Role of *Babesia microti* Surface Antigen 1 and its Interaction with Erythrocytes in Babesiosis

A thesis submitted by

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Abstract

Human babesiosis is a malaria-like disease highly prevalent in North East America that can be fatal to the elderly and immune-compromised subjects. However, relatively little is known about the molecular mechanisms of the disease. *Babesia microti* Surface Antigen 1 (BmSA1) is a membrane/secreted protein that has been previously identified and characterized, but its functional role in parasite invasion and growth as well as its potential as a vaccine candidate remains to be determined. To test the role of BmSA1 in parasite invasion, we re-evaluated published methods of *in vitro* invasion assays to find optimal conditions for the quantification of parasitemia. Surprisingly, despite multiple attempts and shared reagents, we could not reproduce published *in vitro* invasion assays. Alternatively, we established an *in vitro* growth assay to quantify intraerythrocytic development of *Babesia microti*, the most common causative agent of human babesiosis in North America. Using the *in vitro* growth assay, we tested the effects of recombinant BmSA1 protein, Signal Peptide Peptidase (SPP) inhibitor, Z-LL2 ketone, and HIV protease inhibitors, Atazanavir and Lopinavir on parasite development in erythrocytes. Both single and combination protocols were used to test the efficacy of potential inhibitors. Remarkably, the Z-LL2 ketone potently inhibited *B. microti* growth in human erythrocytes, and other test molecules also showed significant inhibitory effects under these conditions. Furthermore, to identify the host erythrocyte receptor for *BmSA1*, I performed multiple phage display screens using the human reticulocyte cDNA library and far western blotting of RBC membrane proteins. Our screens suggest that BmSA1 can recognize two potential receptors in human erythrocyte membranes of molecular mass ranging within ~200-245 kDa and ~48-50 kDa. Together, these studies set the stage

for molecular identification of host receptor for BmSA1 as well as development of potential therapeutics for human babesiosis.

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Empitu M. (2015). *Identifying the red blood cell interacting molecule of Babesia microti* (Master's in Science dissertation) Tufts University. Retrieved from Dissertations and Theses @ Tufts University ProQuest Database.

List of Abbreviations

BLAST	Basic Local Alignment Search Tool
bp	base pair
B. microti	*Babesia microti*
BmSA1	*Babesia microti* Surface Antigen 1
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
ELISA	enzyme linked immunosorbent assay
EDTA	Ethylene diamine tetraacetic acid
ER	endoplasmic reticulum
ECL	Enhanced Chemiluminescence
FITC	Fluorescein Isothiocyanate
FBS	fetal bovine serum
GPI	glycosylphophatidylinositol
GPA	glycophorin A
GPB	glycophorin B
GPC	glycophorin C
HIV	human immunodeficiency virus
i.p	intra peritoneal
iRBC	infected red blood cell
JEM	Journal of Experimental Medicine
LB	lysogeny broth
mAb	monoclonal antibody
MHC	major histocompatibility complex
$NaHCO_3$	sodium bicarbonate
P. falciparum	*Plasmodium falciparum*
PfSPP	Plasmodium falciparum signal peptide peptidase
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PBS	phosphate buffered saline
pfu	plaque forming unit
pH	potential hydrogen
RBC	red blood cell
rBmSA1	recombinant Babesia microti Surface Antigen 1
RPMI	Roswell Park Memorial Institute (medium)
RNA	ribonucleic acid
SPP	signal peptide peptidase
SDS	sodium dodecyl sulfate
TTB	transfusion transmitted babesiosis
TAP	transporter associated with antigen presentation
TBST	Tris buffered saline with Tween
TBS	Tris buffered saline
Trx	thioredoxin

Chapter 1 – Introduction

1.1 Introduction to Babesiosis, Epidemiology, and Different Strains of Pathogens.

Human babesiosis, a parasitic disease caused by an apicomplexan parasite of *Babesia genus*, is transmitted most commonly through tick bites of genus *Ixodes*.[1] It is prevalent in Northeastern and Midwest regions of North America such as Massachusetts, New York state, Wisconsin and New Jersey, with fewer cases reported in Connecticut, Maryland, Virginia and California too.[2] Over 100 species of *Babesia* have been reported, most of which largely affect cattle and domestic animals. The first case of human babesiosis was reported in Yugoslavia in 1957, followed by in California in 1966, and several hundred cases reported in North America thus making this infection a significant topic of research in infectious diseases afflicting humans[3] Demographic studies have shown that among the large number of *Babesia* organisms that infect cattle and domestic animals, *Babesia microti* and *Babesia divergens* are the most common causative agents for human babesiosis[4] *B. divergens*, originally identified as a cattle strain, is mostly prevalent in Europe whereas *B. microti*, a rodent strain, is the most common transfusion-transmitted pathogen (TTP) in North America[4] Due to the high clinical significance of *B. microti*, I elected to investigate the mechanisms of its invasion and growth in host erythrocytes as a topic of my research thesis.

The life cycle of *B. microti* involves multiple hosts; the tick species *Ixodes scapularis* of *Ixodes genus* serves as a host where the sexual cycle occurs (definitive host), commonly white footed mouse *Peromyscus leucopus* serves as a primary

mammalian reservoir host for asexual stage of replication, and the humans become the accidental hosts to the parasite.[4] During a blood meal from humans by the tick, sporozoites get transmitted into the peripheral blood where the merozoites invade multiple erythrocytes and undergo asexual reproduction also known as budding. Intraerythrocytic growth stages of *B. microti* can be identified based on morphological structures which begin with the ring stage progressing into amoeboid stage or binary pyriform bodies. The pyriform bodies undergo further multiplication forming cruciform bodies, identified as tetrad stage or the maltese cross, that mark the characteristic feature of *Babesia* genus differentiating it from the malaria parasite *P. falciparum.* The tetrads burst open from the infected erythrocyte thus sustaining the invasion process by infecting multiple normal erythrocytes[4] Humans are usually the dead-end host to the disease, but a horizontal transmission can take place through blood transfusion that is often fatal to the elderly and immune-compromised patients. Human babesiosis lacks an appropriate diagnostic tool to detect extremely low parasitemia thus increasing parasite infectivity and spread. Moreover, it tends to be confused with *P. falciparum* due to their similar intraerythrocytic ring stage making diagnosis even more challenging and possible to leading to incorrect prognosis and medication for the patient. This limitation makes human babesiosis a significant health risk and an important research topic for the development of new drugs and ultimately a viable vaccine against this unpredictable infectious disease.

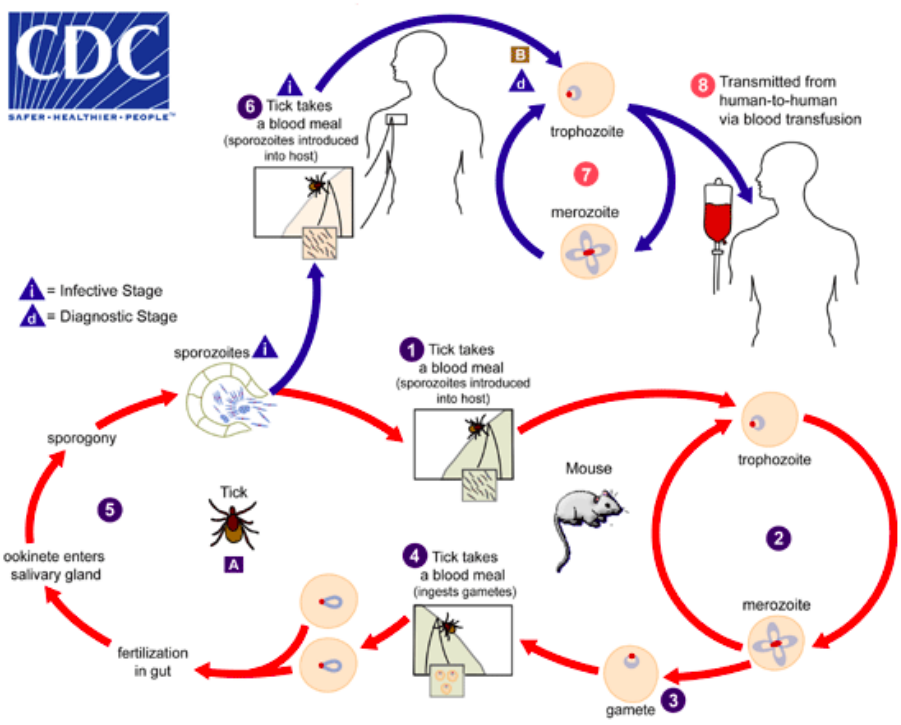


Figure 1.1 ***Babesia Microti*** **Life Cycle:** Reprinted with permission from (Center for Disease Control and Prevention, 2015) Parasites-babesiosis; Retrieved from https://www.cdc.gov/parasites/babesiosis/biology.htmL

1.2 Diagnostic Options for Human Babesiosis

Human babesiosis is a parasitic disease that is transmitted to humans either by a tick (*Ixodes Scapularis*) bite or by blood transfusion from an infected donor. Babesiosis is a zoonotic disease that was prevalent mostly among cattle and domestic animals, but its progression into humans has made it a point of concern due to resulting fatality in the immune-compromised and elderly population[4] Transfusion-transmitted babesiosis (TTB) is a major threat where infected donor blood is given to patients unintentionally mainly due to the lack of sensitive diagnostic tools to detect low parasitemia in donor blood.[5] Since *B. microti* resides within enucleated erythrocytes, its presence can be directly detected by staining with dyes such as Giemsa/Wright Giemsa treated microscopic blood

smears. Similarly, the nuclear staining by dyes such as Hoechst is an effective means of quantifying parasitemia by flow cytometry. In principle, parasitemia even as low as 6 parasites in 1.0 mL volume of blood can be detected through microscopic smears, but the chances of asymptomatic patients carrying even 6 parasites is relatively low, thus making the need for a reliable and efficient diagnostic tool is of utmost importance for preventing TTB[6] Based on a combination of *B. microti* antigen peptides, which included immunodominant peptides from BmSA1, and their reactivity with serum of *B. microti* symptomatic patients, a microplate ELISA was developed for diagnostic purposes[7] The ELISA test showed a high specificity of 99.9% but the cost effectiveness of the screening turned out to be a major limitation for its widespread implementation.

1.3 Characteristics of *Babesia microti* Surface Antigen 1

The extracellular merozoites contain various surface proteins that facilitate recognition, reorientation, and invasion of RBCs.[8] *Babesia microti* Surface Antigen 1 was identified by immunoscreening a *Babesia microti* cDNA library using sera from immunized hamsters and a 33 kDa antigen was recognized using antibodies.[9] To investigate for binding of BmSA1 with RBC proteins, a former researcher in the Chishti Lab, Maulana Empitu, performed a phage display screen by constructing a cDNA library from mouse RBCs infected with *B. microti* RM/NS strain, and phage clones were selected after four rounds of biopanning using RBCs as bait. DNA sequencing results were analyzed for homology using BLAST and a 400 bp cDNA encoding for BmSA1 was identified as a strong candidate protein involved in binding with RBC proteins[10]*Babesia microti* Surface Antigen 1 is a membrane-associated protein present on the surface of the parasite as well

as found to be secreted in the serum of the infected host and known to be involved in the binding with RBCs making it a potential vaccine candidate against babesiosis. BmSA1, also termed BMN1-9, consists of 302 amino acid residues with a predicted molecular weight of 33 kDa. The N-terminus contains a signal peptide and the carboxyl terminus displays a glycosylphosphatidylinositol(GPI) anchor motif (Figure 1.2). A detailed characterization of BmSA1 including its subcellular localization and functional role remains to be established.

(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 1.2. Schematic Representation of Bmsa1 Primary Structure (A) Signal peptide is present on the N-terminus in BmSA1. (B) BmSA1 amino acid sequence. The patterned section in (A) and underlined part in (B) represent the sequence of BmSA1 that was isolated from phage display cDNA library screening. Reprinted with permission from[10] Empitu M. *Identifying the red blood cell interacting molecule of Babesia microti* Tufts University.

1.4 *In vitro* Culture of *Babesia microti* to Investigate the Functional Role of rBmSA1

Two key processes that are essential for the survival of this obligate intraerythrocytic parasite involve invasion in RBCs and its intraerythrocytic growth. When the extracellular parasite contacts host erythrocyte receptor protein, it reorients its apical

membrane forming a tight junction that facilitates invagination to commence invasion[8] Potential vaccine approaches involve a combination of recombinant surface antigen proteins, mostly aimed at hindering the parasite invasion process. The underlying principle is that inoculation of subjects with recombinant BmSA1 would block the recognition of the erythrocyte receptor by the parasite thus hindering invasion *in vivo*. To further understand the molecular mechanism of parasitic invasion and develop novel strategies for vaccine development, the critical host-ligand interaction molecules need to be identified. BmSA1, a novel surface antigen that binds to erythrocytes could be involved in the invasion process thus making recombinant BmSA1 a potential vaccine candidate.[9] To ascertain the functional role of BmSA1 in parasite invasion, it would be necessary to establish an *in vitro* culture system and perform protein-binding assays. It is noteworthy that a paradigm shift breakthrough in the malaria field occurred with the invention of a continuous *in vitro* culture of *Plasmodium falciparum* by Trager and Jensen,[11] which facilitated immense research opportunities and the discovery of many host-pathogen interaction molecules. Although an *in vitro* culture exists for *B. divergens* and *B. bovis*, the lack of a similar culture system for *B. microti* hampers research discoveries pertaining to this particular parasite species that infects a large population in North America. Published evidence shows that successful attempts have been made to establish an *in vitro* or ex vivo culture system for *Babesia microti* using various approaches such as osmotic lysis of infected RBCs, or plating of infected blood from host mice using optimized complete media and incubation of the culture under appropriate conditions[12, 13] These observations provided us a basis to set up similar *in vitro* culture methods in order to test and validate the functional role of rBmSA1 in parasite invasion.

1.5 Role of Signal Peptide Peptidase in Intraerythrocytic Development of *Babesia microti*

Signal peptide peptidase (SPP) is an intramembrane aspartyl protease, and one of the functions include cleaving of transmembrane proteins allowing them to act as signaling molecules for further activation.[14] Studies in *Plasmodium* have confirmed the localization of active SPP within endoplasmic reticulum (ER) that causes the release of secretory antigenic proteins into the cytosol and ER lumen, allowing transporter associated with antigen presentation (TAP) dependent and independent MHC class 1 loading.[14] Our previous studies have also demonstrated that inhibiting *P. falciparum* signal peptide peptidase (PfSPP) using mammalian SPP inhibitors such as Z-LL2 ketone and L-685,458 blocks malarial parasite growth and invasion into human RBCs.[15] Based on these studies, we examined whether there is a substantial sequence homology between *P. falciparum* and *B. microti* signal peptide peptidases and test the effect of SPP inhibitor Z-LL2 ketone on *B. microti* intraerythrocytic growth. Another key step of *B. microti* infection involves intraerythrocytic growth process where post engulfment growth phase initiates with the ring stage proceeding to tetrad shaped merozoites that are eventually released into the extracellular space.[16]Pharmacological inhibition of the intraerythrocytic growth progression is one of the principal approaches to prevent the spread of infection and an attractive point for drug development.

The incidence of malaria and HIV/AIDS being contracted simultaneously is high, primarily in the sub-Saharan Africa, Southeast Asia and South America, leading to patients being given a combination therapy of antimalarial and antiretroviral drugs. When contracted simultaneously, these two infections exist bidirectionally and synergistically with each other.[17] Of the different classes of antiretroviral drugs tested for inhibiting

malaria infectivity, protease inhibitors showed the most promising results in particular Lopinavir showed inhibitory activity within an achievable serum plasma concentration along with Atazanavir also showing fairly good inhibitory activity.[18] Based on these studies and homology between *P. falciparum* and *B. microti* signal peptide peptidase we tested the effect of HIV protease inhibitors as well as SPP inhibitor on *B. microti* growth *in vitro*.

1.6 Potential Erythrocyte Receptors for *Babesia microti* invasion

Identification of host receptors involved in the parasite invasion is a key step for pathogenesis. Published evidence suggests that *B. microti* preferentially invades and grows in mature erythrocytes as compared to immature reticulocytes, further indicating the specificity of ligand-host interactions.[19] There are over 50 major erythrocyte transmembrane proteins with diverse functions and characteristics that can serve as potential receptors for *B. microti* (Figure 1.3). Previous studies have shown that invasion by *B. divergens* in erythrocytes is reduced by 90% when erythrocytes are treated with neuraminidase that removes glycosidic linkages of neuraminic.[20] This finding suggests a key role of sialic acid, a molecule abundantly present in glycophorins, in the invasion of *B. divergens* making GPA, GPB, and GPC's as potential host receptors involved in invasion.[21] Despite the lack of an established *in vitro* assay for *B. microti* and therefore inability to perform direct invasion assays *in vitro*, attempts have been made to identify host transmembrane proteins using RBC binding assays. A former researcher in the Chishti Lab carried out *in vitro* RBC binding assays and tested for the binding of BmSA1 with enzymatically treated human RBCs. These experiments indicated that BmSA1

binding with human RBCs is independent of trypsin, chymotrypsin, and neuraminidase treatment. To build on these studies, we utilized different approaches including the phage display technology and blot overlay assays to identify potential host receptors for *B. microti*.

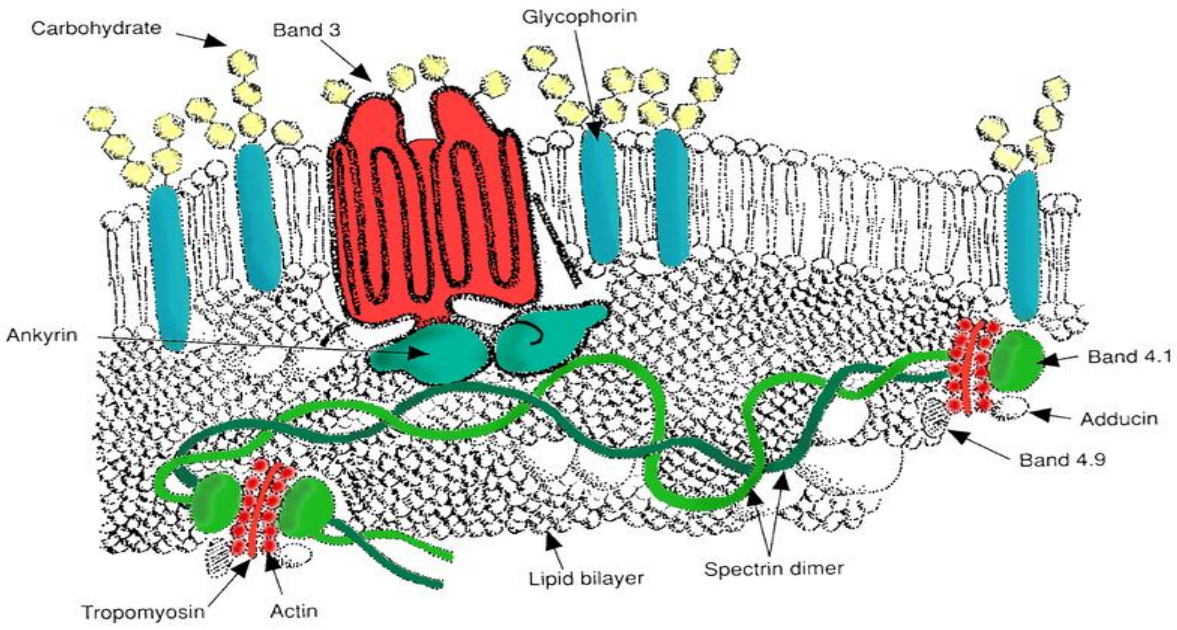


Figure 1.3 Erythrocyte Membrane Representing Major Membrane and Cytoskeletal Proteins: Reprinted with permission from[22]Wikimedia Commons contributors. RBC membrane major proteins.png. https://commons.wikimedia.org/w/index.php?title=File:RBC_membrane_major_proteins.png&oldid=270032062. Accessed 27 March 20:21 UTC, 2018.
Modifications done by Tim Vickers which includes adding all the colors to image.

Chapter 2 – Materials and Methods

2.1 Mouse and Parasite Strains

Immune-deficient Rag-1 KO mice, obtained from Jackson Laboratories, were utilized for maintaining and growing *B. microti* RM/NS strain.[19] The primary male Rag-1 KO mouse was inoculated with frozen stock of RM/NS strain, which became the source of infected cells for inoculation of subsequent normal Rag-1 KO

2.2 Ex vivo Assay for Invasion of Erythrocytes by *Babesia microti*

Injection Conditions: To establish a short-term ex vivo culture, a protocol was adapted from Lawres *et. al* [12] to set up an invasion assay. Rag-1 KO mice were inoculated with 10^7 infected RBCs, which were obtained by adding one drop (~20 µL) of 50% parasitemia to 1 mL of phosphate buffered saline, mixed gently, and 200 µL of the mixture was injected intraperitoneally. The precise volume of infected cell solution to be injected was determined by performing a serial dilution of 1 mL PBS containing one drop of 50% parasitemia blood, and cell counting was done using a hemocytometer.

Blood Smear Preparation: Parasitemia was monitored in pre-defined intervals after the IP injection using Wright Giemsa stained blood smears. Blood smears were prepared by pipetting 1.5 µl of washed, infected, and packed RBCs supplemented with equal volume of FBS on a cleaned glass slide. Blood smears were air-dried for five minutes, fixed for 30 seconds in absolute methanol, followed by methylene blue nuclear staining using Wright-Giemsa for 45 seconds. Stained smears were washed in distilled water for ten minutes, blow dried, and parasitemia was quantified using light microscopy.

Blood Collection, RBC Washing and Plating Conditions: Infected blood was collected when parasitemia reached 10%, by submandibular puncture, in 40 mL RPMI 1640 containing 10% FBS. Diluted blood was centrifuged at 400xg for ten minutes with slow deceleration and washed three times with RPMI 1640 at 800xg for four minutes. Infected blood was diluted with normal syngeneic mouse blood to achieve a final parasitemia of 2% in a 96 well plate incubated in gas mixture of 5% CO_2, 3% O_2 and the remainder nitrogen at 37ºC for 48 hours. The complete media composition used for culturing of *B. microti* was RPMI medium 1640 supplemented with 0.225% $NaHC0_3$, 10% heat-inactivated fetal bovine serum, 30 mg/l hypoxanthine, 25 mM Hepes, Pen Strep (Gibco; 15140-122), 10 µg/mL of Gentamicin, and 100 µg/mL of Kanamycin. Using similar conditions and experimental set up, complete malaria media and culture media of unknown composition sent by Choukri Ben Mamoun *et. al* from Yale group, was also used separately to check for invasion of *B. microti in vitro***.** The composition of the media solution supplied by Choukri Ben Mamoun *et. al* from the Yale group was not disclosed due to propriety limitations.

2.3 Flow Cytometry Assay

Hoechst 33342 Staining: To analyze samples using flow cytometry, each well from the plate was transferred to individual Eppendorf tube and spun down 400xg. Media was aspirated and two washes were carried out with 1 mL PBS. To fix the samples, the RBC pellet was resuspended in 500 µL PBS containing 2% paraformaldehyde and 0.2% glutaraldehyde and rotated at 4ºC for 45 minutes. After fixation, the solution was spun and aspirated, followed by two more washes with 1 mL PBS. Hoechst 33342 dye was

used to stain the parasite nucleus to determine parasitemia. Working stock of Hoechst was prepared from a 2 µM stock solution, and diluted (1:8000 dilutions from stock in RPMI 1640). Samples were stained for an hour at 37ºC in the dark (minimal light exposure). Upon staining, samples were spun and dye was aspirated, followed by two more washes with 1 mL PBS. Samples were transferred into appropriate tubes containing PBS and analyzed using a BD LSR II flow cytometry instrument using the UV laser within the Indo 1 blue region to detect Hoechst and FITC spectra with negative control.[23]

YOYO-1 Dye Staining: Samples were collected in the same manner as in the Hoechst 33342 dye staining protocol, and fixed with 0.01% glutaraldehyde. To do so, 9.9 µL of glutaraldehyde stock was added to 25 mL of 1X PBS and vortexed. 500 µL of diluted glutaraldehyde was added to samples and incubated for 30 minutes at room temperature. Samples were then centrifuged at 300xg for ten minutes, and washed with PBS. Washed packed cells were then incubated with 250 µL of Triton X-100 for 10 minutes, spun, and aspirated followed by 60-minute incubation with 500 µL of DNase free RNase at 37ºC. Packed cells were then stained with 500 µL of YOYO-1 solution that was prepared by adding 20 µL of YOYO-1 solution to 10 mL of staining buffer (RPMI). Cell were incubated with YOYO-1 stain for an hour in the dark, and later analyzed using flow cytometry (BD LSR II).

2.4 Isolation of *B. microti* Parasites from Infected RBCS by Osmotic Lysis

Another approach to establish an ex vivo culture was the "lysis method" adapted from a recently published protocol.[13] This approach involved the utilization of free parasites obtained by RBC lysis by osmotic shock.[24] Infected blood at 40% parasitemia was

collected in RPMI supplemented with 10% FBS, washed three times with PBS, and resuspended in equal volume of RPMI as a 50% v/v solution. Lysis buffer was prepared by adding 9 mL of 0.83% ammonium chloride solution in deionized water to 1 mL of 0.17M Tris-HCl buffer at pH 7.6. The final pH of buffered ammonium chloride solution was at 7.4. 100 µL of resuspended iRBC pellet was treated with 1 mL of lysis buffer for 3 minutes at 37ºC. The resulting lysate was centrifuged at 500g for 10 minutes and supernatant was obtained and washed three times with RPMI 1640. The pellet obtained was incubated with normal RBCs from Rag-1 KO mouse and in separate wells with human RBCs at 4% hematocrit for 24 hours in gas mixture of 5% CO_2, 3% O_2 and the remainder nitrogen at 37ºC.

2.5 Ex vivo Growth Assay

Growth inhibition assays were carried out in 96 well plates containing complete media that was pre-warmed to 37ºC. Blood from infected mouse at 10% parasitemia was collected in RPMI 1640 with 10% FBS, centrifuged at 500g for ten minutes with slow deceleration, followed by three washes with RPMI 1640. Blood smears were prepared using a similar method as stated above to quantify parasitemia, morphological life stages of parasites were identified using light microscope, and 4 µL of remaining packed iRBCs were added to each well. The experimental design included a positive control without any treatment, 20 µM DMSO,18 µM Trx, and 50 µL of PBS as negative control. The four treatment arms included Z-LL2 ketone, Lopinavir, Atazanavir, and rBmSA1 protein at stock concentrations of 5 mM, 10 mM, 10 mM, and 75 µM, respectively. 1 µM working stocks of Z-LL2 ketone, Lopinavir, and Atazanavir were prepared from which 4 µL, 2

µL, and 1 µL was pipetted to achieve 20 µM, 10 µM, and 5 µM final concentration, respectively. 50 µL of 75 µM rBmSA1 was added to obtain 18 µM final concentration. Infected RBCs were incubated with complete media supplemented with 0.1 mM β-mercaptoethanol at 2% hematocrit in gas mixture of 5% CO_2, 3% O_2, and the remainder nitrogen at 37ºC, and harvested after 24 hours to analyze growth through blood smears prepared and stained as described above. Three independent experiments were performed for each measurement.

2.6 Phage Display Screening for Host Receptors using Human Reticulocyte cDNA Library

Human reticulocyte cDNA library, prepared by Dr. Toshihiko Hanada, was screened using recombinant BmSA1 protein as bait.

ELISA Plate Well Coating: ELISA wells were coated by applying 100 µL of 10 µg/mL of Trx tagged BmSA1 and a separate row of only Trx protein. Wells were incubated overnight at 4ºC. Wells were washed with 300µL of 1X TBS to remove unbound protein, blocked with 200 µL of 1% bovine serum albumin in TBS, and incubated for an hour. Plates were washed 5 times with deionized water, and stored at 4ºC with 200 µL of deionized water in each well until further use.

Biopanning: The cDNA screening was performed by utilizing a biopanning protocol based on the interaction between bait and human reticulocyte cDNA clones expressing binding peptides. 20 µL of a phage display cDNA library that corresponds to 1×10^8 plaque forming units, was diluted 1:100 in TBS containing 0.1% Tween-20, applied to each of 2 Trx coated wells, and incubated for 30 minutes with paraffin cover at

room temperature. The diluted phage library was transferred from each well into Trx tagged BmSA1 and incubated for 30 minutes. The unbound phage solution was discarded. Wells were washed five times with 200 µL of TBST, followed by phage elution by addition of 100 µL of 1% sodium dodecyl sulphate solution in distilled water for ten minutes at room temperature. Simultaneously, an overnight culture of *E. coli* BLT5403 grown in M9LB with ampicillin at 37ºC was diluted in a 250-mL flask containing 50 mL of sterile LB supplemented with 2 mL of 20% glucose, 5 mL of 20X M9 salts, and 100 µL of 1 M magnesium sulfate and ampicillin (M9LB). The diluted overnight culture was incubated at 37ºC in a shaker at 280 rpm until OD_{600} had reached 0.6 measured in intervals using the Eppendorf Bio photometer. Upon achieving the desired OD, the culture left at room temperature until the eluted phage library was ready to be added. The eluted phage solutions from the two wells were pooled together into a single 1.5 mL microtube (~200µL) and pipetted into bacterial culture with OD 0.6 and incubated in a shaker at 37ºC until lysis occurred (appearance of white strands ~1-3 hours). 1 mL of lysed culture was centrifuged at 13,000 rpm for ten minutes and the supernatant was saved for consecutive rounds at 4ºC (-20ºC for long term storage). 20 µL of saved supernatant was used as the source of phage library for the next round and 3 such successive rounds were carried out for completion of the biopanning screening.

Plaque Assay, Extraction, and Sequence Analysis: Bacterial culture at OD 1.0 was prepared by inoculating 2 mL of M9LB media containing ampicillin with BLT5403 and shaking at 37ºC. Top agarose was simultaneously prepared by adding 3 mL of melted 4% agarose to 17 mL of LB prewarmed to 50ºC and left in the water bath until the serial dilutions of phage library were ready for use. Supernatant from fourth and final round

was used to perform serial dilutions using LB as diluent where 10 µL of phage library was transferred into 990 µL of LB (1:100) followed by 100 µL of 1:100 dilutions into 900 µL of LB to make 1:1000 dilution until $1:10^{10}$ was achieved. Dilutions ranging from 10^{-7} -10^{-10} were selected to plate containing host *E. coli* BLT5403 in LB agar plates to form plaques. DNA extraction was performed the next day with 10 mM EDTA for 10 minutes at 65ºC, from $1:10^{9}$ dilution plate that contained optimal number of plaques. The extracted DNA was PCR amplified using a set of primers T7-UP-5'-GGAGCTGTCGTATTCCAGTC and T7-DOWN- 5'-AACCCCTCAAGACCCGTTTA. The PCR products were purified using E.Z.N.A. Cycle Pure kit, and DNA sequences were determined with T7-DOWN primer obtained from the Tufts University Genomics Core Facility.

2.7 Far Western Blot (Blot Overlay Assay) Using RBC Membranes (Ghosts)

To measure protein interactions between recombinant BmSA1 and transmembrane protein receptors, a blot overlay assay was performed using human RBC ghosts. Whole human blood was washed three times with RPMI 1640 and centrifuged at 500g for 10 minutes at 4ºC to remove plasma, leukocytes, and platelets. The packed RBC pellet was collected in 7 microtubes each containing 20 µL of packed RBCs with 1 mL ice cold lysis buffer made up of 5 mM sodium phosphate buffer at pH 8.0, 1.0 mM EDTA at pH 8.0, and protease inhibitor cocktail in distilled water. Cell lysate was centrifuged at 10,000 rpm for ten minutes at 4ºC. Supernatant was removed carefully, followed by 3 more washes, and the pellet was resuspended in lysis buffer that can be stored at -80ºC for future use. 300 µL of ghosts were treated with 200 µL of 3X SDS-PAGE sample buffer containing 37.5 mL of 0.5 M Tris-HCl at pH 6.8, 6% SDS, 0.3 volumes of

glycerol, 4-5 drops of bromophenol blue supplemented with 100µM BME in distilled water (stored at -20ºC). Samples were incubated at 37ºC for 15 minutes followed by cooling to room temperature. Samples were loaded onto 12% SDS-PAGE gel and electrophoresis was performed for 1.5 hours at 80 voltage. The RBC ghost proteins were separated based on their molecular weight. Proteins in the gel were transferred onto nitrocellulose membrane and stained with ponceau dye and stored in distilled water at 4°C till further use. Two lanes of membrane proteins were cut out and markings were made for molecular weights ranging from 245 kDa to 25 kDa using a blue stain protein ladder and pencil. The lanes were excised, washed in TBST for 1.5 hours, and incubated overnight with blocking buffer made of TBST containing 10% heat inactivated horse serum (Gibco). The Trx tagged BmSa1 protein at stock concentration of 0.45 mg/mL and Trx protein (negative control) at 0.18 mg/mL were diluted to 0.5 µg/mL in the blocking buffer and incubated overnight with 10 mL of protein solutions respectively. The nitrocellulose membrane was washed 5 times for 15 minutes each to remove unbound proteins, followed by the incubation with anti-Trx mAb (Genscript) at a 1: 10,000 dilutions overnight. The membrane was washed 5 times with blocking buffer to remove the primary antibody, followed by the incubation with alkaline phosphatase goat anti-mouse IgG as the secondary antibody at a dilution of 1: 5,000 in the blocking buffer for one hour. Membrane was washed four times with TBST followed by a final wash with TBS. The signal on the nitrocellulose membranes was detected using Immun-Star Detection Kit (Bio Rad).

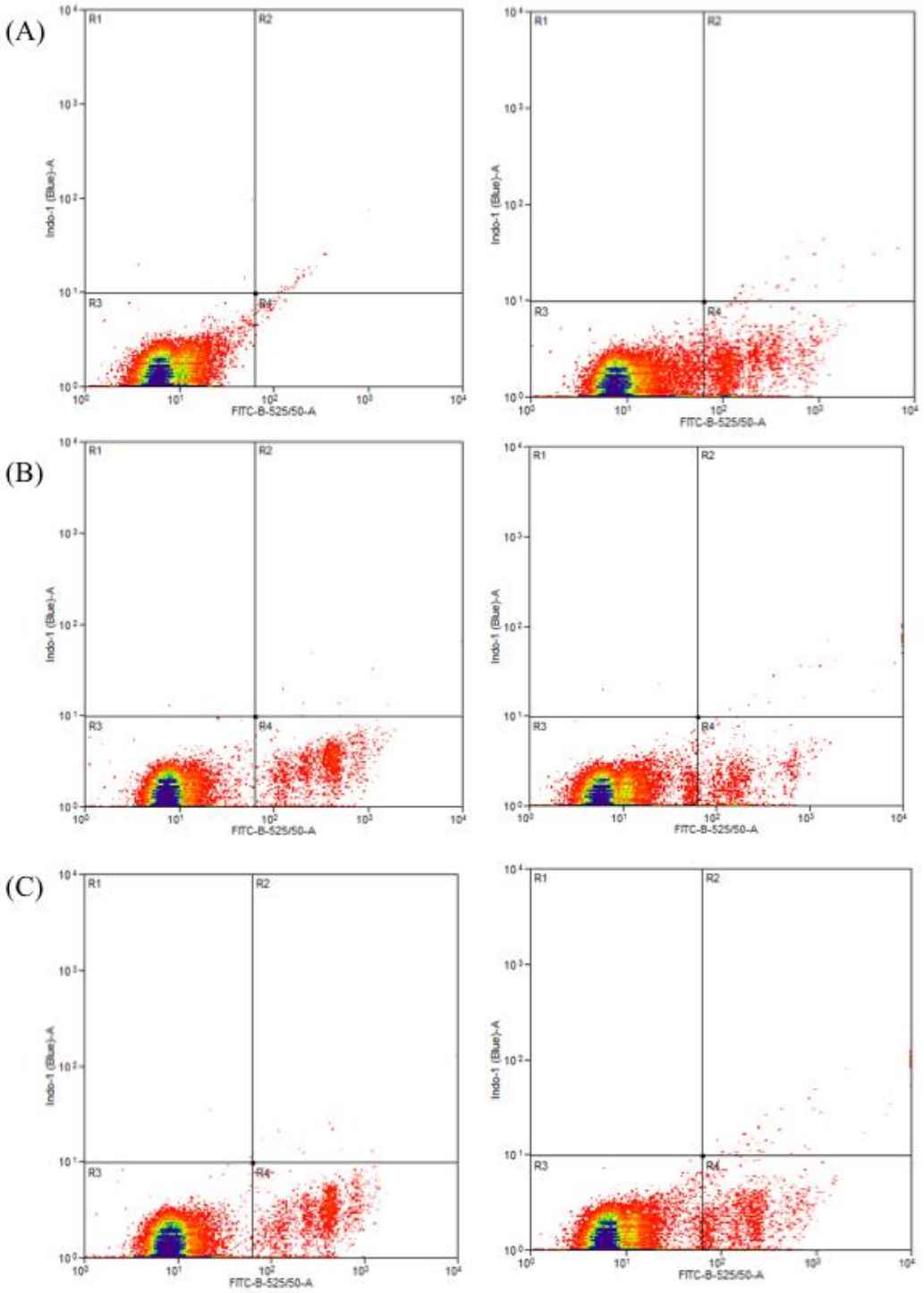
2.8 Statistical Analysis

Statistical analysis was performed using one-way ANOVA independent groups where the mean of each arm was compared to the mean of the control arm and Dunnett's/Tukey’s test was applied for correction of multiple comparisons. Data were considered of statistical significance when p value was less than 0.05.

Chapter 3 – Results

3.1 *In vitro* Culturing Does Not Facilitate Invasion of *Babesia microti* Parasites

The experimental rationale behind our attempt to establish an *in vitro* culture was to set up an invasion assay and investigate the effect of rBmSA1 on invasion. This information is essential for the characterization of the functional role of BmSA1 and its potential as a vaccine candidate. Based on the method described in the JEM paper[12] we replicated the protocol and carried out *in vitro* cell culture of *B. microti*. Parasitemia was adjusted to 2% for the *in vitro* assay. After 24 hours of incubation, the parasitemia remained at 2% or even dropped to 1% in multiple independent experiments. Our attempt at performing *in vitro* cell culturing of *B. microti* from a published method did not show an increase in parasitemia either over 24 or 48 hours. Hence we concluded the method suggested in the JEM paper[12] is not reproducible, and *B. microti* parasites are unable to invade normal erythrocytes. Optimization by testing different conditions was carried out, such as incorporating regular FBS vs heat inactivated FBS in the media to check for an effect on invasion, but there was no significant effect. Hematocrit values of 2% and 10% in 96 well plate were tested for effect on invasion but there was no significant effect. The lysis method reported by Moitra *et. al*[13] was also attempted, but the pellet obtained after centrifugation post ammonium chloride treatment contained RBC shells. This was inconsistent with the goal of obtaining 100% free parasites for overnight incubation with normal human RBCs and provided no definitive data.


(A)
(B)
(C)
R1
R2
R3
R4
Indo-1 (Blue)-A
FITC-B-525/50-A

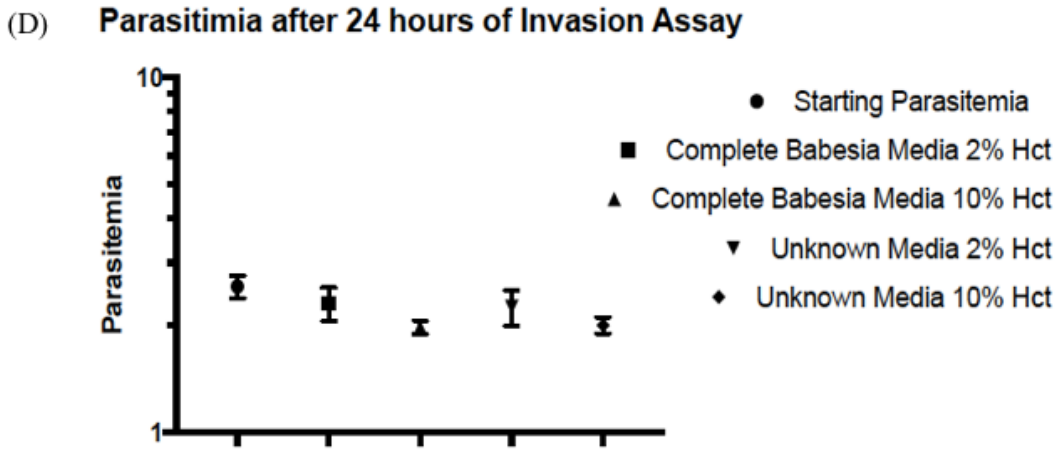


Figure 3.1 Results from Flow Cytometry After 24 Hours of *in vitro* Incubation Under Different Media and Hematocrit Conditions (A) Left-Dot plot represents normal uninfected cells. Right- Dot plot represents infected cells at a starting parasitemia of 2.5% (R4 region). (B) Left- Dot plot represents infected cells incubated at 2% hematocrit with complete Babesia media. Right- Dot plot represents infected cells incubated at 10% hematocrit with complete Babesia media. (C) Left- Dot plot represents infected cells incubated at 2% hematocrit with unknown media. Right- Dot plot represents infected cells incubated at 2% hematocrit with unknown media. (D) Graph indicating percent of infected cells from flow cytometry data under different media and hematocrit condition. Compared the standard mean of different groups with standard mean of starting parasitemia group using ANOVA. Independent t-test with P value > 0.5 and bars indicating SD

3.2 *In vitro Babesia microti* Develops from Ring to Tetrad Stage Within 24 Hours

The experimental rationale behind setting up the growth assay was to quantify the intraerythrocytic growth of *B. microti* through an *in vitro* assay. Parasite growth in erythrocytes is another key process, along with invasion, involved in infection, and permits testing of the effects of rBmSA1 or other pharmacological compounds on the tetrad stage of development. Although there was no discernible increase in parasitemia and therefore no invasion of parasites into normal RBCs, I observed fully developed or

tetrad-like structures after 24 hours of culture through blood smears. To further validate this finding, the number of rings and tetrads were quantified on day 0; i.e., on the day of plating of the infected blood, and on day one; i.e., after 24 hours of incubation.

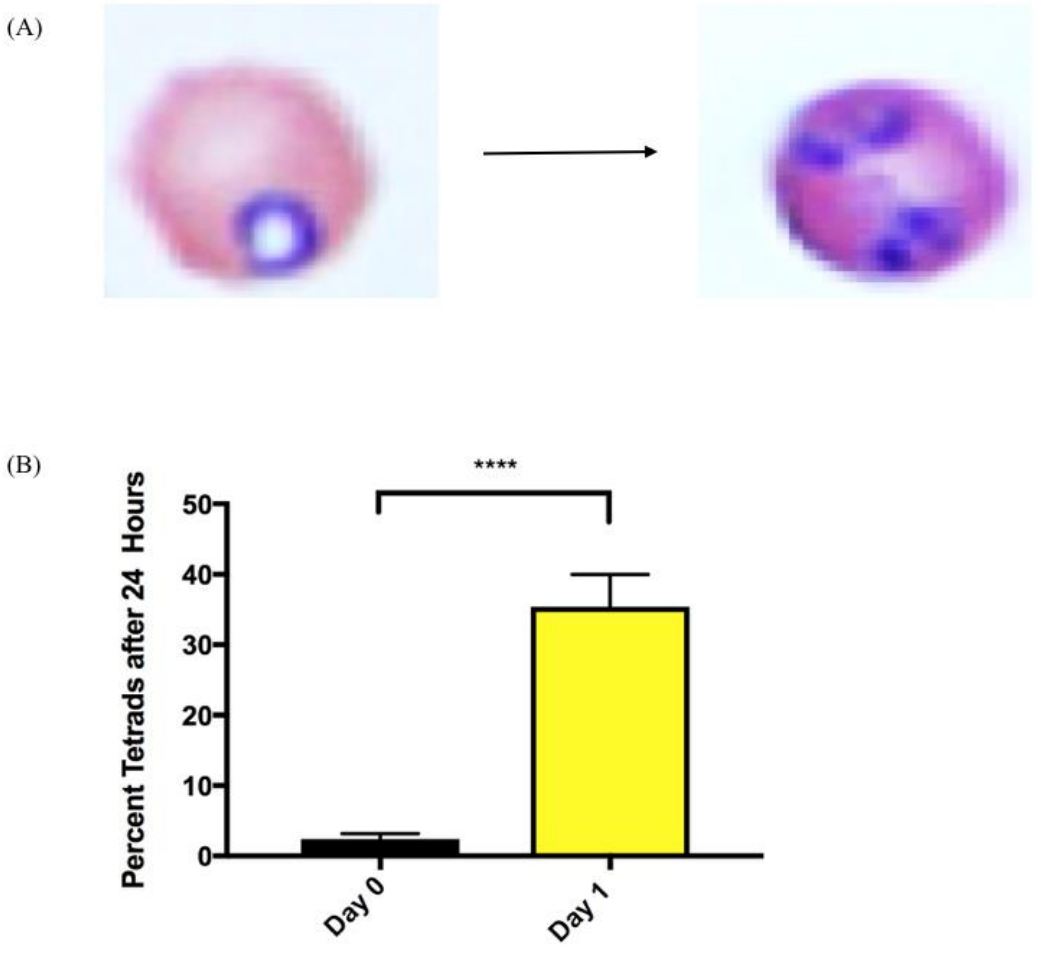


Figure 3.2 Smear Image and Manually Quantified Percent Tetrads Showing Parasite Growth in 24 Hours (A) Blood smear image represents intraerythrocytic growth of *B. microti* from ring to tetrad stage over 24 hours. (B) Statistical analysis of tetrad quantification; data represents percent mean ± SD of triplicate samples. *** indicates significant difference with $p<0.0001$.

Quantification of parasites based on their morphological structures was done manually by counting ten random microscopic fields having an average of 400 cells. These

measurements showed that there was a significant increase in the number of tetrads from 2.4 ±0.4% to 35.4 ± 2.3%. This data confirms that intraerythrocytic growth of *B. microti* occurs within the window of *in vitro* assay over 24 hours. Five independent experiments were carried out to validate this assay and confirm its reproducibility.

3.3 Recombinant *Babesia. microti* Surface Antigen 1 Protein Inhibits Growth

Recombinant BmSA1 has been evaluated as potential diagnostic antigen by ELISA and immunochromatographic tests, and specific antibodies were detected against rBmSA1 post infection.[9] However, the role of BmSA1 in intraerythrocytic growth of the parasite remains to be identified.

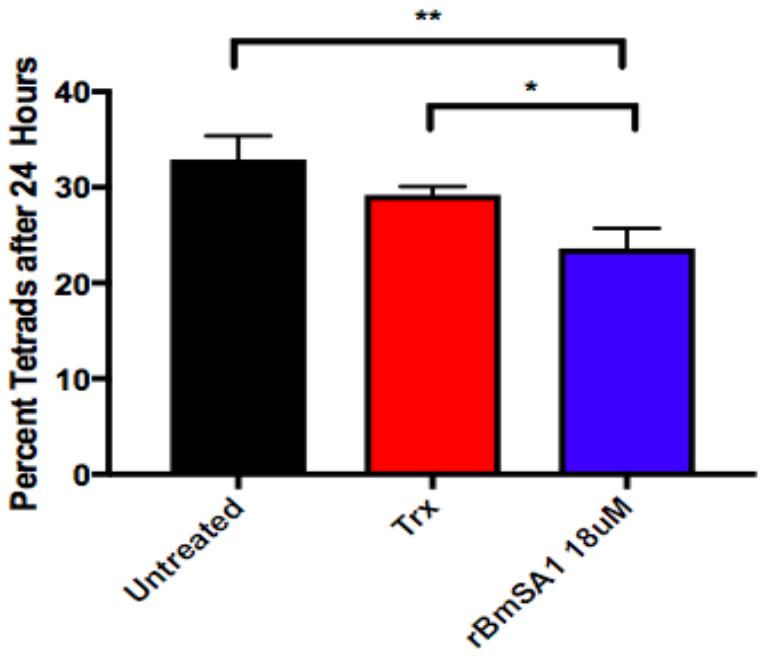


Figure 3.3 Statistical Quantification of Tetrads Post rBmSA1 Protein Treatment
Statistical analysis of tetrad quantification after 18µM rBmSA1 treatment (blue column) and Trx treatment (red column); data represents percent mean ± SD of triplicate samples. ** indicates significant difference with p value of 0.0025 and * indicates significant difference with p value of 0.0283 (independent t-test).

Upon establishment of *B. microti* growth assay, we wanted to test the effect of Trx tagged *B. microti* Surface Antigen 1 (BmSA1) on parasite growth. Quantification of blood smears from rBmSA1 treated samples showed a significantly reduced number of tetrads; i.e., 24.2% as compared to the positive control of 36.7% tetrads under the same culture conditions. To further validate this finding, the same amount of Trx protein (2.0 mg/mL), dialyzed in phosphate buffered saline(PBS), was used as a negative control and plated at 50 µL to test its effect on *B. microti* growth. The Trx protein alone yielding 28.2% showed that there is no significant effect on parasite growth compared as compared to the inhibitory effect of rBmSA1 (Figure 3.3).

3.4 Z-LL2 Ketone, Atazanavir, Lopinavir, and Atazanavir+Lopinavir combination inhibit intraerythrocytic *Babesia microti* Growth

Using protein BLAST analysis, we examined the homology between malaria PfSPP protein sequence and *Babesia microti* signal peptide peptidase (BmSPP) sequence. The primary structure of signal peptide peptidase of *B. microti* showed 81% sequence identity, thus encouraging us to test the effect of PfSPP inhibitors on *B. microti* growth. The SPP inhibitor Z-LL2 ketone, and two HIV protease inhibitors, Lopinavir and Atazanavir, along with a combination of both protease inhibitors were tested to investigate their effect on parasite growth. A triplicate study was designed containing all four treatment arms along with 0.4% DMSO as negative control. Study analyses showed the following means of percentage tetrads under similar conditions after 24 hours - Z-LL2 ketone – 0.073%, Atazanavir – 21.4%, Lopinavir – 14.58%, combination of Atazanavir and Lopinavir – 18.1% and negative controls - 0.4% DMSO – 26.6%.

Statistical studies were carried out using Graphpad prism which showed significant difference between DMSO treated arm and Z-LL2 ketone, Atazanavir, Lopinavir and combination therapy groups.

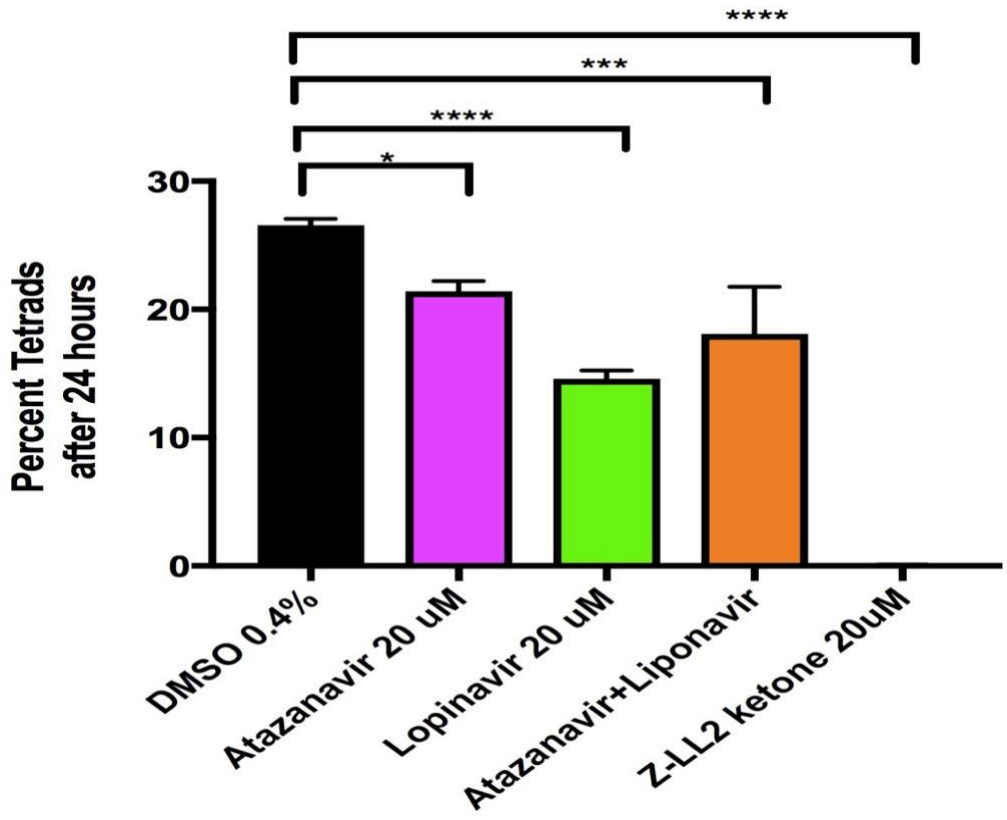


Figure 3.4 Graphical Representation of Effect On Growth After Treatment with Z-LL2 Ketone, Atazanavir, Lopinavir, And Combination of Atazanavir + Lopinavir. Statistical analysis of tetrads quantification of various treated groups compared with corresponding vehicle using one-way ANOVA; F value= 2.54. Data represents percent mean ±SD of triplicate samples. * represent significant difference at p value 0.0139, *** indicates p value 0.0004 and **** indicates p value 0.0001 (independent t-tests).

In conclusion, all 4 treatment arms showed differential inhibitory effects on *B. microti in vitro* growth, but Z-LL2 ketone showed the most dramatic effect of growth inhibition up to 99.8% compared to untreated group (Figure 3.4).

3.5 Human Reticulocyte cDNA Screens to Identify Host Receptors

The total number of cDNA clones in the human reticulocyte cDNA library was determined to be 6 X 10^6 by the plaque assay. Ten independent plaque colonies were selected and DNA was extracted and amplified through polymerase chain reaction(PCR). The amplified cDNA library had a titer of 11X 10^{10} pfu/mL. The selected clones showed a base pair range of 200 bp and 500 bp (Figure 3.5).

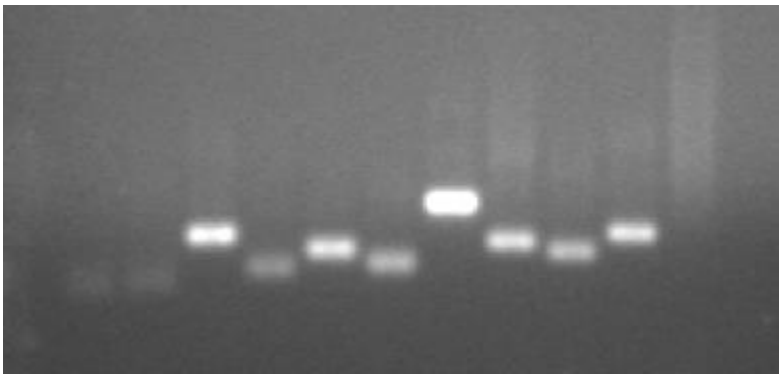


Figure 3.5 Agarose Gel Electrophoresis of Ten Phage Clone DNA Isolated from Plaque Assay. All ten phage clones were sequenced and analyzed for homology with known protein, using BLAST.

DNA sequencing was performed by the Tufts University Genomics Core Facility. Sequencing data of each clone were analyzed by BLAST using the homo sapiens genome database. Ninety percent of the phage clones encoded for hemoglobin chains. One phage clone encoded for protein casein kinase 1 delta (CSNK1D), and another predicted protein shown by BLAST analysis was solute carrier family 16 member 3 (SLC16A3). Both phage clone sequences were outside the coding region for the respective predicted proteins and a protein BLAST did not indicate any homology to known proteins.

3.6 Recombinant Bmsa1 Binds to Potential Host Receptors in Human Erythrocyte Membranes (Ghosts) Detected by Blot Overlay Assay

Upon optimization of the blot overlay protocol, our findings showed a strong interaction between rBmSA1 and a protein with a molecular mass of ~245 kDa. Band 1 and 2 correspond to alpha and beta spectrin chains with molecular weight of 240 and 220 kDa, respectively.[25] Bands 2.1 and 2.2 representing ankyrin also migrate in the same zone. Ankyrin is a peripheral membrane protein located on the cytoplasmic side of the erythrocyte membrane. Spectrin-ankyrin interaction serves a cytoskeletal meshwork providing stability and shape to RBCs. Since spectrin and ankyrin do not possess any features of transmembrane domains or extra facial loops, it is unlikely that they represent the 245 kDa band identified in our blot overlay screen using rBmSA1 (Figure 3.6).

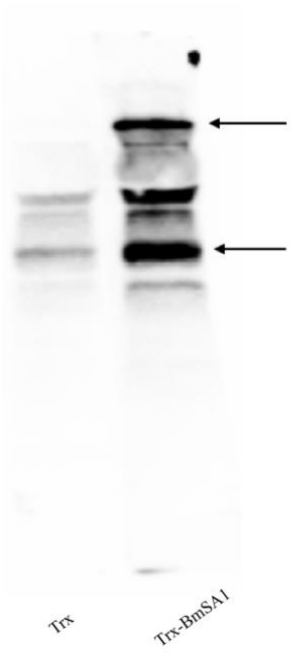


Figure 3.6 Agarose Gel Electrophoresis of Ten Phage Clone DNA Isolated from Plaque Assay. Left- Trx protein alone at 0.5 mg/mL concentration (negative control). Right-Trx tagged BmSA1 at 0.5 mg/mL shows strong binding to bands at 245 kDa and 48 kDa.

Moreover, we also detected another band of ~48 kDa in the blot overlay assay with rBmSA1 (Figure 3.6). The 48 kDa band was particularly robust as compared to the negative control Trx protein band in the blot overlay assay. It is noteworthy that the SLC16A3, a potential host receptor identified in our reticulocyte phage display screens, corresponds to a predicted molecular weight of 49.5 kDa. These observations raise the possibility that BmSA1 may bind to multiple receptors in human erythrocytes.

Chapter 4 – Discussion

The adage "prevention is better than cure" is the underlying principle for the rationale to develop effective vaccines against infectious diseases. To develop an effective vaccine for human babesiosis, it is essential to identify an appropriate combination of antigenic immunodominant peptides against parasite proteins. To achieve this goal, there is a need to establish an *in vitro* culture system that facilitates invasion and growth of *B. microti* parasites. This experimental assay can be utilized to test the effects of various recombinant proteins for vaccine development as well as examine novel pharmacological compounds as potential therapeutics. Published evidence suggests the feasibility of an *in vitro* assay for *B. microti* suitable for invasion studies.[12, 13] These approaches employ ex vivo conditions using specific blood collection and processing protocols, and incubation of infected RBCs under optimized complete media [12]. Another experimental approach is use the osmotic lysis technique to rupture infected RBCs and harvest free parasites, which are then used to infect fresh RBCs in the presence of optimized complete media.[13]

To set up these assays, we first attempted to replicate the lysis method. Despite multiple trials and optimization, we could not replicate this assay for invasion studies. The lysis method may not be experimentally the most convenient and reliable, since the method requires optimization to obtain parasites that are absolutely free of lysed RBC shells. These RBC shells present in the assay system subsequently interfere with data analysis during quantification of *B. microti* invasion from blood smears. The ex vivo method seems to be more adaptable since it utilizes the *B. microti* infected blood during the exponential phase of the infection induced in an immune-compromised inoculated

mouse model. There was no significant increase in invasion as detected by parasitemia, although blood smears prepared after 24 hours of incubation showed more extracellular and developed stages of parasites as compared to smears made at the start of incubation period. These observations suggest that an essential factor required for *B. microti* invasion may be missing from the media under these culture conditions. We also added mouse serum from Rag-1 KO mice in the complete media to increase invasion efficiency. Again, no increase in invasion was observed. In future studies, supplementing complete media with human serum of AB blood group could be tested to increase in *vitro* invasion, since a similar approach was originally utilized by the Trager and Jansen to establish an *in vitro* assay for *Plasmodium falciparum* invasion and growth assay.[11]

Despite the lack of a viable *in vitro* invasion assay for *B. microti*, our experimental system for the intraerythrocytic development of *B. microti* to tetrads was robust, quantitative, and reproducible. Although the growth assay is feasible, it requires laborious counting of tetrads by microscopy. In the future, the tetrad quantification could be optimized using a protocol adapted for flow cytometry by utilizing appropriate nucleus staining dyes such as SYBR green that can differentiate various stage of parasite development. With this approach, the quantification of parasites would not be limited to only tetrad stage and could provide more information on the effect of target molecules on the amoeboid and ring stages of the parasite. Using a manual growth assay, we were able to determine the inhibitory effect of BmSA1 on tetrad development during the 24 hours' incubation period as compared to untreated control. The underlying mechanism by which BmSA1 inhibits parasite growth remains unknown at this stage. A hypothetical rationale could be considered whereby a communicative pathway is established between the ring

stage parasite and host RBC receptor involved in invasion, which facilitates parasite development. This pathway could be affected by rBmSA1 via its recognition of the host receptor thus causing disruption of parasite growth under these conditions.

Babesia microti Surface Antigen 1 (BmSA1) is a major antigenic ligand, and literature suggests its potential functional role via recognition of host RBC receptors during invasion.[10, 9] The identification of host erythrocyte receptors facilitating *B. microti* invasion is of immense importance as it can facilitate mapping the binding interface of the host-ligand interactions, thus leading to specific antibody development as well as elucidating the underlying cellular mechanism and pathways involved in the invasion process that could be potential targets to inhibit parasitic invasion. Despite the potential experimental limitations generally encountered in the search to identify host receptors, we performed phage display screens using human reticulocyte cDNA to identify the host receptor for rBmSA1. Since most of the cDNA clones were derived from hemoglobin chains, future studies will be required to optimize this experimental approach to suppress the abundant expression of hemoglobin clones in such screens. As an alternate strategy, we attempted to purify mRNA isolated from reticulocytes through an agarose beads affinity column containing zinc beads which have high affinity for alpha and beta subunits of hemoglobin.[26] However, this approach was not successful under our conditions. Another potential limitation of the phage display technology is the difficulty in preparing libraries without splicing the mRNA into very small fragments that leads to the expression of proteins with relatively shorter domains expressed on the phage surface that have lost its original conformation. A similar limitation also exists for the blot overlay assay where the SDS-PAGE of the RBC membrane proteins results in the

denaturation of some proteins. In any case, the identification of two potential host receptors migrating at 245 kDa and 48 kDa bands, as detected in this study, raises the possibility that one or both of these bands could serve as physiological host receptors of *B. microti*. Future studies using mass spectrometry and *in vitro* expression assays will be required to test these predictions. Together, the main purpose of this study was to begin to elucidate the underlying cellular mechanisms involved in *Babesia microti* invasion and growth in human erythrocytes, steps that are integral for the development of novel vaccine and therapeutic drug targets.

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