Identifying the Red Blood Cell interacting molecule of *Babesia microti*

A thesis

submitted by

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Abstract

Babesia microti is an emerging human pathogen that infects red blood cells (RBCs) and causing severe disease in immunocompromised population. Despite its clinical importance, relatively little is known about the molecular mechanisms of RBC invasion by *B. microti*. We employed phage display technology to identify molecules that mediate parasite-host interaction of *B. microti*. From this screen, we identified *B. microti* secreted antigen 1 (BmSA1) as a molecule that binds to human RBCs. Recombinant BmSA1 protein expressed in *E. coli* bound to human and mouse RBCs *in vitro*. The RBC binding of BmSA1 was not sensitive to neuraminidase, trypsin or chymotrypsin treatment of human RBCs. The binding of BmSA1 was slightly less to the mouse reticulocytes enriched RBCs as compared to normal RBCs, consistent with the previous observation that *B. microti* primarily invades mature RBCs in mice. Taken together, we propose that BmSA1 is a parasite ligand utilized by *B. microti* to bind host RBCs upon invasion process.

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List of Abbreviations

BLAST	Basic Local Alignment Search Tool
BmSA1	*Babesia microti* surface antigen 1
bp	base pair
cDNA	complementary DNA
DNA	deoxyribonucleic acid
EBL1-F2i	erythrocyte-binding ligand-1, EBL-1 domain F2i
GPI	glycosylphosphatidylinositol
mAb	monoclonal antibody
mg	milligram
ml	milliliter
NCBI	National Center for Biotechnology Information
PBS	phosphate buffered saline
PHZ	Phenylhydrazine
pfu	plaque forming unit
RBC	red blood cell
RNA	ribonucleic acid
Trx	Thioredoxin

Introduction

1.1 Epidemiology and clinical features of Babesiosis

Babesiosis is a malaria-like disease caused by protozoan parasites of genus Babesia that infect host red blood cells [1]. The disease is name after Victor Babes, the microbiologist who discovered intra-erythrocytic organism that caused febrile hemoglobinuria in cattle at 1888[2].

The first documented case of human babesiosis was found in splenectomized Croatian subject attributed to *Babesia divergens* [3]**,** while the first case in immunocompetent host was identified in Nantucket Island, Massachusetts in 1964[4]. *Babesia microti* is the most prevalent cause of human babesiosis in the United States and endemic in the Northeast and upper Midwest of the US [5].

Clinical manifestations of babesiosis commonly include fever, chills, sweats, muscle and joint pain. Laboratory features are notable for hemolytic anemia, thrombocytopaenia, elevated liver function test, and decreased kidney function [6]. While most cases of *B. microti* infection in the healthy human host are mild or asymptomatic, patients over 50 years and immunocompromised population (e.g., patients with HIV, malignancy or asplenia) are at higher risk for complicated disease. Severe babesiosis can present as acute respiratory failure, congestive heart failure, renal failure, and/or disseminated intravascular coagulation [7].

B.microti is transmitted to people either through vectors - the deer tick Ixodes scalpularis, its reservoir - the white-footed mouse, as well as blood transfusion [3]. The Food and Drug Administration reported *B. microti* as the most common transfusion-

transmitted pathogen [8, 9]. Since there is a large pool of asymptomatic human hosts in the endemic area, acquiring *B. microti* infection through blood transfusion is a real and serious threat for susceptible immunocompromised patients. Therefore, an efficient screening system to detect *B. microti* in the blood supply is of urgent priority.

1.2 The use of phage display technology for identifying host-pathogen interaction molecules

Phage display is a system in which the library of peptides or proteins is expressed on the surface of phage particles. The segments of foreign DNA are incorporated into the genome of phages, and expressed as fused peptide/protein on the surface of virions. The phages multiply by infecting standard recombinant DNA host - bacterium *Escherichia coli.* A phage-display "library" is a heterogeneous mixture of large number of phage clones, each carrying a different foreign DNA insert and therefore displaying a different peptide on its surface [10].

The displayed peptide behaves as it would if it were not attached on the surface of phage particle [10]. Therefore, peptide that is a ligand for receptors still retains specificity and affinity to its particular receptors in the form of phage displayed peptide. By selecting phage clones through the binding to particular molecules, it is possible to screen a large number of peptides or proteins for specific molecular interactions [11, 12].

The potential ligands of particular receptors can be screened by affinity purification or biopanning. An immobilized receptor is used to capture the ligand from a heterogeneous mixture of phage-displayed peptides. The captured peptides are amplified by infecting the phages to bacterial culture[10]. The utilization of this method is well demonstrated by Lauterbach et al. and Li et al [11, 12].

Lauterbach et al. (2003) have generated phage displayed cDNA library from *Plasmodium falciparum* FCR3 strain to identify the host-pathogens interaction molecules of the parasite. Seven *P. falciparum* proteins have been identified as specific binders to the immobilized human erythrocyte spectrin and protein 4.1 [11].

The study of Li et al. (2012) used the same method as Lauterbach et al. to generate the phage displayed cDNA library. However, purified human glycophorins and intact RBC were used in biopanning [12].

Since there is available mouse model of *B. microti* infection to be utilized as RNA source to generate a cDNA library [13, 14], we used the phage display technology to identify RBC binding molecules of *B. microti*. Despite using the immobilized receptors to screen for potential host-pathogens interaction molecules, intact RBCs were used for biopanning since little known about the *B. microti* interacting molecules of host cell.

1.3 Mechanism of red blood cell invasion by *Babesia microti*

Babesia, like Plasmodium, is an obligatory intracellular organism that requires to be inside the host cell to grow and reproduce. It is suggested that the mechanism of RBC invasion of Babesia shares some similarities with malaria parasites of Plasmodium genus [15]. The RBC invasion process by Babesia has been studied using interference microscopy as well as electron microscopy. The first step of invasion is the moment when merozoite of Babesia randomly collides with RBC and reorients its apical end leading to formation of a tight junction. The following step is the engulfment of merozoite into RBC, and finally the formation of an intracellular ring by the intracellular parasite [15, 16].

In the case of Plasmodium, a large number of molecules have been identified as the parasite ligands that mediate interaction with the host RBCs and play roles in the invasion process [17]. However, little is known about the molecules of *B. microti* that mediate RBC interaction and invasion. Identification of such parasite molecules is important for developing an efficient screening system to detect *B. microti* and to control its infection.

Published evidence indicates that Babesia does not invade host cells other than red blood cells [13, 18], implyingthe existence of specific receptors on the surface of red blood cell that bind to complementary ligands in Babesia. In this study, we utilized human RBCspre-treated with trypsin, chymotrypsin, and neuraminidase as bait to identify and characterize the potential ligand-receptor interactions of *B. microti* using the phage display cDNA library screening technology.

1.4 B. microti tropism towards different maturation stages of RBCs

Some Babesia species vary in their tropism toward RBCs. For example the canine *B. gibsoni* preferentially invades reticulocytes whereas the murine *B. hylomysci* primarily multiplies inside mature RBCs [19, 20]. However, the tropism of *B. microti* in rodents has not clearly described as yet. Two earlier studies in 1969 and 1989 indicated that *B. microti* is ambivalent, but the later report by Borggraefe et al. using the zoonotic isolate of *B. microti* suggested that the parasite primarily multiplies inside mature RBCs [13]. Here we tested *B. microti* peptide isolated from phage display cDNA library for binding with mouse mature RBCs as well as with reticulocyte rich blood.

Materials and Methods

2.1 Construction of the *B. microti* phage display cDNA library

The *B. microti* RM/NS strain [13] was used as the source of the RNA for library. The immune-deficient RAG1$^{-/-}$ mouse was inoculated with the frozen stock of RM/NS, and the blood was collected when the parasitemia reached >50%. Total RNA was prepared using TRIzol plus RNA purification kit (Ambion) from the infected RBCs after carefully removing the buffy coat containing white blood cells from top of the RBC pellet. The mRNA was isolated from total RNA with two rounds of the purification using Dynabeads mRNA purification kit (Ambion). The phage display cDNA library was constructed using OrientExpress random prime cDNA system (Novagen) and T7Select 10-3b phage display system (Novagen). The cDNA was synthesized from 3.8 μg of the mRNA using 1.0 μg of HindIII random primers. The cDNA was end modified with EcoRI/HindIII linkers and digested with EcoRI and HindIII. Small cDNA products (< 300 bp) and excess linkers were removed by gel filtration. Finally, the cDNA was ligated with T7Selects 10-3b EcoRI/HindIII vector arms, and packaged into phage by using T7Select packaging extract.

2.2 Phage display screening for human RBC binding proteins

B. microti cDNA phage display library was screened by the biopanning system based on human RBC binding. Human RBCs were washed three times with phosphate buffered saline (PBS) and resuspended in the RBC biopanning buffer (RPMI 1640 medium pH stabilized with 10 mM HEPES, pH 7.4 and 10 % fetal bovine serum). Human RBCs of 20 μl packed volume and phage display library corresponding to 1 x 10^8

pfu were mixed in the total of 1.0 ml RBC binding buffer and incubated at room temperature for 1.0 hour on a rotator. After the incubation RBCs were separated from unbound phage by centrifuging through 500 µl of silicon oil 15000 x *g* 1 min. RBCs were further washed three times with PBS, and treated with 1.5 M NaCl to elute the bound phage. Eluted phage were amplified in the liquid culture of the host *E. coli* BLT5403 and used for the further round of biopanning. After four rounds of the biopanning selection, phages were plated on a lawn of the host *E. coli* BLT5403 to form the plaques. The DNA was extracted from each plaque with 10 mM EDTA, pH 8.0 at 65°C for 10 minutes. The inserts were PCR amplified using a set of primers T7UP 5'-GGAGCTGTCGTATTCCAGTC and T7DOWN 5'-AACCCCTCAAGACCCGTTTA. The PCR products were purified using E.Z.N.A. Cycle Pure kit (OMEGA Bio-tech), and DNA sequences were determined with T7DOWN primer obtained from the Tufts University Genomics Core Facility.

2.3 Recombinant protein expression

The cDNA insert of BmSA1 (146-213) was amplified from the phage DNA by PCR using T7UP and T7DOWN primers, digested with EcoRI and HindIII, and cloned in the compatible EcoRI and HindIII site of pET32b plasmid (Novagen) to create pET32b/BmSA1 (146-213). The host *E. coli* BL21 (DE3) was transformed with the plasmid and the recombinant protein was expressed and purified using High Density Nickel affinity beads (Gold Biotechnology) as described [12]. The purified recombinant protein was dialyzed against PBS and used for the RBC binding assays.

2.4 *In vitro* RBC binding assay

The purified recombinant BmSA1 (146-213) protein contains thioredoxin (Trx) tag derived from the pET32b vector in its N-terminus; therefore we call the recombinant protein as Trx-BmSA1. The RBC binding was performed essentially as done in biopanning described above except that the binding was performed in the RBC binding buffer (PBS supplemented with 3% bovine serum albumin). After the Trx-BmSA1 protein was eluted from the RBCs with 1.5 M NaCl, it was resolved in SDS-PAGE and transferred on nitrocellulose membrane, 0.45 μm (BioRad). The Trx-BmSA1 was detected by Western blotting using anti Trx-tag monoclonal antibody (GenScript) and goat anti mouse IgG (H+L)-HRP conjugated secondary antibody (BioRad). The signal was detected using SuperSignal West Pico Chemiluminescent Substrate kit (Thermo).

2.5 Enzymatic treatment of human RBCs

The 20 μl packed volume of human RBCs were resuspended in PBS and incubated with either neuraminidase 100 mU/ml (Sigma), trypsin 1.0 mg/ml (Sigma) or chymotrypsin 1.0 mg/ml (Sigma) at 37°C for 1.0 hour. The trypsin and chymotrypsin treated human RBCs were further treated with soybean trypsin/chymotrypsin inhibitor 1.0 mg/ml (Sigma) and incubated at 37°C for 15 minutes. The enzyme-treated human RBCs were washed three times with PBS prior to the binding assays.

2.6 Preparation of the mouse reticulocytes

To induce reticulocyte production, male C57BL/6 mice were injected with phenylhydrazine hydrochloride (Sigma) at the dose of 60 mg/kg intraperitoneally at day 1

and day 2. Blood was collected via cardiac puncture in EDTA coated tube (BD biosciences) at day 5. Reticulocytes were isolated through two rounds of the Percoll centrifugation. Sterile isotonic Percoll solutions of densities 1.100 and 1.058 g/mL were prepared by diluting Percoll (Pharmacia) with RPMI 1640 medium containing 1.5 mg/ml bovine serum albumin. In the first round of centrifugation, 2 ml of mouse blood was layered over 5 ml of Percoll solution (density 1.058 g/ml) and centrifuged at 1,500 rpm for 30 minutes at 4°C in a swing-out tube holder centrifuge (Beckman model J-6B). The mature RBCs and reticulocytes were collected by centrifugation at the bottom of the tube. In the second round of centrifugation, the RBC pellet was washed two times and resuspended with the RPMI 1640 medium, layered over 5.0 ml of Percoll solution (density 1.100 g/ml) and centrifuged at 1,500 rpm for 30 minutes at 4°C. Reticulocytes were recovered at the top of Percoll solution (density 1.100 g/ml).

Results

3.1 Random primed phage display cDNA library characterization

Total number of the cDNA clones in the *B. microti* library was determined as 1.05 x 10^6 by plaque assay. The library was amplified once using *E. coli* BLT5403 as host by the plate method. The amplified library had a titer of 1 x 10^{10} pfu/ml. Most of the phage clones contained the insert ranging between 300 bp and 1000 bp (Fig. 1).

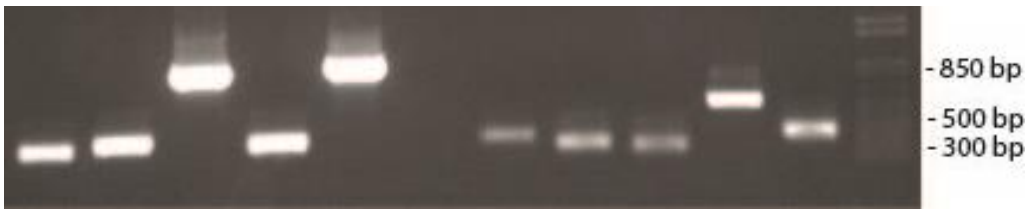

Figure 1. Gel electrophoresis of phage clones DNA that were isolated from plaque assay

3.2 Phage display screening identified BmSA1 as a human RBC binding protein

To search for RBC binding protein of *B. microti*, we constructed a phage display cDNA library from mouse RBCs infected with *B. microti*. We selected phage clones with four rounds of biopanning system based on the human RBC binding. The DNA sequence of selected clones was analyzed for homology using BLAST database of NCBI. Among the selected clones was a 400 bp cDNA (Fig. 2) encoding amino acid 146–213 of BmSA1 (Fig. 3). This screen was originally performed by Gregory Mines in our laboratory under the guidance of Dr. Toshihiko Hanada.

BmSA1, also known as BMN1-9, contains a signal sequence in its amino terminus and glycosylphosphatidylinositol (GPI) anchor motif in the carboxyl terminus

[21, 22]. BmSA1 is expressed as the membrane-associated protein but also found in secreted form present in the serum of infected host animal [21]. It is one of the candidate antigens for the assay system detecting *B. microti* infection [23, 24]. Based on its known properties as a membrane-associated antigen expressed on the parasite, we investigated BmSA1 as a strong candidate for a RBC binding protein of *B. microti*.

Figure 2. Gel electrophoresis of selected phage clones DNA by untreated human RBC biopanning. Lane 1: BmSA1; 2: uncharacterized protein, *B. microti* chromosome III; 3: empty lane; 4: mouse hemoglobin; 5: mouse hemoglobin; 6: uncharacterized protein, *B. microti* chromosome III; L: 1 kb plus DNA ladder

3.3 Recombinant BmSA1 protein binds to both human and mouse RBCs *in vitro*

The cDNA insert coding for BmSA1 (146-213) was cloned in pET32b plasmid vector for recombinant protein expression in *E. coli* (Fig.4). The pET32b vector attaches His-tag and Trx-tag in the amino terminus of the recombinant protein for the convenience of the purification and the detection by Western blotting. Purified recombinant Trx-BmSA1 protein bound specifically to the human RBCs whereas the control Trx protein did not (Fig.5). Since *B. microti* infects both human and mouse RBCs, we tested the binding of recombinant Trx-BmSA1 with mouse RBCs. The recombinant Trx-BmSA1 bound to the mouse RBCs as well (Fig.4).

(B)

SFKPTIITAF AAFLAFGNIS PVLSAGGSGG NGGNGGGHQE QNNANDSSNP TGAGGQPNNE
SKKKAVKLDL DLMKETKNVC TTVNTKLVGK AKSKLNKLEG ESHKEYVAEK TKEIDEKNKK
FNENLVKIEK KKKIKVPADT GAEVDAVDDG VAGALSDLSS DISAIKTLTD DVSEKVSENL
KDDEASATEH TDIKEKATLL QESCNGIGTI LDKLAEYLNN DTTQNIKKEF DERKKNLTSL
KTKVENKDED YVTHFRDMAT EAQNAVGEVK KAIDAVVAHR KAENLDVDDT LFSNLSTLLD
TIIETSRAYL PGVAFALLSS VAMFLF

Figure 3. Schematic representation of the primary structure of BmSA1. (A) BmSA1 consist of signal peptide in its N-terminus. (B) The amino acid sequence of BmSA1. The dotted part in (A) and underlined part in (B) represent the sequence of BmSA1 that was isolated from phage display cDNA library screening.

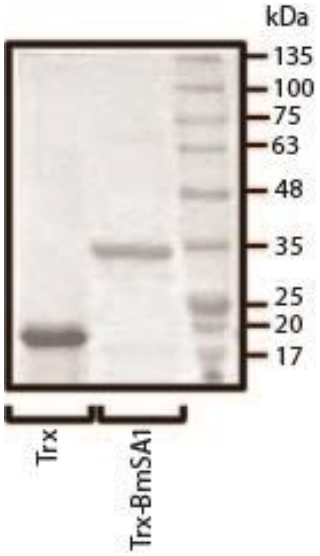

Figure 4. Coomassie stained SDS-PAGE of Trx-BmSA1 and Trx purified recombinant protein expressed in *E. coli* BLT5403. The protein ladder is also shown. Tris-Glycine 4-20%.

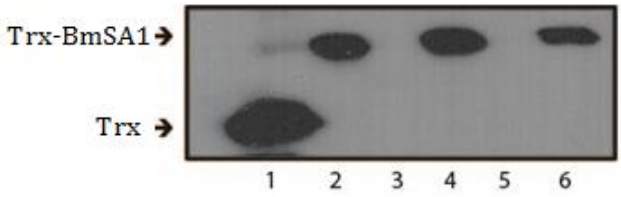

Figure 5. Western blot using Anti-Trx mAb showing the binding of BmSA1 with untreated human and mouse RBCs. Lane 1, Trx control (1µM); lane 2, Trx-BmSA1 control (1µM); lane 3, untreated human RBCs + Trx (1µM); lane 4, untreated human RBCs + Trx-BmSA1 (1µM); lane 5, mouse RBCs + Trx (1µM); lane 6, mouse RBCs + Trx-BmSA1 (1µM).

3.4 BmSA1 binding is not diminished by the enzymatic treatment of RBCs

It has been reported that *B. divergens* invasion of the RBCs was significantly inhibited by the treatment of the RBCs with neuraminidase[17]. Neuraminidase cleaves sialic acid residues from glycoproteins. Since glycophorins are the major sialo-glycoproteins on the RBC surface, it has been suggested that glycophorins serve as the receptor for invasion of *B. divergens*. Other enzymes such as trypsin and chymotrypsin digest certain proteins on RBCs. Therefore treatment of RBCs with these enzymes have been widely used to evaluate the requirement of RBC surface molecules for the interaction and invasion of Babesia and Plasmodium [15]. So far, there is no report concerning the sensitivity of *B. microti* invasion of the enzyme treated RBCs. We tested BmSA1 binding to human RBCs treated with neuraminidase, trypsin, and chymotrypsin. These enzyme treatments did not affect the RBC binding of BmSA1 (Fig. 6a). As shown before, the RBC binding of EBL-1, a *Plasmodium falciparum* protein that binds to glycophorin B [12], was sensitive to neuraminidase and chymotrypsin treatments of

human RBCs (Fig.6b), showing that the enzymatic treatments of RBCs were properly achieved in these experiments. These results suggest that BmSA1 binds to RBCs via a receptor that is not sensitive to neuraminidase, trypsin, and chymotrypsin. The western blot result was a representation of three different experiments.

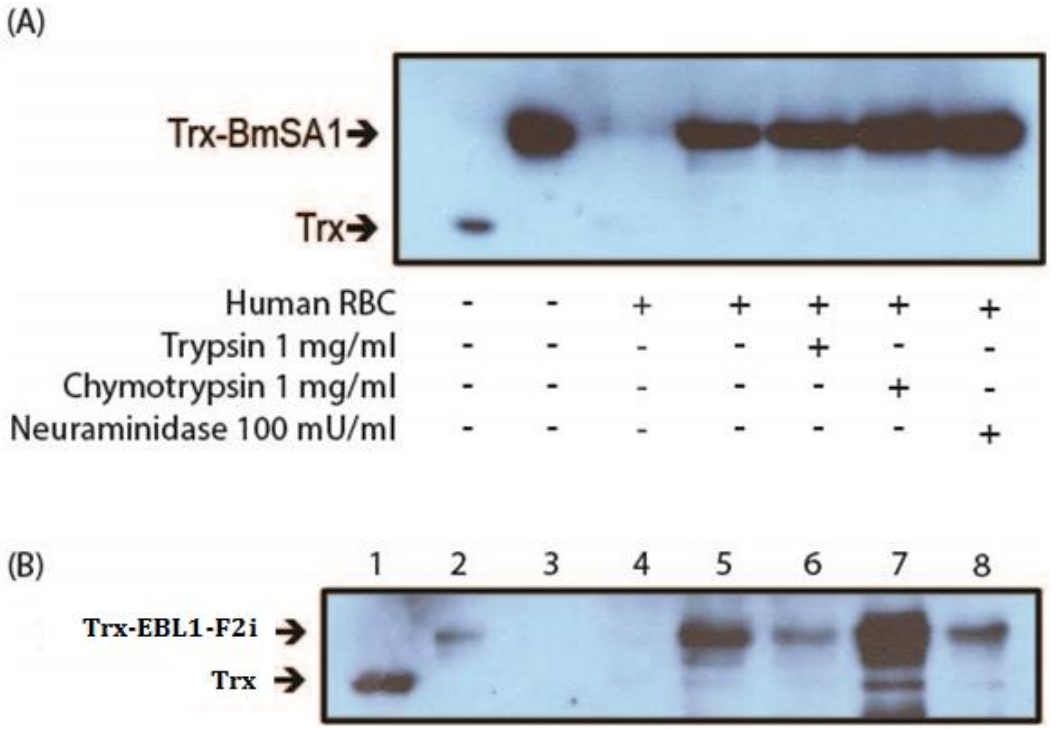

Figure 6. Western blot using Anti-Trx mAb showing the binding of BmSA1 human RBC is not diminished by enzyme treated. (A) The binding of BmSA1 with both untreated and enzyme treated human RBC. (B) Showing that trypsin and neuraminidase treatment diminished the binding of EBL1-F2i to human RBC. Lane 1, Trx control (1µM); lane 2, Trx-EBL1-F2i control (1µM); lane 3, BioRad standard protein ladder; lane 4, untreated human RBCs + Trx (1µM); lane 5, untreated human RBCs + Trx-EBL1-F2i (1µM); lane 6, chymotrypsin treated human RBCs + Trx-EBL1-F2i (1µM); lane 7, trypsin treated human RBCs + Trx-EBL1-F2i (1µM); lane 8, neuraminidase treated human RBCs + Trx-EBL1-F2i (1µM).

3.5 Binding of BmSA1 with mouse reticulocytes

It has been shown that *B. microti* invades preferentially mature erythrocytes rather than immature reticulocytes of mice *in vivo*, although there are other species of Babesia that show preference to reticulocytes [13]. We tested whether BmSA1 binding shows selectivity to either reticulocytes or mature erythrocytes. Reticulocytes were prepared from mice that were injected with PHZ to induce anemia. Injection of PHZ induce the destruction of mature RBC membrane and stimulate the release of immature RBC or reticulocyte to the systemic circulation. After separation with Percoll density gradient centrifugation, reticulocytes proportion appeared to increase as determined by new methylene blue stained blood smears. The reticulocytes differ from mature RBCs in the blood smear by the present of reticula as overlapping "stick shape" inside the RBCs (Fig.7A). The manual cell count on the blood smears showed that the reticulocyte proportion per 1,000 RBCs in PHZ treated and Percoll centrifugation group was 76.6% (SD ± 11.4) compared to untreated C57BL/6 mice 9.3% (SD ± 0.8) .

Using 60% preparation of reticulocytes, the binding of BmSA1 to reticulocytes was slightly less as compared to normal RBCs (Figure 8). The difference of the binding was observed qualitatively by comparing the difference of bands' intensity on the western blot film. This result is consistent with the previous report that *B. microti* primarily invades mature erythrocytes in mice [13].

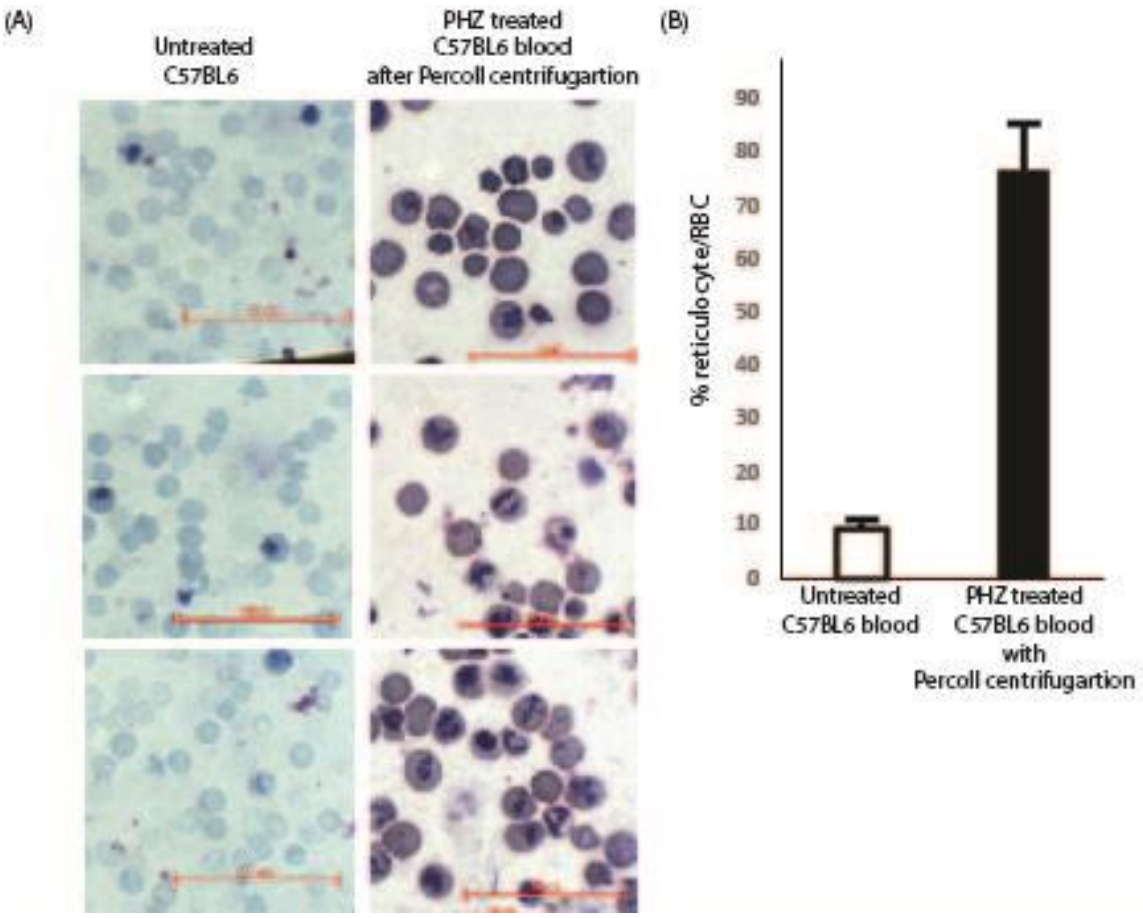

Figure 7. Reticulocyte proportion in C57BL/6 mice was increased after PHZ injection and Percoll gradient density centrifugation. (A) New methylene blue stained blood smear of untreated group and PHZ treated with Percoll centrifuged C57BL/6 mice blood, from 3 independent experiments. (B) The comparison of reticulocyte proportion between untreated group (n=3) and PHZ treated with Percoll centrifuged C57BL6 mice blood (n=3).

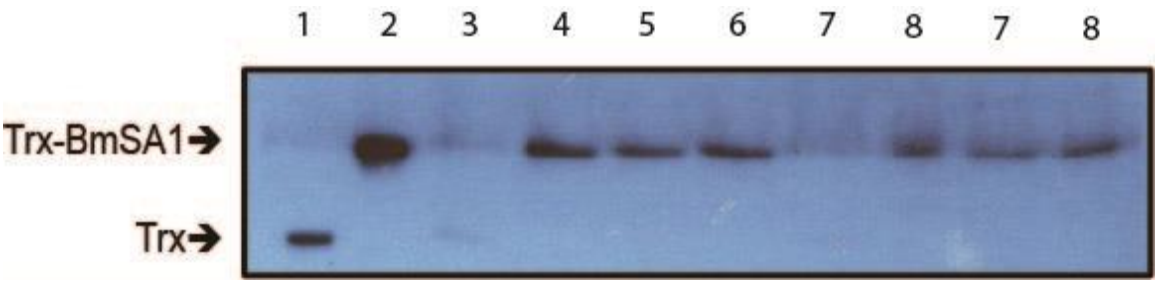

Figure 8. Western blot using Anti-Trx mAb showing the binding of BmSA1 with mouse mature RBC and mouse reticulocyte. Lane 1, Trx control (1μM); lane 2, Trx-BmSA1 control (1μM); lane 3, mouse mature RBCs + Trx (1μM); lane 4-6, mouse mature RBCs + Trx-BmSA1 (1μM) from 3 experiments; lane 7, mouse reticulocytes + Trx (1μM); lane 8-10, mouse reticulocytes + Trx-BmSA1 (1μM) from 3 separate experiments.

Discussion and Future Directions

The RBC invasion of Babesia is a critical step in the life cycle of the parasite. It is known that Babesia and Plasmodium share similarities in the process of RBC invasion [15]. First step is the initial attachment of the parasite to the RBC surface before reorienting its apical ends towards the RBC. In Plasmodium, the proteins expressed on the surface of the merozoite, termed merozoite surface proteins (MSPs) mediate the initial attachment step. One characteristic feature of Plasmodium MSPs is that they are GPI-anchored proteins. Since MSPs are expressed uniformly on the surface, the parasite can attach to the RBC in any orientation. Following the initial attachment, the parasite orients itself to face its apical side to the RBC surface, and the junction is formed with the apical membrane of the parasite with RBC membrane to facilitate the entry. These steps are mediated by sequential release of the proteins stored in the apical organelles, including micronemes, rhoptries, and the dense granules or spherical bodies.

In this study, BmSA1 was identified as a potential RBC binder through the phage display cDNA library screening. The precise location where BmSA1 is expressed in the merozoite is not clearly known. BmSA1 was first identified as a secreted protein found in the serum of the infected host [23]. Detergent extraction with Triton X-114 from *B. microti* infected RBCs demonstrated that it is associated with the membrane [24]. Confocal immunofluorescence microscopy showed that BmSA1 protein found was in the cytoplasm of the parasite within RBC [15]. As a GPI-anchored protein, it is likely that BmSA1 plays a role in the initial attachment step as a merozoite surface protein. However, the functional role of BmSA1 in the parasite invasion of RBC remains to be

determined. Invasion mechanisms of Plasmodium and in some Babesia species have been aided by the availability of quantitative parasite invasion assay systems. However, a major obstacle for studying invasion mechanism of *B. microti* is the lack of established *in vitro* culture system. Development of quantitative invasion assay system of *B. microti* will be needed to characterize the function of BmSA1 in the RBC invasion process. In our laboratory, currently we are testing the function of recombinant BmSA1 to interfere with *in vivo* RBC invasion of *B. microti* using the RAG1 deficient mouse model system as a reservoir of the parasite.

Borggraefe et al (2006) first demonstrated that the isolate of zoonotic *B. microti* infection shows a tropism towards mature red blood cells. Our finding that BmSA1 binds more avidly to mature RBCs as compared to 60% reticulocyte-enriched RBCs is consistent with the earlier finding. However, the binding of BmSA1 to 60% reticulocyte in this study cannot distinguish whether BmSA1 has more affinity towards mature RBCs or reticulocytes. The 60% reticulocyte contains a large fraction of mature red blood cells, which could also contribute to the positive binding against BmSA1. Additionally, the decreased binding of BmSA1 to 60% reticulocyte compared to mature RBC could be attributed to the difference of pre-treatment of the mice blood before the binding assay. Percoll centrifugation and oxidative stress environment that followed PHZ treatment might affect the membrane structure of the RBC as well as reticulocyte and interfere with the binding. Currently we are testing the binding of BmSA1 with mature RBCs and reticulocytes by immunofluorescence microscopy to resolve this intriguing feature of the Babesia invasion process. The fluorescence flow cytometry can also be use in this purpose. By labeling BmSA1 with fluorescein isothiocyanate (FITC) and sorting the

RBC based on its maturation stage, the binding of BmSA1 to mature RBC as well as reticulocyte can be quantitatively compared.

Another important question is the identity of the cognate receptor for BmSA1 on the host RBCs. The enzymatic treatment experiments demonstrated that the binding of BmSA1 is not sensitive to neuraminidase, trypsin, and chymotrypsin treatment of the RBCs. These results exclude some of the major RBC membrane proteins as potential BmSA1 receptors, such as Glycophorin A (neuraminidase and trypsin sensitive), Glycophrin B (neuraminidase and chymotrypsin sensitive), and Band 3 (chymotrypsin sensitive) (Gauer 2004). An effort is ongoing in our research group to screen for BmSA1 binding protein using the phage display cDNA library from human fetal liver. The library contains heterogeneous peptide population from hepatocyte as well as the hematopoietic precursor cells. From drug development point of view, the identification of RBC interacting molecule of *B. microti*, its receptors of the RBC surface and its role in the RBC invasion process will give a clue to the strategy of interrupting the transmission. Antagonistic blocking of infection by recombinant protein of known ligand of the particular receptor would possibly be an approach. However, currently little is known about the prophylactic candidate for Babesia infection [3]. The ultimate goal of these studies is to define the molecular mechanism(s) of the *Babesia microti* invasion process in red blood cells, and identifying critical components of the multi-subunit vaccine against *Babesia microti* infection in humans.

References

1. Hunfeld, K.-P., A. Hildebrandt, and J. Gray, *Babesiosis: recent insights into an ancient disease.* International journal for parasitology, 2008. **38**(11): p. 1219-1237.
2. Babes, V., *Sur l'hemoglobinurie bacterienne du boeuf.* CR Acad. Sci, 1888. **107**: p. 692-694.
3. Vannier, E. and P.J. Krause, *Human babesiosis.* New England Journal of Medicine, 2012. **366**(25): p. 2397-2407.
4. Western, K.A., et al., *Babesiosis in a Massachusetts resident.* New England Journal of Medicine, 1970. **283**: p. 854-856.
5. Cornillot, E., et al., *Sequencing of the smallest Apicomplexan genome from the human pathogen Babesia microti.* Nucleic acids research, 2012. **40**(18): p. 9102-9114.
6. Nathavitharana, R.R. and J.A. Mitty, *Diseases from North America: focus on tick-borne infections.* Clinical Medicine, 2015. **15**(1): p. 74-77.
7. Hatcher, J.C., et al., *Severe babesiosis in Long Island: review of 34 cases and their complications.* Clinical infectious diseases, 2001. **32**(8): p. 1117-1125.
8. *Biological Product and HCT/P Deviation Reports: Annual Summary for Fiscal Year 2008*. 2008; Available from: http://www.fda.gov.ezproxy.library.tufts.edu/BiologicsBloodVaccines/SafetyAvailability/ReportaProblem/BiologicalProductDeviations/ucm169990.htm.

9. Asad, S., J. Sweeney, and L.A. Mermel, *TRANSFUSION COMPLICATIONS: Transfusion-transmitted babesiosis in Rhode Island.* Transfusion, 2009. **49**(12): p. 2564-2573.

10. Smith, G.P. and V.A. Petrenko, *Phage display.* Chemical reviews, 1997. **97**(2): p. 391-410.

11. Lauterbach, S.B., R. Lanzillotti, and T.L. Coetzer, *Construction and use of Plasmodium falciparum phage display libraries to identify host parasite interactions.* Malaria journal, 2003. **2**(1): p. 47.

12. Li, X., et al., *Identification of a specific region of Plasmodium falciparum EBL-1 that binds to host receptor glycophorin B and inhibits merozoite invasion in human red blood cells.* Molecular and biochemical parasitology, 2012. **183**(1): p. 23-31.

13. Borggraefe, I., et al., *Babesia microti primarily invades mature erythrocytes in mice.* Infection and immunity, 2006. **74**(6): p. 3204-3212.

14. Vannier, E., et al., *Age-associated decline in resistance to Babesia microti is genetically determined.* Journal of Infectious Diseases, 2004. **189**(9): p. 1721-1728.

15. Lobo, C.A., M. Rodriguez, and J.R. Cursino-Santos, *Babesia and red cell invasion.* Current opinion in hematology, 2012. **19**(3): p. 170-175.

16. Jack, R.M. and P.A. Ward, *Babesia rodhaini interactions with complement: relationship to parasitic entry into red cells.* Journal of Immunology, 1980. **124**(4): p. 1566-1573.

17. Lobo, C.-A., *Babesia divergens and Plasmodium falciparum use common receptors, glycophorins A and B, to invade the human red blood cell.* Infection and immunity, 2005. **73**(1): p. 649-651.

18. Homer, M.J., et al., *Babesiosis.* Clinical microbiology reviews, 2000. **13**(3): p. 451-469.

19. Hussein, H., *Babesia hylomysci in mice: preference for erythrocytes of a particular age-group and pathogenesis of the anaemia.* Zeitschrift für Parasitenkunde, 1976. **50**(2): p. 103-108.

20. YAMASAKI, M., et al., *The cause of the predilection of Babesia gibsoni for reticulocytes.* Journal of Veterinary Medical Science, 2000. **62**(7): p. 737-741.

21. Luo, Y., et al., *Identification and characterization of a novel secreted antigen 1 of Babesia microti and evaluation of its potential use in enzyme-linked immunosorbent assay and immunochromatographic test.* Parasitology International, 2011. **60**(2): p. 119-125.

22. Lodes, M.J., et al., *Serological expression cloning of novel immunoreactive antigens of Babesia microti.* Infect Immun, 2000. **68**(5): p. 2783-90.

23. Luo, Y., et al., *A double antibody sandwich enzyme-linked immunosorbent assay for detection of secreted antigen 1 of Babesia microti using hamster model.* Experimental Parasitology, 2012. **130**(2): p. 178-182.

24. Priest, J.W., et al., *Multiplex assay detection of immunoglobulin G antibodies that recognize Babesia microti antigens.* Clin Vaccine Immunol, 2012. **19**(9): p. 1539-48.