A thioredoxin homologous protein of *Plasmodium falciparum* participates in erythrocyte invasion.

1. Wei Wang¹#, Peng Huang¹#, Ning Jiang², Huijun Lu¹, Dongchao Zhang¹, Dawei Wang², Kai Zhang², Mats Wahlgren³, Qijun Chen²³*

1. Key Laboratory of Zoonosis, Jilin University, Xi An Da Lu 5333, Changchun 130062, China.
2. Key Laboratory of Zoonosis, College of Veterinary Medicine, Shenyang Agricultural University, Dongling Road 120, Shenyang 110866, China.
3. Institute of Microbiology, Tumor- and Cellular Biology Center, Karolinska Institutet, Nobel väg 16, 171 77 Stockholm, Sweden.

#These authors contribute equally to the manuscript.

*Corresponding author: Professor Qijun Chen

Key Laboratory of Zoonosis, College of Veterinary Medicine, Shenyang Agricultural University
Dongling Road 120, Shenyang 110866, China.
Tel: +86 15802409566
Email: qijunchen759@svau.edu.cn.
Abstract

Invasion into erythrocytes of merozoites is required in the life cycle of malarial parasites. Proteins derived from the invasive merozoites are essential ligands for erythrocyte recognition and penetration. In this study, we report a novel protein which possesses a Trx domain-like structure of the thioredoxin family and is expressed on the surface of merozoites of the malaria parasite *Plasmodium falciparum*. This protein, namely PfTrx-mero protein, displayed a mutated sequence character at the Trx domain, but with a specific binding activity to human erythrocytes. Specific antibodies to the protein inhibited merozoite invasion into human erythrocytes. Immunization of a homologous protein of *P. berghei* ANKA strain also showed significant protection against lethal infection in mice. These results suggested that the novel PfTrx-like-mero protein expressed on the surface of merozoites is an important ligand participating in erythrocyte invasion and a potential vaccine candidate. (142 words)

Keywords: Plasmodium; erythrocyte; invasion; thioredoxin; ligand.

Malaria, a severe infectious disease, is caused by the parasites of *Plasmodium* genus. *Plasmodium falciparum*, the most virulent malarial parasite that infects humans, causes higher morbidity and mortality than other species (1). The emergence and wide dissemination of drug-resistance strains of *P. falciparum* and insecticide-resistant mosquitoes propel the search for effective vaccines (2, 3). Merozoites are the invasive form of the parasite in the blood, and the infection is initiated by adhering to and
penetration into erythrocytes (4). Blocking merozoite invasion is an effective way for prevention of parasite infection (5). Up to date, a number of proteins expressed on merozoite surface or associated have been put into vaccination studies (6-10), though the clinical performance of the vaccine candidates has not been very satisfactory.

The process of erythrocyte invasion of a merozoites contains multiple receptor-ligand interactions. Several glycosaminoglycans (GAG), including sialic acid and heparin sulfate-like moieties on the surface of human erythrocytes have been proved as receptors for merozoite derived proteins such as MSP-1 (11, 12). Recently, we revealed the heparin-binding proteome of *P. falciparum* (13). Apart from MSP-1, a number of proteins expressed at early developmental stage of the parasite showed specific binding activity to heparin (13). These proteins all contain one or several GAG-binding motifs with a characteristic amino acid context as [-X-B-B-X-B-X-] or [-X-B-B-X-B-X-], where B is a basic residue such as lysine, arginine, or histidine (14, 15). Among the heparin-binding proteins, one of the protein encoded by PF3D7_1104400 attracted our attention. The amino acid sequence of the encoded protein showed high similarity to Thioredoxin (Trx). The Trx family in *Plasmodium* contained three Trx protein (Trx1, 2 and 3) and a number of Trx-like proteins. The Trx proteins are critical to maintain the intracellular redox balance of the parasite and the infected erythrocyte (16), but the function of the Trx-like proteins is still speculative. The intracellular antioxidant function of thioredoxins is mediated by the conserved Cys-Gly-Pro-Cys motif (CGPC) (17). Additionally, there are three other non-catalytic cysteine residues in Trx, Cys62, Cys69 and Cys73 (18), and Cys62 and Cys69 can
form an extra disulfide bond to form a hydrophobic pocket, and the hydrophobic region often plays an important role in extracellular protein–protein interaction (19, 20). Further, most of the proteins in the Trx family are functioning in the cytoplasm, but some of them have been found to be secreted by an unknown route and function extracellularly like protein binding (21, 22). For instance, the Trx-like protein in *Trichuris suis* has been reported to bind to intestinal epithelial cells, and a shorter form of Trx can be secreted outside the parasite and binds to the outer membrane of human U937 cells and MP-6 cells (23-25).

In this study, a novel protein encoded by PF3D7_1104400 was found to possess a conserved sequence feature of Trx family. It was mainly expressed at the merozoite surface and participated in erythrocyte invasion by binding to heparin sulfate receptor on the erythrocytes. The data revealed a novel function of the Trx family proteins in the malaria parasite *P. falciparum*.

**MATERIALS AND METHODS**

**Ethical statement**

All procedures performed on the animals (mice and rabbits) in this study were conducted according to the animal husbandry guidelines of Jilin University. Study protocols have been reviewed and approved by the Experimental Animal Committee and the Ethical Committee of Jilin University, Changchun, China.

**Parasites**
The *P. falciparum* 3D7 strain was cultured according to standard methods with 0.25\% w/v Albumax II and 5\% v/v human B+ serum added to the buffered medium (RPMI 1640 supplemented with HEPES, hypoxanthine, and sodium bicarbonate) with 5\% hematocrit (26). The Development of the parasites were synchronized with 5\% w/v sorbital. The parasites were harvested respectively at the time points of 8, 16, 24, 32, 40 and 48 h post erythrocyte invasion, which were preserved at -80°C before use.

The *P. berghei* ANKA strain parasites were obtained by respectively infection of C57BL/6 and BALB/c female mice by intraperitoneally injection 1×10^6 parasitized erythrocytes (27).

**Bioinformatic analysis of the amino acid sequence encoded by PF3D7_1104400**

In our earlier study (13), a protein encoded by gene sequence (PF3D7_1104400) was identified. The whole sequences of the gene and the encoded protein (PfTrx-like-mero) were obtained from PlasmoDB (http://plasmodb.org/) and the signal peptide and transmembrane domain were predicted via the SignalP 4.1 server and TMHMM Server v.2.0, respectively (28, 29). The conserved domain of PfTrx-like-mero was identified by Basic Local Alignment Search Tool (BLAST) (30), and the glycosaminoglycan (GAG) binding motifs (XBBXBX or XBBBXXBX) were determined as described (12).

The amino acid sequence of the PfTrx-like-mero protein was firstly aligned with homologues in other *Plasmodium* spp (including *P. reichenowi, P. berghei, P. knowlesi, P. vivax, P. chabaudi* and *P. yoelii*) via Multiple Sequences Alignment Tool provided
by DNAMAN, and subsequently compared with Trx domain-containing proteins in
_Homo sapiens, Mouse, Drosophila melanogaster, Saccharomyces cerevisiae,
_Arabidopsis thaliana_ and _Schistosoma japonicum_ to determine the functional sites of
the Trx domain.

**Transcription analysis of the gene (PF3D7_1104400) with real-time quantitative PCR**

Briefly, RNA of synchronized parasites in the 6 time points of the blood-stage was
extracted with Trizol solution according to the manufacturer’s instructions (Invitrogen,
CA, USA). After removing the DNA remnant by DNase I (TaKaRa, Dalian, China)
treatment, reverse transcription was carried out immediately in the system containing
oligo(dT) primer and reverse transcriptase (31). RT-qPCR with cDNA template and
specific primers (Forward 5’-CCC ATA CAA AAG AAT CAG ATA TGC-3’, Reverse
5’-GGG TCT TGT ATG AAT TCT GG-3’) was performed in the 7500 Real-Time
PCR System (Applied Biosystems, USA) using SYBR Premix Ex Taq™ (TaKaRa,
Dalian, China). The data was analyzed by the \(2^{-\Delta\Delta C_T}\) method (32) in which the
amount of target RNA was compared to an internal control gene, seryl-tRNA
synthetase (PF3D7_1205100), which is stably expressed during the erythrocytic stage
of the parasite (33). The transcription levels were determined by the mean of the three
repeated experiments and the error bars represent the mean \(\pm\) SEM.

**Expression of recombinant proteins and preparation of polyclonal antibodies**
Based on the positions of GAG binding motifs and Trx domain in the sequence, the PfTrx-like-mero protein was expressed in three fragments (I: PF3D7_1104400-N888, II: PF3D7_1104400-C522, and III: PF3D7_1104400-1101, as illustrated in Figure 1). His-tagged and GST-tagged recombinant proteins of the three fragments were expressed separately for obtaining polyclonal antibodies and further functional analysis. Briefly, the gene fragments encoding the three regions (I, II, III regions) of the gene were amplified with specific primers (I: Forward 5'-gaattc ACT TCG TCC TTA CTA GAA ACC-3', Reverse 5'-ctcgag TAA GGA GAG TAA CAT GTC TAT TTC-3'; II: Forward 5'-gatcc TCT GAA ACT TTT GTC CTA GG-3', Reverse 5'-ctcgag CAA TTC ATC TTT TTC ATT TGC TTT TAT GA-3') by PCR. The amplicons were respectively cloned into pET-28a and pGEXT-4T-1 expression vectors. The recombinant plasmids were expressed in *Escherichia coli* BL21 (DE3) (34, 35) and both His- and GST-tagged recombinant proteins were purified using His GraviTrap™ affinity columns (GE Healthcare) and Gluthathione-Sepharose™ 4B (GE Healthcare) according to the manufacturer instructions. SDS-PAGE and Western blot were used to evaluate the purified recombinant proteins. Polyclonal antibodies against the three fragments of PF3D7_1104400 (I, II, III) were prepared by immunization of rabbits (New Zealand white strain) with His-tagged recombinant proteins emulsified with Freund's adjuvant. The rabbits were immunized subcutaneously for four times with 2 weeks intervals, and the antisera were collected 10 days after the fourth immunization. IgG was
purified from the immune sera using Protein A Sepharose™ 4 Fast Flow (GE Healthcare), the specificity and quality of the antibodies against the natural protein were verified by Western blot as previously describe (31).

Expression and localization analysis of the protein in parasites by Western blot, IFA and IEM

The parasites collected at the 6 time-points post erythrocyte invasion were dissolved in SDS-PAGE loading buffer (250 mM Tris, 1.92 Mglycine, and 1% SDS), and run on SDS-PAGE gel and transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad, CA, USA) using a semi-dried blotting system (Bio-Rad, CA, USA) in a condition of constant 24 V voltage. After being blocked with 5% skim milk (Sigma, St Louis, USA) for 1 h at 37°C, the membrane was incubated in TBST containing the rabbit anti-PfTrx-like-mero-1101 IgG (1:1,000 dilution) for 12 h at 4°C. Afterwards, the membrane was washed 4 times with TBST buffer and further incubated with HRP-conjugated goat anti-rabbit IgG (H+L) (Abcam, Shanghai, China) (1:3,000 dilution). The membrane was developed after washing with an ECL enhanced chemiluminescence reagent kit (Thermo) with a LAS 4000 mini luminescent image analyzer (GE Healthcare) (36).

To localize the expression of the PfTrx-like-mero protein, thin blood smears of infected red blood cells (iRBC) at late schizont stage and free merozoites were fixed with cold methanol in -80°C for 5 min, and air-dried. The smears were washed three times using sterilized PBS and blocked in 5% skim milk (Sigma, St Louis, USA) for 1
h at 37°C, and the slides were incubated in PBS containing a rabbit anti-PfTrx-like-mero protein polyclonal antibody (1:50 dilution) as well as a rat anti-MSP-1.42 antibody (1:100 dilution) for 12 h in 4°C. The slides were washed with PBS and further incubated with Alexa Fluor 488 goat conjugated goat anti-rabbit IgG (1:1,000) (Life technologies) and Alexa Fluor 594 goat anti-rat IgG (Life technologies) at 37°C for 1 h. The parasite nuclei were stained with Hoechst (Hoechst AG, German) at 1:1,000 dilution for 5 min before image capturing with a fluorescence microscope (Olympus, BX 53, Japan).

To further reveal the location of PfTrx-like-mero protein on the parasite, immune electron microscopy (IEM) was performed. Briefly, free merozoites and parasites at late schizont stage were fixed in 0.25% glutaraldehyde and 1% paraformaldehyde for 30 min at 4°C. Then the samples were washed, dehydrated and embedded in LR White resin (Sigma, St Louis, USA) at 50°C for 24 hours. Ultra-thin sections were blocked in 3% skim milk and then incubated with rabbit anti-PfTrx-like-mero protein antibody (1:100 dilution) at 4°C overnight as described (37). Subsequently, these sections were incubated in goat anti-rabbit IgG conjugated to 5 nm gold (1:50 dilution) (Sigma, St Louis, USA) at 37°C for 1 h. Eventually, samples were examined with a transmission electronic microscope (Hitachi H-7650, Japan).

**Analysis of heparin-binding activity of recombinant proteins**

The binding activity of the PfTrx-like-mero recombinant proteins to heparin was studied as previously described (26). Briefly, the GST-tagged soluble recombinant
proteins (fragment I, II, III) and GST protein in the concentration of 0.4 μM were mixed with 40 μl Heparin-Sepharose and uncoupled Sepharose 4B (GE Healthcare) and incubated for 2 h at 4°C. The mixtures were centrifuged and washed 3 times with cold PBS after incubation. The Sepharose pellets were mixed with loading buffer for SDS-PAGE and Western blot and a GST specific monoclonal antibody (Sungene Biotech, Tianjin, China) to determine the binding activity of the recombinant proteins with heparin.

Adhesion of recombinant proteins to human erythrocytes
To investigate the erythrocyte-binding activity of the PfTrx-like-mero protein, 10 μl human erythrocytes were respectively mixed with 0.4 μM of the GST-tagged recombinant proteins (fragments I, II, III) and incubated for 2 h at 4°C as described (5). The GST protein with equal molarity was incubated with erythrocytes as a negative control and 10 μl erythrocyte alone was used as blank. After incubation, the RBCs were washed 3 times with PBS. Subsequently, the erythrocytes were mixed with loading buffer and boiled for SDS-PAGE. Western blot was performed with an anti-GST monoclonal antibody as previously described.

Invasion inhibition assay in vitro
The invasion inhibition activity of the PfTrx-like-mero protein specific antibodies was tested in vitro as previously described (38). Briefly, synchronized parasites at early ring stage were diluted to 0.2% parasitaemia and were incubated respectively with
purified rabbit IgG to the three fragments of the PfTrx-like-mero protein in concentrations of 1:250, 1:150, 1:100, 1:50 and 1:10 μg/ml in 96-well cell culture plates. The rabbit anti-MSP-1.42 IgG and a healthy rabbit IgG were used as positive and negative controls. After incubation for 48 h at 37 °C, the medium in each well containing corresponding IgG was changed once. Parasitaemia was determined by flow cytometry and 50,000 cells were analyzed in each experiment. The average parasitaemia were determined after 3 repeated experiments. The invasion inhibition efficiency of the negative control group was set as 0%, and the inhibition efficiencies of the PfTrx-like-mero protein specific IgGs and that of the MSP-1.42 specific IgG were calculated relative to the parasitemia of the negative control group. The results are calculated from 3 independent experiments, error bars represent mean ±SEM. One-factor analysis of variance (ANOVA) were used in this study, asterisks indicate the significant difference between groups (* P<0.05, **P<0.01).

**Immunization and challenge experiment**

Firstly, the recombinant PbTrx (encoded by PBANKA_0942500) protein of the *P. berghei* ANKA strain, homologous to the PfTrx-like-mero protein (Supplementary Figure 1), was expressed and purified as above. In the first immunization, each C57BL/6 or BALB/c mouse in the immunization group (N=10) was intramuscularly injected with 50 μg His-tagged recombinant protein (rPBANKA_0942500-His) emulsified with complete Freund’s adjuvant. For the following three times immunization, the mice were injected with recombination protein emulsified with
incomplete Freund’s adjuvant with 2-week intervals. Mice of the control group were
injected only with Freund’s adjuvant and the naive group was also set without any
immunization (N=10 in each group). The antibody titres were determined by the
indirect ELISA after 4 immunizations. Subsequently, each mouse was challenged
intraperitoneally with 10^6 iRBCs (37). The parasitaemia of the mice was measured
daily by counting 3,000 cells per blood smear stained by Giemsa. SPSS 19.0 and
one-factor analysis of variance (ANOVA) were used in this study. Mean ± standard
deviation (SD) was used to express the data. Kaplan-meier/log rank test has been used
to assess the survival data between the groups.

RESULTS
The amino acid sequence encoded by PF3D7_1104400 possesses a conserved
thioredoxin domain
The amino acid sequence of the protein encoded by PF3D7_1104400 identified in the
heparin-binding proteome (13) was bioinformatically analyzed. Interestingly, the
sequence was found to contain a conserved domain with a high similarity to the
thioredoxin (Trx) family. However, the motif (“-CXXC-”) which determines the
function of anti-oxidation was missing, though the three non-catalytic conserved
cysteine residues (Cys51, Cys55 and Cys62) were kept (Fig. 1a, b). Further, this
sequence exhibited a considerable conservation in the parasites of Plasmodium genus
with up to 85.38% similarity. It is phylogenically more related to the annotated
Trx-like proteins in other Plasmodium species than to the P. falciparum PfTlps
(Supplementary Figure 1). Additionally, the N-terminus of the sequence contained a classical signal peptide domain (Fig. 1c) indicating that the protein is eventually secreted outside the parasite. Further, two GAG binding motifs were also identified in the molecule (Fig. 1a, c).

The protein is expressed in the blood-stage and localized on the surface of *P. falciparum* merozoite

The transcription and expression of the gene (PF3D7_1104400) encoding the Trx-like protein during the blood-stage of *P. falciparum* 3D7 strain was studied by real-time quantitative PCR and Western blot with synchronized parasites at 6 developmental time-points post erythrocyte invasion. The results showed that the transcription and expression of the PF3D7_1104400 gene began early in the ring stage and reached a maximal level at 32 h (Fig. 2a, b).

Indirect immunofluorescence assays (IFA) revealed that the PfTrx-like-mero protein was expressed on the surface of both invading and schizontic merozoites colocalized with the MSP-1 protein (Fig. 3a, b). After merozoite invasion, this protein is mostly distributed inside the parasitophorous vacuole in a similar manner of MSP-1 (Fig. 3b). Further, in the immune-electronic microscopy (IEM) assay with PfTrx-like-mero protein specific antibodies, gold particles were clearly observed on the surface of both free and schizontic merozoites (Fig. 3c, d). Thus, the results of both IFA and IEM suggested that the PfTrx-like-mero protein is expressed on the surface of *Plasmodium* merozoites.
The protein adhered heparin and human erythrocytes

In order to determine the role of the PfTrx-like-mero protein in merozoite invasion, GST-tagged recombinant proteins (fragment I, II and III) (Supplementary Figure 2a, b) were respectively incubated with Heparin-Sepharose and erythrocytes. All of the 3 fragments of the PfTrx-like-mero protein could bind Heparin-Sepharose, the GST protein did not show any binding to heparin (Fig. 4a). However, only the fragment I and III showed binding to human erythrocytes, but not fragment II and the GST control (Fig. 4b).

The protein specific antibodies inhibited parasite invasion in vitro

Polyclonal antibodies to the 3 fragments of the PfTrx-like-mero protein which can recognize the native protein (Supplementary Fig. 3) were prepared and tested for their ability to inhibit merozoite invasion into erythrocytes. The result showed that all antibodies inhibited merozoites invasion in a similar manner as that of anti-MSP1.42 antibodies (Fig. 4c). The data further suggested that the PfTrx-like-mero protein is an important parasite ligand participating in the interaction of invading merozoite with erythrocytes.

Immunization of a recombinant PbTrx-like protein protected mice against parasites infection

Both HIS- and GST-tagged recombinant P. berghei Trx-like proteins (PbTrx-like
protein) encoded by PBANKA_0942500 was generated (Supplementary Fig. 4), and the HIS-tagged PbTrx-like protein was immunized in C57BL/6 mice. As shown in Figure 5, a significant reduction in parasitaemia and prolonged surviving time were observed in the immunized groups compared to that of the control groups after challenge with $10^6$ infected red blood cells (iRBCs). The experiment was repeated with BALB/c mice and similar results were obtained (Supplementary Fig. 5). These data indicated that PbTrx-specific antibodies protected the immunized mice against parasite infection.

DISCUSSION

Parasitation in erythrocytes has provided malarial parasites evolutionary advantages to evade host recognition. Erythrocytes only have basal metabolic activity and no antigen presentation pathway, which are ideal hiding places for the parasite to thrive. However, due to their unique function in transportation of oxygen and carbon dioxide, erythrocyte cells need to keep a complete antioxidative system for efficiently detoxifying the radicals and release oxidation stress. On the other hand, oxygen radicals, as components of the host innate immune system, play an import role in defending against invading pathogens, especially microbes. Anti-oxidation is, thus, critical for self-defense or self-protection. Thioredoxins (Trx), existed in nearly all known organisms, are proteins that act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Erythrocytes contain a functional Trx system comprising Trx reductase, Trx, and at least three peroxiredoxins. Further,
three Trx proteins (Trx 1, 2 and 3) and 2 Trx-like proteins (Tlp1 and Tlp2) have previously been reported in *P. falciparum* to have antioxidation activity (39). In a recent study (13), from the heparin-binding proteome we identified a novel Trx-like protein in *P. falciparum*, which showed a general sequence similarity and characteristics to Trx proteins but lacks the “-CXXC-" motif which is the core for anti-oxidation function (Figure 1). Further analysis with sequences extracted from the PlsmoDB database, a number of Trx-like proteins in *P. falciparum* and other malaria parasites which lack the “-CXXC-" motif, but keep a general structure as Trx proteins (Figure 1, Supplementary Figure 1 and data not shown) were identified. The recombinant PfTrx-like-mero protein did not show any anti-oxidation activity also supported the essential role of “-CXXC-" motif (data not show). However, the presence of this protein in all parasites of the *Plasmodium* genus indicating that it may possess an essential function for all *Plasmodial* parasites. Furthermore, we have found the PfTrx-like-mero protein contains GAG binding motif, which is essential for binding to the heparin sulfate-like receptor on the surface of erythrocytes, and the capacity of its binding to heparin was proven in our previous study, from which we concluded that the PfTrx-like-mero protein may play a role in merozoite stage for invasion. However, it could not be ruled out that the PfTrx-like-mero may perform other function considering its distribution inside the erythrocyte after invasion.

A classical signal peptide sequence was predicated at the N-terminus of the PfTrx-like-mero protein (Figure 1c) suggested that the protein may be secreted outside the parasite after expression. Real-time PCR and Western blot assays
indicated that the protein is constantly expressed throughout the entire erythrocytic stages (Figure 2). However, IFA and IEM results clearly showed that this protein is expressed on the surface of merozoites overlapping with MSP-1 protein (Figure 3). The pattern of immuno-EM is different from that of immunofluorescence, which is most likely due to the technical reason. Antigens on the merozoite surface could be lost during sample processing. Since the protein was initially identified by heparin-binding with proteins extracted from late stage parasites (13), its surface location strongly indicated that it may participate in erythrocyte invasion process.

The involvement of the PfTrx-like-mero protein in merozoites invasion was confirmed in several analyses. Firstly, GAG binding motifs were identified in the molecule and the recombinant proteins indeed bound heparin, which supported the previous heparin-based affinity purification and proteomic analysis results. Importantly, the N-terminal region containing the Trx domain as well as the full length protein bound human erythrocytes, but not the C-terminal region (Figure 4a,b) suggesting that the erythrocyte-binding activity was mediated by the N-terminal Trx domain. However, antibodies against three proteins (fragment I, II and III, Figure 1) showed similar inhibitory effect on merozoites invasion (Figure 4c), indicating that the C-terminal region was functionally essential to the molecule during merozoite invasion.

To further prove that the PfTrx-like-mero protein is involved in parasite invasion, homologous protein (Supplementary Figure 1) of P. berghei encoded by PBANKA_0942500 was immunized mice of both C57BL/6 and BALB/c strain. All
immunized mice showed significant protection against parasite challenge, whereas the
mice in the control group were not protected at all (Figure 5 and Supplementary
Figure 5). The data collectively suggested that the novel Trx-like protein of *P. falciparum* is a merozoite-associated molecule participating in erythrocyte invasion.

In summary, we have identified and characterized a novel thioredoxin-like protein
in *P. falciparum*, which was expressed on the surface of merozoites and had an
erthrocyte-binding property. Specific antibodies to the molecule displayed
significant invasion inhibition effect. Further, mice immunized with the homologous
protein showed significant protection against parasite infection. The data facilitate the
understanding of the complexity of *P. falciparum* erythrocyte invasion mechanism
and the malaria vaccine development. (3435 words)

**Figure legends**

**Figure 1.** Sequence analysis and schematic representation of protein encoded by
*PF3D7_1104400*. A, The sequence alignment of the PfTrx-like-mero protein with
homologous protein in other *Plasmodium* species. A predicted Trx domain is shown in
yellow box (38-247 aa) while two GAG-binding motifs are identified in red box
(residues FKKSNKA and FKKADKG). Red stars indicate the three conserved
non-catalytic cysteine residues in the Trx domain. *B*, Trx domain sequence alignment
of the PfTrx-like-mero protein with other Trx proteins. A gap in the PfTrx-like-mero
protein sequence due to the missing of the “-CXXC-” motif is labelled in the red box.
*C*, The schematic illustration of the PfTrx-like-mero protein (encoded by
The PfTrx-like-mero protein consists of 424 amino acid residues with a predicted molecular weight of 49 kDa. A signal peptide (SP, 1-22 aa) is shown in black box with slashed lines. The two GAG-binding motifs were indicated with grey boxes. The three fragments (I, II and III) for expression as recombinant proteins (PfTrx-like-mero protein-N888, PfTrx-like-mero protein-C522, PfTrx-like-mero protein-1101) are illustrated underneath.

Figure 2. Transcription and expression of PF3D7_1104400 gene in the blood-stage of the P. falciparum 3D7 strain. A, Transcriptional analysis of PF3D7_1104400 gene by real-time PCR. The transcription of the gene at 8, 16, 24, 32, 40, and 48 h post erythrocyte invasion is shown. Results are the means and standard deviations of three separate experiments. B, The expression the PfTrx-like-mero protein at the 6 time-points was analyzed by Western-blot assay. HSP70 was used as an internal loading control.

Figure 3. Distribution of the protein on merozoite surface. A, The distribution of the PfTrx-like-mero protein on merozoite in the schizont and before erythrocyte invasion was localized by indirect immunofluorescence. Nuclei are stained with Hoechst (blue color). The PfTrx-like-mero protein (PF3D7_1104400) is illustrated by green fluorescence-labelled antibodies, which was mostly seen on the periphery of the merozoites. B, Colocalization of the PfTrx-like-mero protein with MSP-1 protein. The distribution of the PfTrx-like-mero protein is co-localized with MSP-1 during the
asexual stage. After merozoite formation, the two proteins are distributed on the surface of both free and schizontic merozoites. At the trophozoite stage, the two proteins are distributed on the parasitophorous vacuole (PV) membrane and also inside the PV. Scale bar, 5 µm. C and D, Localization of the PfTrx-like-mero protein by immune-electronic microscopy. Parasites at late schizont (C) and free merozoites (D) were labeled using a protein specific antibody, and detected with a secondary antibody conjugated with gold particles. Gold particles were highlighted with red dashed frames. The protein is mostly localized on merozoite surface. Scale bar, 200 nm.

**Figure 4. Association of the protein (PF3D7_1104400) with merozoite invasion.** A, The recombinant PfTrx-like-mero proteins bind to heparin. The GST-tagged recombinant proteins of the three fragments of the PfTrx-like-mero protein (Figure 1C and Supplementary Figure 2) were respectively incubated with Heparin-Sepharose and their binding activity to heparin was detected by Western blot. GST protein (IV) and uncoupled Sepharose (V) were used as controls. All three fragments bound to heparin, but not to the Sepharose beads. GST did not show any binding to heparin. B, Adhesion of recombinant PfTrx-like-mero proteins to human erythrocytes. The three fragments of the PfTrx-like-mero protein were respectively incubated with human erythrocytes, and the adhesion were detected by Western blot. GST protein (IV) was used as a control. Only the fragments I and III showed binding activity to human erythrocytes, but not the second fragment (fragment II) and the GST control. C, The
PfTrx-like-mero protein (PF3D7_1104400) specific antibodies inhibited parasite invasion. The bar chart shows the inhibitory effect on erythrocyte invasion by the specific antibodies to the three protein fragments (I, II and III) as well as MSP-1. Antibodies from healthy rabbits were used as a negative control and did not show inhibitory effect.

**Figure 5. Protection against parasite infection generated by immunization with rPBANKA_0942500 in C57BL/6 mice.** A, Parasitemia variations in immunized C57BL/6 mice after challenge. The parasitemia of the mice in the naïve group (without any immunization) and control group (immunized only with Freund’s adjuvant) climbed more quickly and were 2.26-fold higher at the 13th day post infection than that of the immunized group with rPBANKA_0942500. The final parasitemia in each group are means of ten mice and the error bars indicate standard deviation (SD). B, The survival rates of the immunized mice after challenge, the data was analyzed by Kaplan-meier test, mice immunized with rPBANKA_0942500 can prolong live time significantly than other groups (p<0.01) post challenge injection.

**Acknowledgement**

This work was financially supported by the National Natural Science Foundation of China (grants 81420108023 and 81772219).

**Conflicts of interest:** All authors have declared that there is no conflict of interest.
References

1. Nkumama IN, O’Meara WP, Osier FH. 2017. Changes in Malaria Epidemiology in Africa and New Challenges for Elimination. Trends in parasitology 33:128-140.

2. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, Battle K, Moyes CL, Henry A, Eckhoff PA, Wengen EA, Briet O, Penny MA, Smith TA, Bennett A, Yukich J, Eisele TP, Griffin JT, Fergus CA, Lynch M, Lindgren F, Cohen JM, Murray CLJ, Smith DL, Hay SI, Cibulskis RE, Gething PW. 2015. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. Nature 526:207-211.

3. White NJ. 2016. Can new treatment developments combat resistance in malaria? Expert opinion on pharmacotherapy 17:1303-1307.

4. Cowman AF, Crabb BS. 2006. Invasion of red blood cells by malaria parasites. Cell 124:755-766.

5. Baum J, Chen L, Healer J, Lopaticki S, Boyle M, Triglia T, Ehlgen F, Ralph SA, Beeson JG, Cowman AF. 2009. Reticulocyte-binding protein homologue 5 - an essential adhesin involved in invasion of human erythrocytes by Plasmodium falciparum. International journal for parasitology 39:371-380.

6. Beeson JG, Drew DR, Boyle MJ, Feng G, Fowkes FJ, Richards JS. 2016. Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. FEMS microbiology reviews 40:343-372.

7. Ockenhouse CF, Angov E, Kester KE, Diggs C, Soisson L, Cummings JF, Stewart AV, Palmer DR, Mahajan B, Krzych U, Tornieporth N, Delchambre M, Vanhandenhove M, Ofori-Anyinam O, Cohen J, Lyon JA, Heppner DG, Group MSPW. 2006. Phase I safety and immunogenicity trial of FMP1/AS02A, a Plasmodium falciparum MSP-1 asexual blood stage vaccine. Vaccine 24:3009-3017.

8. McCarthy JS, Marjason J, Elliott S, Fahey P, Bang G, Malkin E, Tierney E, Aked-Hurditch H, Adda C, Cross N, Richards JS, Fowkes FJ, Boyle MJ, Long C, Druilhe P, Beeson JG, Anders RF. 2011. A phase 1 trial of MSP2-C1, a blood-stage malaria vaccine containing 2 isoforms of MSP2 formulated with Montanide(R) ISA 720. PloS one 6:e24413.

9. Audran R, Cachat M, Lurati F, Soe S, Leroy O, Corradin G, Druilhe P, Spertini F. 2005. Phase I malaria vaccine trial with a long synthetic peptide derived from the merozoite surface protein 3 antigen. Infection and immunity 73:8017-8026.

10. Sheehy SH, Duncan CJ, Elias SC, Biswas S, Collins KA, O’Hara GA, Halstead FD, Ewer KJ, Mahungu T, Spencer AJ, Miura K, Poulton ID, Dicks MD, Edwards NJ, Berrie E, Moyle S, Colloca S, Cortese R, Gantlett K, Long CA, Lawrie AM, Gilbert SC, Doherty T, Nicosia A, Hill AV, Draper SJ. 2012. Phase I clinical evaluation of the safety and immunogenicity of the Plasmodium falciparum blood-stage antigen AMA1 in ChAd63 and MVA vaccine vectors. PloS one 7:e31208.

11. Boyle MJ, Richards JS, Gilson PR, Chai W, Beeson JG. 2010. Interactions with heparin-like molecules during erythrocyte invasion by Plasmodium falciparum merozoites. Blood 115:4559-4568.

12. Vogt AM, Winter G, Wahlgren M, Spillmann D. 2004. Heparan sulphate identified on human erythrocytes: a Plasmodium falciparum receptor. The Biochemical journal 381:593-597.

13. Zhang Y, Jiang N, Lu H, Hou N, Piao X, Cai P, Yin J, Wahlgren M, Chen Q. 2013. Proteomic
analysis of *Plasmodium falciparum* schizonts reveals heparin-binding merozoite proteins.

Journal of proteome research **12**:2185-2193.

14. Cardin AD, Weintraub HJ. 1989. Molecular modeling of protein-glycosaminoglycan interactions. Arteriosclerosis **9**:21-32.

15. Hata A, Ridinger DN, Sutherland S, Emi M, Shuhua Z, Myers RL, Ren K, Cheng T, Inoue I, Wilson DE, et al. 1993. Binding of lipoprotein lipase to heparin. Identification of five critical residues in two distinct segments of the amino-terminal domain. The Journal of biological chemistry **268**:8447-8457.

16. Jortzik E, Becker K. 2012. Thioredoxin and glutathione systems in *Plasmodium falciparum*. International journal of medical microbiology: IJMM **302**:187-194.

17. Arner ES, Holmgren A. 2000. Physiological functions of thioredoxin and thioredoxin reductase. European journal of biochemistry **267**:6102-6109.

18. Hashemy SI, Holmgren A. 2008. Regulation of the catalytic activity and structure of human thioredoxin 1 via oxidation and S-nitrosylation of cysteine residues. The Journal of biological chemistry **283**:21890-21898.

19. Rubartelli A, Sitia R. 1991. Interleukin 1 beta and thioredoxin are secreted through a novel pathway of secretion. Biochemical Society transactions **19**:255-259.

20. Zhang X, Lu J, Ren X, Du Y, Zheng Y, Ioannou PV, Holmgren A. 2015. Oxidation of structural cysteine residues in thioredoxin 1 by aromatic arsenicals enhances cancer cell cytotoxicity caused by the inhibition of thioredoxin reductase 1. Free radical biology & medicine **89**:192-200.

21. Rubartelli A, Bajetto A, Allavena G, Wollman E, Sitia R. 1992. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. The Journal of biological chemistry **267**:24161-24164.

22. Hirota K, Nakamura H, Masutani H, Yodoi J. 2002. Thioredoxin superfamily and thioredoxin-inducing agents. Annals of the New York Academy of Sciences **957**:189-199.

23. Ditgen D, Anandarajah EM, Hansmann J, Winter D, Schramm G, Erttmann KD, Liebau E, Brattig NW. 2016. Multifunctional Thioredoxin-Like Protein from the Gastrointestinal Parasitic Nematodes Strongyloides ratti and Trichuris suis Affects Mucosal Homeostasis. Journal of parasitology research **2016**:8421597.

24. Silberstein DS, McDonough S, Minkoff MS, Balcewicz-Sablinska MK. 1993. Human eosinophil cytotoxicity-enhancing factor. Eosinophil-stimulating and dithiol reductase activities of biosynthetic (recombinant) species with COOH-terminal deletions. The Journal of biological chemistry **268**:9138-9142.

25. Sahaf B, Soderberg A, Spyrou G, Barral AM, Pekkari K, Holmgren A, Rosen A. 1997. Thioredoxin expression and localization in human cell lines: detection of full-length and truncated species. Experimental cell research **236**:181-192.

26. Chen Q, Barragan A, Fernandez V, Sundstrom A, Schlichtherle M, Sahlen A, Carlson J, Datta S, Wahlgren M. 1998. Identification of *Plasmodium falciparum* erythrocyte membrane protein 1 (PFEMP1) as the rosetting ligand of the malaria parasite P. falciparum. The Journal of experimental medicine **187**:15-23.

27. Sanni LA, Fonseca LF, Langhorne J. 2002. Mouse models for erythrocytic-stage malaria. Methods in molecular medicine **72**:57-76.

28. Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal
peptides from transmembrane regions. Nature methods 8:785-786.

29. Chaudhuri R, Ahmed S, Ansari FA, Singh HV, Ramachandran S. 2008. MalVac: database of malarial vaccine candidates. Malaria journal 7:184.

30. O’Driscoll A, Belogrudov V, Carroll J, Kropp K, Walsh P, Ghazal P, Sleator RD. 2015. HBLAST: Parallelised sequence similarity--A Hadoop MapReduceable basic local alignment search tool. Journal of biomedical informatics 54:58-64.

31. Zhao X, Chang Z, Tu Z, Yu S, Wei X, Zhou J, Lu H, Jiang N, Chen Q. 2014. PRON3 is an erythrocyte-binding protein and a potential blood-stage vaccine candidate antigen. Malaria journal 13:490.

32. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

33. Moll K, Chene A, Ribacke U, Kaneko O, Nilsson S, Winter G, Haeggstrom M, Pan W, Berzins K, Wahlgren M, Chen Q. 2007. A novel DBL-domain of the P. falciparum 332 molecule possibly involved in erythrocyte adhesion. PloS one 2:e477.

34. Smith DB, Johnson KS. 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene 67:31-40.

35. Flick K, Ahuja S, Chene A, Bejarano MT, Chen Q. 2004. Optimized expression of Plasmodium falciparum erythrocyte membrane protein 1 domains in Escherichia coli. Malaria journal 3:50.

36. Du F, Wang S, Zhao C, Cao YM, Luo EJ. 2016. Immunogenicity and immunizing protection effect of GAMA gene DNA vaccine on Plasmodium berghei. Asian Pacific journal of tropical medicine 9:158-163.

37. Chang Z, Jiang N, Zhang Y, Lu H, Yin J, Wahlgren M, Cheng X, Cao Y, Chen Q. 2016. The TatD-like DNase of Plasmodium is a virulence factor and a potential malaria vaccine candidate. Nature communications 7:11537.

38. Hui G, Hashimoto C. 2007. Plasmodium falciparum anti-MSP1-19 antibodies induced by MSP1-42 and MSP1-19 based vaccines differed in specificity and parasite growth inhibition in terms of recognition of conserved versus variant epitopes. Vaccine 25:948-956.

39. Nickel C, Rahifs S, Deponte M, Koncarevic S, Becker K. 2006. Thioredoxin networks in the malarial parasite Plasmodium falciparum. Antioxidants & redox signaling 8:1227-1239.

40.
