} enhanced the Th1 type immune responses against this antigen, which signifies that advax is a proper adjuvant for this antigen. However, evaluation of the growth-inhibitory activity among sera obtained from different vaccine groups reflected those mice receiving rPfMSP-1_{42} in combination with poly(I:C)/advax induced the strongest inhibitory antibodies. This result denotes that poly(I:C) is a potent adjuvant in inducing antibodies

against inhibitory epitopes, and advax could assist poly(I:C) in increasing the induced immune responses, suggesting that advax is a suitable co-adjuvant for increasing the immune responses raised to most of malaria vaccine formulations.

Taken together, advax is a favorable adjuvant for combining with poly(I:C), and this combination of adjuvant could induce Th1 immune responses and growth inhibitory antibodies against rPfMSP-1_{42}, despite decreasing the dose of poly(I:C). Thus, this combination of adjuvant could be suggested for clinical trials of PfMSP-1_{42}-based malaria vaccine and even in other malaria vaccine candidate antigens. Moreover, advax, as a safe adjuvant, could be used in other formulations of vaccines instead of liposomes or oils.

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