Genetic Diversity and Immunogenicity Analysis of 6-Cysteine Protein Family Members in *Plasmodium ovale curtisi* Imported from Africa to China: P12, P38, and P41

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Abstract

Background: The s48/45 six-cysteine proteins exist throughout the *Plasmodium* life cycle and play important roles in the interaction between *Plasmodium* parasites and their hosts. P12, P38, and P41, which containing specific s48/45 domains, have recently attracted widespread attention due to their unique molecular structure. In this study, we aimed to characterize the genetic diversity and immunogenicity of P12, P38, and P41 in order to evaluate their potential protective efficacy against malaria in mice.

Methods: Blood samples were collected from 37 patients infected with *P. ovale curtisi* and genomic DNA was extracted for sequencing and protein expression. After expression and purification of the recombinant PocP12, PocP38, and PocP41 proteins, they were used for the assessment of immune responses and antigen-specific T-cell responses in BALB/c mice.

Results: The sequences of *pocp12*, *pocp38*, and *pocp41* were found to be highly conserved. The recombinant PocP12, PocP38, and PocP41 proteins were expressed successfully with high purity. Antibodies against these proteins in mice were first detected two weeks after the primary immunization, and the levels increased over the entire immunization period. When immunized with recombinant PocP38, the mice had higher levels of IFN-γ in $CD4^+$ and $CD8^+$ T cells, and even showed effective lymphoproliferation.

Conclusions: Low genetic polymorphism of *pocp12*, *pocp38*, and *pocp41,* and

appropriate immunoreactivity in mice support their efficient immune defence against malaria.

Keywords: Malaria, *Plasmodium*, immune response, polymorphism, six-cysteine

1. Introduction

Malaria is one of the most harmful and deadliest infectious diseases caused by the *Plasmodium* parasite worldwide, which severely threatens human health. In 2018, malaria had caused 405,000 deaths out of 228 million cases of malaria (WHO, 2019). Five *Plasmodium* species (*Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae*, and *Plasmodium knowlesi*) are known to cause malaria in humans. Among these five causative agents, *Plasmodium falciparum* is the most widely distributed human malaria parasite in Africa, which accounting for more than 99%, and the one that leads to the malaria morbidity and mortality. (WHO, 2019) [1]. *Plasmodium ovale* was firstly described by Stevens in 1922 according to its Typical oval morphology in infected red blood cells (Stevens JWW, 1922). Due to its low parasitemia and low prevalence in limited areas, *P. ovale* has been given relatively little attention compared with the other species (Roucher C et al., 2014; Mueller I et al. 2007). But what is noticeable is that in some sub-Saharan Africa countries such as The Republic of Angola, The Republic of Equatorial Guinea, Democratic Republic of the Congo and so on, *Plasmodium ovale* remains a non-negligible threat as many Chinese workers returning from these countries are infected with ovale malaria (Rui-min Z et al., 2018). It is not hard to see although the prevalence of *Plasmodium ovale* is generally less than *Plasmodium falciparum* and *Plasmodium vivax*, it is still an important cause of the disease, with subsequent economic loss. Despite the several international control strategies and powerful policies conducted in the past, only limited success has been achieved till date, and malaria remains a major factor in harming human health (Baird JK, 2000; Dixit A, 2016). Although no malaria case due to *P. ovale* was reported in China in 2017 (Li Z et al., 2018), the increasing number of imported *P. ovale* cases,

often misdiagnosed as *P. falciparum* or *P. vivax*, has been demanding more attention recently (Zhongjie L et al., 2016; Jun F et al., 2017; Chavatte JM et al., 2015). Furthermore, there is no effective means yet to completely block the transmission and development of malaria, owing to the complex life cycle of malarial parasites, diversity of their invasion pathways, and rapid evolution of new antigenic variants.

Proteins secreted by the parasite play important roles when the latter invades the host cells (Cowman AF et al., 2016). Proteins containing the s48/45 domains have recently attracted widespread attention due to their unique molecular structure. This domain exhibits an approximate β-sandwich structure, and generally contains six positionally conserved cysteines (6-cys), due to which they have been classified as 6-cys protein family (Arredondo SA et al., 2016). This family has 14 members that occur during the life cycle of the malarial parasite *P. falciparum*, and eight of them have glycosylphosphatidylinositol (GPI) anchors or predicted GPI that anchors them to the plasma membrane (Arredondo SA et al., 2016). Members lacking GPI anchors may play an indirect role by interacting with other membrane proteins to maintain contact with the surface of the parasite (Taechalertpaisarn T et al., 2012).

Currently, the pathogens causing malaria are blood-stage parasites since, in the asexual stage, they can cause not only serious anemia but also splenomegaly in infected patients. Therefore, more focus should be placed on blood-stage parasite antigens, which may have an important role in the process of invading erythrocytes (Richards JS et al., 2009; Zhang X et al., 2019; Van Schaijk BC et al., 2008). In asexual erythrocytic stages, P12, which is highly conserved and could be strongly recognized by immune sera from infected patients, has been shown to be a GPI-anchored protein localizing on merozoite surface (Gilson PR, et al.,2006; Tonkin ML, et al.,2013). P41, which lacks a GPI-anchor, is effective indirectly in the process of invading erythrocytes by interacting with P12 to form a stable heterodimer (Crosnier C et al., 2010). In *P. vivax*, P41 is also shown to be strongly recognized by individual sera (Cheng Y et al., 2013). In *P. falciparum*, P38 is shown to have a 20–33% inhibition of erythrocyte invasion in vitro (García J et al., 2009). Moreover, the immune response to Pf38 is comparable to that to

AMA1 and $MSP1_{19}$, two protein antigens recognized to have satisfactory immunogenicity, in patients with *P. falciparum*-infection (Feller T et al., 2013).

For *Plasmodium* infection, the immune response to malaria parasite is mainly based on cellular immunity against individual malaria antigen. IFN-γ produced by T cells has been reported to be associated with protection against malaria infection and CD4+ T cells were the main source of IFN-γ (King T et al., 2015). Moreover, CD8+ T cells are also critical mediators of protection against *Plasmodium* infection just like previous study has shown that no obvious protection of *Plasmodium* vaccine was observed on account of the poor CD8+ T-cell responses induced (Doll KL et al., 2013). Here, we focused on verifying the function of P12, P38, and P41 in *P. ovale* by characterizing their genetic diversity and immunogenicity. Since *P. ovale curtisi* and *P. ovale wallikeri* are two closely related, yet distinct, subspecies of *ovale* parasites, assessment of *P. ovale wallikeri* will also be conducted if the assessment immunizing protection of *P. ovale curtisi* is found advantageous.

In this study, cloning and expression of three members of the 6-cys protein family in *P. ovale curtisi* (PocP12, PocP38, and PocP41) were demonstrated, and their immunogenicity analyzed in BALB/c mice. We found that *pocp12*, *pocp38*, and *pocp41* were highly conserved in each isolate and the recombinant PocP12, PocP38, and PocP41 (rPocP12, rPocP38, and rPocP41) could induce strong immune response in mice. Considering that many members of 6-cys protein family have been shown to play important roles in inhibiting the progress of parasites through their life cycle (Taechalertpaisarn T, et al., 2012; Van Schaijk BC, et al., 2008), the function of PocP12, PocP38, and PocP41 in merozoite invasion will be worth studying in future.

2. Materials and methods

2.1. Study areas and sample collection

Samples of *P. ovale curtisi* were obtained from patients with fever, who worked in

tropical malaria-prevalent sub-Saharan Africa, and were admitted in local hospitals in Jiangsu Province of China between 2012 and 2016. A total of 37 *P. ovale*-infected blood samples were collected, isolates were confirmed by polymerase chain reaction (PCR), and parasite species were distinguished with real-time TaqMan PCR (Cao Y et al., 2016). The exact origin of these isolates is provided in Supplementary Table S1.

2.2. Nested PCR for identification and microscopic examination of Plasmodium species

Nested PCR, based on the SSU rRNA genes, was conducted as previously described (Snounou G et al., 1993). The first round of DNA amplification primers, rFAL1-Forward (5′-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3′) and rFAL2-Reverse (5′-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3′), were used for *P. falciparum*, and rOVA3-Forward (5′-CGG GGA AAT TTC TTA GAT TGC-3′) and rOVA4-Reverse (5′-GAG AAA CAG CAT GAA TTG CG-3′) were used for *P. ovale*. In the second round of DNA amplification, primers P1-Forward (5′-ACG ATC AGA TAC CGT CGT AAT CTT-3′) and Pf-Reverse (5′-CAA TCT AAA AGT CAC CTC GAA AGA TG-3′) were used for *P. falciparum*, and P1-Forward (5′- ACG ATC AGA TAC CGT CGT AAT CTT -3′) and Po-Reverse (5′-ACT GAA GGA AGC AAT CTA AGA AAT TT-3′) were used for *P. ovale*. The 20-µL PCR mixture contained 10 µL premix (2× Phanta® Max Master Mix, Vazyme, China), 1 µL genomic DNA solution, 0.8 µL of each primer, and 7.4 µL double-distilled water. The PCR program involved: 95 ºC for 3 min, followed by 35 cycles of 95 ºC for 15 s, 55 ºC for 30 s, and 72 ºC for 30 s, and a final extension at 72 ºC for 5 min. PCR products were run on 2.5% agarose gels. The thin smears for malaria, which collected from Jiangsu Institute of Parasitic Diseases, were observed under an inverted microscope (Nikon, China) at a thousand-time magnification, and the image was edited with Adobe Photoshop CC 2015.

2.3. PCR amplification and sequencing of pocp12, pocp38, and pocp41

Genomic DNA extracted from *P. ovale*-infected patients was used as templates for PCR. The primers *pocp12*-Forward (5′-ATG GTA GGG GCT AAA GGA GTG-3′) and *pocp12*-Reverse (5′-CAA TAT GGT GAA AAA AAC TGA GGA C-3′), *pocp38*-Forward (5′-ATG TTT CGC GTT AGA AAT GCA A-3′) and *pocp38*-Reverse (5′-ATT GAA AGC AAA AAA TGA GAG AAA A-3′), and *pocp41*-Forward (5′-ATG TTT TTT TTT CTT ATC CTT TTA-3′) and *pocp41*-Reverse (5′-TTC TGG AAA CGA TTT TGC AAT TGT C-3′) were designed based on the reference gene sequences *pocp12* (PlasmoDB, PocGH01_11044200), *pocp38* (PlasmoDB, PocGH01_10033500), and *pocp41* (PlasmoDB, PocGH01_03012400) obtained from *Plasmodium* Genome Resource database. The 20-μL reaction system included 1 μL genomic DNA, 0.8 μL of each primer, 7.4 μL double-distilled water, 0.5 units DNA polymerase, and 10 μL of premix (2× Phanta® Max Master Mix, Vazyme, China). PCR program included: denaturation at 95 ºC for 3 min; 35 cycles of 95 ºC for 15 s, 50 ºC for 30 s, and 72 ºC for 30 s; and a final extension at 72 ºC for 5 min. Amplified fragments were sequenced by GENEWIZ (Suzhou, China).

2.4. Sequence alignment

The primary structure of PocP12, PocP38, and PocP41 proteins was predicted using bioinformatics tools (http://smart.embl-heidelberg.de/ and http://mendel.imp.ac.at/gpi/gpi_server.html). The sequences of *pocp12*, *pocp38*, and *pocp41* were used as templates and GeneDoc v.2.7.0. was used to evaluate genetic polymorphism. Phylogenetic trees of p12, p38, and p41 were constructed using the neighbor-joining method, as previously described (Chu R et al., 2018).

2.5. Recombinant protein expression, purification, and western blot analysis

The *pocp12*, *pocp38,* and *pocp41* genes were cloned into pET30a expression vector, which contains six-histidine tags at both the N- and C-terminal ends, and expressed using *E. coli* expression system. Proteins were purified by YouLong Biotech

(Shanghai, China). Protein products were verified by 10% (SDS-PAGE) using Coomassie blue staining and western blot analysis under both reducing and nonreducing conditions. To determine the recombinant proteins, specificity of mouse anti-serum, and immunogenic proportion, anti-His antibody (Southern Biotech, Tuscaloosa, USA) anti-rPocP12, rPocP38, and rPocP41 mouse immune sera were used, respectively, as primary antibodies for western blot analysis. Results were visualized using a chemiluminescence detection assay (ECL, New Cell & Molecular Biotech, China), and images were merged using ImageJ software.

2.6. Mouse immunizations

Each group of five female BALB/c mice at 6–8 weeks of age were immunized with 50 µg of rPocP12, rPocP38, and rPocP41 proteins with complete Freund's adjuvant (CFA; Sigma, San Francisco, USA), via intraperitoneal route of administration, for the first time. Equal volume of antigen with incomplete Freund's adjuvant (IFA; Sigma, San Francisco, USA) was injected twice, after three and six weeks. Control mice received an equal amount of phosphate buffered saline (PBS) and indicated adjuvant. Mouse blood samples were collected once a week after each immunization.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well plates were coated overnight with 50 ng proteins in 100 µL coating buffer (15 mM Na_2CO_3, 35 mM NaHCO3 in 1,000 ml ultrapure water) at 4 °C. The antigen solution was removed and plates were blocked with TBST (TBS containing 0.1% Tween 20) containing 5% skim milk at room temperature for 2 h. Next, 100 µL of serum samples, diluted at different gradients, were added to the plates and incubated at room temperature for another 2 h. Thereafter, 100 µL of horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Southern Biotech, Tuscaloosa, USA) were added to the plate at 1:5,000 dilution in TBS, containing 0.1% bovine serum albumin (BSA;

Beyotime Biotechnology, China) and 0.05% Tween20, and incubated at room temperature for 1 h and 30 min. Plates were washed thrice with TBST for each step. Bound antibodies were detected by adding 100 μL of 3, 3′, 5, 5′-tetramethylbenzidine (Invitrogen, Waltham, USA) as the substrate solution at room temperature for 2 min, followed by 50 μL of 2 M H_2SO_4 to stop the reaction. The plates were read by absorbance at 450 nm.

The avidity of anti-rPocP12, rPocP38, and rPocP41 IgG antibodies was evaluated as previously described (Mehrizi AA et al., 2018). ELISA was performed in duplicate plates. After 90-min incubation of sera, one of the plates was washed thrice with TBST, and the other with TBST containing 6 M urea. Thereafter, both the plates were washed once with TBST. The other steps were performed as described above. Avidity index (AI) was calculated as follows:

AI = (OD_{450} of a sample treated with 6 M urea / OD_{450} of a sample not treated with 6 M urea) × 100%.

2.8. Ex-vivo stimulation and analysis of cytokine production by flow cytometry

Ex-vivo stimulated T cells, derived from splenocytes, were analyzed by flow cytometry. Approximately 2 weeks after the last immunization, spleens were harvested and single cell suspensions were prepared. RBCs were lysed with erythrocyte lysate, and the membrane debris was removed by filtering the cell suspension through a sterile filter. The collected cells were diluted to a concentration of 5×10^5/mL in complete Roswell Park Memorial Institute (RPMI) 1640 (Biosharp, China) medium and 100 μL of the cell suspension were dispensed into 96-well plate in duplicate. Spleen lymphocytes were stimulated with 10 μL protein antigens per well for 72 h, followed by the addition of 10 μL Cell Counting kit-8 (CCK-8, Biotime, China) for 4 h before detection, and finally, measurement of OD at 450 nm. One-milliliter of the cell suspension was dispensed into the 24-well plate in duplicate. Cells were stimulated with 5 μg/mL rPocP12, rPocP38, and rPocP41 for 24 h in presence of phorbol 12-myristate

13-acetate (50 ng/mL PMA; sigma, USA), ionomycin (1 µg/mL; Solarbio, China), and brefeldin A (5 µg/mL; Solarbio, China) For cytokine measurement, cells were incubated with APC-anti-mouse CD4 (BioLegend, China) and FITC-anti-mouse CD8 (BioLegend, China) for the cell surface stain; after fixation and permeabilization (BioLegend, China), cytokin IFN- γ was stained with PE-anti-mouse IFN- γ (BioLegend, China). Data were acquired using BD Accuri C6 Flow Cytometer (Shanghai, China).

2.9. Statistical analysis

Statistical analysis and graph preparation were performed using GraphPad Prism software v.5.0 (GraphPad Software Inc., San Diego, USA). To analyze antibody responses, data were compared using a two-tailed, unpaired Student's *t* test, with probability (*p*) values < 0.05 representing statistically significant differences. Phylogenetic trees for P12, P38, and P41 were estimated with neighbor-joining method based on the nucleotide sequences. Evolutionary relationships of the aligned sequences were determined using MEGA v.7.0. The three-dimensional structure of P12, P38, and P41 were constructed with SWISS-MODEL tools (https://swissmodel.expasy.org/) and edited with PyMol stereo 3D Zalman v.1.8.

3. Results

3.1. Characterization of PocP12, PocP38, and PocP41

Six samples were selected randomly for nested PCR and only the primer pair rOVA3-F/rOVA4-R amplified a 456-bp sequence of *P. ovale* while the primers rFAL1-F/rFAL-R failed to amplify the 206-bp sequence of *P. falciparum*. The PCR results confirmed that our collected samples were positive for *P. ovale* (Fig. 1A). Furthermore, result from the thin smear also reflected the existence of *P. ovale* (Fig. 1B).

PocP12, which contains two s48/45 domains (amino acids 23–139 and 171–284), was found to begin with the N-terminal secretion signal peptide sequence and end with C-terminal GPI-anchor signal sequence (Fig. 2A). An N-terminal signal peptide and a C-terminal GPI-anchor were predicted in PocP38, which also contains one s48/45 domain (amino acid 153–284) (Fig. 2B). The predicted primary structure of PocP41 contained a coiled-coil domain and two s48/45 domains (amino acids 16–115 and 238–349), but no GPI-anchor (Fig. 2C).

Three-dimensional (3D) models of P12, P38, and P41 in *P. falciparum* and *P. ovale* were constructed to verify their similarity. Results showed the structures of P12, P38, and P41 in *P. falciparum* and *P. ovale* to have high similarity with a large number of overlapping areas upon merging (Supplemental: Figure S1). Furthermore, the similarity comparison based on the amino acid between *P. falciparum* and *P. ovale* is also shown in Supplementary Figure S2.

3.2. Phylogenetic analysis

Phylogenetic analysis of *pocp12*, *pocp38*, and *pocp41*, from human, nonhuman primate, murine, and avian malarial species, was performed using the neighbor-joining method. Analysis showed that *pocp12* and *powp12* have extremely high consistency of 99%, and that of *p41* in *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* was the same as that of *p12* (Fig. 2D, 2E). Interestingly, the consistency of *pocp38* and *powp38* reached 100% (Fig. 2F). The *pocp12*, *pocp38*, and *pocp41* gene ID numbers of other *Plasmodium* species included in the analyses are provided in Additional file: (Supplemental: Table S2.).

3.3. Gene analysis of pocp12, pocp38, and pocp41

In order to verify the predicted gene structure of these proteins, genomic DNA from patients infected with *P. ovale* was prepared and used as the PCR template. On the

basis of the parasitemia of each isolate and primer specificity, we successfully amplified 35 *pocp12*, 24 *pocp38*, and 19 *pocp41* gene samples by PCR and obtained the correct size (approximately 1 kb, 1 kb, and 1.1 kb respectively). No superimposed signal was displayed on the electropherograms for *pocp12, pocp38*, and *pocp41* (Fig. 3A). In general, full-length alignments of *pocp12, pocp38*, and *pocp41* showed the occurrence of only one amino acid mutation across all the *pocp12, pocp38*, and *pocp41* samples. Results suggested that *pocp12, pocp38*, and *pocp41* genes are highly conserved in each isolate.

3.4. Expression and purification of PocP12, PocP38, and PocP41

The recombinant PocP12, PocP38, and PocP41 proteins were predicted to consist of 291 amino acid (aa) residues, 296 aa residues, and 344 aa residues, respectively. After excluding the N-terminal secretion signal peptide sequence and C-terminal GPI-anchor signal sequence, the recombinant protein PocP12, Pocp38 and PocP41 were successfully expressed and purified as soluble form. The purity of the purified recombinant PocP12, PocP38, and PocP41 were assessed and the proteins migrated as a single band of 43 kDa, 44 kDa, and 49 kDa respectively from SDS-PAGE and Coomassie blue staining analysis (Fig. 3E). Purified rPocP12 migrated as a single band of 43kDa under reducing conditions, under nonreducing conditions, a higher-molecular-weight band of 120kDa was observed, which possibly indicates the formation of triploids (Fig. 3B). Purified rPocP38 migrated as a single band of 44kDa under reducing conditions and exhibited a slightly faster mobility under nonreducing conditions with no higher molecular weight aggregates observed (Fig. 3C). Purified rPocP41 also exhibited similar patterns of migration under reducing and nonreducing conditions (Fig. 3D). The corresponding immunoblots probed with mouse immune sera and anti-His antibody revealed a similar and specific pattern of migration for the proteins, whereas there was no specific band in PBS-immune sera and pre-immune sera that were used as negative controls (Fig. 3F, 3G, 3H).

3.5. Immune responses against rPocP12, rPocP38, and rPocP41 in mice

The levels of immune responses against rPocP12, rPocP38, and rPocP41 in mice were measured by ELISA using the same proteins as the solid-phase coating antigen, with PBS as a negative control. Two weeks after final immunization, mouse sera were collected and diluted in different proportions; significant linear downtrends were observed and the ratio of 1:10,000 was found to be the best among all dilution gradients (Fig. 4A, 4B, 4C). We suspected the possibility of a better choice if diluted in a smaller ratio; however, no linear trend was observed in the 6 gradients set from 1:1,000 to 1:10,000. Therefore, a dilution of 1:10,000 was chosen to detect antibody levels in the serum of immunized mice.

Antibodies against rPocP12 and rPocP41 in the serum of mice were first detected two weeks after the primary booster, and reached a distinctly high level after 49 days of immunization; the time of producing antibodies against rPocP38 was slightly longer than that against rPocP12 and rPocP41. On the 28th day after first immunization, a week after the mice received their second immunization, the antibody levels of these three antigens in the mouse serum significantly improved and continued to rise throughout the immunization process, only slightly declining at the last time point (Fig. 4D, 4E, 4F).

High-avidity IgG antibodies were also induced in mice immunized with rPocP12, rPocP38, and rPocP41. The IgG avidity response induced in rPocP41-immunized mice was the highest in all immune formulations (mean: 83.38%). The responses detected in rPocP12- and rPocP38-immunized mice were 67.86% and 67.51% respectively.

3.6. Antigen-specific T-cell response

Considering that these three protein antigens are capable of inducing high antibody levels in mice, we wanted to verify whether they can also induce effective T cell

immune responses. Lymphocyte-secreting cytokine levels of the mice immunized with different proteins were evaluated by flow cytometry. Significantly higher levels of IFN-γ from $CD4^+$ T cells were observed in mice immunized with rPocP38, compared to that in mice immunized with PBS ($P < 0.01$; Fig. 5B). However, the ones immunized with rPocP12 and rPocP41 showed no significant difference when compared to the PBS group ($P > 0.05$; Fig. 5A, 5C). Moreover, the percentage of IFN-γ in $CD8^+$ T cells was 23.04% in rPocP38-immunized mice, which was higher than that in PBS-immunized mice ($P < 0.05$; Fig. 5E). Neither of rPocP12 and rPocP41 could stimulate the secretion of an effective level of IFN-γ from $CD8^+$ T cells in mice ($P > 0.05$; Fig. 5D, 5F). After a 72-h culture, we observed significantly higher lymphocyte proliferation in rPocP38-immunized mice compared to that in PBS group ($P < 0.001$). rPocP41 could also induce higher lymphocyte proliferation ($P < 0.05$) while no significance was found between the rPocP12 immunized group and PBS group (Fig. 5G, $P > 0.05$).

4. Discussion

A quick and effective diagnosis is necessary as the imported cases of malaria and the damage it has caused in China. The PCR method and conventional microscopic examination have been widely accepted due to their accuracy and validity. In this study, the samples collected from the infected patients were confirmed by PCR-based technique and microscopic examination of peripheral thin blood smears. Not surprisingly, we successfully amplified target fragments with species-specific primers and clearly observed the existence of parasites in peripheral blood. The results not only confirmed that samples we collected were from *P. ovale*-infected patients, but also showed the highly efficiency and superiority of microscopy and PCR-based technique.

Similar protein structures are often associated with similarities in function. Previous studies had shown P12, P38, and P41 to be highly conserved in both *P. falciparum* and *P. vivax* (Forero-Rodríguez J, et al., 2014; Reeder JC, et al.,2011; Forero-Rodriguez J, et al.,2014). Considering that the genetic diversity might have an important role in the

transmission and control strategies of malaria, we analyzed nucleotide polymorphism of some clinical samples in sub-Saharan Africa. The full-length *pocp12*, *pocp38*, and *pocp41* gene sequences were found to be highly conserved (Additional file 5: Figure S3), which probably indicates functionally important roles for them in the process of erythrocyte infection.

Till date, IgG is the only immune effector known to contribute to natural acquisition of malarial immunity in humans (Cohen S et al., 1961). To further verify the IgG levels produced in mouse serum, we diluted the serum at different gradients and identified the abilities of rPocP12, rPocP38, and rPocP41 to induce high levels of IgG antibody in mice (Fig. 4). This is encouraging, since high level of specific IgG antibody response often has a positive association with low parasitemia density, and immune IgG could rapidly degrade the parasitemia in children infected with malaria (Pitabut N et al., 2007).

Considering that effective malaria vaccines would be able to induce balanced $CD4^+$ and $CD8^+$ T cell responses, we tested the content of IFN-γ in $CD4^+$ and $CD8^+$ T cells in the serum of mice immunized with rPocP12, rPocP38, and rPocP41 (Fig. 5). Results showed no significant difference between the PBS-immunized mice and rPocP12-immunized mice, thus indicating that rPocP12 could not induce significant T cell response. This was similar to the result of erythrocyte invasion inhibition test for P12, shown in previous studies, in which antibodies against rPf12 did not show any invasion inhibition activity (Tonkin ML, et al., 2013). However, the detailed mechanism is yet to be elucidated. P41, which could form a stable heterodimer with P12 on the surface of infective *Plasmodium* merozoite, is similar in structure to P12 and acts indirectly in the process of parasites invading erythrocytes (Parker ML, et al., 2015). As expected, the expression and localization profiles for P41 in *P. falciparum* are similar to that of P12 (Hall N, et al.,2005; Sanders PR, et al.,2005; Van Dijk MR, et al.,2010). This may explain why the results of rPocP41 closely resemble those of rPocP12. When the mice were immunized with rPocP38, significantly higher levels of IFN-γ in $CD4^+$ and $CD8^+$ T cells were revealed. This finding is noteworthy since it

reflects that CD4+ and CD8+ T cells play a critical role in the immune response against this antigen and the immune response of PocP38-specific T cells is ready induced following *P. ovale* infection. When the host infected with malaria, CD4 + T cells rapidly induce effector cells, which are responsible for the secretion of intense but transient peaks of TNF-α and IFN-γ. These proinflammatory cytokines could not only improve the phagocytosis of macrophages and dendritic cells, but also activate the immune system, which is beneficial to the hypersensitivity of the host to Toll-like receptor (TLR) agonists (Ing R, et al., 2009; Franklin BS, et al., 2009). Furthermore, previous studies had shown IFN-γ to be protective during malaria infection (King T et al., 2015; Roestenberg M, et al., 2009; White MT, et al., 2013). The percentage of IFN-γ in $CD4^+$ and $CD8^+$ T cells was also associated with a greater likelihood of uncomplicated malaria and could reduce severe malarial anemia in humans (Van Den Broek MF et al., 1995; Murray HW, et al., 1983). There are various effector functions of IFN-γ that enable it to enhance immunological recognition and elimination of different lifecycle stages of the parasite (McCall MB, et al., 2010). Although three members of this 6-cysteine protein family, P12, P38, and P41, were found to localize on the merozoite surface, P38 was distributed more specifically at the apical end than P12 and P41 in *P. falciparum* (Reeder JC, et al., 2011). In addition, peptides of P38 in *P. falciparum* were shown to have a higher affinity for erythrocytes and could restrict their invasion by the parasites (Feller T et al., 2013). P38 in *P. falciparum* is expressed during both the asexual and sexual stages, indicating its indispensable role in preventing asexual blood-stage symptoms as well as blocking the transmission of malaria (Sanders PR, et al.,2005). The explanation regarding *P. falciparum* may also be applicable to *P. ovale*, which explains why the effect of rPocP38 is superior to that of rPocP12 and rPocP41. Proliferation of spleen lymphocytes in the rPocP38-immunized mouse group was also obviously higher than in the PBS group. All the results indicated rPocP38 could cause better immune protection in mice; the function of rPocP12, rPocP38, and rPocP41 in merozoite invasion would be interesting to study and may be explored in a future investigation.

5. Conclusions

The findings of this study demonstrated the remarkable conservation and high immunogenicity of rPocP12, rPocP38 and rPocP41. The highly similar structures of P12, P38, and P41 in *P. falciparum* and *P. ovale* may also indicate high similarity in their function. These represent an important advance in the understanding of blood-stage immunity to *P. ovale*, at least in part. Their promising effect of immune protection give us interests to explore their function in merozoite invasion.

Supplementary datas

Supplementary data 1: Table S1 The exact origin of *P. ovale curtisi* isolates.

Supplementary data 2: Figure S1 Homology model building of P12, P38, and P41 in *P. falciparum and P. ovale*. (A, D, G) 3D structure of Pf12, Pf38 and Pf41. (B, E, H) Predicted 3D model of PocP12, PocP38 and PocP41. (C, F, I) Orthogonal view of Pf12 (green) overlapping with PocP12 (magenta), Pf38(green) overlapping with PocP38 (magenta) and Pf41(green) overlapping with PocP41 (magenta).

Supplementary data 3: Figure S2 The similarity comparison based on the amino-acid between *P. falciparum* and *P. ovale*.

Supplementary data 4: Table S2 The *p12*, *p38* and *p41* Gene ID number of other *Plasmodium* species.

Abbreviations

PocP12: *P. ovale curtisi* P12; PocP38: *P. ovale curtisi* P38; PocP41: *P. ovale curtisi* P41; rPocP12: recombinant PocP12; rPocP38: recombinant PocP38; rPocP41: recombinant PocP41; 6-cys: 6-cysteines; PCR: Polymerase chain reaction; ECL: chemiluminescence

detection assay; ELISA: Enzyme-linked immunosorbent assays; AI: Avidity index; RPMI: Roswell Park Memorial Institute; CCK-8: Cell Counting kit-8; PMA: phorbol 12-myristate 13-acetate.

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Authors' contributions

YC and JC conceived this study. FHS designed the research protocol and wrote the manuscript. RLC, XXZ, JG, XDY and GDZ collected the samples. FHS, RLC, WXY and XXZ performed the acquisition of data and data analysis. FHS, RLC, YL and HTF conducted the laboratory work, data handling and analysis and reviewed the manuscript. HTF, JG, GDZ, XDY and YL contributed to interpret the results and assisted in writing the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee, Jiangsu Provincial Key Laboratory on Parasite and Vector Control Technology, Jiangsu Institute of Parasitic Diseases (JIPD) (IRB00004221), Wuxi, China. Informed consent was obtained from all of the participants, and the animal trial was approved by the Animal Ethics Committee, Jiangnan University (JN. No20180615t0900930 [100]).

Declaration of Competing Interest

The authors declare that they have no competing interests.

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References:

Arredondo S.A., Kappe S.H.I. 2016. The s48/45 six-cysteine proteins: mediators of interaction throughout the *Plasmodium* life cycle. Int J Parasitol. 47, 409-423.

Baird J.K. 2000. Resurgent malaria at the millennium: control strategies in crisis. Drugs. 59, 719-743.

Cao Y, Wang W, Liu Y, Cotter C, Zhou H, Zhu G, Tang J, Tang F, Lu F, Xu S, Gu Y, Zhang C, Li J, Cao J. 2016. The increasing importance of *Plasmodium ovale* and *Plasmodium malariae* in a malaria elimination setting: an observational study of imported cases in Jiangsu Province, China. Malar J. 15, 459.

Chavatte J.M., Tan S.B., Snounou G., Lin R.T. 2015. Molecular characterization of misidentified *Plasmodium ovale* imported cases in Singapore. Malar J. 14, 454.

Cheng Y, Lu F, Tsuboi T., Han ET. 2013. Characterization of a novel merozoite surface

protein of *Plasmodium vivax*, Pv41. Acta Trop. 126, 222-228.

Chu R, Zhang X, Xu S, Chen L, Tang J, Li Y, Chen J, Xuan Y, Zhu G, Cao J, Cheng Y. 2018. Limited genetic diversity of N-terminal of merozoite surface protein-1 (MSP-1) in *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* imported from Africa to China. Parasit Vectors. 11, 596.

Cohen S., Mcgregor I.A., Carrington S. 1961. Gamma-globulin and acquired immunity to human malaria. Nature. 192, 733-737.

Cowman A.F., Berry D., Baum J. 2012. The cellular and molecular basis for malaria parasite invasion of the human red blood cell. J Cell Biol. 198, 961-971.

Crosnier C., Wanaguru M., McDade B., Rayner J., Wright GJ. 2010. A library of functional recombinant *Plasmodium falciparum* merozoite surface proteins. Malar J. 9, 08.

Dixit A. 2016. Preventive Strategies to Reduce Malaria Burden: Epidemiological Surveillance and Modeling for New Control and Elimination Methodologies. Preprint at https://escholarship.org/uc/item/04r8z8hq

Doll K.L., Butler N.S., Harty J.T.. 2013. Tracking the total CD8 T cell response following whole *Plasmodium* vaccination. Methods Mol Biol. 923, 493-504.

Feller T., Thom P., Koch N., Spiegel H., Addai-Mensah O., Fischer R., Reimann A., Pradel G., Fendel R., Schillberg S., Scheuermayer M., Schinkel H. 2013. Plant-Based Production of Recombinant *Plasmodium* Surface Protein Pf38 and Evaluation of its Potential as a Vaccine Candidate. PLoS One. 8, e79920.

Forero-Rodríguez J., Garzón-Ospina D., Patarroyo MA. 2014. Low genetic diversity and functional constraint in loci encoding *Plasmodium vivax* P12 and P38 proteins in the Colombian population. Malar J. 13, 58.

Forero-Rodriguez J., Garzon-Ospina D., Patarroyo M.A. 2014. Low genetic diversity in the locus encoding the *Plasmodium vivax* P41 protein in Colombia's parasite

population. Malar J. 13, 388.

Franklin B.S., Parroche P., Ataíde M.A., Lauw F., Ropert C., de Oliveira R.B., Pereira D., Tada M.S., Nogueira P., da Silva L.H., Bjorkbacka H., Golenbock D.T., Gazzinelli R.T. 2009. Malaria primes the innate immune response due to interferon-gamma induced enhancement of toll-like receptor expression and function. Proc Natl Acad Sci U S A. 106, 5789-94

García J., Curtidor H., Pinzón C.G., Vanegas M., Moreno A., Patarroyo M.E. 2009. Identification of conserved erythrocyte binding regions in members of the *Plasmodium falciparum* Cys6 lipid raft-associated protein family. Vaccine. 27, 3953-62.

Gilson P.R., Nebl T., Vukcevic D., Moritz R.L., Sargeant T., Speed T.P., Schofield L., Crabb B.S. 2006. Identification and Stoichiometry of Glycosylphosphatidylinositol-anchored Membrane Proteins of the Human Malaria Parasite *Plasmodium falciparum*. Mol Cell Proteomics. 5, 1286-1299.

Hall N., Karras M., Raine J.D., Carlton J.M., Kooij T.W., Berriman M., Florens L., Janssen C.S., Pain A., Christophides G.K., James K., Rutherford K., Harris B., Harris D., Churcher C., Quail M.A., Ormond D., Doggett J., Trueman H.E., Mendoza J., Bidwell S.L., Rajandream M.A., Carucci D.J., Yates J.R., Kafatos F.C., Janse C.J., Barrell B., Turner C.M., Waters A.P., Sinden R.E. 2005. A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. Science. 307, 82-86.

Ing R., Stevenson M.M. 2009. Dendritic cell and NK cell reciprocal cross talk promotes gamma interferon-dependent immunity to blood-stage *Plasmodium chabaudi* AS infection in mice. Infect Immun. 77, 770-782.

Li Z, Jun F, Shao-Sen Z, Zhi-gui X, Shui-Sen Z. 2017. Malaria epidemiological characteristics in China, 2005–2015. China Tropical Medicine. 17, 325–335.

King T., Lamb T. 2015. Interferon-γ: The Jekyll and Hyde of Malaria. PLoS Pathog. 11,

e1005118.

Li Z, Jun F, Shao-Sen Z, Zhi-gui X, Shui-Sen Z. 2018. The progress of national malaria elimination and epidemiological characteristics of malaria in China in 2017. Chin J Parasitol Parasit Dis Apr. 36, 201–209.

McCall M.B., Sauerwein R.W. 2010. Interferon-γ—central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. J Leukoc Biol. 88, 1131-1143.

Mehrizi A.A., Rezvani N., Zakeri S., Gholami A., Babaeekhou L. 2018. 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. Med Microbiol Immunol. 207, 151-166.

Mueller I., Zimmerman P.A., Reeder J.C. 2007. *Plasmodium malariae* and *Plasmodium ovale*--the "bashful" malaria parasites. Trends Parasitol. 23, 278-283

Murray H.W., Rubin B.Y., Rothermel C.D. 1983. Killing of intracellular Leishmania donovani by lymphokine-stimulated human mononuclear phagocytes. Evidence that interferon-gamma is the activating lymphokine. J Clin Invest. 72, 1506-1510.

Parker M.L., Peng F, Boulanger M.J. 2015. The Structure of *Plasmodium falciparum* Blood-Stage 6-Cys Protein Pf41 Reveals an Unexpected Intra-Domain Insertion Required for Pf12 Coordination. PLoS One. 10, e0139407.

Pitabut N., Panichakorn J., Mahakunkijcharoen Y., Hirunpetcharat C., Looareesuwan S., Khusmith S. 2007. IgG antibody profile to C-terminal region of *Plasmodium vivax* merozoite surface protein-1 in Thai individuals exposed to malaria. Southeast Asian J Trop Med Public Health. 38, 1-7.

Reeder J.C., Wapling J., Mueller I., Siba P.M., Barry A.E. 2011. Population genetic analysis of the *Plasmodium falciparum* 6-cys protein Pf38 in Papua New Guinea reveals domain-specific balancing selection. Malar J. 10, 126.

Richards J.S., Beeson J.G. 2009. The future for blood-stage vaccines against malaria. Immunol Cell Biol. 87, 377-390.

Roestenberg M., McCall M., Hopman J., Wiersma J., Luty A.J., van Gemert G.J., van de Vegte-Bolmer M., van Schaijk B., Teelen K., Arens T., Spaarman L., de Mast Q., Roeffen W., Snounou G., Rénia L., van der Ven A., Hermsen C.C., Sauerwein R. 2009. Protection against a malaria challenge by sporozoite inoculation. N Engl J Med. 361, 468-477.

Roucher C., Rogier C., Sokhna C., Tall A., Trape J.F. 2014. A 20-Year Longitudinal Study of *Plasmodium ovale* and *Plasmodium malariae* Prevalence and Morbidity in a West African Population. PLoS One. 9, e87169.

Rui-min Z, Su-hua L, Dan Q, Cheng-yun Y, Ying L, Yu-ling Z, Bian-li Xu, Hong-Wei Z. 2018. Epidemiological characteristics of imported ovale malaria in Henan Province during 2010-2016. Chin J Parasitol Parasit Dis Apr. 36,193-203

Sanders P.R., Gilson P.R., Cantin G.T., Greenbaum D.C., Nebl T., Carucci D.J., et al. Distinct protein classes including novel merozoite surface antigens in Raft-like membranes of *Plasmodium falciparum*. J Biol Chem. 2005;280(48):40169-40176.

Sanders P.R., Gilson P.R., Cantin G.T., Greenbaum D.C., Nebl T., Carucci D.J., McConville M.J., Schofield L., Hodder A.N., Yates J.R., Crabb B.S. 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol. 61, 315-320.

Stevens J.W.W. 1922. A New Malaria Parasite of Man. Ann Trop Med Parasitol. 16, 383–388

Taechalertpaisarn T., Crosnier C., Bartholdson S.J., Hodder A.N., Thompson J., Bustamante L.Y., Wilson D.W., Sanders P.R., Wright G.J., Rayner J.C., Cowman A.F., Gilson P.R., Crabb B.S. 2012. Biochemical and Functional Analysis of Two *Plasmodium falciparum* Blood-Stage 6-Cys Proteins: P12 and P41. PLoS One. 7, e41937.

Tonkin M.L., Arredondo S.A., Loveless B.C., Serpa J.J., Makepeace K.A., Sundar N., Petrotchenko E.V., Miller L.H., Grigg M.E., Boulanger M.J. 2013. Structural and Biochemical Characterization of *Plasmodium falciparum* 12 (Pf12) Reveals a Unique Interdomain Organization and the Potential for an Antiparallel Arrangement with Pf41. J Biol Chem. 288, 12805-12817.

Van Den Broek M.F., Müller U., Huang S., Zinkernagel R.M., Aguet M. 1995. Immune defence in mice lacking type I and/or type II interferon receptors. Immunol Rev. 148, 5-18.

van Dijk M.R., van Schaijk B.C., Khan S.M., van Dooren M.W., Ramesar J., Kaczanowski S., van Gemert G.J., Kroeze H., Stunnenberg H.G., Eling W.M., Sauerwein R.W., Waters A.P., Janse C.J. 2010. Three members of the 6-cys protein family of *Plasmodium* play a role in gamete fertility. PLoS Pathog. 6, e1000853.

van Schaijk B.C., Janse C.J., van Gemert G.J., van Dijk M.R., Gego A., Franetich J.F., van de Vegte-Bolmer M., Yalaoui S., Silvie O., Hoffman S.L., Waters A.P., Mazier D., Sauerwein R.W., Khan S.M. 2008. Gene Disruption of *Plasmodium falciparum* p52 Results in Attenuation of Malaria Liver Stage Development in Cultured Primary Human Hepatocytes. PLoS One. 3, e3549.

White M.T., Bejon P., Olotu A., Griffin J.T., Riley E.M., Kester K.E., Ockenhouse C.F., Ghani A.C. 2013. The relationship between RTS, S vaccine-induced antibodies, CD4+ T cell responses and protection against *Plasmodium falciparum* infection. PLoS One. 8, e61395.

WHO. World Malaria Report. 2019. Geneva: World Health Organization. http://www.who.int/malaria/publications/world_malaria_report/en/.

Zhang X, Chu R, Xu S, Fu H, Tang J, Chen L, Shi X, Chen J, Li Y, Zhu G, Han ET, Xuan Y, Cao J, Cheng Y. 2019. Immunogenicity analysis of genetically conserved segments in *Plasmodium ovale* merozoite surface protein-8. Parasit Vectors. 12, 164.

Li Z, Zhang Q, Zheng C, Zhou S, Sun J, Zhang Z, Geng Q, Zhang H, Wang L, Lai S, Hu W, Clements A.C., Zhou XN, Yang W. 2016. Epidemiologic features of overseas imported malaria in the People's Republic of China. Malaria Journal. 15, 141.

Figures

Fig. 1 Agarose analysis of nested PCR detection of *P. ovale* malaria and thin blood smear for *P. ovale* malaria. (A) Amplification for *P. falciparum* and *P. ovale* with species-specific primers. 1-6: Amplification for *P. falciparum*. 7-12: Amplification for *P. ovale*. M: DNA size standard. (B) Microscopic image of thin smear. Arrow indicates the presence of parasite in erythrocyte.

Fig. 2 Schematic diagram of PocP12, PocP38 and PocP41. (A) structure of PocP12. The PocP12 protein comprises 338 amino acids. The GPI anchor (amino acid [aa] position 313-338), the s48/45 domains (aa 24-139 and 171-284), and the signal peptide (aa 1-23) are indicated. (B) structure of PocP38. The PocP38 protein comprises 351 amino acids. The GPI anchor (aa 329-351), the s48/45 domain (aa 153-284), and the signal peptide (aa 1-24) are indicated. (C) structure of PocP41. The PocP41 protein comprises 375 amino acids. The s48/45 domains (aa 16-115 and 238-349), the coiled-coil region (aa 170-225), and the signal peptide (aa 1-15) are indicated. The N-terminal signal peptide and the C-terminal GPI anchor are removed when the proteins are expressed. (D, E, F) Phylogenetic relationship of *pocp12*, *pocp38* and *pocp41* genes within ortholog *Plasmodium* species *P. vivax*, *P. knowlesi*, *P. falciparum* and *P. cynomolgi* etc. based on neighbor-joining method.

Fig. 3 PCR, SDS-PAGE and western blot analysis of P12, P38 and P41. (A) PCR amplification for *pocp12*, *pocp38*, *pocp41*. (B, C, D) Immunoblot analysis of purified

rPocP12, rPocP38 and rPocP41 under reducing (R) and nonreducing (NR) conditions probed with anti-His antibody. (E) Coomassie stain after SDS-PAGE separation of purified rPocP12, rPocP38 and rPocP41. Molecular weight is indicated to the left. (F, G, H) Immunoblot analysis of purified rPocP12, rPocP38 and rPocP41 incubated with serum samples from mice immunized with rPocP12, rPocP38 and rPocP41(S), anti-His antibody (H), serum samples from mice immunized with PBS (P) and pre-immune sera (PI) respectively. M, Molecular size marker.

Fig. 4 Immune responses in mice immunized with rPocP12, rPocP38 and rPocP41 respectively. (A, B, C) The sera obtained from immunized mice were diluted from 1:10,000 to 1:5,120,000 and the data were presented as the geometric mean OD expressed as the reciprocal of the serum dilution. (D, E, F) IgG levels in rPocP12, rPocP38, rPocP41-immunized mice. IgG was detected at 14 days after immunization and the levels increased throughout the whole immunization period.

Fig. 5 T cell immunogenicity of rPocP12, rPocP38 and rPocP41. (A, B, C) The levels of IFN-γ from CD4+ T cells in mouse spleen lymphocytes after stimulated with rPocP12, rPocP38 and rPocP41 respectively. (D, E, F) The levels of IFN-γ from CD8+ T cells in mouse spleen lymphocytes after stimulated with rPocP12, rPocP38 and rPocP41 respectively. Values represent the percentage of individual cytokines to CD4+ or CD8+ T cells. A non-parametric Tukey test was used to determine the difference in the production of IFN-γ in response to stimulation with the corresponding proteins. Significant differences between the groups are indicated on the graph: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Non-significant (ns) difference was shown ($P > 0.05$). (G) The proliferation of splenocytes after stimulated *ex vivo* for 72h with rPocP12, rPocP38 and rPocP41 respectively.