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Measuring of IgG2c isotype instead of IgG2a in immunized C57BL/6 mice with *Plasmodium vivax* TRAP as a subunit vaccine candidate in order to correct interpretation of Th1 versus Th2 immune response

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Abstract

Evaluation of the murine isotype antibodies is essential in subunit vaccine development because inbred mouse strains with diverse genetic backgrounds respond different to recombinant proteins. In this regard, the main goal of this study was to measuring and comparing the profile of IgG isotype responses in C57BL/6 mice. For this purpose, the extracellular region of *plasmodium vivax* thrombospondin-related adhesive protein (PvTRAP) gene was expressed in *Escherichia coli* Rosetta (DE3)-pET23a. Then, the recombinant PvTRAP alone or emulsified with Freund's complete adjuvant were applied for immunization of the C57BL/6 mice. The role of antibodies and cellular immune responses induced by recombinant PvTRAP were evaluated. The results showed the level of anti-rPvTRAP IgG2c was significantly higher than IgG2a in the groups that received rPvTRAP alone (mean OD₄₉₀ = 0.758 ± 0.11 and 0.309 ± 0.1, respectively) and emulsified with CFA/IFA (mean OD₄₉₀ = 1.37 ± 0.06 and 0.605 ± 0.13, respectively; *P* < 0.05, independent sample t-test). Additionally, the immunized mice with rPvTRAP and rPvTRAP + CFA/IFA had an intermediate-avidity IgG2a antibody but high-avidity IgG2c antibody as well as the mean of serum antibody titers results exhibited that in both rPvTRAP and rPvTRAP + CFA/IFA mouse groups, IgG2a end-point titer (1:3200 and 1:25600, respectively) was noteworthy lower than IgG2c (1:25600 and 1:102400, respectively). Moreover, the results revealed the eliciting significant levels of IFN- γ (*P* < 0.05, independent sample t test) and no detectable level of IL-4 in the mouse groups received rPvTRAP alone and emulsified with CFA/IFA as compared to the mouse control groups. In general, our results showed that for correctly interpreting of Th1 immune responses in C57BL/6 mouse strain it is critical to measure IgG2c instead of IgG2a along with IFN- γ .

Keywords: C57BL/6 mouse; IgG2a isotype; IgG2c isotype; recombinant PvTRAP

1. Introduction

Malaria, human immunodeficiency (AIDS), and tuberculosis account for a 5.6 million deaths and the loss of 166 million disability-adjusted life years annually (Palombi and Moramarco, 2018). Diseases for which vaccination has been successful are caused by pathogens that are either extracellular or disease mediated through toxins but vaccination against intracellular pathogens including those causing diseases such as malaria and tuberculosis has proven more difficult (Matuschewski, 2017; Pitt et al., 2013) owing to a lack of understanding in the pathogenesis of the infecting organism, the lack of correlates of protection as well as the absence of a clear understanding of the immune responses that help to suppress the pathogens growth in the infected host.

Regarding malaria disease, parasite-specific antibodies, CD4⁺ T cells, CD8⁺ T cells, contribute to protection against infective sporozoites and parasites that initially develop in hepatocytes (Hollingdale and Krzych, 2002). It is noted that the production of Th1-type cytokines appears to play a central role in the protective response to pre-erythrocytic-stage malaria parasites and may involve the synthesis of IFN- γ . The useful effect of IFN- γ on antibody production has also been shown and has correlated with increased levels of parasite-specific IgG2a in mice (White et al., 1991).

Since, mouse IgG heavy chain constant region are encoded by several tightly linked genes which are inherited as Igh haplotypes (Sikorav et al., 1980; Yamawaki et al., 1981), an earlier publication (Morgado et al., 1989) suggested that Igh-1a and Igh-1b could derive from two distinct isotypes, IgG2a and IgG2c, which are organized in tandem on the same chromosome as two distinct loci in MAI strain and BALB/c and C57B/6 mice carry different IgG2a isotypes. Moreover, expression of only IgG2c in C57B/6 and SJL mice was discovered and was assumed to be caused by a deletion of IgG2a gene in those strains (Martin et al., 1997). In this regard, for developing a subunit vaccine candidate, it is essential that with respect to a mouse strain the relative levels of convenient subtype antibodies were measured in the preclinical phase for preventing incorrect interpretation of the murine humoral immune responses.

TRAP is an antigen from the pre-erythrocyte stage of the malaria parasite *Plasmodium*. It is a transmembrane protein that is required for sporozoite gliding motility, and it has been found that this protein with circumsporozoite protein is essential for the process of malaria sporozoite infection of the hepatocyte (Muller et al., 1993; Sinnis and Sim, 1997). These findings have important implications for the potential use of this antigen as a subunit vaccine against *P. vivax* parasite. Since, the measuring of IgG2a or IgG2c production levels along with IFN- γ and IL-4 in an immunized mice strain with a subunit vaccine candidate is essential for assessing of the relative contribution of Th1 versus Th2 immune responses, thus PvTRAP in the present study was selected as a subunit vaccine candidate. Therefore, the main goal of this study was the measurement and comparison of IgG2a and IgG2c antibody isotypes, their avidities, titration as well as cytokines induced by recombinant PvTRAP antigen in C57BL/6 mouse in order to correct interpretation of Th1/Th2 immune responses.

2. Materials and methods

2.1 Recombinant protein production, purification and confirmation

The ectodomain of PvTRAP from amino acids 25 to 488 (regions II to IV; GenBank Accession No. KT207838) (Nazeri et al., 2015), was inserted in frame with C-terminal hexa-His tag (as a fusion protein) in the plasmid expression vector pET23a and expressed in *Escherichia coli* Rosetta (DE3) as described previously (Nazeri et al., 2017). Briefly, a single colony (*E. coli* Rosetta (DE3) pET23a-PvTRAP) was inoculated into 5 ml of LB medium containing 100 µg/ml of ampicillin and grown overnight at 37°C and 180 rpm. Then, 3 ml of this pre-culture was transferred into 200 ml Terrific broth medium (pH 7.2) containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and incubated on a continuous shaker (180 rpm) at 37°C for 4 h. The protein expression was induced by the addition of IPTG (Thermo Scientific, Waltham, Massachusetts, USA) to a final concentration of 0.2 mM when OD600 reached 0.6-0.8.

After incubation, the cells were harvested by centrifugation at 12,000 g at 4 °C for 15 min. As the rPvTRAP expressed in inclusion bodies (insoluble protein), the bacterial pellets was lysed by sonication (Ultraschallprozessor, Stuttgart, Germany) under denaturing conditions after being suspended in 6 M urea, 2 M Guanidine thiocyanate, 20 mM Tris-HCl, 750 mM NaCl, and 15 mM imidazole. The bacterial lysate was centrifuged at 12,000 g at 4 °C for 15 min, and the supernatant was incubated with Nickel-Nitriloacetate acid agarose resin (Ni-NTA Agarose; Qiagen) at room temperature for 90 min. The resin-lysate suspension was poured to a column and the unbound proteins were allowed to pass down with a wash buffer containing 6 M urea, 20 mM Tris-HCl, 1 M NaCl, and 60 mM imidazole. The bound rPvTRAP was eluted successfully with an elution buffer containing 4 M urea, 20 mM Tris-HCl, 300 mM NaCl, and 250 mM imidazole.

In the end, elution of the purified rPvTRAP desalted against 0.5 × PBS using an Econo-Pac 10 DG columns (BioRad, Hercules, CA, USA) and concentrated with a concentrator (Eppendorf, Hamburg, Germany). Protein concentration was detected by Bradford's assay with a spectrophotometer (DeNovix, Wilmington, DE, USA). The desalted rPvTRAP were evaluated by SDS-PAGE for purity and the identity of the product expressed was confirmed by immunoblotting with an anti-His antibody (Penta His Antibody; Qiagen) and with *P. vivax*-infected human sera.

2.2 Circular Dichroism of rPvTRAP

Circular dichroism (CD) spectroscopy was used for estimating the secondary structures of the recombinant protein. CD spectra of purified and desalted rPvTRAP were recorded on a Jasco model J-810 spectropolarimeter at 250 µg/ml protein in 1 × PBS pH 7.2. All measurements were carried out in a 0.1-mm path length cylindrical cuvette at room temperature at 190-240 nm. The number of spectra was recorded at a scan speed of 200 nm/min with a step resolution of about 0.1 nm. We applied the crystal structure of *P. vivax* TRAP (Protein Data Bank [PDB] code 4HQO) to calculate the secondary structure of the protein based on experimental values for domains II and III. Since the data for domain IV did not exist in PDB, we used the predicted secondary structure information of PvTRAP by SOPMA server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) (Combet et al., 2000) as a standard protein in our study. Finally the two sets of data were used to calculate the overall secondary structure elements for the whole protein.

2.3. Animals

Inbred female C57BL/6 mice (6–8 weeks old) from the Laboratory Animal Science Department, Pasteur Institute of Iran, (Karaj, Iran), were used. Animals were overseen by a veterinarian and were housed under pathogen-free circumstances for 1 week prior to the experiment. The animal testing protocols were submitted and approved by the Animal Ethics Committee of Pasteur Institute of Iran.

2.4. Immunization of C57BL/6 mice with to rPvTRAP

To assess the immunologic activity of the rPvTRAP, sixty C57BL/6 mice were separated in four groups of fifteen animals each: the experimental groups was immunized on days 0, 14, and 28 subcutaneously at the base of tail with 5 µg at prime and 2.5 µg at boosts of rPvTRAP alone (group 1) or formulated in Freund's adjuvants (Sigma-Aldrich Co., St. Louis, MO, USA) (group 2) at a 1:1 proportion (Table 1). The control groups was immunized with PBS (group 3) or PBS formulated in the Freund's adjuvants (group 4) following similar immunization schedule (Table 1). The first vaccination dose was formulated in complete Freund adjuvant (CFA), whereas the other two doses were formulated in incomplete Freund adjuvant (IFA). Moreover, samples were collected from tail vein on days 0 (preimmune) and 38 (Table 1).

2.5. Immunofluorescence

The similarity of epitopes in the recombinant protein to its corresponding native form was checked by indirect immunofluorescence antibody test (IFAT). In this regard, the sera of immunized mice were used to recognize the *P. vivax* native TRAP on the sporozoite surface, following a standardized procedure (Andolina et al., 2015). Briefly, multi-spot slides were coated with 1×10^3 sporozoites, air-dried and then fixed in cold acetone for 10 min. The slides were blocked and then were rinsed with PBS. The spaces between the wells were dried and then the serial dilution of anti-rPvTRAP mice sera from 1:50 to 1:25600 in PBS were added to the duplicated wells and incubated for 60 min. After rinsing with PBS, the spaces between the wells were dried and reaction was developed with the FITC-labelled rabbit anti-mouse IgG (1:40) for 45 min. Again, after rinsing with PBS, each slide examined by a fluorescence microscope (Nikon E200, Tokyo, Japan). The sera gained from control groups were used as negative controls.

2.6. Enzyme immunoassay for total IgG

Immunized mouse sera were analyzed by an indirect ELISA for measurement of the specific IgG against rPvTRAP as earlier described (Nazeri et al., 2018). In brief, each well of microtiter plates (Jet Biofil, Guangzhou, China) were coated with 50 ng/well of affinity-purified rPvTRAP (based on checkerboard titrations), incubated at 4°C for 16 h and then rinsed in wash buffer (0.05% Tween 20 in PBS; PBS-T). Then, the plates incubated with blocking buffer (1% BSA in PBS) at room temperature for 120 min. After rinsing, serum samples were added to duplicate wells at 1:200 dilution in PBS-T containing 0.5% BSA. After 90 min of incubation at room temperature, unbound material was washed away, and enzyme-labeled secondary antibody (horseradish peroxidase-labeled anti-mouse immunoglobulin, 1:25,000 in PBS-T, Sigma-Aldrich Co., St. Louis, MO, USA) was added to each well. After 60 min of incubation at room temperature, excess labeled antibody was removed during rinsing and the reaction was developed by using the OPD substrate system (Sigma-Aldrich Co.). Plates were read at 490 nm

on an ELISA reader (Biotech, Winooski, VT, USA). The mean plus 3 standard deviations (SD) of optical density (OD) values for the pre-immune mouse sera ($n = 20$) was used to define cutoffs for positive and negative sera.

2.7. Enzyme immunoassay for IgG subclass antibodies

An ELISA to detect the IgG subclasses of the anti-rPvTRAP antibodies was performed. Plates were coated with antigen, blocked, and incubated with sera as in the ELISA for total IgG. After rinsing, the secondary antibodies that were specific to mouse IgG1, IgG2a, IgG2b, IgG2c and IgG3 antibodies (Sigma-Aldrich Co.) diluted 1:1000 and incubated at room temperature for 60 min. After rinsing, anti-goat IgG HRP (Sigma-Aldrich Co.) was added at a dilution of 1:10,000. After incubation at room temperature for 60 min, plates were rinsed, and developing enzyme reaction was done as mentioned above for IgG ELISA.

2.8. Avidity and titer measurements of IgG2a and IgG2c antibodies

The avidity of antibodies were assessed with urea elution-based ELISA as described by Hedman et al. (Hedman et al., 1989) with some modifications. An ELISA was done as described above for subclasses, but in the rinsing step before adding the secondary antibodies, one of the plates was rinsed three times with PBS-T, and the other plate incubated with the same serum was rinsed three times with dissociation buffer containing PBS-T-urea (8 M) as a chaotropic agent. Then, both plates were rinsed again one time with PBS-T buffer. In the end, following steps were performed as mentioned above for the subclasses ELISA. The avidity index (AI) was calculated as the ration of the OD value of urea-treated samples to that of the untreated samples, multiplied by 100. We interpreted AI as follows: < 30%, low avidity; 30%–50%, intermediate avidity; and > 50%, high avidity.

Antibody titration was done to assess the end-point titers of IgG2a and IgG2c antibodies by ELISA as described above for subclasses. The sera of immunized mice in each group were pooled and used in serial dilutions from 1:200 to 1:409,600. The last dilution of serum that had an OD_{490 nm} above the cut-off was measured as the titration endpoint of IgG2a and IgG2c antibodies.

2.9. Cellular proliferation assay

The induction of specific anti-rPvTRAP cellular responses was evaluated through splenocyte proliferation detection. In brief, on day 38 after the first immunization three mice from each group were sacrificed and the spleens were removed. Then, single-cell suspensions were prepared in RPMI 1640 medium (Gibco, Invitrogen, Scotland, UK) and red blood cells were eliminated with ammonium chloride-potassium lysis buffer (pH 7.2). Then, the cells washed twice and resuspended in RPMI 1640 medium, containing 5% fetal bovine serum, 2.3×10^{-2} mM 2-mercaptoethanol (2-ME), penicillin–streptomycin (100 U–100 μ g/ml), and 10 mM HEPES (Sigma-Aldrich Co.). In the next step, the splenocytes (2.5×10^5 cell/well) were cultured in a flat-bottom 96-well tissue culture plate (Jet Biofil) in triplicates and received 5 μ g/ml of rPvTRAP as stimulus, while 5 μ g/ml of concanavalin A (ConA,) was added to the positive controls and RPMI to the negative controls. In addition, the cultivation of splenocytes from non-immunized mice was used as a negative control. All plates were incubated in a 5% CO₂ at 37 °C

for 48 h. Subsequently, the supernatants were removed, and cell proliferation was measured by the MTT (3 (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In this assay, 100 μ l MTT (0.5 mg/ml, Sigma-Aldrich Co.) was added to each well, and the plates were further incubated at 37 °C for 4 h. Afterwards, the supernatant was removed, and the formazan crystals were dissolved in 0.04 N HCl in absolute isopropanol, and the OD was measured at 550 nm.

2.10. Cytokine production assay

The production of specific cytokines (IFN- γ and IL-4) in the supernatant of rPvTRAP stimulated splenocytes was measured using murine cytokine immunoassay kits (R&D system, Minne-apolis, USA). After optimization at 24 and 120 h, the supernatants of splenocyte cultures, stimulated with rPvTRAP antigen, were collected and stored at -80 °C until use to measure IL-4 and IFN- γ productions by ELISA. For the quantitative estimation of IFN- γ and IL-4, the frozen samples were taken out and analyzed using the standard curve. To assay cytokine release profiles in immunized mice at day 38, three mice from the treated and control groups were sacrificed, and the spleens were isolated. A single-cell suspension of splenocytes was seeded in complete RPMI 1640 medium, supplemented with 5% FCS, 2 mM glutamine, 2.3×10^{-2} mM 2-ME, 10 mM HEPES, and 100 U-100 μ g/ml of penicillin–streptomycin. The cells were incubated in flat-bottomed, 96-well microtitre plates (Jet Biofil) at a density of 2.5×10^5 cells/well in the presence of rPvTRAP (5 μ g/ml). The positive and negative controls were Con A (5 μ g/ml) and medium alone, respectively. All plates were incubated in the presence of 5% CO₂ at 37 °C, and culture supernatants were collected after 24 h for IL-4 and 120 h for IFN- γ . The concentration of cytokines was calculated based on the standard curves performed in parallel with known concentrations of recombinant mouse IL-4 and IFN- γ cytokines for each experiment. All tests were performed in duplicate, and the mean of concentration \pm SD was recorded for each set of samples.

2.11. Statistical analysis

A database was created with IBM SPSS 21.0 for Windows (Armonk, NY: IBM Corp, USA). Independent sample t-test was used to analyze between two groups on day 38. P values < 0.05 were considered statistically significant.

3. Results

3.1. Expression and confirmation of rPvTRAP

Transfection of pET23a-PvTRAP into *E. coli* Rosetta (DE3) competent bacteria resulted in production of the recombinant protein with the expected sizes of ~70 kDa as revealed by SDS-PAGE and the optimum conditions for rPvTRAP expression was gained and reported in the previous study [Data not shown]. The confirmation of rPvTRAP was verified by Western blot assay using anti-His antibody and *P. vivax*-infected human sera [Data not shown].

3.2. Secondary structure evaluation of rPvTRAP by CD

Circular dichroism (CD) spectra data produced the fractional composition of each secondary structure element using Yang database implemented in Jasco-810 instrument. The spectrum of rPvTRAP (Fig. 1) was recorded at pH 7.2 showed 14% α -Helix, 33.5% β -Sheet, 1.5% β -turn and 51% random coil (Table 2). The CD data were close to the calculated data for secondary structure based on the crystal structure of PvTRAP (PDB code: 4HQO) and SOPMA server (Table 2).

3.3. Native protein recognition by specific anti-rPvTRAP antibodies in IFAT

Mouse polyclonal anti-rPvTRAP antibody produced in mouse groups 1 and 2 (rPvTRAP and rPvTRAP + CFA/IFA, respectively) were able to recognize sporozoites in IFAT assays with high intensity (Fig. 2A and B, respectively). Moreover, the control mouse sera (PBS and CFA/IFA, respectively) did not recognize sporozoites (Fig. 2C and D, respectively).

3.4. Analysis of humoral immune response by ELISA

As shown in Figure 3, the mouse group induced with rPvTRAP + CFA/IFA showed higher and statistically significant specific IgG antibody ($OD_{490} \text{ nm}=2.4\pm 0.06$) compared with the immunized mice group received rPvTRAP alone ($OD_{490} \text{ nm}=1.39\pm 0.03$) on day 38 ($P < 0.05$, independent sample t-test). None of the control mice that were injected with PBS ($OD_{490} \text{ nm}=0.05\pm 0.003$) and CFA adjuvant ($OD_{490} \text{ nm}=0.07\pm 0.005$) showed detectable levels of specific antibodies (Fig. 3).

Analysis of specific subtype antibodies to rPvTRAP in immunized mouse groups that received rPvTRAP and rPvTRAP + CFA/IFA on day 38 exhibited that both IgG2a and IgG2c were detected (Fig. 3A and B). However, the level of anti-rPvTRAP IgG2c was significantly higher than IgG2a in the groups that received rPvTRAP alone (mean $OD_{490} = 0.798\pm 0.12$ and 0.39 ± 0.1 , respectively) and emulsified with CFA/IFA (mean $OD_{490} = 1.48\pm 0.07$ and 0.605 ± 0.13 , respectively) ($P < 0.05$, independent sample t-test, Fig. 3A and B). It is noted that there weren't significant differences between the mean level of anti-PvTRAP IgG1 (mean $OD_{490} = 0.41\pm 0.2$) and IgG3 (mean $OD_{490} = 0.262\pm 0.12$) with IgG2a (mean $OD_{490} = 0.39\pm 0.1$) in rPvTRAP mouse group as well as between the mean level of anti-PvTRAP IgG1 (mean $OD_{490} = 0.640\pm 0.05$) and IgG3 (mean $OD_{490} = 0.475\pm 0.08$) with IgG2a (mean $OD_{490} = 0.605\pm 0.13$) in rPvTRAP + CFA/IFA mouse group ($P > 0.05$, independent sample-t test Fig. 3A). However, there were significant differences between the mean level of anti-PvTRAP IgG1 (mean $OD_{490} = 0.41\pm 0.2$) and IgG3 (mean $OD_{490} = 0.262\pm 0.12$) with IgG2c (mean $OD_{490} = 0.798\pm 0.12$) in rPvTRAP mouse group as well as between the mean level of anti-PvTRAP IgG1 (mean $OD_{490} = 0.640\pm 0.05$) and IgG3 (mean $OD_{490} = 0.475\pm 0.08$) with IgG2c (mean $OD_{490} = 1.48\pm 0.07$) in rPvTRAP + CFA/IFA mouse group ($P < 0.05$, independent sample-t test Fig. 3B). Besides, more analysis of specific IgG subtypes exhibited that the rPvTRAP alone and rPvTRAP + CFA/IFA mouse groups, presented the pretty lower IgG1/IgG2c (0.51 and 0.43, respectively) ratio (Th1 response) than IgG1/IgG2a (1.05 and 1.06, respectively) ratio (a mixture of Th1 and Th2 response).

3.5. Determination of avidity and titer

The immunized mice with rPvTRAP and rPvTRAP + CFA/IFA had an intermediate-avidity IgG2a antibody but high-avidity IgG2c antibody (Table 3). Moreover, the mean of serum

antibody titers exhibited that in both rPvTRAP (Fig. 4A) and rPvTRAP + CFA/IFA (Fig. 4B) mouse groups, the pretty lower IgG2a end-point titer (1:3200 and 1:25600, respectively) than IgG2c (1:25600 and 1:102400, respectively).

3.6. Evaluation of cell-mediated immune response

Splenocytes from immunized and non-immunized mice were stimulated, in vitro, with purified rPvTRAP. As expected, ConA-stimulated controls showed high levels of splenocyte proliferation (mean OD550nm 1.32–1.38) in all mouse groups, whereas the RPMI-stimulated controls showed low levels (mean OD550 nm 0.42–0.49). Furthermore, the proliferation of splenocytes among the immunized mice with rPvTRAP/CFA (mean OD550 nm 1.12) was significantly different from the mouse group that received rPvTRAP alone (mean OD550 nm 0.82) ($P < 0.05$, independent sample t test). Moreover both groups showed significantly higher levels of splenocyte proliferation in comparison to negative controls ($P < 0.05$, independent sample t test).

In this investigation, cytokine production in the mice immunized with rPvTRAP antigen were determined in vitro. The results revealed the eliciting significant levels of IFN- γ (as Th1 response) in the mouse groups that received rPvTRAP alone and emulsified with CFA/IFA as compared to the mouse control groups on day 38 after first immunization ($P < 0.05$, independent sample t test, Fig. 5A). Concerning the levels of IL-4 secretions (as Th2 response), there was no significant difference in IL-4 production in different groups as compared with that in the control groups immunized with PBS and CFA/IFA ($P > 0.05$, independent sample t test, Fig. 5B).

4. Discussion

The clones of murine CD₄ T cells can be classified into two subsets that are distinguished by the cytokines they produce upon stimulation with antigen, and the classes/subclasses of antibodies whose production they support (Mosmann et al., 1986; Mosmann et al., 1989). Cells of the Th1 subset can enhance the production of murine IgG2a or IgG2c antibodies. The delivery of IL-4 to B cells by Th2 cells can enhance their production of IgG1 antibody (Mosmann et al., 1989; Bretscher et al., 1992). In mice, high level of IL-4 and IgG1 responses (higher IgG1/IgG2a or IgG1/IgG2c ratios) are usually associated with Th2 responses, whereas high levels of IFN- γ and IgG2a or IgG2c (lower IgG1/IgG2a or IgG1/IgG2c ratios) are thought to reflect Th1 responses (Ahlborg et al., 2000). It is worthy of notice that subtype antibodies in mice with various genetic background are different. Hence, due to the modulatory effects of mouse strain on the Th1/Th2 balance of immune responses, it is essential that either IgG2a or IgG2c subtype antibody along with cytokines are evaluated in order to correct interpretation of Th1/Th2 immune responses in subunit vaccine development.

It is clear that in the subunit vaccine development, the correct folding of the recombinant protein must be considered since antibody recognition could rely on conformational epitopes. In this regard in our previous study, the results of reduced and non-reduced SDS-PAGE and Western blot analysis (Nazeri et al., 2017) partly confirmed that the bacterial rPvTRAP has been correctly folded. Moreover in the current study, CD of rPvTRAP exhibited to some extent ordered secondary structure of the recombinant purified and desalted PvTRAP as well as IFAT results revealed that anti-rPvTRAP antibodies produced in mice recognized the native protein expressed on the surface of *P. vivax* sporozoite that shows the presence of common epitopes in

recombinant and native forms of PvTRAP antigen and partly confirmed the correct structural of rPvTRAP.

After confirming the proper protein folding, the analysis of IFN- γ and IL-4 levels, specific IgG subtypes distribution frequencies of anti-rPvTRAP antibodies and IgG1/IgG2a and IgG1/IgG2c ratios in immunized C57BL/6 mice with rPvTRAP or rPvTRAP + CFA/IFA showed that the measurement of IgG2c subclass antibody along with IFN- γ compared to IgG2a was more appropriate and accurate for evaluating Th1 immune response. Since, there is a link between IFN- γ and IgG2a or IgG2c production, our observation showed high level of IgG2c than IgG2a antibody in different mouse groups that had a high detectable IFN- γ . Our results were in consistent with some studies (Martin et al., 1998; Petrushina et al., 2003; Petritus and Burns, 2008; Fiorino et al., 2017) that showed subtype response in C57BL/6 is IgG2c biased. As a result, in the advance of clinical trials, the measuring of an appropriate subtype antibody along with cytokine with respect to mouse strain is very important in the determining of Th1/Th2 polarization against a subunit vaccine candidate. However, there are conflicting data that have measured IgG2a (Petritus and Burns, 2008; Fiorino et al., 2017; Vasconcelos et al., 2004; Echeverria et al., 2006, Patricia et al., 2006, Laguía-Becher et al., 2010, Lucca et al., 2014) in C57BL/6 mice that maybe result in misleading from correct immune response against a subunit vaccine candidate and more studies are required.

The evaluation of the avidity of antibody is one of the important factors that should involve in prevention and protection from infections (Ferreira et al., 1996). Our results showed that the immunized mice with rPvTRAP and rPvTRAP + CFA/IFA had an intermediate-avidity IgG2a antibody and high-avidity IgG2c antibody in the same C57BL/6 mice. Thus evaluating IgG2a avidity in C57BL/6 mice probably end in incorrect interpretation of being convenient vaccine candidate and consequently informing about protection from infection. Moreover, the mean of serum antibody titers were evaluated and the results exhibited that in both rPvTRAP and rPvTRAP + CFA/IFA mouse groups, IgG2a end-point titer was significantly lower than IgG2c. This result showed that the measuring of IgG2a end-point titer in the C57BL/6 mice partly result in to misinterpretation of the potency of sporozoite blocking antibodies to hepatocytes.

In general, our results showed that for correctly interpreting of Th1 immune response, prevention and protection from infections and potency of blocking antibodies in the preclinical phase of a subunit vaccine development in C57BL/6 mouse strain it is critical to measure IgG2c instead of IgG2a along with IFN- γ .

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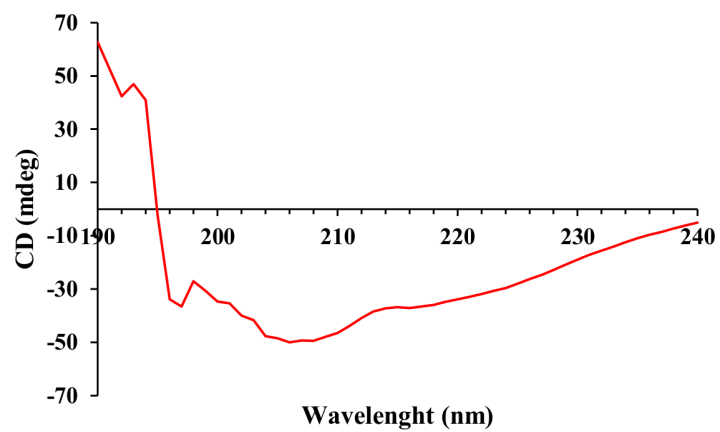


Fig. 1. The CD spectrum of recombinant PvTRAP.

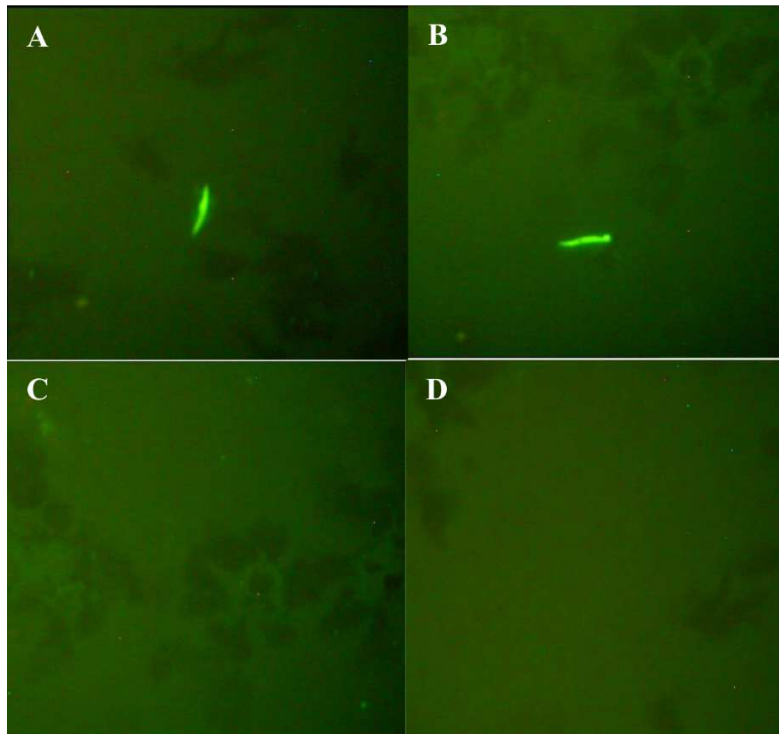


Fig. 2. Photographs of IFAT.

The upper pictures show the recognition of native form of PvTRAP in the surface of *P. vivax* porozoite using polyclonal antibodies (1:100) produced in mice against rPvTRAP (A) and rPvTRAP CFA (B). None of the control mice sera means CFA (C) and PBS (D), recognized the native protein on *P. vivax* parasite thus ensuring that those obtained responses are specific to rPvTRAP.

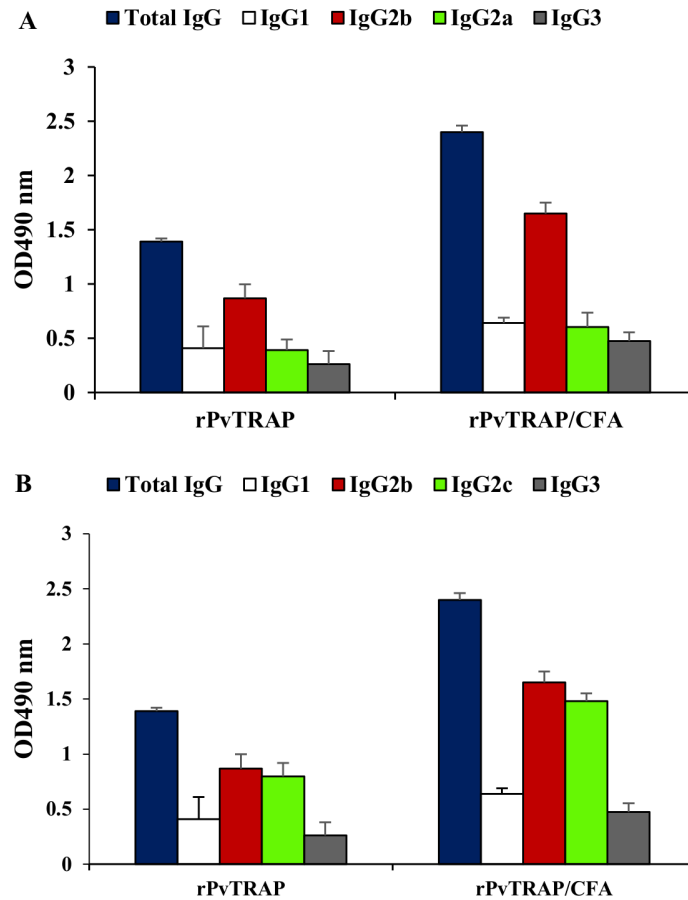


Fig. 3. Antibody responses of immunized mice with rPvTRAP alone or in combination with CFA.

(A) Assessment of the total IgG, IgG1, IgG2b, IgG2a and IgG3 antibodies to rPvTRAP in the sera of immunized mice on day 38 of the first immunization. (B) Assessment of the total IgG, IgG1, IgG2b, IgG2c and IgG3 antibodies to rPvTRAP in the sera of immunized mice on day 38 of the first immunization. The bars and error bars show the mean OD₄₉₀ nm and standard deviation (SD) in each mouse group (n = 15), respectively. The ELISA cut-offs were calculated as the mean OD₄₉₀ nm of pre-immune mice sera (as negative controls, n = 20) plus 3SD. The cut-offs for IgG, IgG1, IgG2a, IgG2b, IgG2c and IgG3 were OD₄₉₀ nm = 0.161, 0.070, 0.077, 0.091, 0.082 and 0.059 respectively.

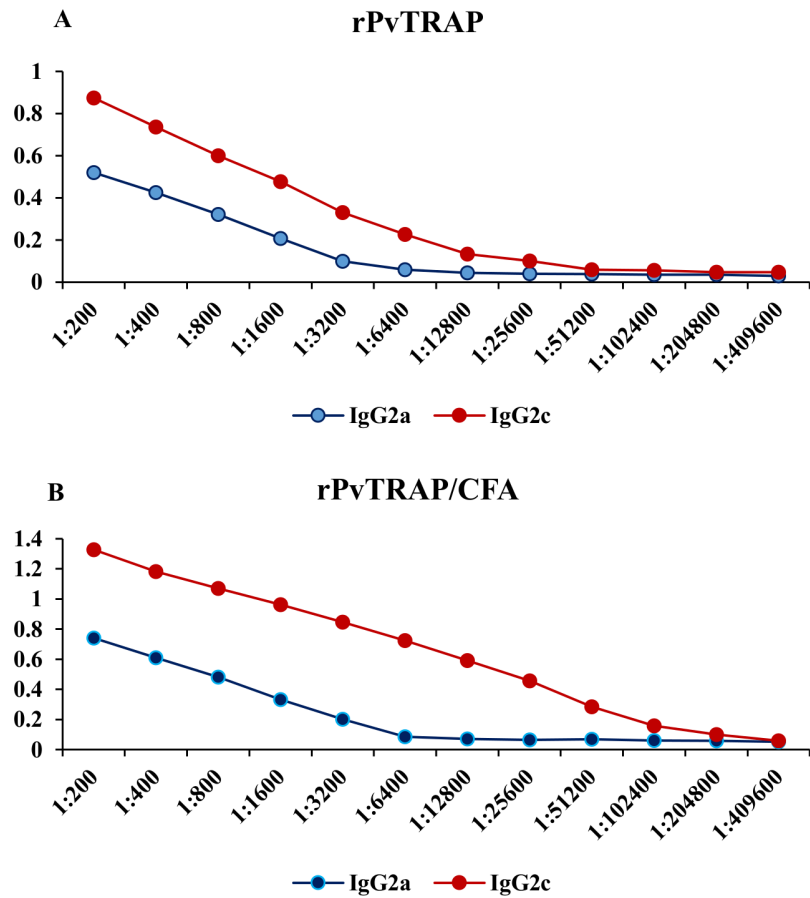


Fig. 4. Titration evaluation of IgG2a and IgG2c antibodies in immunized mice with rPvTRAP.

Significant difference was observed in the endpoint titres of IgG2a and IgG2c antibodies among mouse groups immunized with either rPvTRAP (A) or rPvTRAP/CFA (B) on day 38 after the first immunization. The cut-offs for IgG2a and IgG2c were 0.077 and 0.082, respectively.

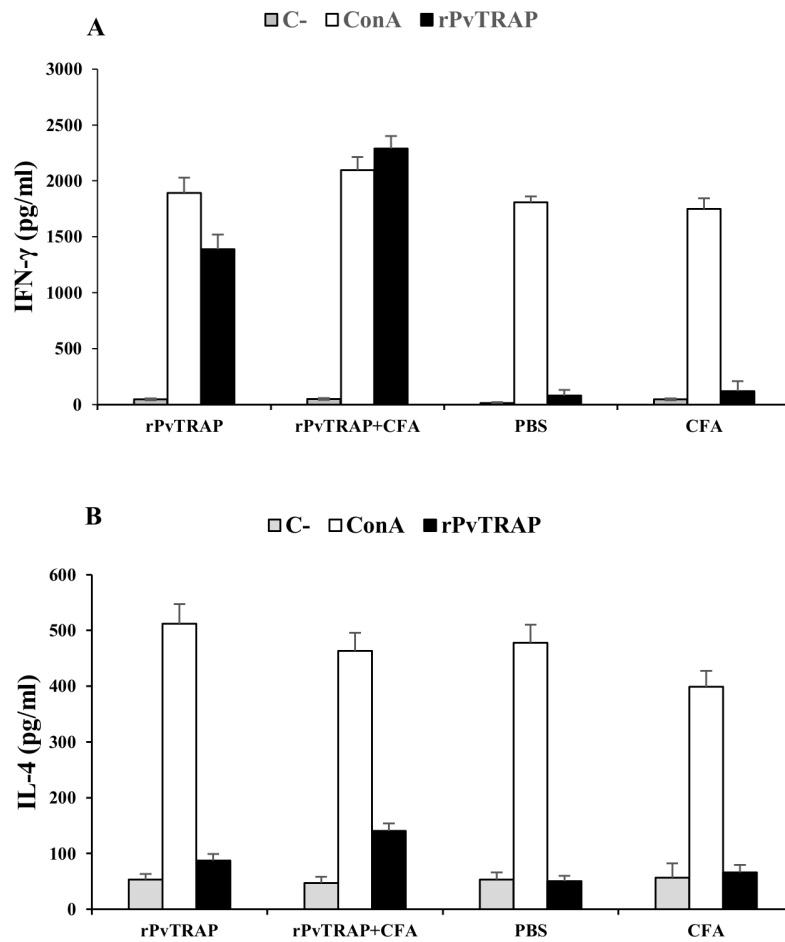


Fig. 5. Evaluation of the IFN- γ (A) and IL4 (B) in mice immunized with rPvTRAP alone or emulsified in CFA/IFA. The bars show the mean concentration of elicited IFN- γ and IL-4 from pooled lymphocytes of immunized mice ($n = 3$) in each group, and error bars indicate SD. IFN- γ responses of ConA as the positive control and no antigen as the negative control were in the range of 1822–2154 and 15–67 pg/ml, respectively, among different examined groups. IL-4 responses of ConA as the positive control and no antigens as the negative control were in the range of 1879–2087 and 14–18 pg/ml, respectively, among different examined groups.

Table 1

Mice immunization approach and bleeding.

| Group | Prime (day 0) | | Boost 1 (day 14) | | Boost 2 (day 28) | | Bleeding (day 38) |
|--------|---------------|----------|------------------|----------|------------------|----------|----------------------|
| | Antigen | Adjuvant | Antigen | Adjuvant | Antigen | Adjuvant | |
| 1 | rPvTRAP | - | rPvTRAP | - | rPvTRAP | - | |
| 2 | rPvTRAP | CFA | rPvTRAP | IFA | rPvTRAP | IFA | |
| 3 (NC) | - | PBS | - | PBS | - | PBS | |
| 4 (NC) | - | CFA | - | IFA | - | IFA | |

CFA: Complete Freund's adjuvant, **IFA:** Incomplete Freund's adjuvant, **NC:** negative control,Mice received 200 μ l rPvTRAP (5 μ g at prime and 2.5 μ g at boost) alone and in the presence of CFA/IFA adjuvant subcutaneously at the base of tail. Control mice received PBS or CFA/IFA.

Table 2

The evaluation of rPvTRAP folding via CD, PDB/SOPMA Server and SOPMA alone.

| | CD results | PDB (4HQO) and SOPMA server results | SOPMA server results |
|----------------------------------|-------------------|--|-----------------------------|
| α Helix | 14% | 19.18% | 31.11% |
| β sheet | 33.5% | 18.33% | 8.56% |
| β turn | 1.5% | 10.34% | 4.80% |
| Random coil | 51% | 52.15% | 55.53% |
| Total | 100% | 100% | 100% |

CD: Circular dichroism

PDB: Protein data bank

Table 3

Avidity analysis of IgG2a and IgG2c antibodies to rPvTRAP.

| Group | Avidity index % \pm SD | |
|--------------------------|--------------------------|------------------|
| | IgG2a | IgG2c |
| | Day 38 | Day 38 |
| 1-rPvTRAP | 30.66 \pm 0.28 | 51.07 \pm 0.18 |
| 2-rPvTRAP+CFA/IFA | 43.46 \pm 0.24 | 93.72 \pm 0.25 |

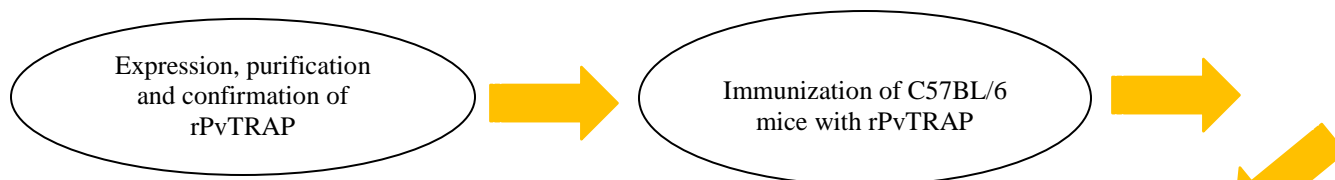
SD: Standard Deviation; **CFA:** complete Freund's adjuvant; **IFA:** incomplete Freund's adjuvant. An Avidity index (AI) less than 30 % corresponds to low-avidity antibodies, AI between 30 % and 50 % to an intermediate avidity and AI greater than 50 % to high avidity.

Measuring of IgG2c isotype instead of IgG2a in immunized C57BL/6 mice with *Plasmodium vivax* TRAP as a subunit vaccine candidate in order to correct interpretation of Th1 versus Th2 immune response

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Aim of Study

The main goal of this study was to measuring and comparing the profile of IgG isotype responses in C57BL/6 mice to recombinant PvTRAP as a subunit vaccine candidate.



1- The level of anti-rPvTRAP IgG2c was significantly higher than IgG2a in the groups that received rPvTRAP alone and emulsified with CFA/IFA ($P < 0.05$, independent sample t-test)

2- The immunized mice with rPvTRAP and rPvTRAP + CFA/IFA had an intermediate-avidity IgG2a antibody but high-avidity IgG2c antibody.

3- The mean of serum antibody titers exhibited that in both rPvTRAP and rPvTRAP + CFA/IFA mouse groups, the pretty lower IgG2a end-point titer (1:3200 and 1:25600, respectively) than IgG2c (1:25600 and 1:102400, respectively).

4- The results revealed the eliciting significant levels of $\text{IFN-}\gamma$ ($P < 0.05$, independent sample t test) and no detectable level of IL-4 in the mouse groups received rPvTRAP alone and emulsified with CFA/IFA as compared to the mouse control groups.

In general, our results showed that for correctly interpreting of Th1 immune response, prevention and protection from infections and potency of blocking antibodies in the preclinical phase of a subunit vaccine development in C57BL/6 mouse strain it is critical to measure IgG2c instead of IgG2a along with $\text{IFN-}\gamma$.

Humoral immune response analysis by ELISA against rPvTRAP

