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ORIGINAL INVESTIGATION

Poly(I:C) adjuvant strongly enhances parasite-inhibitory antibodies and Th1 response against *Plasmodium falciparum* merozoite surface protein-1 (42-kDa fragment) in BALB/c mice

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Abstract
Malaria vaccine development has been confronted with various challenges such as poor immunogenicity of malaria vaccine candidate antigens, which is considered as the main challenge. However, this problem can be managed using appropriate formulations of antigens and adjuvants. Poly(I:C) is a potent Th1 inducer and a human compatible adjuvant capable of stimulating both B- and T-cell immunity. *Plasmodium falciparum* merozoite surface protein 1_{42} (PfMSP-1_{42}) is a promising vaccine candidate for blood stage of malaria that has faced several difficulties in clinical trials, mainly due to improper adjuvants. Therefore, in the current study, poly(I:C), as a potent Th1 inducer adjuvant, was evaluated to improve the immunogenicity of recombinant PfMSP-1_{42}, when compared to CFA/IFA, as reference adjuvant. Poly(I:C) produced high level and titers of anti-PfMSP-1_{42} IgG antibodies in which was comparable to CFA/IFA adjuvant. In addition, PfMSP-1_{42} formulated with poly(I:C) elicited a higher ratio of IFN-γ/IL-4 (23.9) and IgG2a/IgG1 (3.77) with more persistent, higher avidity, and titer of IgG2a relative to CFA/IFA, indicating a potent Th1 immune response. Poly(I:C) could also help to induce anti-PfMSP-1_{42} antibodies with higher growth-inhibitory activity than CFA/IFA. Altogether, the results of the current study demonstrated that poly(I:C) is a potent adjuvant that can be appropriate for being used in PfMSP-1_{42}-based vaccine formulations.

Keywords Malaria vaccine · *Plasmodium falciparum* · MSP-1 · Poly(I:C) adjuvant · Growth-inhibitory antibody

Introduction

Despite successful reduction in the rates of mortality and clinical cases of malaria in the recent years, virtually 3 billion people in 91 countries are still at the risk of malaria, with an estimated 212 million malaria cases and 429,000 deaths annually [1]. Besides, the presence and appearance of drug-resistant parasites [2] and insecticide-resistant mosquitoes [3] are serious issues that interfere with malaria elimination and eradication strategies. Therefore, administration of all available tools in parallel with complementary intervention tools, such as an efficient malaria vaccine, is in a high priority to achieve the elimination and eradication of malaria throughout the world [4].

For development of a malaria vaccine, protective cellular and humoral immune responses against target antigens are highly required. In an attempt for malaria vaccine development, scientists started with vaccination of individuals via repeated bites by *Plasmodium falciparum*-infected and -irradiated mosquitoes 30 years ago [5]. Although this approach could be effective due to the use of whole parasite, it seems that production and application of sporozoites for vaccination of a large number of target populations (3.3 billion) is impractical. Indeed, for a large population, a protective, safe and cost-effective vaccine with easy production and administration is needed. New generation of vaccines based on recombinant antigens can cover many of these characteristics for malaria vaccine; however, one of the challenges is poor immunogenicity of recombinant antigens, which should be considered in vaccine developments [6].

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To overcome the poor immunogenicity of *Plasmodium* recombinant antigens, researchers have investigated the application of various strategies with different antigens to achieve an efficient malaria vaccine. One of these strategies is the addition of some components such as adjuvants, which has been used during the development of RTS,S/AS01 [7]. Safe, strong and appropriate adjuvants are able to improve the magnitude and the type of immune responses to increase functional antibody titer and to induce long-lasting protective immune responses and robust cell-mediated immunity [8]. Until now, only five adjuvants have received license for being used in humans, including alum, MF59, virosome, AS03 (α-tocopherol + squalene + polysorbate 80 in an oil-in-water emulsion), and AS04 (alum and MPL) [8]. Most of the licensed adjuvants induce humoral arm of immune responses that is not suitable for intracellular pathogens such as malaria, in which both cellular and humoral immune responses are required to diminish the infecting parasites [9, 10]. Other non-licensed adjuvants with an acceptable safety that are used in clinical trials include CpG [11], flagellin [12, 13], poly(I:C) [14], AS01 (MPL, QS-21 and Liposome) [15], AS02 (MPL and QS-21 in an emulsion) [16], ISCOMs, and ISCOMATRIX [17]. Some of these adjuvants such as flagellin and ISCOMs induce both Th1 and Th2 immune responses [8] that are not appropriate for malaria vaccines. Concerning development of PfMSP-1_{42}-based vaccines, AS01 and AS02 that had encouraging results with RTS,S were not promising in clinical trials [18, 19] and CpG in combination with Alhydrogel could not induce the protective immune responses in clinical trials [20].

Polyriboinosinic acid-polyribocytidylic acid, known as poly(I:C), is a double-stranded RNA (dsRNA) that binds to TLR3 and induces type I interferon (IFN), cytokine production, and dendritic cell (DC) maturation [21]. This adjuvant stimulates both T- and B-cell lymphocytes of Th1 type response [22] and is used as an adjuvant for vaccine developments, especially in cancer and virus infections [14, 23, 24]. With regard to malaria vaccine development, poly(I:C) in combination with *P. falciparum* circumsporozoite protein (PfCSP) induced long-lived antibody and Th1 immune response in primates [25]. In a study, this adjuvant was used with three allelic forms of *P. vivax* circumsporozoite protein (PvCSP) and this formulation induced high and long-lasting serum IgG titers comparable to those produced by proteins emulsified in complete Freund's adjuvant [26]. However, dsRNA compounds, such as poly(I:C), have short half-life and might be rapidly degraded in body fluids by RNases [27–29], thereby, combination of this adjuvant with a delivery system or depot adjuvant may prevent of degrading the poly(I:C).

Several antigens of different stages of *Plasmodium* life cycle have been considered as malaria vaccine candidates [30, 31]. The 42-kDa fragment of merozoite surface protein 1 (MSP-1_{42}) is a leading vaccine candidate for the blood stage of *P. falciparum* [30, 31]. Based on our knowledge, in preclinical studies, CFA/IFA is the only adjuvant that is able to induce anti-PfMSP-1_{42} antibodies with high efficiency for parasite growth inhibition [32–34]. However, due to the toxicity of CFA/IFA adjuvant, in the clinical trials with PfMSP-1_{42}, human compatible adjuvants such as alum [35], AS02 [18], alum/CpG [20], AS01 [19], and ISA720 [36] have been used, though no promising results were obtained with these adjuvants. Therefore, to improve the efficiency of vaccine formulations based on PfMSP-1_{42} antigen, potent and strong human compatible adjuvants are required.

Previous studies have shown that protective immune responses against PfMSP-1_{42} antigen are associated with strong B- and T-cell immune responses of Th1 pathway and growth-inhibitory antibodies [37–39]. Hence, in the present study, poly(I:C), as a potent Th1 inducer and a human compatible adjuvant, was investigated to evaluate whether it is able to enhance and to improve the desired immune responses against this antigen. In this light, the immunogenicity of recombinant PfMSP-1_{42} (rPfMSP-1_{42}) was studied in mouse groups that received this antigen alone or in combination with poly(I:C) adjuvant or CFA/IFA, as the reference adjuvant. Furthermore, regarding the short half-life of poly(I:C) in body fluids [27, 28], in the current study, a group of mice was immunized with rPfMSP-1_{42} antigen in combination with poly(I:C)/alum (as depot effect). Finally, to determine the best vaccine formulation, the titer of antibodies, profiles of IgG isotypes, avidity of the IgG and its subclasses as well as growth-inhibitory activity of antibodies to rPfMSP-1_{42} were evaluated in immunized mice.

Materials and methods

Cloning and sub-cloning of PfMSP-1_{42}

For cloning and expression of the recombinant PfMSP-1_{42}, K1 strain with Q/KNG/L haplotype of 19 kDa fragment, as the predominant haplotype in Iranian *P. falciparum* isolates [40] was selected. The primers were designed based on the sequence of the *P. falciparum* K1 strain (accession no. X03371) to amplify the nucleotides 3763–4836 (amino acids 1255–1612). To use these primers for sub-cloning of *pfmsp-1_{42}* into the pQE30 plasmid, *Bam*HI and *Hind*III restriction sites were designed in 5′ end of the oligonucleotide primers as follows:

MSP1-42F: 5′ TAACTGGATCCGCAGTAACTCCTTCC 3′ (underlined: *Bam*HI site; nt: 3763–3777).

MSP1-42R: 5′ ATGCCAAGCTTGTTAGAGGAACT GCAG 3′ (underlined: *Hind*III site; 4821–4836).

For amplification of the K1 allelic type of *pfmsp-1*$_{42}$ gene, the template DNA was obtained from an Iranian individual with patent *P. falciparum* infection (Chabahar, Sistan and Baluchistan Province, South-East Iran) and a known sequence of K1 allelic type of PfMSP-1_{42} (GenBank accession no. DQ489585, [41]). PCR amplification was performed in 25 µL of reaction buffer containing 1 µL of DNA, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 250 nM of oligonucleotide primers, 0.25 µL of BSA (10 mg/mL, New England BioLabs Inc.) and 0.5 U of high fidelity Taq DNA polymerase (Roche, Germany).

PCR product was purified from 1% agarose gel using a DNA Extraction Kit (Qiagen, Germany) and then cloned and sub-cloned into the pGEM-T easy (Promega, Madison, WI, USA) and pQE30 (Qiagen, Germany) plasmids, respectively, as described previously [42]. The difference was that the *pfmsp-1*$_{42}$ fragment was excised from recombinant pGEM-T-easy-PfMSP-1_{42} plasmid using *Bam*HI and *Hind*III restriction enzymes and inserted into the corresponding sites in pQE30 plasmid. Confirmation of clones was performed by plasmid extraction followed by *Bam*HI–*Hind*III digestion. For final confirmation, the recombinant plasmid was sequenced using pQE-F and pQE-R universal primers.

The expression and purification of rPfMSP-1_{42}

For expression of the rPfMSP-1_{42} protein with an N-terminus His-tag fusion, the *E. coli* M15-pQE30-PfMSP-1_{42} clone was expanded in Terrific Broth (TB) containing ampicillin (100 µg/mL) with continuous shaking (180 rpm) at 37 °C. When the absorbance at OD_{600nm} reached 0.6–0.8, protein expression was induced by 0.2 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Fermentas GmbH, St. Leon-Rot, Germany). The pellet of recombinant clones was collected 4 h after induction and analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of the His-Tag fused rPfMSP-1_{42} was performed using Ni^{2+}-nitrilotriacetic acid agarose resin (Ni–NTA agarose, Qiagen, Germany) under denaturing conditions. Purification was carried out following the protocol described before [42] with some modifications. Briefly, the cells containing the inclusion bodies of rPfMSP-1_{42} were resuspended in a lysis buffer (5 M Guanidin thiocyanate, 20 mM Tris–HCl, 10 mM imidazole and 500 mM NaCl, pH 7.9) and incubated at 4 °C for 90 min. After disrupting the cells by sonication, the bacterial lysate was centrifuged and the supernatant containing the recombinant protein was incubated with equilibrated Ni–NTA agarose using lysis buffer for 2 h. The resin was then washed with wash buffer (6 M urea, 20 mM Tris–HCl, 20 mM imidazole and 500 mM NaCl, pH 7.9). The bound protein was then eluted and analyzed by 12% SDS-PAGE gel. The expressed and purified rPfMSP-1_{42} protein was confirmed by Western blotting analysis using both monoclonal penta-His antibody (Qiagen, Germany) and *P. falciparum*-infected patient's sera. The eluted rPfMSP-1_{42} was desalted with Econo-Pac 10DG columns (BioRad, USA) according to the manufacturer's instructions and concentrated with a concentrator (Eppendorf, Germany). The concentration of the purified protein was determined by Bradford's assay at 595 nm using spectrophotometer (Denovix DS-11, Wilmington, USA). The level of bacterial endotoxin was also determined using the LAL chromogenic kit (Lonza, Walkersville, MD, USA) according to the manufacturer's instructions.

Mice immunization

Inbred female BALB/c mice were obtained from Laboratory Animal Science Department, the Pasteur Institute of Iran (Karaj, Iran). All the experimental protocols were approved by the Committee of Animal Ethics of the Pasteur Institute of Iran and performed accordingly. For immunization, 10 groups of 15 mice at 6–8 weeks of age were inoculated on days 0, 14 and 28 via subcutaneously at the base of tail with 25 µg of purified rPfMSP-1_{42} alone (group 1) or formulated with poly(I:C) (vaccine grade type, InvivoGen, USA, San diego; group 2), combination of poly(I:C)/alum (Alhydrogel^R adjuvant 2%, vaccine grade type, InvivoGen; group 3), alum (group 4) or CFA/IFA (Sigma, USA; group 5). The dose of poly(I:C) was 50 µg/mouse, while the alum, CFA or IFA were mixed with antigen by 1:1 (volume/volume) ratio prior to immunization. In group 5 (rPfMSP-1_{42}/CFA/IFA), mice received the antigen emulsified in CFA for the first immunization and IFA was used for second and third immunization. The control mouse groups received PBS 1× (pH 7:2) alone (group 6) or in combination with adjuvants including poly(I:C) (group 7), combination of poly(I:C)/alum (group 8), alum (group 9) or CFA/IFA (group 10) (Table 1). For evaluation of the antibody responses to rPfMSP-1_{42} and their persistence, sera samples were collected from tail vein before immunization (as pre-immune sera) as well as 12, 26, 40 and 205 days after primary immunization. All sera were stored at −20 °C until use. In addition, the cellular immune responses were evaluated 40 and 205 days after primary immunization.

Antibody responses

Antibody responses to rPfMSP-1_{42} at 12, 26, 40 and 205 days after primary immunization were measured by enzyme-linked immunosorbent assay (ELISA), as described previously, with some modifications [43]. Briefly, 100 ng of rPfMSP-1_{42} antigen was coated in Maxisorp flat-bottom

Table 1 Mouse groups for immunization in this study

Mouse groups	Antigen	Adjuvant
Immunized with rPfMSP-1_{42}		
1	rPfMSP-1_{42}	–
2	rPfMSP-1_{42}	poly(I:C)
3	rPfMSP-1_{42}	poly(I:C)/alum
4	rPfMSP-1_{42}	alum
5	rPfMSP-1_{42}	CFA/IFA
Controls		
6	–	PBS 1×
7	–	poly(I:C)
8	–	poly(I:C)/alum
9	–	alum
10	–	CFA/IFA

Mice were immunized with rPfMSP-1_{42} (25 µg) antigen alone or in combination with poly(I:C), poly(I:C)/alum, alum or CFA/IFA adjuvants subcutaneously at base of tail. Control mice received PBS 1× alone or in combination with poly(I:C), poly(I:C)/alum, alum or CFA adjuvants. The amounts of used adjuvants were as follows: 50 µg of poly(I:C)/mouse, 100 µL of alum, CFA or IFA (1:1 ratio of adjuvant/antigen)

Poly(I:C) polyinosinic:polycytidylic acid, *alum* Alhydrogel^{R} adjuvant 2%, *CFA* complete freund adjuvant, *IFA* incomplete freund adjuvant

96-well ELISA plates (Grainer; Labortechnic, Nurtingen Germany). After blocking with PBS 1×-1% Bovin Serum Albumin (BSA), sera samples were diluted 1:200 in PBS 1×-Tween 20 (PBS-T) containing 0.5% BSA and incubated in duplicate wells for 90 min. Goat anti-mouse IgG antibodies conjugated with horseradish peroxidase (HRP) (Sigma, USA) with 1:20,000 dilution was used to recognize the bound IgG antibodies to rPfMSP-1_{42}. The reaction was developed by adding the 3,3′,5,5′-tetramethylbenzidine (Sigma, USA) as substrate and then was stopped by 2 N H_2SO_4, and the absorbance was measured at 450 nm. For evaluation of the specific rPfMSP-1_{42} IgG subclasses response, the experiment was performed as described above except for the secondary antibodies that were goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 antibodies (Sigma, USA) diluted 1:1000 and incubated at RT for 1 h. After washing, HRP-conjugated anti-goat antibody was used at 1:10,000 dilutions. Pre-immune sera were used as negative control to determine the cut-off that calculated as the mean ELISA units + 3 standard deviation (SD). For ELISA unit calculation, a pool of sera ($n = 15$) with high antibody titers was used to prepare a standard curve by twofold serial dilutions. The absorbance of individual test samples was converted into ELISA units using the standard curve in the same plate [44]. The relation between reciprocal number of the dilution and OD_{450nm} was approximated by a 4-parameter logistic curve (http://www.myassays.com/four-parameter-logistic-curve.assay). To assign the antibody units with OD_{450nm}, the dilution giving an $OD_{450nm} = 1$ was considered as 10,000 ELISA units. Moreover, to evaluate the titration end-point of specific anti-PfMSP-1_{42} antibodies (IgG, IgG1, IgG2a, IgG2b and IgG3), sera from individual mice in each group were pooled and used in serial dilutions (1:200 to 1:204,800) in ELISA. The titration end-point that was determined as the last dilution of serum had an ELISA unit ≥ cut-off.

Moreover, the avidity of anti-PfMSP-1_{42} IgG antibodies and cytophilic antibodies (IgG2a and IgG2b) was estimated as described earlier [45] with minor modifications. Briefly, ELISA test was performed as mentioned above except that the test was performed in duplicate plates. After sera (1:200 dilution) incubation for 90 min, one of the plates was washed three times with PBS-T and the other duplicate, incubated with the same sera, was washed three times with PBS-T-urea (8 M) dissociation buffer. Then, the plates were washed once with additional wash with PBS-T buffer. Incubation with secondary antibody, washing steps and developing enzyme reaction were performed as mentioned above for ELISA. The avidity index (AI) was calculated as follows:

$$\mathrm{AI} = \frac{\text{OD450 of treated sample with urea 8 M}}{\text{OD450 of sample without treatment with urea 8 M}} \times 100.$$

Lymphocyte proliferation assay

Lymphocyte proliferation assay was measured on days 40 and 205 after the first immunization. To perform this assay, the mice ($n = 4$ from each group) in each time point were euthanized under sterile conditions. The spleens were transferred to complete culture medium containing RPMI 1640 medium (Gibco, Invitrogen, Scotland, UK), 5% fetal calf serum (FCS, Sigma, USA), 2.3×10^{-2} mM 2-mercaptoethanol, penicillin–streptomycin (100 U–100 µg/mL) and 10 mM HEPES and single cell suspension of spleen cells were prepared, as described previously [43]. Then, 100 µL of cell suspension containing 2×10^6 cell/mL were cultured in a flat-bottom 96-well tissue culture plate (Orange Scientific, EU, Belgium) in the presence of rPfMSP-1_{42} (5 µg/mL), concanavalin A (ConA, 5 µg/mL, as positive control) and medium alone (as negative control) in tetraplicates. All cells were cultured at humidified atmosphere in a 5% CO_2 incubator at 37 °C for 72 h. Afterwards, the supernatant was removed, and cell proliferation was determined by 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, thiazolyl-blue (MTT) dye assay, as described previously [43].

Cytokine analysis

Extracellular cytokine profiles were measured in supernatants of stimulated splenocytes of immunized mice with

rPfMSP-1_{42} as antigen using murine cytokine immunoassay kits (R&D system, Minneapolis, USA). In the first step, the splenocytes of each group ($n = 4$) on days 40 and 205 after primary immunization were cultured as described above. Next, the supernatants of splenocyte cultures, which were stimulated with target antigen (5 µg/mL) were collected at 24 and 72 h for interleukin-4 (IL-4), and interferon-γ (IFN-γ) measurement, respectively. For the cytokine analysis, the supernatants were analyzed for the quantitative estimation of IFN-γ and IL-4 using the standard curve, obtained with known concentrations (15.7–2000 pg/mL) of recombinant cytokines. The concentration of cytokines were calculated from the standard curves performed in parallel with known concentrations of recombinant mouse IFN-γ and IL-4 cytokines for each experiment. All tests were performed in duplicate, and the mean of concentration was recorded for each set of samples.

Indirect immunofluorescence antibody test (IFAT)

In this investigation, IFAT was performed to test the ability of anti-PfMSP-1_{42} (K1) mice sera of the immunized mice for recognizing the native form of the PfMSP-1_{42} antigen on merozoite surface of *P. falciparum* K1 strain. The procedure was performed as described previously [43]. The polyclonal mice sera were tested in 1:50 to 1:51,200 dilutions.

P. falciparum culture

K1 strain of *P. falciparum* was cultured using human group O^+ erythrocytes, at 10% hematocrit, in the presence of RPMI 1640 medium (Gibco, Invitrogen, Scotland, UK) supplemented with 1 mM HEPES (Sigma, USA), 25 mM $NaHCO_3$, 0.2% Albumax I (Gibco, Invitrogen, Scotland, UK), 1.96 gr/L Glucose (Sigma, USA), 60 µg/mL gentamicin and 12% pooled human sera of AB^+ blood group of healthy donors from Iran, Tehran. The culture was maintained in an atmosphere of 3% O_2, 6% CO_2, and 91% N_2 at 37 °C, as previously described [46]. The culture was synchronized two times in 96 h interval using 5% D-sorbitol (Sigma, St Louis, MO).

IgG purification

IgG was purified from the sera of all immunized mouse groups. To purify the IgGs, 100 µL immunized mice sera obtained on day 40 of the first immunization were dialyzed against PBS 1× using Micro DispoDIALYZER (Sigma, USA, Holliston) with 50K MW cut-off. Then, the dialyzed IgGs were purified using 150 µL of Protein G agarose beads (ABT, Madrid, Spain). The purified IgGs were dialyzed using Dialysis tubing cellulose of 14K MW cut-off against RPMI and concentrated to the original starting volume (100 µL).

Growth-inhibitory assay (GIA)

Parasite growth-inhibitory assay was performed 1 day after last synchronization, when the majority of parasites were at the late trophozoite or early schizont. Parasites were cultured in sterile flat-bottom 96 well plates (Orange Scientific, EU, Belgium) in 200 µL malaria culture media in 1% hematocrit. The volume of culture was optimized to obtain the best volume to grow the parasites in micro-wells. The purified IgGs were inactivated 20 min in 56 °C and then, were absorbed with human O^+ erythrocytes by adding 5 µL of 50% O^+ hematocrit. To assay the growth-inhibitory activity, purified IgGs from different immunized mouse groups as well as pre-immune mice were tested in four repeats in final concentrations of 0.3, 0.225, 0.15 and 0.075 mg/mL in 200 µL culture media. After 48 h, the thin blood smears were prepared from one of repeats for microscopy assessment and the other triplicates were applied for lactate dehydrogenase (LDH) assay [47, 48] to evaluate the growth-inhibitory rate. Infected RBCs were considered in each plate as positive control. Besides, un-infected RBCs were applied in each plate in 1% hematocrit as negative control.

To perform LDH assay, the parasite culture samples were freezed and thawed three times to lyse cells. Then, the parasites were resuspended and aliquots of 20 µL were transferred to 100 µL of the Malstat reagent containing 0.11% v/v Triton-100, 115.7 mM lithium L-lactate, 30.27 mM Tris, 0.62 mM 3-acetylpyridine adenine dinucleotide (APAD; Sigma-Aldrich), adjusted to pH 9 with 1 M HCl [47, 48] in a 96-well microtiter plate and 25 µL of a solution of 1.9 µM nitro blue tetrazolium (NBT) and 0.24 µM phenazine ethosulphate (PES) was added to this mixture. The samples were incubated 30 min at 37 °C to allow color development and absorbance was measured at 650 nm using ELISA reader (Biotek, USA). The rate of growth inhibitory (GI) was measured with the following formula.

$$\mathrm{GI} = \left(1 - \frac{(\mathrm{OD650\ of\ infected\ RBCs\ with\ test\ IgG} - \mathrm{OD650\ of\ normal\ RBCs\ only})}{(\mathrm{OD650\ of\ infected\ RBCs\ with\ preimmune\ sera} - \mathrm{OD650\ of\ normal\ RBCs\ only})}\right) \times 100.$$

Statistical analysis

A database was created with IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp, USA. Comparisons between the groups for antibody levels and cellular responses were analyzed by one-way ANOVA. Independent sample *t* test was used for comparison between two groups. Paired sample *t* test was used to analyze the persistence of humoral and cellular immune responses in each group on days 40 and 205. For all tests, P values < 0.05 were considered significant.

Results

Expression and confirmation of rPfMSP-1_{42} in *E. coli* M15-pQE30 expression system

The rPfMSP-1_{42} protein was successfully cloned and expressed in *E. coli* M15-pQE30 expression system after 4 h of induction using 0.2 mM IPTG at OD_{600nm}: 0.6–0.8. The lysate of induced recombinant bacteria on SDS-PAGE indicated a ~ 42-kDa protein. This protein was purified and desalted in a large scale for immunization. The purity of rPfMSP-1_{42} was confirmed by SDS-PAGE, as it moved as a single band (supplementary Fig. A1). Moreover, Western blot assay with anti-His antibody (Penta-His antibody, Qiagen) and with *P. falciparum*-infected human sera confirmed the purified recombinant protein (supplementary Fig. A1). The confirmed protein was used for mice immunization.

Recognition of native MSP-1_{42} on *P. falciparum* parasites

Mouse polyclonal antibodies to rPfMSP-1_{42} recognized the native protein expressed on the surface of *P. falciparum* merozoite at late schizont stage. This result showed the presence of common epitopes in recombinant and native forms of MSP-1_{42} antigen. However, none of the control mouse sera (groups 6–10) recognized the native protein expressed by parasite (Fig. 1). Interestingly, the last sera dilution capable of recognizing the native protein was the highest in group 2 that received rPfMSP-1_{42}/poly(I:C) (1:3200), followed by mouse groups immunized with rPfMSP-1_{42}/CFA/IFA (1:1600), rPfMSP-1_{42}/poly(I:C)/alum (1:1600), rPfMSP-1_{42} (1:800), and rPfMSP-1_{42}/alum (1:800).

Anti-rPfMSP-1_{42} IgG antibody responses and persistence

The IgG antibody responses against rPfMSP-1_{42} in mice immunized with rPfMSP-1_{42} in combination with different adjuvant formulations were measured using ELISA and calculated as ELISA units. After first immunization, on day 12, in comparison with control groups a significant anti-rPfMSP-1_{42} IgG antibody was detected in mice immunized

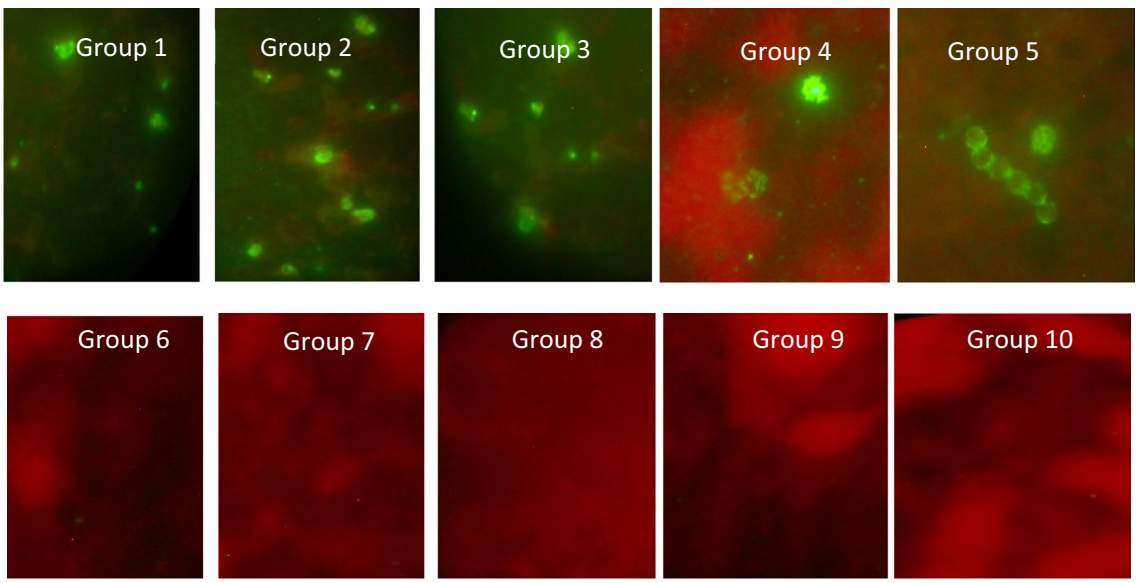


Fig. 1 The figure shows the recognition of native MSP-1_{42} on the surface of *P. falciparum* K1 parasites using mouse polyclonal antibodies to rPfMSP-1_{42} in mouse groups that received rPfMSP-1_{42} antigen alone (group 1) or in combination with poly(I:C) (group 2), poly(I:C)/alum (group 3), alum (group 4), or CFA/IFA (group 5) adjuvants. The parasite detection is observed by green color of fluorescence. None of the control mouse sera receiving PBS 1× (group 6), poly(I:C) (group 7), poly(I:C)/alum (group 8), alum (group 9), or CFA/IFA (group 10) adjuvants recognized the native protein expressed by parasite

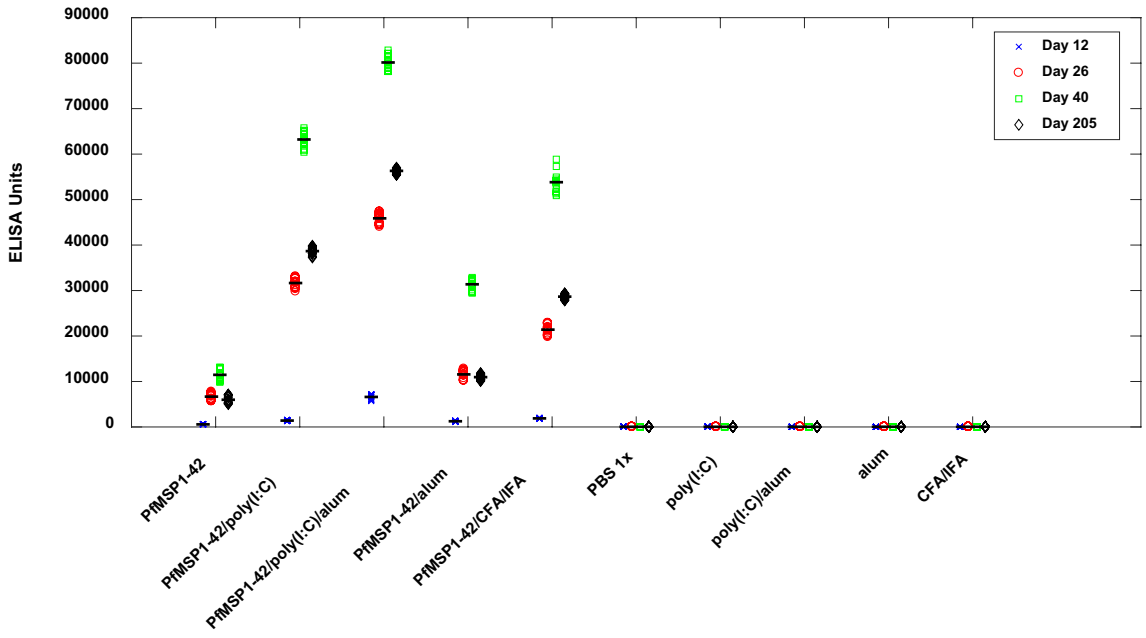


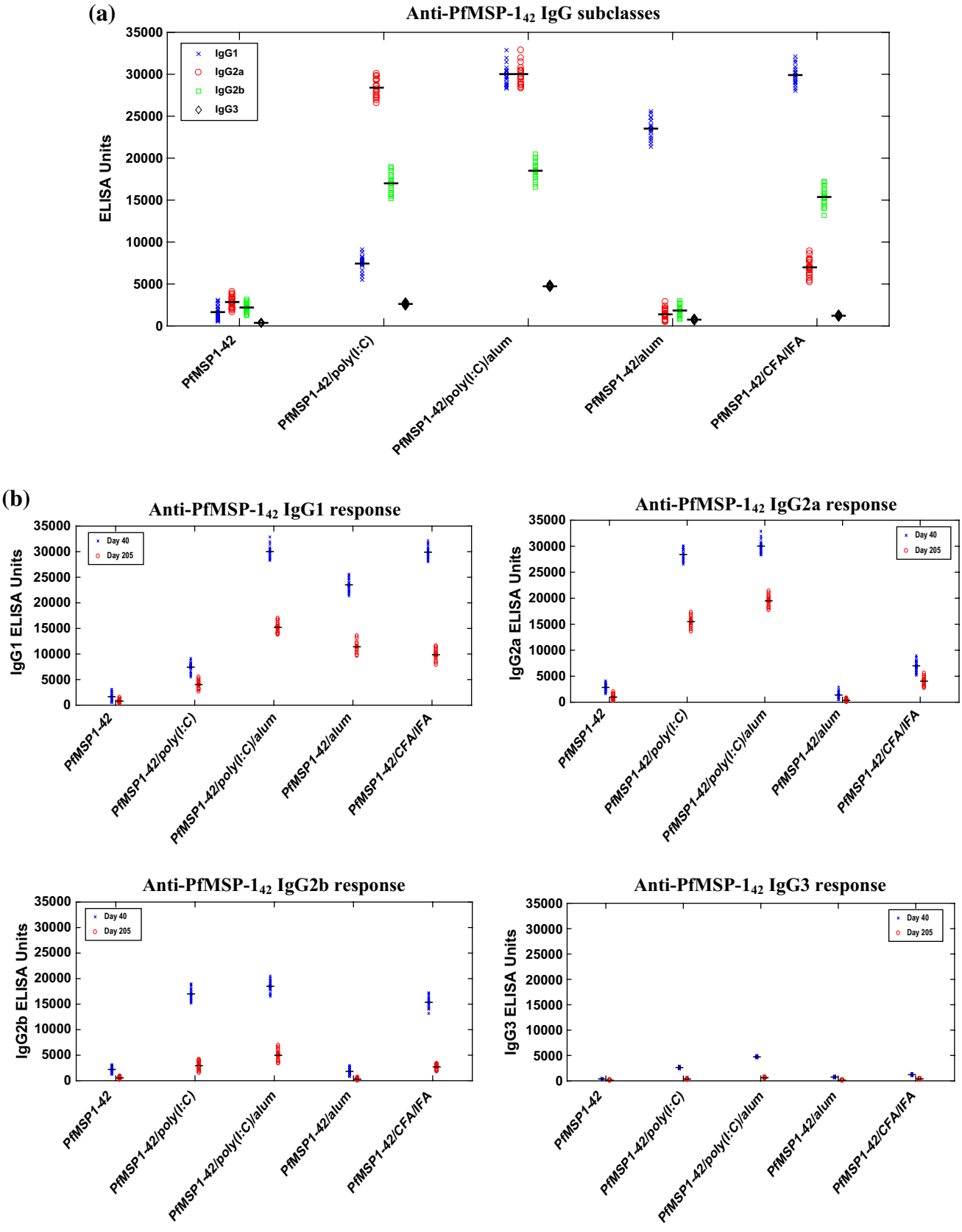
Fig. 2 Anti-rPfMSP-1_{42} IgG antibody responses and longevity in immunized mouse groups receiving rPfMSP-1_{42} antigen alone or emulsified in poly(I:C), poly(I:C)/alum, alum, and CFA/IFA. Control groups received PBS 1× alone or in combination with poly(I:C), poly(I:C)/alum, alum, and CFA/IFA adjuvants. Anti-rPfMSP-1_{42} IgG antibodies in immunized mice were measured on days 12 (after primary immunization), 26 (after the first boost), 40 (after the second boost), and 205 (after 6 months) of the first immunization. All experiments were performed with 1:200 dilution of the tested sera. Each point shows the ELISA unit of an individual mouse serum, and the horizontal lines show the mean ELISA units in each group

with rPfMSP-1_{42} alone or in different adjuvant formulations (ranging from 586 to 6611 U, one-way ANOVA, $P < 0.05$; Fig. 2). After the first (on day 26) and the second (on day 40) boost, the level of anti-rPfMSP-1_{42} IgG antibodies were significantly elevated in all test groups relative to the previous immunization ($P < 0.05$, paired sample *t* test). On day 40, after the second boost, the mean ELISA units of anti-rPfMSP-1_{42} IgG antibodies in mouse group receiving rPfMSP-1_{42}/poly(I:C) (63,340 U) was comparable to rPfMSP-1_{42}/CFA/IFA-immunized mice (53,460 U) (Fig. 2). Comparing to mouse group that received only rPfMSP-1_{42}, poly(I:C) could significantly increase the level of anti-rPfMSP-1_{42} IgG antibodies. The mean ELISA units of anti-rPfMSP-1_{42} IgG antibodies in mouse group receiving rPfMSP-1_{42}/poly(I:C)/alum was 80,000 U. No detectable anti-rPfMSP-1_{42} IgG antibodies were measured in control groups 6–10 (Fig. 2).

To determine the persistence of anti-rPfMSP-1_{42} IgG in immunized mice, ELISA test was performed using the collected sera on day 205 after primary immunization. The results revealed significant levels of anti-rPfMSP-1_{42} IgG antibodies in all test groups as compared to the control groups 6–10 (Fig. 2, one-way ANOVA, $P < 0.05$). However, the levels of anti-rPfMSP-1_{42} IgG had been significantly decreased in comparison with the collected sera on day 40 (Fig. 2, paired sample *t* test, $P < 0.05$). The percentage of reduction in IgG antibody ELISA units in mouse group 2 (38.8%) receiving rPfMSP-1_{42}/poly(I:C) was significantly lower than that in mouse group immunized with rPfMSP-1_{42} antigen alone (46.4%) or emulsified in reference adjuvant CFA/IFA (46.3%). The amount of reduction in IgG antibody ELISA units in mouse group immunized with rPfMSP-1_{42}/poly(I:C)/alum was 29.4% (Fig. 2).

Anti- rPfMSP-1_{42} IgG subclasses profile and persistence

Analysis of the anti-rPfMSP-1_{42} IgG subclasses was performed in immunized mice sera collected from mouse groups 1–10 on day 40 following the first immunization. As shown in Fig. 3, the mean IgG1 ELISA units was 7445 U in rPfMSP-1_{42}/poly(I:C)-immunized mouse group, which shows the increased level of this isotype relative to mouse group receiving this antigen without any adjuvant (mean 1619 U). It was notable that combination of rPfMSP-1_{42} antigen with poly(I:C) induced significantly less IgG1 antibody relative to CFA/IFA (mean 30,000 U), poly(I:C)/alum (mean 30,000 U) and alum (mean 22,170 U) adjuvants. Instead, poly(I:C) induced a high level of IgG2a (mean 28,120 U) and IgG2b (mean 17,000 U/mL) when compared to mouse group immunized with rPfMSP-1_{42} antigen alone (Fig. 3a). The level of IgG2a isotype induced by rPfMSP-1_{42}/poly(I:C) was


(a)
Anti-PfMSP-1$_{42}$ IgG subclasses
ELISA Units
IgG1
IgG2a
IgG2b
IgG3
PfMSP1-42
PfMSP1-42/poly(I:C)
PfMSP1-42/poly(I:C)/alum
PfMSP1-42/alum
PfMSP1-42/CFA/IFA
(b)
Anti-PfMSP-1$_{42}$ IgG1 response
IgG1 ELISA Units
Anti-PfMSP-1$_{42}$ IgG2a response
IgG2a ELISA Units
Anti-PfMSP-1$_{42}$ IgG2b response
IgG2b ELISA Units
Anti-PfMSP-1$_{42}$ IgG3 response
IgG3 ELISA Units
Day 40
Day 205

◂**Fig. 3** IgG subclass antibodies to rPfMSP-1_{42} in the sera of immunized mice. **a** Comparison of the anti-rPfMSP-1_{42} IgG1, IgG2a, IgG2b, and IgG3 antibodies in the examined mice groups on day 40 (after the second boost). A significant difference was observed in the ELISA units of anti-rPfMSP-1_{42} IgG1, IgG2a, IgG2b, and IgG3 antibodies among mouse groups received different adjuvant formulations (one-way ANOVA, $P < 0.05$). **b** Analysis of the sera 40 and 205 days after primary immunization for evaluating the longevity of the anti-rPfMSP-1_{42} IgG isotypes. All assays were carried out with 1:200 dilution of mouse sera. Each point shows the ELISA unit of an individual mouse serum, and the horizontal lines show the mean ELISA units in each group

significantly higher than that induced by rPfMSP-1_{42}/CFA/IFA (mean 6950 U) adjuvant ($P < 0.05$, independent sample *t* test; Fig. 3a). However, the level of elicited IgG2b antibodies in mouse group immunized with rPfMSP-1_{42}/poly(I:C) was comparable with the level of induced IgG2b by CFA/IFA adjuvant ($P > 0.05$, independent sample *t* test; Fig. 3a). No significant difference was observed in IgG2a and IgG2b levels in mouse groups immunized by rPfMSP-1_{42} antigen in combination with poly(I:C) or poly(I:C)/alum adjuvants ($P > 0.05$, independent sample *t* test). The highest level of IgG3 antibody was detected in mouse groups who received the antigen in combination with poly(I:C) or poly(I:C)/alum adjuvants, and this difference was not statistically significant ($P > 0.05$, independent sample *t* test). Regarding the mouse group immunized with rPfMSP-1_{42}/alum, in comparison with mouse group receiving rPfMSP-1_{42}/CFA/IFA, high level of IgG1 but low levels of IgG2a, IgG2b, and IgG3 ELISA units were detected (Fig. 3a).

The results of the longevity of the anti-rPfMSP-1_{42} isotypes in immunized mice revealed that both rPfMSP-1_{42}/poly(I:C) and rPfMSP-1_{42}/CFA/IFA combinations could induce IgG2a antibodies after 6 months with almost the same reduction (44.8 and 42.11%, respectively; Fig. 3b). Both IgG1 and IgG2b antibodies were reduced in mice immunized with rPfMSP-1_{42} antigen together with poly(I:C) (45.9 and 83.2%) or CFA/IFA (67.1 and 82.6%) adjuvants, respectively. The reduction of antibody level for IgG1, IgG2a, and IgG2b was 49.2, 35.1, and 72.3% in mouse group receiving rPfMSP-1_{42}/poly(I:C)/alum. The most reduction in the level of isotypes was found in rPfMSP-1_{42}/alum-immunized mouse group (Fig. 3b).

Antibody titers

The mean of serum antibody titers were evaluated 40 days after first immunization by ELISA. The rPfMSP-1_{42} antigen in combination with poly(I:C) and CFA/IFA adjuvants could induce comparable IgG end-point titers (25,600), which was significantly higher than the IgG end-point titer in rPfMSP-1_{42}-immunized mice without any adjuvant (6400). The highest anti-rPfMSP-1_{42} IgG end-point antibody titer was detected with poly(I:C)/alum adjuvant (51,200). The titer of anti-PfMSP-1_{42} IgG1 antibodies was the highest with this adjuvant, the same as CFA/IFA and alum adjuvants (102,400; Fig. 4). However, among different formulations, the lowest IgG1 end-point titer was detected with poly(I:C) (25,600; Fig. 4). Concerning IgG2a, an end-point titer of 51,200 was detected for poly(I:C) which was more than CFA/IFA (12,800) adjuvant or when antigen was used alone for immunization (6400). Anti-PfMSP-1_{42} IgG2a end-point titer was comparative (51,200) when poly(I:C) or poly(I:C)/alum were used as adjuvants. In the mouse group that received antigen with poly(I:C) adjuvant, IgG2b end-point titer was 51,200, which was higher than mouse groups immunized with antigen alone (6400) or emulsified in CFA/IFA (25,600) adjuvant (Fig. 4). The highest end-point titer of IgG3 was observed in mouse group 3 (25,600) receiving antigen in combination with poly(I:C)/alum (Fig. 4).

Avidity of anti-rPfMSP-1_{42} IgG, IgG2a and IgG2b

In this study, high-avidity IgG antibodies were induced in all mouse groups immunized with different adjuvant formulations (mean AI 62.81–83.24%, Table 2). Concerning AI of cytophilic IgG isotypes, poly(I:C) adjuvant induced the highest AI of anti-PfMSP-1_{42} IgG2a antibodies (mean AI ± SD 82.72% ± 1.49) among different adjuvant formulations. In addition, poly(I:C) adjuvant could induce anti-PfMSP-1_{42} IgG2b antibodies with a similar avidity to those elicited by CFA/IFA adjuvant (Table 2). The highest avidity index of anti-PfMSP-1_{42} IgG2b (mean AI ± SD 72.21% ± 2.82) was induced by poly(I:C)/alum adjuvant (Table 2).

Cellular immune responses in immunized mice

For determination of both proliferation and cytokine production in immunized mice groups, four mice from each group were anesthetized on days 40 and 205 after primary immunization, and the splenocytes were used for the analysis. The significant proliferation of splenocytes was observed in mouse groups 1–5 (mean OD_{550nm} 0.35–0.5), but not in control mouse groups 6–10 (mean OD_{550nm} 0.07–0.15, $P < 0.05$, one-way ANOVA). The stimulated splenocytes with ConA had high proliferation (mean OD_{550nm} 0.4–0.7). However, no proliferation was observed with splenocytes of control mouse groups 6–10 (data not shown).

The cytokine profiles of the immunized mice with different adjuvant formulations were determined using murine cytokine immunoassay kits. The results revealed eliciting significant levels of IFN-γ in mouse groups received rPfMSP-1_{42} antigen/adjuvants as compared to mouse control groups (6–10) on day 40 after primary immunization ($P < 0.05$, one-way ANOVA, Fig. 5a). On day 40, the low level of IFN-γ (mean: 51 pg/mL) was produced by mouse group that received rPfMSP-1_{42} antigen without any

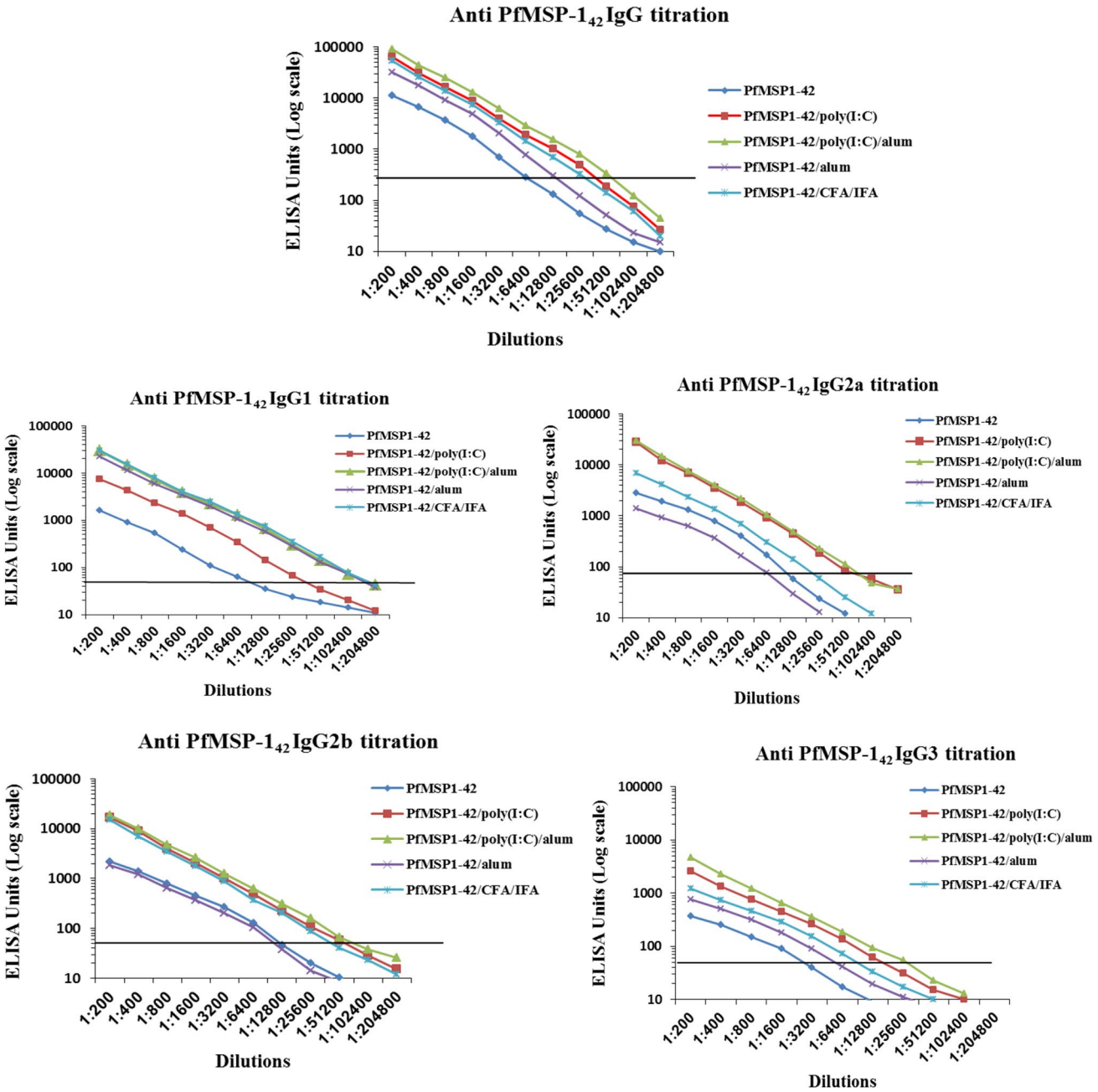


Fig. 4 Titration of anti-rPfMSP-1$_{42}$ IgG, IgG1, IgG2a, IgG2b, and IgG3 antibodies in mouse groups receiving different adjuvant formulations with rPfMSP-1$_{42}$ antigen. For titration, 1:200 to 1:204,800 dilutions of mouse sera were analyzed. The difference between the titration of antibodies was detected in mouse groups that received rPfMSP-1$_{42}$ antigen alone or in combination with poly(I:C), poly(I:C)/alum, alum, and CFA/IFA adjuvants. The horizontal lines show the cut-off values. The cut-off values for IgG, IgG1, IgG2a, IgG2b, and IgG3 antibodies were 250, 50, 80, 48 and 50 ELISA units, respectively

adjuvant, while comparable levels of IFN-γ was elicited in mouse groups immunized with rPfMSP-1$_{42}$/poly(I:C) (mean 263 pg/mL) or rPfMSP-1$_{42}$/CFA/IFA (mean 298 pg/mL) ($P > 0.05$, independent sample t test). Besides, in comparison to other groups, a significant level of IFN-γ (mean 358 pg/mL) was elicited in mice received the antigen together with poly(I:C)/alum ($P < 0.05$, one-way ANOVA, Fig. 5a). On day 205 after primary immunization, the level of IFN-γ was significantly decreased in response to rPfMSP-1$_{42}$ in all mouse groups ($P < 0.05$, paired sample t test) and no detectable IFN-γ was observed in group 1 that immunized with rPfMSP-1$_{42}$ antigen alone (Fig. 5a). The most reduction (35.7%) in the level of IFN-γ was observed in mice immunized with rPfMSP-1$_{42}$ plus poly(I:C)/alum.

Table 2 Avidity indices of anti-rPfMSP-1_{42} IgG, IgG2a and IgG2b antibodies among mouse groups immunized with rPfMSP-1_{42} alone or in combination with poly(I:C), poly(I:C)/alum, alum, or CFA/IFA

Groups (antigen/adjuvant)	Mean AI ± SD		
	IgG	IgG2a	IgG2b
1 (rPfMSP-1_{42})	48.45 ± 2.15	47.19 ± 1.81	45.43 ± 1.65
2 (rPfMSP-1_{42}/poly(I:C))	80.23 ± 3.17	82.72 ± 1.49	66.83 ± 2.26
3 (rPfMSP-1_{42}/poly(I:C)/alum)	83.24 ± 1.06	78.2 ± 1.94	72.21 ± 2.82
4 (rPfMSP-1_{42}/alum)	62.81 ± 1.43	48.41 ± 1.22	48.11 ± 1.09
5 (rPfMSP-1_{42}/CFA/IFA)	75.25 ± 2.28	65.71 ± 2.02	62.35 ± 1.38

AI avidity index, *SD* standard deviation

Besides, in mouse groups that received the antigen in combination with poly(I:C) and CFA/IFA adjuvants, 22% and 20.5% reduction in the levels of IFN-γ was observed on day 205, respectively ($P < 0.05$, one-way ANOVA).

Regarding the levels of IL-4 secretion as Th2 type response, no significant levels were produced in mouse groups immunized with antigen alone or in combination with poly(I:C). However, significant levels of IL-4 were elicited in immunized mice with rPfMSP-1_{42}/alum (mean 258 pg/mL), rPfMSP-1_{42}/CFA/IFA (mean 173 pg/mL), and rPfMSP-1_{42}/poly(I:C)/alum (mean 150 pg/mL) in comparison to the control groups on day 40 of the first immunization ($P < 0.05$, one-way ANOVA). On day 205, no significant decrease was detected in the IL-4 levels in mice immunized with rPfMSP-1_{42}/poly(I:C)/alum (128 pg/mL), rPfMSP-1_{42}/alum (230 pg/mL), or rPfMSP-1_{42}/CFA/IFA (146 pg/mL), when compared to day 40 of the primary immunization ($P > 0.05$, paired sample *t* test, Fig. 5b).

Growth-inhibitory assay evaluation

The inhibitory effect of anti-PfMSP-1_{42} IgG antibodies against the growth of *in vitro* culture of *P. falciparum* K1 strain was evaluated among the sera obtained from different mouse groups on day 40 following the first immunization. As illustrated in Fig. 6, the highest growth-inhibitory activity (mean GI 99.5, 94.6, and 82.3%) was observed in mouse group 2, which immunized with rPfMSP-1_{42}/poly(I:C) in the final concentrations of 0.3, 0.225, and 0.15 mg/mL of the purified IgGs in culture media, respectively. In final concentrations of 0.3, 0.225, and 0.15 mg/mL of the purified IgGs, the inhibitory rate of antibodies induced by rPfMSP-1_{42} antigen in combination with poly(I:C) was higher than that induced by rPfMSP-1_{42} antigen alone or in combination with CFA/IFA or poly(I:C)/alum adjuvants (Fig. 6). The growth-inhibitory rate of purified IgGs from group 4 (rPfMSP-1_{42}/alum) was low (mean GI 21.6–1.8% in the presence of 0.3–0.15 mg/mL of purified IgGs) in which this rate was even less than group 1 that received rPfMSP-1_{42} antigen alone. None of the immunized test groups showed significant growth-inhibitory activity in 0.075 mg/mL of purified IgG antibodies in culture media (Fig. 6). Additionally, no growth-inhibitory activity was observed in antibodies of control mouse groups. The growth-inhibitory rate measured by LDH assay was confirmed with microscopy (data not shown).

Discussion

Despite extensive efforts in the field of malaria vaccine, no licensed malaria vaccine is available yet. Malaria vaccine development has faced with several challenges such as poor immunogenicity of candidate malaria vaccine antigens, as the main challenge. However, this problem can be solved by appropriate combinations of antigens and adjuvants. Studies have shown that PfMSP-1_{42} is the promising vaccine candidates for blood stage of malaria [49–55], and based on preclinical studies, only CFA/IFA adjuvant is able to induce highly efficient antibodies for inhibition of parasite growth [32–34]. However, more potent and strong human-compatible adjuvants are required to improve the efficiency of PfMSP-1_{42}-based vaccine formulation. In this regard, in the current study, poly(I:C) was evaluated to improve the immunogenicity of recombinant PfMSP-1_{42} and was compared with CFA/IFA, as the reference adjuvant.

The mice receiving antigen in combination with poly(I:C) produced high levels and titers of IgG antibodies, which was comparable to CFA/IFA adjuvant after the second boost. Besides, poly(I:C) induced anti-PfMSP-1_{42} IgG antibodies with more growth-inhibitory activity than those induced by CFA/IFA adjuvant. The results of the current study showed no association between the anti-PfMSP-1_{42} IgG antibody titer and growth-inhibitory activity. Contrary to this result, other investigations found an association between IgG antibody titer against this antigen and growth-inhibitory activity or protection [56–59]. In concordance with our result, the finding of a previous study indicated that among different adjuvant formulations, only monophosphoryl lipid A (MPL) is the inducer of growth-inhibitory antibody against PfMSP-1_{42}, despite similar antibody titers induction [60]. Hui et al. [60] proposed that inhibitory epitopes are weak; hence, only potent adjuvants are able to induce clonal expansion of rare B-cells specific for parasite-inhibitory epitopes to obtain sufficient growth-inhibitory antibodies. This explanation may be true for the poly(I:C), as a potent B-cell and $CD4^+$ T-cells inducer [22, 61, 62], to help in increasing the growth-inhibitory antibodies against PfMSP-1_{42} antigen.

Results from the present study also revealed that poly(I:C) had remarkable effects on the type of immune responses,

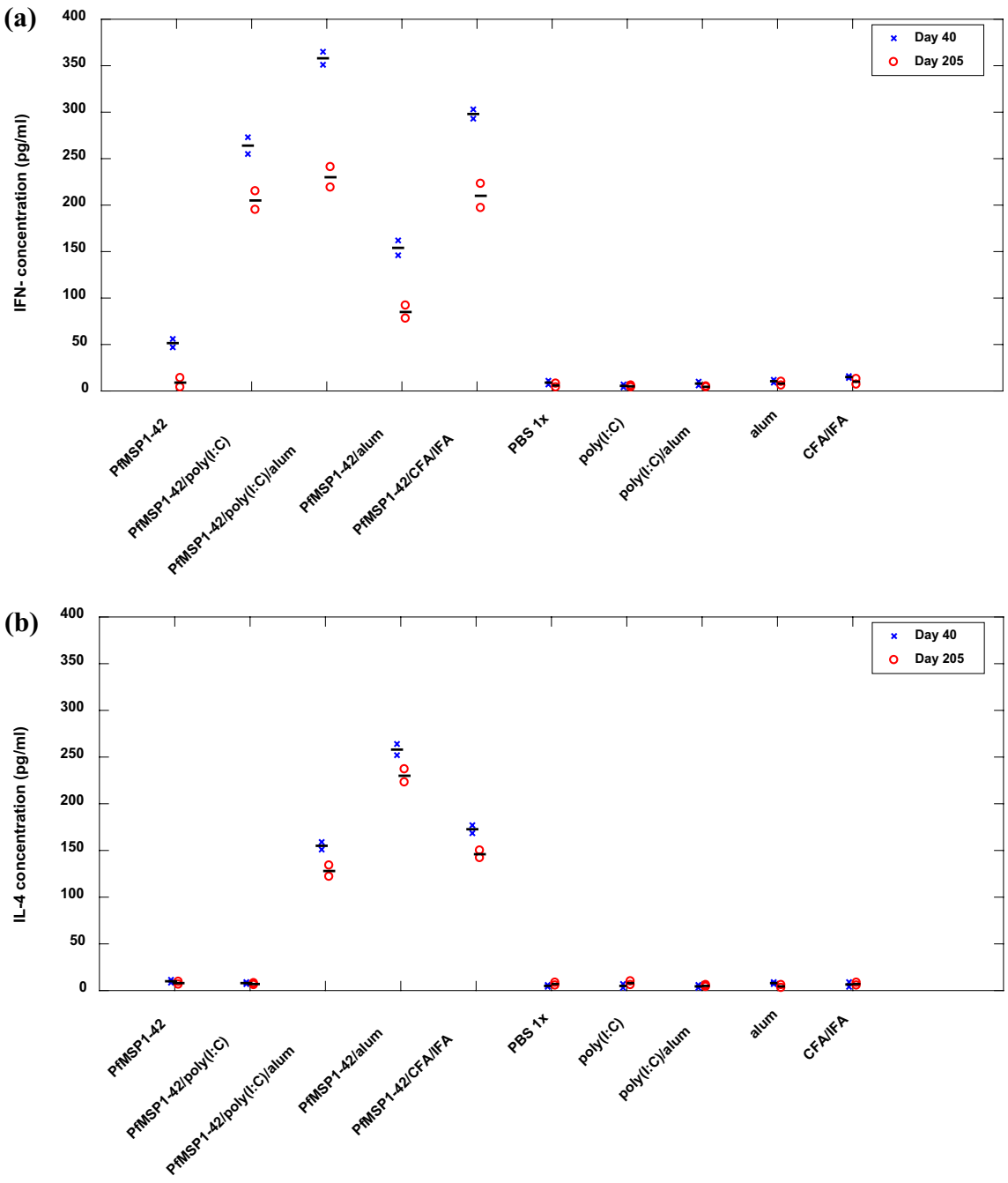


Fig. 5 Assessment of the cytokine production in immunized mice with rPfMSP-1_{42} emulsified in different adjuvant formulations, including poly(I:C), poly(I:C)/alum, alum, and CFA/IFA on days 40 and 205 after primary immunization. **a** IFN-γ production among examined mouse groups. IFN-γ responses of Con A, as the positive control, and no antigen, as the negative control were in the range of 1200–2100 and ~5–30 pg/mL, respectively, among different examined groups. **b** IL-4 production among the examined mouse groups. IL-4 responses of ConA, as the positive control, and no antigen, as the negative control, were in range of 100–200 and < 15 pg/mL, respectively, among different examined groups. Each point shows the concentration of cytokine in each experiment, and the horizontal lines show the mean concentrations of cytokines in each group

cytokines, and the profile of IgG subclasses. This adjuvant elicited the highest ratio of IFN-γ/IL-4 (23.9) and IgG2a/IgG1 (3.77) with high avidity and titer of anti-PfMSP-1_{42} IgG2a antibody, indicating a potent Th1 immune response. Previously, it has been shown that Th1 type immune response, IFN-γ production [38, 39], and IgG2a [38, 39, 63, 64] is associated with protection against *Plasmodium* infection. In addition, both opsonizing isotypes, IgG2a and

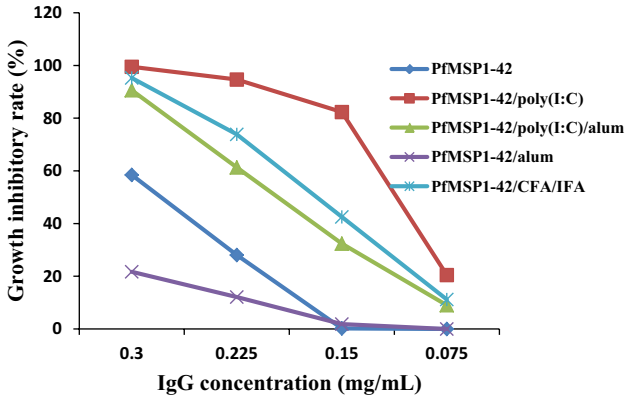


Fig. 6 Growth inhibition of *P. falciparum* K1 parasites by purified IgG antibodies elicited with rPfMSP-1_{42} antigen alone or in combination with poly(I:C), poly(I:C)/alum, alum, or CFA/IFA adjuvants in BALB/c mice. The plot shows the growth-inhibitory activity of pooled purified IgG antibodies produced in different mouse groups. LDH assay was used to measure the growth-inhibitory rate. LDH assay was performed with purified IgG mice sera in final concentrations of 0.3, 0.225, 0.15, and 0.075 mg/mL

IgG2b, bind to activating FcγR receptors [65], and IgG2a, IgG2b, and IgG3 antibodies fix the complement [66]. However, IgG1 does not fix the complement and binds well to FcγRIIB as an inhibitory receptor [65]. Therefore, with regard to inducing high titers of IgG2a and IgG2b antibodies to rPfMSP-1_{42} as well as low titer of IgG1 antibody along with eliciting significant IFN-γ, poly(I:C) is suggested as potent Th1 inducer in malaria vaccine formulations based on PfMSP-1_{42}.

It has been reported that production of high-avidity antibodies is essential for prevention of a severe disease in malaria infections [67]. In the current study, the highest IgG avidity response was detected in mice immunized with rPfMSP-1_{42}/poly(I:C)/alum (mean: 83.65%), while mouse group receiving rPfMSP-1_{42}/poly(I:C) had the highest avidity (mean: 82.72) for IgG2a implying on a strong Th1 immune response. However, the reference adjuvant, CFA/IFA, induced anti-PfMSP-1_{42} IgG2a antibodies with lower avidity (mean: 65.71) than poly(I:C). In sum, among different adjuvant formulations, poly(I:C) was the most potent adjuvant in inducing the high-avidity antibodies especially anti-PfMSP-1_{42} IgG2a antibodies.

Regarding the longevity of immune responses, the persistence of anti-MSP-1_{42} IgG and IgG2a antibodies as well as high levels of Th1 cytokines in mouse group receiving antigen plus poly(I:C) after 6 months has likely revealed the presence of memory T- and B-cell, or long-lived plasma cells. This is an important issue to consider as the success of an efficient vaccine heavily relies on the persistence of antibodies and also on memory cells that are able to proliferate upon re-exposure to the same antigen [68, 69]. Therefore, the presence of significant levels of anti-PfMSP-1_{42} IgG antibodies 6 months after last immunization suggests the ability of poly(I:C) adjuvant to generate memory cells and persistent antibodies in PfMSP-1_{42} based vaccine.

Different studies have been demonstrated that PfMSP-1_{42} vaccine-induced protective immunity is associated with the ability of the induced anti-MSP-1 antibodies to inhibit parasite growth in vitro [34, 70, 71]. In this view, there are reports suggesting the role of adjuvant formulations that could influence in the induction of parasite-inhibitory antibodies by MSP-1 vaccines [32, 60]. Interestingly, the present results demonstrated and confirmed that the inhibitory anti-MSP-1_{42} antibodies induced by poly(I:C) adjuvant was the highest among different examined vaccine formulations. This result may explain by action of poly(I:C) that could expand minor B-cell clones specific for an inhibitory epitope(s), that may increase the level of inhibitory antibodies for in vitro biological activities that significantly affect parasite growth. Therefore, the results of this study encourage to use poly(I:C) as an appropriate adjuvant in malaria vaccine formulations based on PfMSP-1_{42}.

Although PfMSP-1_{42} antigen formulated with poly(I:C) induced high growth-inhibitory antibodies, alum plus poly(I:C) induced more non-inhibitory antibodies despite eliciting the high levels and titers of IgG antibodies. This decrease in the growth-inhibitory antibodies may be due to the ability of alum in inducing antibodies against non-inhibitory epitope(s). Furthermore, in this group of immunized mice [PfMSP-1_{42}/alum/poly(I:C)], the level of IgG1 was the highest in the detected IgG subclasses. As previously demonstrated, IgG1 is a weak and non-protective antibody in malaria infection, and this was also confirmed in a study of epitope-matched mouse IgG isotypes specific for rPfMSP-1_{19} [72]. Hence, it can be drawn to the conclusion that poly(I:C) without alum as depote could be an appropriate adjuvant in inducing a high level of growth-inhibitory antibodies against rPfMSP-1_{42}.

In conclusion, poly(I:C) is a potent adjuvant for eliciting persistent and high-avidity Th1 antibodies as well as IFN-γ and also for inducing the growth-inhibitory antibodies against rPfMSP-1_{42}, a leading malaria vaccine candidate. Therefore, it has potential to improve the immunogenicity of the rPfMSP-$1_{42,}$ in malaria subunit vaccine. Concerning the differences in the immune system of mouse and human, it is suggested that the potential of this adjuvant needs to be investigated in human clinical trials of PfMSP-1_{42}-based vaccine. Furthermore, the results of this study showed that alum as a depot adjuvant can affect the magnitude of the immune responses but not its inhibitory quality and maybe poly(I:C) should combine with another depot adjuvant with the ability in increasing of Th1 immune responses.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal handling was in accordance with the ethical standards of the Laboratory Animal Science Department, Pasteur Institute of Iran.

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