A Novel Thioredoxin Homologous Protein of *Babesia Microti* Involved in Erythrocyte Invasion

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Abstract

Background: Erythrocyte invasion by merozoites is an important stage in the life cycle of *Babesia microti*. Several merozoite proteins have previously been demonstrated to play important roles in this process.

Methods and Results: We identified a novel merozoite protein of *B. microti* with structural characteristics similar to those of the thioredoxin (Trx)-like domain of the Trx family and named it as *Bmi*Trx-like protein. Western blot assays demonstrated that this protein was continuously expressed throughout the erythrocyte phase and peaked on the 11^{th} day post-infection. Immunofluorescence assay showed that the protein might mainly be expressed on the membrane of *B. microti* merozoites. *Bmi*Trx-like protein had both heparin- and erythrocyte-binding properties, which are critical functions of the invasion-related proteins. Immunization with recombinant *Bmi*Trx-like protein imparted considerable protection against *B. microti* infection in mice.

Conclusions: These results suggest that the novel merozoite protein, *Bmi*Trx-like protein, is an important molecule in the invasion process of *B. microti* and may be a possible target for the design of babesiosis vaccines.

Introduction

Babesiosis is a parasitic disease of the blood caused by *Babesia*, a pathogenic protozoa transmitted between humans and animals by hard ticks. *Babesia*, belonging to the phylum Apicomplexa, comprises more than 100 species, which infect a wide array of wild and domestic animals, but only a few have been shown to infect humans, including *Babesia microti, B. venatorum, B. bovis, B. canis*, and *B. divergens* [1]. Nonetheless, for the majority of the reported cases of human babesiosis, the major etiological species is *B. microti*.

Babesiosis poses a serious threat to immunocompromised individuals. Asymptomatic, mild, and moderate infections generally occur in individuals who are immunocompetent [2–4]. However, severe infections are common among immunocompromised patients and the elderly. Patients with primary or secondary immunodeficiency due to splenectomy and suffering from human immunodeficiency virus infection; cancer; hemoglobinopathy; and chronic heart, lung or liver disease [5–9] tended to have fulminating disease caused by *B. microti*. The fatality rate is up to 21% among those with immunosuppression and 6 to 9% among hospitalized patients [5, 9]. Since Skrabalo and Deanovic first reported a case of human infection with *Babesia* in Yugoslavia in 1957 [10], thousands of human cases have been reported worldwide [11–16]. Indeed, babesiosis is now considered a global parasitic disease of humans and is attracting significant attention. Because of improved detection techniques, increased public awareness, and an increase in the number of individuals with immunodeficiency, many more human cases of *Babesia* are expected to be reported. In view of the prevalence and severity of babesiosis and lack of effective drugs for the treatment of *Babesia* infection, there is an urgent need to identify new vaccines and new drug targets.

Invasion into erythrocytes is the critical step for the successful proliferation and transmission of *Babesia* parasites. During the blood stage of their life cycle, *Babesia* merozoites invade the host erythrocytes, where they develop and multiply. After the divided trophozoites egress from the RBC by rupturing the host cells, the progeny merozoites invade new RBC again [17–19]. Furthermore, extracellular merozoites are directly exposed in the peripheral blood of hosts and can be eliminated by humoral immunity, while the antibodies could never reach the parasites inside the RBCs [19]. Thus, merozoite proteins are potential antigen candidates in babesiosis vaccine development. However, the detailed molecular interactions between *Babesia* merozoites and the host RBCs are incompletely understood. Identification of novel host-microbe molecular interactions during the invasion process is required for the development of preventive measures against babesiosis.

Surface molecules coating the extracellular merozoites attach to target RBC and play key roles in erythrocyte invasion. Several surface-coating molecules of merozoites have been discovered in *Babesia* parasites, such as merozoite surface antigen (MSA)-1, MSA-2a1, MSA-2a2, MSA-2b, and MSA-2c in *B. bovis* [20], surface antigen of *B. microti* merozoites 44 (BmSP44) [21], *B. microti* surface antigen 1 (BmSA1) [22] in *B. microti.* Several glycosaminoglycans (GAG), including sialic acid and heparin sulfate-like moieties on the surface of human erythrocytes have been shown to be receptors for merozoite-derived proteins of *Plasmodium falciparum* [23, 24]. In babesiosis, heparin-binding molecules on the surface of merozoites also play crucial role in the erythrocyte invasion process [25]. The heparin-binding proteome of *P. falciparum* has been elucidated in our previous studies. A novel protein of the thioredoxin (Trx) family, named *Pf*Trx-mero protein, was revealed to have specific binding activity to heparin and judged to be an important ligand participating in erythrocyte invasion by *P. falciparum* [26]. Interestingly, the sequence alignment of *Pf*Trx-mero protein showed a homolog in *B. microti*. Due to the similar mechanism of host-parasite interaction between the two Apicomplexan parasites *Plasmodium* and *Babesisa*, the Trx-like protein of *Babesia* could play an important role in the erythrocyte invasion stage of *Babesia*. Thus, in the present study, we report the discovery of a novel Trx-like protein in *B. microti* and further studied its function and expression. In addition, we assessed the feasibility of using this novel protein as a candidate antigen for *B. microti* vaccines.

Materials And Methods

Ethics Statement

All animal procedures were conducted in accordance with the animal husbandry guidelines of the Chinese Academy of Medical Sciences and with permission from the Experimental Animal Committee of the Chinese Academy of Medical Sciences with the Ethical Clearance Number BYS20010.

Parasite and animals

B. microti strain ATCC® PRA-99™ was obtained from the American Type Culture Collection (Manassas, VA, USA) and stored in liquid nitrogen. Six-week-old male BALB/c mice (special pathogen free) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The parasites were

cultured according to standard methods [27]. Briefly, BALB/c mice were intraperitoneally injected with the immunosuppressant dexamethasone (0.5 mg) every day for five consecutive days to suppress immunity in the mice, which were subsequently intraperitoneally inoculated with frozen *B. microti*-infected blood suspension. Smears were prepared with the tail blood from mice, and Giemsa staining was performed to observe the growth and proliferation of *B. microti.* For the following experiments, BALB/c mice were intraperitoneally injected with 1×10^6 parasitized erythrocytes (parasitemia was approximately 20%). Parasitemia was examined every two days.

Parasites were isolated from infected erythrocytes. The peripheral blood of infected mice was collected and filtered using Plasmodipur (EuroProxima, Amhem, The Netherlands) to remove leukocytes, according to the manufacture's instruction. Saponin (10% in PBS) was used to lyze erythrocytes, and the parasite precipitate was collected for the following experiments.

Sequence analyses of B. microti Trx-like protein

The gene sequence (https://www.ncbi.nlm.nih.gov/nuccore/XM_021482140.1) and amino acid sequence (https://www.ncbi.nlm.nih.gov/protein/1206245601/) of *Bmi*Trx-like protein were obtained from NCBI. The signal peptide and transmembrane regions were predicted using Signal P 4.1 Servery and TMHMM 2.0, respectively [28]. The overall domain structure was then predicted using the CDD protein annotation resource (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The amino acid sequence of *Bmi*Trx-like protein was aligned with *Pf*Trx-like-mero protein using the DNAMAN V6 tool. Further alignments were carried out with *Bmi*Trx-like protein, *Pf*Trx-like-mero protein, and other Trx domain-containing proteins from *Homo sapi*ens (NP_001273876.1), *Mus musculus* (AY243534.1), *Drosophila melanogaster* (NP_572212.1), *Arabiodopsis thaliana* (AAF04439.1), and *Schistosoma japonicum* (CAX71381.1) to determine the functional sites of the Trx domain; all these protein sequences were obtained from NCBI.

Molecular cloning and expression of recombinant proteins

Total RNA was extracted from *B. microti* parasites using TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, USA) as previously described [29]. Genomic DNA was removed from total RNA using the TURBO DNA-free TM Kit (Thermo Fisher Scientific, Waltham, MA, USA), and reverse transcription was performed using SuperScript III Reverse Transcriptase (Thermo Fisher) according to the manufacturer's instructions. The gene fragment encoding BmiTrx-like protein was amplified using high fidelity Phusion DNA polymerase (Finnzymes Oy, Finland). All primers used in this study were designed using Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), as shown in Table 1.

Table 1
List of primers used in this study

Primer name	Primer sequence
*Bmi*Trx –F for clone	GGATCC TCGCTG GTGAATAGGA TGGATC
*Bmi*Trx –R for clone	CTCGAG CTATAG TTCAGAGCGC ACGAC
*Bmi*Trx –F for QPCR	CCCTAGGGCTAAAACGCCAA
*Bmi*Trx –R for QPCR	TTGCAGTGTTTGCAGGTTGA
Bmi-18S–F for QPCR	GTTATAGTTTATTTGATGTTCGTTT
Bmi-18S–R for QPCR	AAGCCATGCGATTCGCTAAT

Bmi: *B. microti*. Trx: Trx-like protein-encoding gene

The amplified product was purified using a DNA Gel Extraction Kit (Axygen, CA, USA), cloned into PGEX-4T-1 and PET-28a expression vectors, respectively, and expressed in *Escherichia coli* BL21 (DE3) [30, 31]. The GST and His-tagged recombinant proteins were purified using glutathione-Sepharose 4B and His Gravi Trap affinity columns (both from GE Healthcare, Uppsala, Sweden), respectively, according to the manufacturer's instructions. All proteins were analyzed by running on a 12% SDS-PAGE and western blotting with monoclonal antibodies to the His-tag or GST-tag (all from Cell Signaling Technology, MA, USA).

Relative expression analysis of BmiTrx-like protein-encoding gene at different stages post-infection

B. microti parasites were collected at 0, 1, 3, 5, 7, 9, and 11 days post-infection. Total RNA was extracted, and reverse transcription was performed as described above. qRT-PCR experiments were performed using the TAKARA SYBR® Premix Ex Taq TM II (Takara, Dalian, China), according to the manufacturer's instructions, on a 7300 Real-Time PCR System (Applied Biosystems Carlsbad, USA). Each reaction was performed in a final volume of 25 µL, containing 12.5 µL of 2 X Brilliant II SYBR green QPCR master mix, 100 ng cDNA, and 1 µL 10 µM paired primers. The PCR program was performed for 40 cycles with denaturation at 95 °C for 30 s, followed by annealing and extension at 60 °C for 1 min on the 7300 Real-time PCR System. Relative expression was analyzed using the SDS 1.4 software (Applied Biosystems). 18S ribosomal RNA of *B. microti* (Bmi18S) (GenBank ID: XM_021481625.1) was used as an internal control. 18S rRNA and BmiTrx-like specific primers for RT-qPCR are listed in Table 1.

Western blot analysis of the recombinant BmiTrx-like and native proteins

To confirm the expression of *Bmi*Trx-like proteins in the parasites, rabbit polyclonal antibodies were prepared at Beijing Protein Innovation (Beijing, China) by immunizing New Zealand white rabbits with

recombinant BmiTrx-like protein. Total protein was extracted from parasites at the indicated time points (0, 1, 3, 5, 7, 9, 11, and 13 days post-infection) with RIPA buffer (Solarbio LIFE SCIENCES, Beijing, China). Protein concentrations were quantified by using a BCA kit (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. The extracted protein was separated on a 12% SDS-PAGE gel and analyzed by western blotting. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). Rabbit anti-r*Bmi*Trx-like protein IgG (1:1,000 dilution) was used as the primary antibody, and IRDye 800 CW conjugated goat anti-rabbit IgG (H + L) antibody (1:10,000 dilution, Li-COR Biosciences, Lincoln, Nebraska, USA) was used as the secondary antibody. In this study, we performed parallel experiments using rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology) as the primary antibody. Detections were then made using Odyssey (Li-COR).

Heparin-binding assay with recombinant proteins

The binding of *Bmi*Trx-like proteins to heparin was studied as previously described [32]. Sixteen micrograms *Bmi*Trx-like protein and equal amounts of GST protein were mixed with 100 μL heparin-sepharose or uncoupled sepharose 4B (all from GE Healthcare) in a 20-μL reaction system in PBS at 25 °C for 2 h. Binding proteins were eluted by using 1 M sodium chloride buffer and detected by western blotting using monoclonal antibodies to the GST-tag.

Binding assay of recombinant proteins to human and mouse erythrocytes

The binding activity of *Bmi*Trx-like proteins to human and mouse erythrocytes was studied as previously described [33]. Thirty micrograms of *Bmi*Trx-like protein and equal amount of GST protein were mixed with 100 μL human or mouse erythrocyte precipitate in a total of 300 μL system in RBC binding buffer (50 mM Tris HCl, 200 mM NaCl, 1 mM EDTA, 2.5 mM $MgCl_2$, 2.0 mM DTT, 1% glycerin, pH 8.0), respectively, at 25 °C for 2 h. The mixture was centrifuged after incubation and washed three times with RBC binding buffer. Binding proteins were subsequently eluted with 1 M sodium chloride. The bound proteins were then detected by western blotting using monoclonal antibodies to GST-tag.

Immunization and challenge experiments

To assess the protective role of *Bmi*Trx-like protein immunization against babesiosis infection, immunization and challenge experiments were carried out with His-tagged recombinant *Bmi*Trx-like protein. Twenty mice were evenly divided into immunization and control groups. In the first immunization, each mouse in the immunization group was subcutaneously injected with 60 μg His-tagged *Bmi*Trx-like proteins emulsified with complete Freund's adjuvant. In the three subsequent immunizations, each mouse was injected with 30 μg recombinant proteins with incomplete Freund's adjuvant every two weeks. Mice in the control group were immunized with PBS and adjuvant only. The antibody titer was measured using ELISA as previously described [33]. After successful immunization, mice were challenged with 10^6 infected iRBCs. Thin blood smears were made with tail blood from mice and stained with Giemsa to

assess parasitemia every other day by counting 1000 red blood cells per smear as previously described [33].

Detection of BmiTrx-like protein by immunofluorescence

To further study the location of *Bmi*Trx-like protein in the parasite, immunofluorescence assay was performed. Blood smears were prepared with tail blood from *B. microti*-infected mice with a parasitemia of 20%. After fixing with methanol for 10 min at 4 °C, the slides were incubated in BSA for 1 h and then washed three times with PBS. Rabbit anti-*Bmi*Trx-like protein serum (diluted 1:500 in 3% BSA) was applied as the primary antibody at 4 °C overnight. The slides were then incubated with Alexa-Fluor®488 conjugated goat anti-rabbit IgG antibody and 4′,6-diamidino-2-phenylindole (DAPI) (all from Invitrogen, Carlsbad, USA). The samples were examined using a confocal laser-scanning microscope (Zeiss LSM 880, Oberkochen, Germany).

Statistical analysis

The data were analyzed using GraphPad Prism 5.0 (GraphPad, San Diego, CA). Two-tailed unpaired Student's t-test was used to compare between two groups. Values of $p < 0.05$ were considered to indicate significant differences.

Results

A novel Babesia protein was found to be a homologue of a Plasmodium heparin-binding merozoite protein.

In our earlier study on heparin-binding merozoite proteins of *P. falciparum*, a novel protein, *Pf*Trx-mero protein, containing a conserved domain of *Pf*Trx-like-mero was identified [26, 33]. In the present study, we report the finding of a novel protein encoded by XM_021482140.1 in *B. microti* (Bmi_XP_021338692.1) (Fig. 1a), which was homologous to *Pf*Trx-like-mero of *P. falciparum*. The amino acid sequence of this *Babesia* protein contains two Trx-like domains (Fig. 1c); thus, we named it as *B. microti (Bmi*) Trx-like protein. The '-CXXC-' motif, which is essential for antioxidant function, was identified within the sequence of most of the Trx-like proteins. However, the '-CXXC-' motif was missing in the sequence of both, *Pf*Trx-mero protein and *Bmi*Trx-like protein (Fig. 1b). The sequence of *Bmi*Trx-like protein contains a 17-aa long signal peptide (Fig. 1c), indicating that the protein may have a role outside of the organism.

Recombinant **Bmi**Trx-like protein could be identified by anti-Babesia serum from **B. microti**-infected mice.

Recombinant *Bmi*Trx-like protein with a His-tag or a Glutathione S-transferase (GST)-tag were expressed, respectively, in *E. coli* and subsequently purified. The molecular weight is 46 kDa for recombinant His-tagged *Bmi*Trx-like protein (r*Bmi*Trx-like protein) and 72 kDa for the GST-tagged 72 protein (Fig. 2a). Anti-*B. microti* serum, obtained from mice 21 days post-infection of *B. microti*, was used to detect the r*Bmi*Trx-like protein by western blotting. As shown in the western blot analyses, recombinant r*Bmi*Trx-like protein could be identified by infected serum but not by serum from uninfected mice (Fig. 2b).

Dynamic transcriptional and protein expression of BmiTrx-like protein in vivo.

Expression of mRNA from *Bmi*Trx-like protein-encoding gene XM_021482140.1 was detected by real-time quantitative PCR (RT-qPCR) at 1, 3, 5, 7, 9, 11, and 13 days post-infection. Specific gene expression was higher on days 5, 7, and 9 post-infection, peaking on the 7th day and then decreasing gradually (Fig. 3a). Rabbit anti-*Bmi*Trx-like protein serum was used to detect native protein; specific bands could be detected in parasite lysates at 1, 3, 5, 7, 9, 11, and 13 days post-infection (Fig. 3b). Again, a protein band with an apparent molecular weight of 46 kDa was observed. The protein expression gradually increased and peaked on the 11th day; a sharp decline of the expression was observed on the 13th day.

BmiTrx-like protein adhered to heparin and host erythrocytes.

To elucidate the role of *Bmi*Trx-like protein in the invasion process, GST-tagged recombinant proteins were separately incubated with heparin-sepharose, human erythrocytes, or mouse erythrocytes, with GST used as the control. As shown in Fig. 4, GST-tagged r*Bmi*Trx-like protein could bind both heparin and host erythrocytes, while GST protein did not show any binding to heparin or erythrocytes.

Immunization with recombinant **Bmi**Trx-like protein protected mice against B. microti infection.

Immunization with *Pf*Trx-mero protein has been proven to provide significant protection against *Plasmodium* infection [33]; thus, we studied whether this is also the case for *Bmi*Trx-like protein against *B. microti* infection *in vivo*. His-tagged *Bmi*Trx-like protein was used to immunize BALB/C mice. After successful immunization (Fig. 5a), a challenge with 1×10^6 *B. microti*-infected red blood cells (iRBCs) was implemented. The parasitemia was significantly lower in the immunized groups; especially at day 9 post-infection, the parasitemia decreased more than 50%, compared to that in the control group (immune group vs control group, 2.43% ± 0.33% vs 5.95% ± 0.30%, $p < 0.0001$, Fig. 5b). The results demonstrate that *Bmi*Trx-like protein immunization could partially protect mice from parasite attack.

BmiTrx-like protein might locate on the surface of merozoites.

The subcellular location of *Bmi*Trx-like protein was detected by indirect immunofluorescence, while DAPI was used to show the nuclear chromatin (Fig. 3c). *Bmi*Trx-like protein (green fluorescence) was observed on the membrane of merozoites that were inside the iRBCs. No green fluorescence was observed when normal rabbit serum was incubated with infected erythrocytes. Likewise, only blue fluorescence was detected when the primary antibody was omitted (only the secondary antibody was incubated with the infected erythrocytes). In addition, no fluorescence was detected when uninfected erythrocytes were probed with rabbit anti-r*Bmi*Trx-like protein serum. Results indicate that the anti-r*Bmi*Trx-like protein serum could specifically identify *Bmi*Trx-like protein that might be expressed on the surface of the merozoite of *B. microti*.

Discussion

Babesiosis imposes an enormous threat on immunocompromised individuals; death from babesiosis occurs in up to 20% individuals from this group. The incidence of *B. microti* infections has increased, and the geographic range has expanded globally in the past two decades [1, 34]. Treatment of babesiosis generally depends on antibiotics, such as atovaquone and azithromycin [35, 36]. However, antibiotic therapy may not adequately treat immunocompromised patients [1]. A potent vaccine based on microbial recombinant antigens would be the most effective tool to prevent the disease. Effective *B. bovis* and *B. bigemina* vaccines have been developed for use in cattle and a *B. rossi* vaccine for dogs [37], but no human *Babesia* vaccine has been developed. Progress has been limited due to the small quantity of identified parasite proteins in human *Babesia*. The exploration of novel proteins that play critical roles in the development stages of *Babesia* will provide candidates for human babesiosis vaccine development.

In the erythrocytic phase of human *Babesia*, merozoite is the only development stage that directly contacts with host peripheral blood [1, 34]. The remaining stages all occur within the erythrocytes. Erythrocytes have only basic metabolic activity and no antigen-presenting pathways; therefore, parasitism of erythrocytes provides *Babesia* with an evolutionary advantage in evading host recognition [1]. Furthermore, merozoite proteins play key roles in erythrocyte invasion by adherence to and penetration into erythrocytes [38]. Thus, the proteins of merozoite, especially surface molecules coating the extracellular merozoites were optimal antigens to be candidates of babesiosis vaccine.

In the present study, a gene encoding a Trx-like homologous protein was identified in *B. microti*. This protein is similar to *Pf*Trx-like-mero protein from *P. falciparum* and named *Bmi*Trx-like protein. A specific band for *Bmi*Trx-like protein in *B. microti* parasites with a molecular weight of approximately 46 kDa was detected by western blotting (Fig. 3b), consistent with the molecular weight of the recombinant *Bmi*Trx-like protein with His-tag (Fig. 2). Western blotting and real-time PCR assays indicated that the protein was continuously expressed in the erythrocyte phase (both sets of expression results were consistent), peaking on the eleventh day post infection (Fig. 3a and b).

*Pf*Trx-like-mero protein is an important ligand participating in erythrocyte invasion [33]. The erythrocytic phase of human *Babesia* is quite similar to that of the malaria parasite, which is also a protozoan belonging to the phylum Apicomplexa [1]. Thus, the *Bmi*Trx-like protein would also be involved in the invasion process. The experiments in our study provided several evidence for the hypothesis. Firstly, *Bmi*Trx-like protein could probably locate on the surface of Babesia merozoite. A classical signal peptide sequence was predicted at the *N*-terminus of *Bmi*Trx-like protein (Fig. 1c), indicating that the protein may be secreted outside the parasite or transported to the surface membrane. Immunofluorescence assays showed that this protein was mainly expressed on the membrane of the merozoite (Fig. 3c). Wang *et al.* reported that the *Pf*Trx-like-mero protein is located on the merozoite surface [33]. Due to the homology of *Bmi*Trx-like protein to the *Pf*Trx-like-mero protein, *Bmi*Trx-like protein was expected to be located on the *Babesia* merozoite surface. Secondly. *Bmi*Trx-like protein has erythrocyte-binding activity. *Bmi*Trx-like protein contains two Trx domains and two GAG-binding motifs (Fig. 1c), which were both speculated to be related to invasion or adhesion of the parasite [33]. *In vitro* experiments showed that the recombinant protein could bind to host erythrocytes (Fig. 4b and c). Further study revealed that *Bmi*Trx-like protein had

heparin-binding activity (Fig. 4a). Heparin-like molecules on the surface of erythrocytes are receptors for merozoite-derived proteins in *Plasmodium* [23, 24]. *T. gondii* surface antigen also has a heparin-binding property and mediates attachment of the tachyzoite to the cellular heparin sulfate proteoglycans of host cells [39]. Thus, the erythrocyte-binding activity of *Babesia* merozoites may be mediated by *Bmi*Trx-like protein and heparin-like molecules interaction. Finally, in vivo assays were carried out to demonstrate the role of *Bmi*Trx-like protein in parasite invasion. BALB/c mice were immunized with purified recombinant protein. All the immunized mice showed significant protection against parasite invasion, while the control group showed no protection at all (Fig. 5b). These data indicate that the *Bmi*Trx-like protein is a merozoite-related molecule involved in *B. microti* invasion.

Conclusion

In conclusion, we have identified a novel Trx-like protein in *B. microti*, named *Bmi*Trx-like protein, which may be mainly expressed on the membrane of the merozoites and demonstrates heparin and erythrocyte-binding properties. Antibodies against *Bmi*Trx-like protein provide significant protection against parasite infection, suggesting the potential value of this novel protein for the development of babesiosis vaccines.

Declarations

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Author Contributions

NH and QC conceived the study. XP and YM designed and performed the majority of the experiments. SL and PC performed some of the experiments. XP, NH, and QC analyzed the data and wrote the manuscript.

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Availability of data and materials

All data supporting the conclusions of this article are included in the article.

Ethics approval and consent to participate

All animal procedures were conducted in accordance with the animal husbandry guidelines of the Chinese Academy of Medical Sciences and with permission from the Experimental Animal Committee of

the Chinese Academy of Medical Sciences with the Ethical Clearance Number BYS20010.

Consent for publication

Not applicable.

Competing interests

All the authors in this study declare that they have no conflict of interests.

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Figures

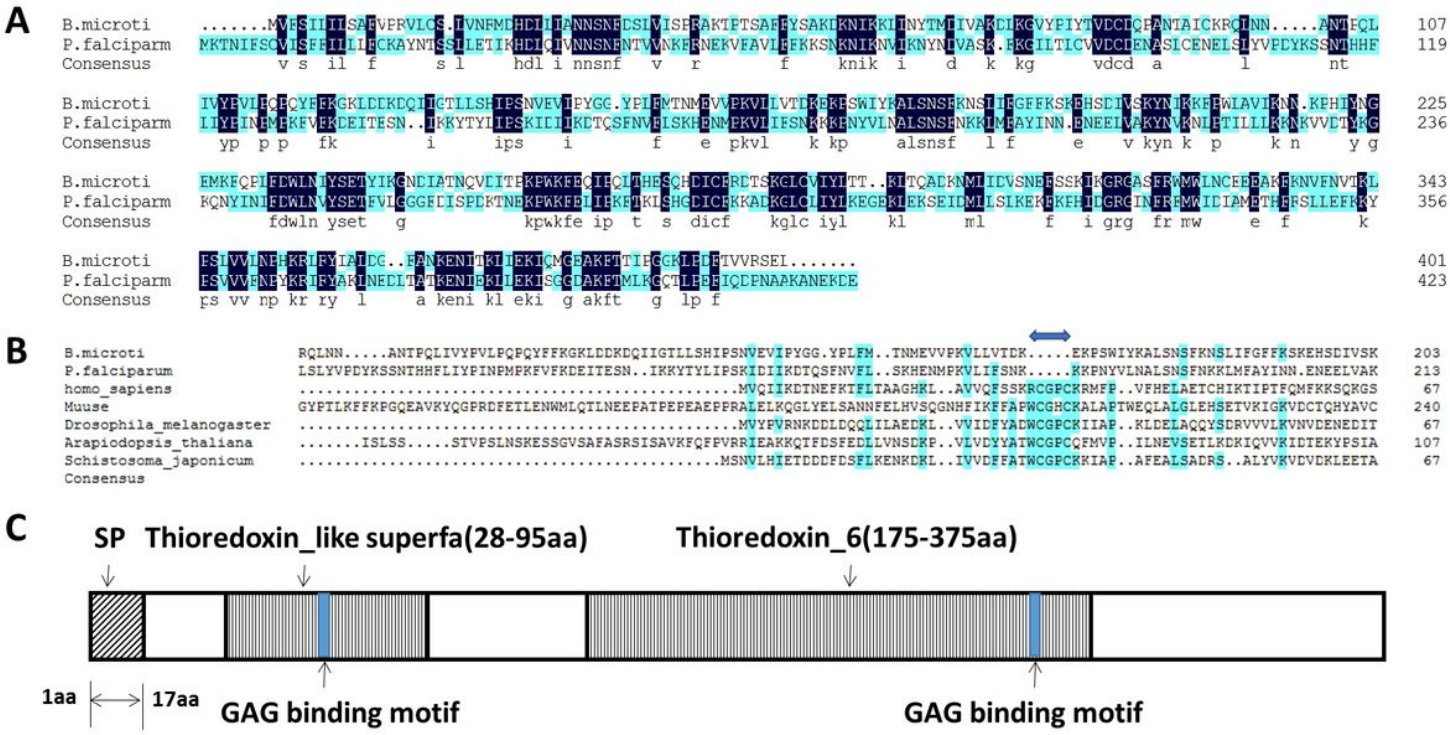

Figure 1

Amino acid sequence alignments and schematic diagram showing the generalized structure of BmiTrx-like protein. (a) Amino acid sequence alignment of BmiTrx-like protein and the homologous PfTrx-like-mero protein. (b) Sequence alignment of the BmiTrx-like protein with other Trx proteins. A gap (blue double-headed arrow) is observed in both the BmiTrx-like protein sequence and PfTrx-like-mero protein sequence due to the deletion of the -CXXC- motif. (c) Schematic diagram of the BmiTrx-like protein. This protein contains a signal peptide (SP; aa 1–17) at the N-terminus and two conserved Trx domains (aa 28–95; aa 175–375). The blue boxes indicate the two glycosaminoglycan (GAG)-binding motifs.

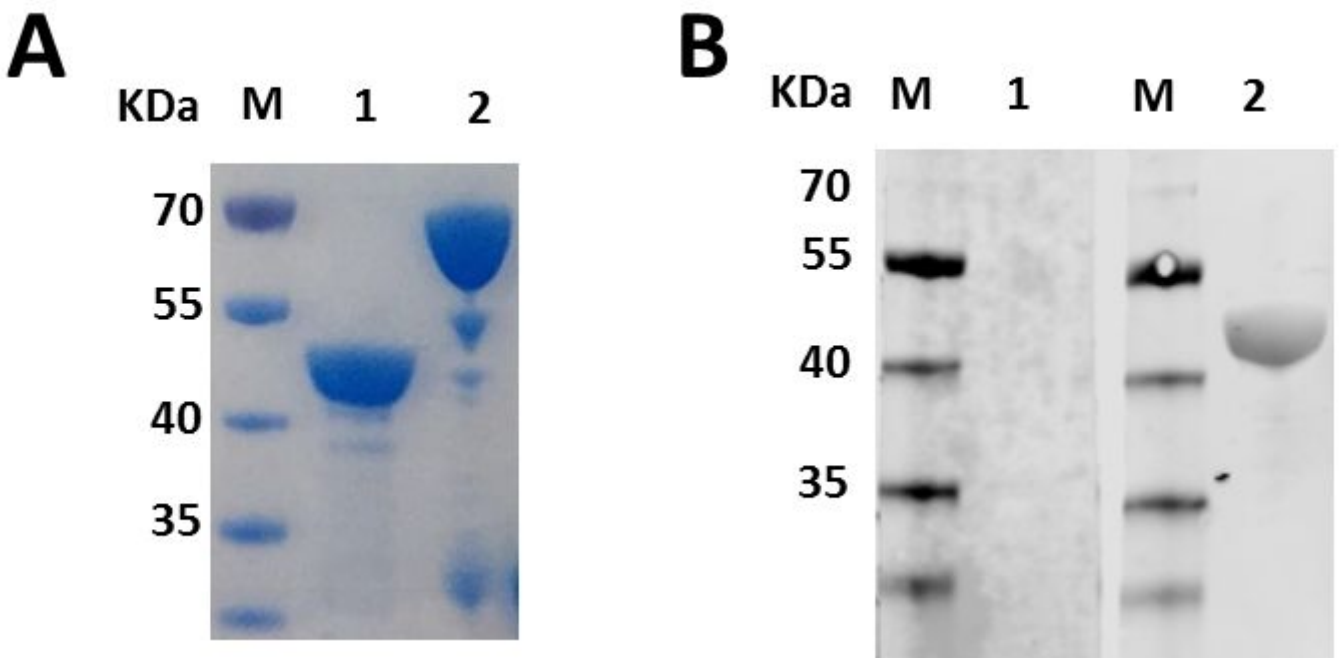

Figure 2

In vitro expression and purification of recombinant BmiTrx-like protein. (a) Recombinant proteins detected by SDS-polyac1ylarnide gel electrophoresis and Coomassie brilliant blue staining. Lane M, standard protein molecular weight marker; Lane 1, His-tagged rBmiTrx-like protein; Lane 2, GST-tagged rBmiTrx-like protein. (b) BmiTrx-like protein-specific antibodies in anti-B. microti serum were detected by western blotting. Lane M, standard protein molecular weight marker; Lane 1, serum from normal mice; Lane 2, serum from mice 21-day post infection of B. microti.

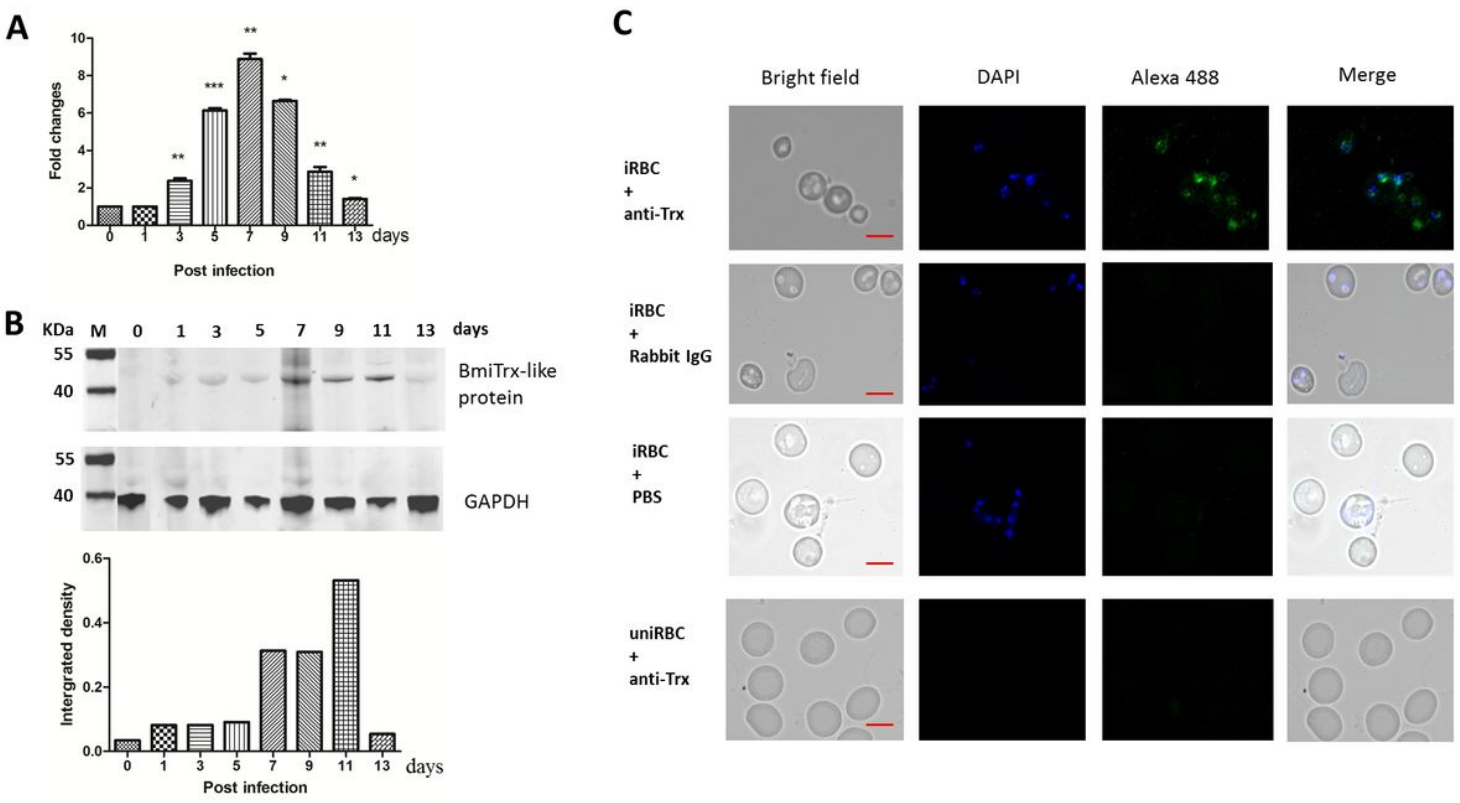

Figure 3

The dynamic transcriptional and protein expression of BmiTrx-like protein in vivo. (a) Transcriptional expression of BmiTrx-like protein-encoding gene mRNA in B. microti parasites from infected mice detected by RT-qPCR. The results are representative of three independent experiments, with data indicating the mean + SD. *, **, ***, Indicate comparison with the previous group *, $p<0.05$, **, $p<0.01$, and ***, $p<0.0001$. (b) Expression of BmiTrx-like protein detected by western blotting. Lane M, standard protein molecular weight marker; Lanes 1–7, Lysates of parasite-infected erythrocytes from Babesia-infected mice on indicated days post-infection. Rabbit anti-BmiTrx-like protein serum was used as primary antibody in this analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference. The column diagram showed the ratio of the bands' density of BmiTrx-like protein compared to that of GAPDH. (c) Intracellular expression of BmiTrx-like proteins detected by immunofluorescence assay. Thin blood smears were prepared with tail blood with infected RBCs (iRBC) from B. microti-infected BABL/c mice with a parasitemia at about 20% or with tail blood with uninfected RBCs (uRBC) from uninfected mice. BmiTrx-like protein was treated with rabbit anti-rBmiTrx-like protein serum (anti-Trx), rabbit IgG control, or PBS control, followed by anti-rabbit IgG secondary antibody conjugated with Alexa-488 (green). Nuclei were stained with DAPI (blue). Scale bar, 5 μm.

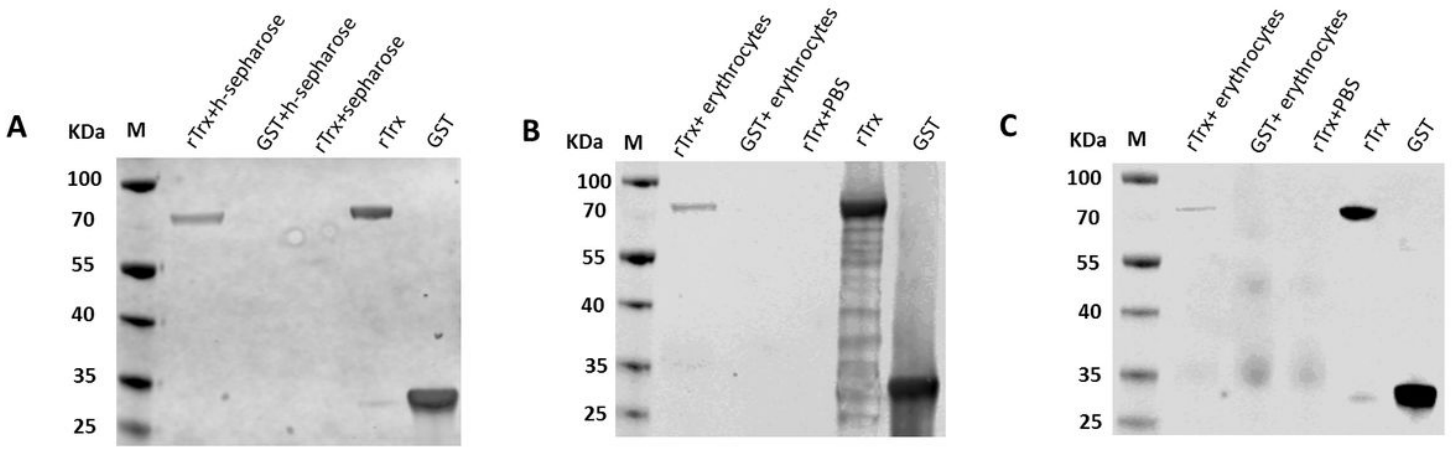

Figure 4

Binding activity of BmiTrx-like protein. rBmiTrx-like protein (rTrx) and GST protein (GST) were incubated with (a) heparin-sepharose (h-sepharose), (b) human erythrocytes, or (c) mouse erythrocytes at 25°C for 2 h. rBmiTrx-like protein incubated with sepharose without heparin or PBS without erythrocytes as negative control. Eluted proteins were collected and detected by western blotting. The recombinant proteins (rTrx, lane 4; GST, lane 5) were used as positive controls. Rabbit anti-GST Tag monoclonal antibody was used as primary antibody. The results are representative of three independent experiments.

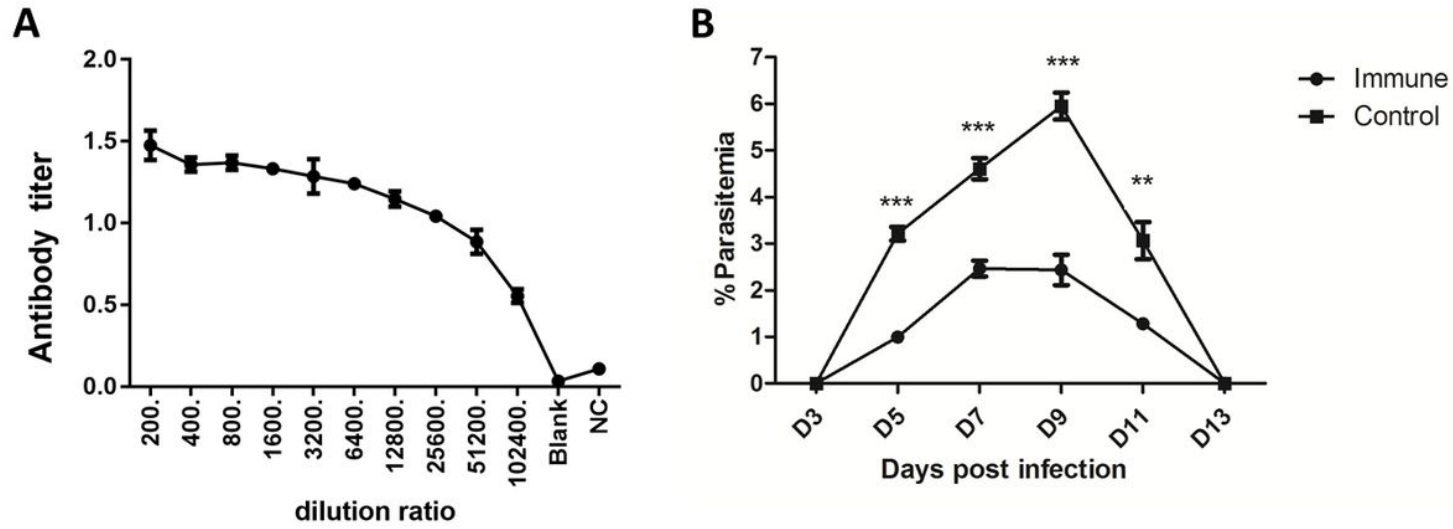

Figure 5

Immunization with recombinant BmiTrx-like protein protected mice against B. microti infection. His-tagged BmiTrx-like protein (immune group) or PBS control (control group) was used to immunize BALB/C mice. (a) The titer of antibodies in mice sera was detected by ELISA after the fourth booster immunization and defined as the dilution that indicated half of the highest optical density. The blank and negative control (NC) wells were incubated with PBS and negative serum, respectively. (b) After successful immunization, a challenge with 1 × 106 B. microti-infected red blood cells (iRBCs) was implemented. Parasitemia was examined with tail blood by performing Giemsa staining. The results are

representative of two independent experiments. Data indicate the mean ± SD. *, **, ***, Indicate comparison with the previous group, *, $p<0.05$, **, $p<0.01$, and ***, $p<0.0001$.

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