Susceptibility to Babesiosis, an Infectious Disease of Erythrocytes

Senior Honors Thesis for the Department of Biomedical Engineering

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TABLE OF CONTENTS

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ABSTRACT

Babesiosis is an emerging infectious disease caused by the intraerythrocytic parasite *Babesia microti*. Young, healthy individuals usually experience asymptomatic infection or mild illness, whereas elderly or immunocompromised individuals are at risk for severe disease. In these cases, symptoms and parasitemia may persist for several months. The genetic and immunologic determinants of resistance to *B. microti* infection are poorly understood. Using the susceptible DBA/2 (D2) strain and the resistant C57Bl/6 (B6) strain, the laboratory mapped the main determinant of resistance in young mice to an interval in the mid/proximal region of chromosome 9. I infected B6.D2 strains that vary by the lengths of their congenic intervals, and narrowed the main determinant to a list of 14 genes. The laboratory had observed that lack of the *cd4* gene renders B6 mice susceptible to babesiosis, but that parasitemia eventually resolves. In depletion experiments, I confirmed that B cells are required for resolution of parasitemia in *cd4*-/- B6 mice, but not in wild type B6 mice. I developed a flow cytometry based assay to quantify IgG subisotypes in plasma samples from *B. microti* infected mice. I observed that IgG1 and IgG3 are specific of *B. microti* whereas IgG2b and IgG2c also recognize moieties of erythrocytes. I established that the antibody response is delayed, but concomitant to resolution of parasitemia in *cd4*-/- mice. I established that B cells are critical for resolution of parasitemia in young, but less so in aged DBA/2 mice. These studies have yielded insights on host resistance/susceptibility to babesiosis.

INTRODUCTION

Babesiosis is an infectious disease caused by parasites of the genus *Babesia*. In the United States, the first confirmed case occurred in 1969 on Nantucket, an island off the coastline of southern Massachusetts.[1] Since, hundreds of cases have been documented in southern New England, New York, New Jersey, Wisconsin and Minnesota.[2-4] Most cases are due to *Babesia microti*, a parasite of small rodents. Owing to the steady increase in the number of cases reported each year over the last two decades, babesiosis is now considered an emerging infectious disease. Most cases of *B. microti* infection are acquired during the bite of an infected tick (*I. scapularis*). Transfusion-transmitted babesiosis is on the rise whereas transplacental transmission remains anecdotal.[3, 5, 6] Infections with *B. microti* range in severity from asymptomatic to fatal. Mild cases present with a flu-like illness. Symptoms include malaise, fatigue, fever, chills, sweats, myalgia, arthralgia, headache, and cough.[7-12] Mild cases usually do not require treatment.[9] On the other hand, severe disease, which is characterized by a parasitemia greater than 4%, requires hospitalization and antimicrobial therapy.[7, 13-15] Despite therapy, the fatality rate among hospitalized patients is 5-10%.[13, 16]

Individuals over age 50 and immunocompromised patients are at risk for severe babesiosis. The number and duration of symptoms do not differ by age, but older individuals are hospitalized more often than younger cases.[17] In one study, the average age at admission was 53 years.[14] In another study, the average age at admission was 63 years for previously healthy individuals, but 48 years for patients with underlying health conditions.[7] Seroprevalence does not differ between age groups, implying that exposure to *B. microti* does not differ with age.[18] These observations suggest that the susceptibility of the elderly results from age-acquired decline in host resistance. Insights into the mechanisms of host resistance come from the epidemiology of

immunocompromised patients. Regardless of age, conditions that render individuals highly susceptible to babesiosis include asplenia, B cell malignancy, HIV/AIDS, and immunosuppressive therapy.[2, 9, 10] One fifth of immunocompromised patients who experience severe babesiosis eventually die.[8] Thus, the susceptibility of the elderly to babesiosis may result from one or several immune deficiencies.

Our knowledge of the immune response to babesiosis is limited, but our knowledge of the genetic basis of host resistance is nil. In mice, resistance to *B. microti* infection depends on the host genetic background, but does not strictly depend on MHC haplotype.[19] To uncover genetic determinants of host resistance, the laboratory developed a mouse model. Parasitemia in young C57Bl/6 (B6) mice is modest and quickly resolves whereas parasitemia in young DBA/2 (D2) mice is severe but transient.[20] The susceptibility of D2 mice increases with age whereas the resistance of B6 mice remains unaffected. Resistance to babesiosis in young mice maps to the proximal region of chromosome 9, whereas resistance in old mice maps to the middle region of chromosome 9.[21] Using B6.D2 congenic strains, the laboratory mapped the main determinant of resistance in young mice to a 1.5 Mb interval in the mid/proximal region of chromosome 9. This interval contains 18 genes. The first aim of my thesis was to shorten the list of candidate genes, and possibly identify the main genetic determinant of resistance to *B. microti* infection.

The spleen is essential for resistance to *Babesia* infection as asplenia is a major risk factor for severe babesiosis in people.[10] In mice, the adoptive transfer of spleen cells from immune donors protects naïve recipients from *B. microti* infection.[22] T cells are critical for protection. Transfer of spleen cells depleted of B cells protects naïve recipient mice that had been depleted of T cells.[23] Athymic mice experience high, persistent parasitemia.[24] Adoptive transfer of thymocytes protects athymic mice as well as T less, B less deficient mice.[25] Antibody-based

cell depletion experiments have established that $CD4^+$ T cells are essential for resistance, whereas $CD8^+$ T cells are dispensable.[26, 27] These observations in mice point to the central role of $CD4^+$ T cells in host resistance to babesiosis, and are in agreement with the susceptibility of HIV/AIDS patients to *B. microti* infection.[28] To study the susceptibility of hosts that have a compromised CD4 T cell compartment, the laboratory has relied on c*d4*-deficient mice. In this model, parasitemia is intense but eventually resolves. The second aim of my thesis was to identify mechanisms that promote resolution of parasitemia in *cd4*-deficient mice.

The role of humoral immunity in babesiosis is unclear. Studies in mice do not support a role for B cells in host resistance to *B. microti* infection. Depletion of B cells by lifelong administration of anti-IgM does not increase susceptibility in BALB/c mice.[29] Likewise, lack of B cell maturation by *igh6* gene deletion does not alter the resistance of B6 mice.[30] Last, transfer of immune serum does not prevent *B. microti* parasitemia in athymic mice or in T less, B less mice.[25, 29, 31] Clinical data suggest otherwise. Persistent or relapsing babesiosis is often diagnosed in patients who have a B cell malignancy, for which they were treated with rituximab, a monoclonal antibody directed against the B cell surface marker CD20.[8] Considering that B cells may be critical in hosts that present an immune deficiency, I investigated the role of B cells in the resolution of parasitemia in *cd4*-deficient mice. Given that young DBA/2 mice are susceptible to *B. microti* infection, and even more so in old age, I also investigated the importance of B cells in the resolution of parasitemia in this strain.

MATERIALS AND METHODS

M1 – Mouse strains

Mice were maintained at the Division of Laboratory Animal Medicine at Tufts University School of Medicine in Boston, Massachusetts. Mice, housed in clean cages holding one to five mice each, had unlimited access to water and chow. C57BL/6 (B6) mice, DBA/2 (D2) mice, and mice deficient for a single gene were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice deficient for two genes were created by: i) mating males from a single gene deficient strain with females from the other single gene deficient strain, ii) intercrossing F_1 males and females, and iii) genotyping the F_2 offspring. Mice with double gene deficiencies were maintained by brother-sister mating. Prior to my arrival, the laboratory had created several strains of congenic mice using an approach detailed in the results section (see section R1). Briefly, mice that carry a D2 congenic interval were backcrossed onto the B6 background. Their offspring were intercrossed, and recombination events identified. Each recombinant mouse of interest was backcrossed, and heterozygous offspring intercrossed to create a new congenic strain.

M2 – Mouse DNA extraction and genotyping

Two punches of ear tissue were obtained from each mouse. The tissue was minced with a razor blade, and digested overnight in proteinase K (QIAGEN, Valencia, CA). Genomic DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN). DNA was amplified by polymerase chain reaction (PCR) using the HotStar *Taq* polymerase. The forward and reverse primers recognized unique sequences in the mouse genome that flank simple sequence length polymorphisms (SSLP) between the B6 and D2 strains. Amplicons were electrophoresed in a 2%

Nusieve agarose gel at 120 V. Parental origin of the amplified DNA was determined based on the size of the amplicons.

M3 – Infection of mice with *Babesia microti*

The clinical isolate of *Babesia microti*, RM/NS, is maintained by Dr. Samuel R. Telford III at Tufts University School of Veterinary Medicine (North Grafton, MA). He maintains the isolate by alternate passages in *Ixodes scapularis* ticks and *rag1*-deficient mice. *Rag1*-deficient mice experience high, sustained parasitemia (~40%) for at least three months, and are the source of parasitized red blood cells for the infection of experimental mice. Briefly, Dr. Telford provided us with aliquots of blood collected in Alsever's solution. Back in our laboratory, we infected a naïve *rag1*-deficient mouse with 10^5 parasitized red blood cells (day 0). Starting on day 14, an aliquot of blood was obtained daily, and parasitemia was determined by flow cytometry (see section M4). When parasitemia approached peak value (~55%), several drops of blood were collected in heparinized phosphate buffered saline (PBS). The concentration of red blood cells was determined using a hematocytometer. The blood sample was diluted in Dulbecco's phosphate buffered saline (D-PBS) so that 10^5 parasitized red blood cells were contained in 0.2 mL. Mice were infected intraperitoneally using a tuberculin syringe mounted with a 25G needle. All experimental mice were males, as the laboratory, in agreement with an earlier report using (B6 x BALB/c) F_1 mice,[32]observed that males from the D2 and B6 strains are more susceptible than strain matched females (unpublished observation). Note that the gender difference is less pronounced in the B6 strain than in the D2 strain, thereby yielding a phenotypic difference large enough for mapping purposes.

M4 – Determination of parasitemia

The protocol to determine parasitemia by flow cytometry had been developed in the laboratory by Borggraefe and colleagues.[33] Briefly, a drop of blood was obtained at the tip of the mouse tail every day to every three days, starting seven days post-infection. The drop of blood was collected in 250 μL heparinized PBS. Blood cells were fixed in glutaraldehyde (0.0097% in PBS) for 30 minutes at room temperature, permeabilized with Triton X-100 (0.25% in PBS) for five minutes at room temperature, and treated with DNase-free RNase A (100 μg/mL in PBS) for 60 minutes at 37° C. All subsequent steps took place at room temperature. Following each incubation period, cells were centrifuged at 200 g for 5 minutes. Following RNase treatment, cells were resuspended in staining buffer (i.e., PBS containing 1% normal rabbit serum and 0.1% sodium azide). Cells from a given sample were transferred into two tubes, and exposed to either a rat anti-mouse CD71 IgG_1 monoclonal antibody (0.5 μg/mL in staining buffer) or a rat anti-keyhole limpet hemocyanin (KLH) IgG_1 monoclonal antibody (0.5 μg/mL in staining buffer) for 30 minutes. Cells were washed, and exposed to Alexa647-coupled goat anti-rat IgG antibody (1.25 μg/mL in staining buffer) for 20 minutes. Cells that had been exposed to the rat anti-KLH antibody were added with staining buffer. Cells that had been exposed to the rat anti-CD71 antibody were added with a solution of YOYO-1, a DNA/RNA fluorescent probe, at a final concentration of 2 μM. After at least 60 minutes of incubation in the dark, samples were analyzed using the FACSCalibur flow cytometer. YOYO-1 was excited by the Argon laser at 488 nm whereas Alexa647 was excited by the red diode laser at 635 nm. Emitted fluorescence was acquired using CellQuest. Data were analyzed using FlowJo. Parasitemia is defined as the fraction of blood cells (expressed in %) that are CD71 negative (i.e., mature erythrocytes) and

YOYO-1 positive (due to parasite DNA) among the 10,000 cells acquired per sample. An overview of the staining protocol and a dot plot of stained blood cells are provided in Figure 1.

M5 – Depletion of B cells

B cells were depleted by repeated injections of 18B12, an IgG2a monoclonal antibody directed against mouse CD20. The isotype control was 2B8, a monoclonal antibody directed against human CD20, as this antibody does not recognize mouse CD20.[34] Both antibodies were injected intraperitoneally every other week, starting two weeks prior to infection. Throughout the course of infection, the frequency of B cells among peripheral blood leukocytes was monitored each week. Briefly, two drops of blood were obtained at the tip of the mouse tail, and collected in 250 µL heparinized PBS. Blood samples were added with 750 µL red blood cell lysis buffer (140 mM ammonium chloride and 18 mM Trizma base in deionized water), and incubated for 10 minutes at room temperature. All steps took place at room temperature. Cells were centrifuged at 200 g for 5 minutes, and resuspended in staining buffer (i.e., D-PBS containing 1% normal rabbit serum and 0.1% sodium azide). Cells were centrifuged, and resuspended in staining buffer containing FITC-conjugated rat anti-mouse CD45 antibody and Alexa647-conjugated rat anti-mouse CD45R/B220 antibody (both at 0.5 µg/mL in staining buffer). Cells were incubated for at least 30 minutes in the dark. Cells were washed once, and resuspended in staining buffer. Samples were analyzed using a LSRII flow cytometer. FITC was excited at 488 nm by the Argon laser whereas Alexa647 was excited by the red diode laser at 633 nm. Emitted fluorescence was acquired using BD FACSDiva. Data were analyzed using FlowJo. Mice in which B cells (detected as $CD45^+B220^+$ cells) accounted for >10% of blood leukocytes (detected as $CD45^+$ cells) at one time point during the course of infection were excluded from the entire analysis.

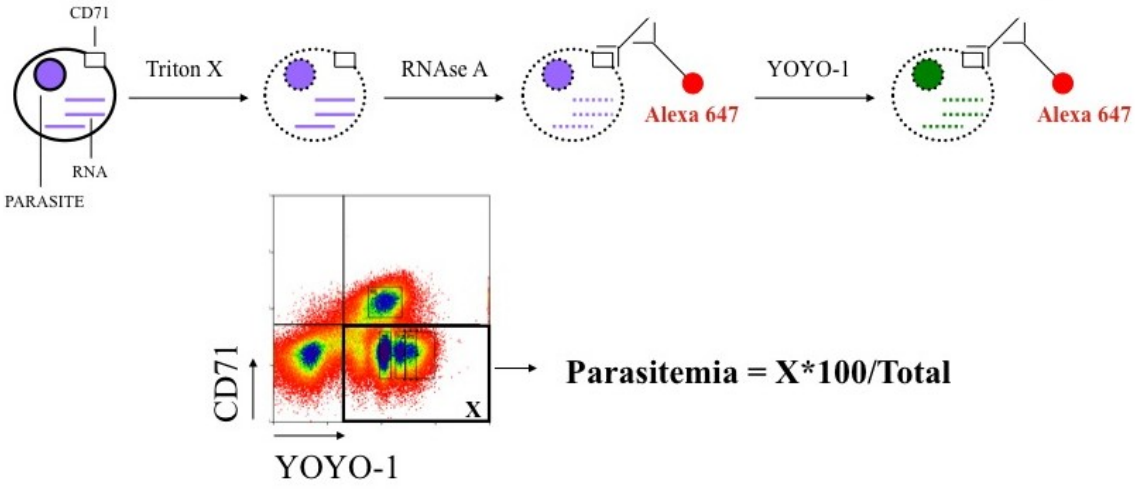

Figure 1 – Measurement of parasitemia using flow cytometry.

A) One drop of blood is collected at the tip of the mouse tail. Cells are fixed in glutaraldehyde, permeabilized in Triton-X 100, and treated with DNase-free RNase A. Reticulocytes are identified using a rat IgG1 monoclonal antibody specific for mouse CD71, a reticulocyte surface marker, and an Alexa 647-coupled goat anti-rat IgG antibody. YOYO-1, a nucleic acid dye, detects parasite DNA.

B) Cells subjected to the Argon and the red diode lasers are visualized in a dot plot. Reticulocytes, rarely infected by *B. microti*,[33] are CD71 positive. Although reticulocytes are exposed to RNase A, residual RNA is detected by YOYO-1 (upper right quadrant). Mature erythrocytes do not express CD71, have no nucleus and no RNA (lower left quadrant). CD71 negative, YOYO-1 positive cells are mature erythrocytes infected with *B. microti* (lower right quadrant). Intensity of fluorescence is a function of parasite number. The most left vertical streak in the lower right quadrant consists of cells that harbor one parasite. [33] The second streak (immediately on the right of this first streak) consists of cells that predominantly contain two parasites. The third streak consists of cells that mostly contain three parasites. Cells containing at least four parasites are rare. Parasitemia is the fraction of cells that are $CD71^{-}$ and $YOYO\text{-}1^{+}$.

The average frequency of B cells in 18B12 treated mice was <5% of total leukocytes at any given time point.

M6 – Preparation of plasma from mouse blood

Starting on day 12 post-infection, plasma was prepared every three to five days. Briefly, mice were heated under a heat lamp for one to two minutes to induce vasodilatation. The tip of the tail was nicked. Three to four drops of blood were collected in a glass capillary tube coated with heparin. The height of the blood column was recorded. The blood column was immediately flushed with heparinized PBS (5 times the volume of the blood column) into a microcentrifuge tube that already contained 100 µL heparinized PBS. The capillary tube was washed several times with the flow through. Additional heparinized PBS was added to the microcentrifuge tube so that the blood sample was diluted by a factor of 15. Diluted blood samples were centrifuged at 200 g for 10 minutes at 4°C. Supernatants were carefully removed to minimize the uptake of blood cells. Supernatants were centrifuged at 13,000 g for 5 minutes at 4°C. Cell-free supernatants were collected while carefully avoiding cell pellets. Diluted plasma samples were stored at -20°C. Samples were thawed only once (on the day of the experiment) as we had observed that antibody-antigen recognition is altered by repetitive thawing (unreported observation).

M7 – Detection and quantification of *Babesia*-specific antibodies

M7.1 – Low-throughput Assay

The flow cytometry based assay to measure parasitemia (see section M4) was modified to detect antibodies bound to red blood cells. Drops of blood were obtained from *Babesia* infected *rag1*-deficient mice, as these mice do not produce antibodies and experience high and persistent

parasitemia (~40%) after the second month of infection. Blood cells were fixed in glutaraldehyde, permeabilized in Triton X-100, and treated with DNase-free RNase A, as described in section M4. The Triton X-100 solution, however, was prepared in PBS containing 1% normal rabbit serum, as we observed that rabbit serum minimizes the stickiness of blood cells to plastic tubes during the RNase step. Once resuspended in staining buffer, cells were exposed for 30 minutes to diluted plasma (prepared in staining buffer). Cells were washed, and exposed for 30 minutes to a biotin-coupled goat antibody directed against a given mouse immunoglobulin isotype (1 μg/mL in staining buffer). Cells were washed, and exposed for 10 minutes in the dark to Alexa647-coupled streptavidin (1.25 μg/mL in staining buffer). Last, YOYO-1 (final concentration of 2 μM) was added to cells that had been exposed to diluted plasma. Cells that had been incubated in staining buffer (instead of diluted plasma) were added with staining buffer (instead of YOYO-1). After at least 60 minutes in the dark, samples were subjected to the Argon and the red diode lasers of the FACSCalibur. Fluorescence was acquired using CellQuest. Data were analyzed using FlowJo. Data are presented as dot plots.

M7.2 – High-throughput Assay

The FACS Calibur is not amenable to high-throughput as samples are prepared in tubes and fed manually to this flow cytometer. The LSRII, however, is more versatile as it reads samples prepared in either tubes or 96 well plates. The flow cytometry based assay described in the above paragraph was scaled up to titer antibody isotypes in plasma samples obtained from a single mouse at nine time points. Blood was obtained from seven infected *rag1*-deficient mice, as described in the above paragraph. Cells were fixed in glutaraldehyde, permeabilized in Triton X-100 (0.25% in PBS containing 1% normal rabbit serum), washed and resuspended in staining

buffer. All steps took place at room temperature. The seven blood samples were pooled, and the cell concentration adjusted to reach 6 million cells per mL. Plasma samples were serially diluted by a factor of 3 in staining buffer. The lowest dilution was 1/100 whereas the highest dilution was 1/72,900. Plasma dilutions (50 µL) were added individually to 96 V-shaped well plates. Cells (50 µL) were added and mixed with the plasma dilutions using a multichannel pipette. After 30 minutes of incubation, staining buffer was added using the multichannel pipette. Cells were resuspended, and plates spun at 200 g for 5 minutes. Supernatants were removed, and the appropriate biotinylated goat anti-mouse isotype antibody (αIgM, αIgG1, αIgG2b, αIgG2c, or αIgG3 at 1 µg/mL in staining buffer) was added using a repeat pipette. Cells and antibodies were mixed for 5 seconds at a frequency of 480 strokes per minute using an ELISA plate reader. Cells were exposed to these antibodies for 30 minutes. Plates were centrifuged, and supernatants removed. A solution containing Alexa647-conjugated streptavidin (1.25 µg/mL), FITC-conjugated rat anti-mouse CD71 (0.5 µg/mL), and DAPI dilactate (1.3 µM) was added with a repeat pipette. Cells were resuspended using the ELISA plate reader, and incubated for 60 minutes in the dark. Cells were washed and resuspended in staining buffer. Samples were analyzed using a LSRII flow cytometer. DAPI was excited at 350 nM, FITC at 488 nM and Alexa647 at 640 nM. (The LSRII allows for the simultaneous detection of three fluorochromes using three different lasers without a need for spectral compensation.) Emitted fluorescence was acquired using BD FACSDiva. Data were analyzed using FlowJo. Note that the data pertaining to CD71 have yet to be analyzed, and are not presented herein. An overview of the staining protocol is provided in Figure 2.

Figure 2 – Detection of antibody isotypes by flow cytometry. Blood is obtained from *Babesia* infected *rag1*-deficient mice, as they do not have mature B cells and cannot produce immunoglobulins. Blood cells are fixed in glutaraldehyde and permeabilized in Triton X-100. Blood cells, the source of *B. microti* antigen, are exposed to serial dilutions of plasma samples, the source of antibodies. Mouse antibody isotypes that are bound to red blood cells are detected by use of biotinylated goat anti-mouse IgM, IgG1, IgG2b, IgG2c, or IgG3. These biotinylated antibodies are detected by use of A647-conjugated streptavidin. Parasite DNA is bound by DAPI. Reticulocytes are distinguished from mature red blood cells by use of a FITC rat anti-mouse CD71 antibody.

M8 – Statistical analysis

M8.1 – Susceptibility to B. microti infection

Parasitemia values are reported as mean ± SEM. For the fine mapping experiment, SEM is omitted for sake of clarity. For each mouse, the parasite burden, defined as the integral of parasitemia over time, was derived. Parasite burden values are presented as mean ± SEM. Differences in parasite burden were tested for statistical significance using the unpaired Student's *t*-test, and considered significant when the two-tailed p-value was ≤ 0.05. Statistical analysis was performed using Microsoft Excel.

M8.2 – Antibody isotype levels

One set of nine plasma samples obtained from a given mouse was tested for the five antibody isotypes on a given day. Each of the nine plasma samples was tested at seven dilutions. Diluted plasma samples were tested for a given isotype on a single 96-well plate. Each plate included a set of three wells containing cells that had been stained with DAPI (for parasite DNA), but not exposed to plasma dilutions. Among these cells, as expected, some were infected with one parasite; others were infected with two, three or more parasites. As DAPI staining was recorded on the X axis, a horizontal threshold was set across the populations of infected cells to divide the overall population of infected cells into two equal halves. This threshold was applied to all samples that had been stained with DAPI and that had been exposed to plasma dilutions (Figure 3, horizontal red dotted line). The fraction of infected cells above the threshold was calculated. For each isotype, means and SEM were derived. The fraction of antibody-bound *infected* cells was obtained by subtracting 0.5 from the mean value of a given group. The threshold set to halve the population of infected cells did not equally divide the population of uninfected cells that had

not been exposed to plasma dilutions. In fact, 60% of these uninfected cells were above the threshold whereas 40% were below. Thus, the fraction of antibody-bound *uninfected* cells was obtained by subtracting 0.6 from the mean value of a given group. When plasma samples were tested at the final dilution of 1/5400, differences in the fractions of antibody-bound cells between wild type mice and *cd4*-/- mice were tested for statistical significance using the unpaired Student's *t*-test, and considered significant when the two-tailed p-value was ≤ 0.05. Statistical analysis was performed using Microsoft Excel.

Figure 3 – Quantification of antibody-bound red blood cells. Plasma samples obtained at nine time points (from day 12 to day 45 post-infection) were serially diluted by a factor of three from 1/100 to 1/72,900. Diluted plasma samples (50 µL) were aliquoted into a 96 well plate. Red blood cells obtained from *rag1*-deficient mice were added (50 µL). Isotypes were detected by a biotin-conjugated goat anti-mouse antibody and Alexa647-conjugated streptavidin. Parasite DNA was detected by DAPI. For each well/sample, the fluorescence emitted in the far red channel (Alexa647; Y axis) was plotted against the fluorescence emitted in the violet channel (DAPI; X axis). For this figure, the matrix of dot plots was generated by analysis of IgG2c contained in plasma samples obtained from a *cd4*-/- mouse. Using cells that had been stained with DAPI but that had not been exposed to plasma dilutions (not shown), a horizontal threshold was set to divide the cloud of infected cells into two halves. The highest dilution of plasma (1/145,800, far right column) typically did not yield antibody staining, i.e., the horizontal threshold (red dotted line) remained centered across the cloud of infected cells (to the right of the vertical red dotted line). As the antibody concentration increased (i.e., the dilution factor decreased), a greater fraction of infected cells (Q2/Q2+Q3) resided above the horizontal threshold. The fraction of antibody-bound infected red blood cells is defined as the upper fraction of infected cells that have been exposed to a plasma dilution minus 0.5 (i.e., the upper fraction of infected cells that have not been exposed to plasma dilutions). Likewise, the fraction of antibody-bound *uninfected* red blood cells is defined as the upper fraction of *uninfected* cells that have been exposed to a plasma dilution (Q1/Q1+Q4) minus 0.6 (i.e., the upper fraction of *uninfected* cells that have not been exposed to plasma dilutions).

RESULTS

▼▼▼▼▼▼R1 – Genetic Basis of Resistance to *Babesia microti* Infection

A few years ago, the laboratory established that host resistance to babesiosis primarily maps to three contiguous loci in the proximal and mid regions of mouse chromosome 9.[21] One of these loci, referred to as *Bmir1C*, overlaps the main locus (24-30 cM) predicted by linkage analysis. The laboratory created several B6 strains that are congenic for D2 intervals in the proximal/mid region of chromosome 9. These B6.D2 strains are referred to by the numerals of the two markers (D9Mit##) that flank the known centromeric and telomeric ends of the D2 congenic interval, and at which DNA is of B6 origin. The laboratory established that the strains 93/262 and 130/262 are susceptible to *B. microti* infection whereas the strain 99/262 is resistant (Figure 4). Given that the actual centromeric end of the 99/262 interval resides between D9Mit99 and D9Mit26, the laboratory conservatively concluded that a main determinant of resistance resides between D9Mit130 and D9Mit26. This interval encompasses 18 genes (Figure 4). To

further narrow the region in which the determinant resides, additional congenic strains were created.

R1.1 – Creation of the congenic strains 93/256 and 331/262

Prior to my arrival, the laboratory had backcrossed the congenic strains 93/144 and 130/262 onto the resistant B6 background, and intercrossed the progeny. The laboratory had identified two novel recombination events: one occurred near D9Mit256 during the backcross of the strain 93/144 (Figure 5). The other occurred near D9Mit331 during the backcross of the strain 130/262 (Figure 6). Taking advantage of these recombination events, I participated in creating the congenic strains 93/256 (Figure 5) and 331/262 (Figure 6). Briefly, the two pups with recombination events of interest were aged to two months, and backcrossed onto the B6 background. Offspring that had heterozygous genotypes across the regions of interest were aged to two months and intercrossed. Among their progeny, offspring that had D homozygous genotypes in the regions of interest were aged to two months and intercrossed to maintain and expand the strains.

R1.2 – Fine mapping of host resistance to B. microti infection

In the summer of 2010, males from the newly created strains (93/256 and 331/262) and males from three congenic strains that had previously been created (93/262, 130/262, 99/262) were infected with 10^5 parasitized red blood cells at two to three months of age. DBA/2 (D2) and C57BL/6 (B6) mice served as parental susceptible and resistant controls, respectively. Parasitemia was measured every day to every three days from day 7 to day 35 post-infection (Figure 7A). Consistent with earlier experiments in the laboratory, I observed that the time-

course of parasitemia in 99/262 mice was similar to that in B6 mice, but that peak parasitemia was higher in 93/262 mice and 130/262 mice than in 99/262 mice (both $p < 0.05$; Figure 7A). I also observed that the time course of parasitemia in 99/262 mice was similar to those in 331/262 mice and 93/256 mice. Likewise, the time course of parasitemia in B6 mice was similar to that in 331/262 mice or 93/256 mice. Parasite burden, defined as the integral of parasitemia over time, was derived for each mouse within a given group, and group averages calculated (Figure 7B). Parasite burdens in 93/262 and 130/262 mice did not statistically differ from each other ($p = 0.42$), but were significantly higher than in 99/262 mice (both $p < 0.01$). Importantly, parasite burdens in 93/262 and 130/262 mice were higher than in 331/262 mice ($p = 0.027$ and $p = 0.014$, respectively). Accordingly, parasite burden in 331/262 mice did not differ from those in 99/262

Figure 4 – Candidate genetic determinants of host resistance to *B. microti*. B6.D2 strains are named after the numerals of the two markers on mouse chromosome 9 (D9Mit##) at which DNA is of B6 origin (black line) and that are immediately proximal and distal to the known centromeric and telomeric ends of the congenic D2 interval (green box). Genomic regions of unknown genotype are depicted as blue boxes. The 93/262 and 130/262 strains are susceptible to *B. microti* infection whereas the 99/262 strain is resistant. When interpreted conservatively, these results indicate that a main determinant of resistance to *B. microti* resides between D9Mit130 and D9Mit26. This interval contains 18 genes. Given that D9Mit99 is located within the *Tmprss5* intragenic region, *Tmprss5* is listed among the genes located on either side of this marker, and is denoted by a single asterisk. D9Mit26 is located within the *Ncam1* intragenic region, but near its transcriptional start site. *Ncam1* is denoted by a double asterisk.

Figure 5 – Creation of the congenic strain 93/256. Mice of the 93/144 strain were backcrossed onto the B6 background and intercrossed. F2 pups were genotyped. In the mouse #3198, a recombination event was identified near D9Mit256. This male was mated with B6 females. Offspring that had heterozygous genotypes at D9Mit130 (i.e., between D9Mit93 and D9Mit256; purple line) were intercrossed at two months of age. Among their progeny, offspring that had

D/D genotypes at D9Mit130 were intercrossed to expand the strain. Since, the 93/256 strain has been maintained by brother-sister mating.

Figure 6 – Creation of the congenic strain 331/262. Mice of the 130/262 strain were backcrossed onto the B6 background, and intercrossed. F2 pups were genotyped. In the mouse #2776, a recombination event was identified near D9Mit331. This female was mated with a B6 male. Offspring that had heterozygous genotypes at D9Mit256 and D9Mit331 and D/D genotypes from D9Mit99 to D9Mit144 were intercrossed at two months of age. Among their progeny, offspring that had B/B genotypes at D9Mit256 and D9Mit331 and D/D genotypes from D9Mit99 to D9Mit144 were intercrossed to expand the strain. Since, the 331/262 strain has been maintained by brother-sister mating.

mice and in B6 mice (both $p > 0.05$). Last, parasite burden in 93/256 mice was lower than in 93/262 mice ($p = 0.002$), but did not differ from that in B6 mice ($p > 0.05$).

The centromeric and telomeric ends of the congenic intervals have been fine mapped to the extent made possible by the use of the D9Mit markers. In addition to the congenic interval defined by D/D genotypes, the regions of unknown genotypes that immediately flank a given congenic interval also may contain determinants of resistance. Given that the 130/262 strain is susceptible whereas the 331/262 strain is resistant, we conclude that a determinant of host resistance to babesiosis resides between D9Mit130 and D9Mit99 (Figure 7C). The 93/256 strain is resistant to babesiosis, and yet has D/D genotypes at and presumably in the immediate vicinity of D9Mit130. Owing to the fact that the telomeric end of the congenic interval in the 93/256 strain has yet to be mapped precisely, we cannot rule out as candidate determinants some of the genes that reside between D9Mit130 and D9Mit256 (Figure 7C). We conclude that at least one of the following 14 genes is a determinant of host resistance to babesiosis: *Fam55d*, *Fam55b*, *Fam55a*, *Nhp211*, *Rexo2*, *Rbm7*, *Nnmt*, *Zbtb16*, *Htr3a*, *Htr3b*, *Usp28*, *Cldn 21*, *Zw10*, and *Tmprss5* (Figure 7C). In the case of *Tmprss5*, only its 5'end, its promoter and putative upstream regulatory regions may contribute to the phenotypic difference between D2 and B6 mice, as D9Mit99 resides within the *Tmprss5* intragenic region. The present experiment rules out the genes residing between D9Mit99 and D9Mit26, namely, *Drd2*, *Ankk1*, *Ttc12* and *Ncam1*. It also rules out the 3'end of *Tmprss5*.

Figure 7 – A main determinant of resistance to *B. microti* resides between D9Mit130 and D9Mit99.

A) Two to three month old males from five congenic strains, the D2 strain and the B6 strain were infected with 10^5 parasitized red blood cells on day 0. Parasitemia was monitored every day to every other day from day 7 to day 35 post-infection. Data are reported as means (n = 7 – 10 per group). SEM is omitted for clarity.

B) Parasite burden, the integral of parasitemia over time, was derived for each mouse in a given experimental group. Data are reported as means + SEM. The newly created strains, i.e., the 93/256 and 331/262 strains were resistant to *B. microti* infection. ns = $p > 0.05$, * = $p < 0.05$; ** = $p < 0.01$.

C) B6.D2 strains are referred to by the numerals of the two markers (D9Mit##) that flank the known centromeric and telomeric ends of the D2 congenic interval (green box), and at which DNA is of B6 origin (solid line). I participated in the creation of the 93/256 and 331/262 strains, and tested all five strains for susceptibility/resistance to *B. microti* infection. The results indicate that a main determinant of resistance resides between D9Mit130 and D9Mit99. This interval contains 14 genes. Our experiment has ruled out the genes residing between D9Mit99 and D9Mit26, namely, the 3' end of *Tmprss5*, *Drd2*, *Ankk1*, *Ttc12* and *Ncam1*.

R2 – Immunologic Basis of Resistance to *Babesia microti* Infection

R2.1 – B cells are necessary for resolution of parasitemia in susceptible hosts

In HIV positive individuals, low $CD4^+$ T cell count is a risk factor for persistent or relapsing babesiosis.[8, 28, 35-38] Likewise, anti-B cell therapy in individuals with B cell malignancies has been associated with persistent or relapsing babesiosis.[8] Taken together, these clinical observations suggest that babesiosis persists or relapses in hosts that present qualitative or quantitative defects in their T cell or B cell population. Prior to my arrival, the laboratory had observed that parasitemia is intense but transient in two strains of mice, namely the *cd4*-/- B6 strain and the D2 strain. In these susceptible hosts, I tested the hypothesis that B cells are critical for resolution of *B. microti* infection.

R2.1.1 – Genetic evidence supports a role for B cells, but not CD8$^+$ T cells in the resolution of parasitemia in cd4-deficient mice

Prior to my arrival, the laboratory had examined the contribution of B cells using *igh6*-/- mice (also known as μMT mice). As the *igh6* gene encodes the heavy chain of IgM, *igh6*-/- mice fail to develop mature B cells. The laboratory observed that *igh6*-/- mice are resistant to babesiosis. To test the hypothesis that B cells are critical for resistance to babesiosis only in hosts that have defects in their T cell compartment, the laboratory created mice that lack both *cd4* and *igh6* genes. *Cd4*-/- mice, *igh6*-/- mice and *cd4*-/- *igh6*-/- mice were infected with 10^5 parasitized red blood cells at two to three months of age. *Rag1*-/- mice, which lack T and B cells, were used as susceptible controls. Wild-type B6 mice served as resistant controls. I monitored parasitemia every day to every three days from day 7 to day 74 post infection (Figure 8A). Consistent with an earlier observation in the laboratory, *cd4*-/- mice experienced modest but transient parasitemia whereas *cd4*-/- *igh6*-/- mice experienced intense and sustained parasitemia.[21] In fact, the time course of parasitemia in *cd4*-/- *igh6*-/- mice was similar to that of *rag1*-/- mice. These results indicate that B cells are necessary for resolution of parasitemia in *cd4*-/- mice, but dispensable in wild-type B6 mice.

The laboratory had observed that both CD8$^+$ T cell population and CD4$^-$CD8$^-$ T cell population expand over the course of infection in *cd4*-/- mice (data not shown). To gain insight into the role of these T cell subsets, the laboratory created mice that lack both *cd4* and *cd8* genes. *Cd4*-/- mice, *cd8*-/- mice and *cd4*-/- *cd8*-/- mice were infected with 10^5 parasitized red blood cells at two to three months of age. Athymic (nude) mice, which carry the *Foxn1*^{nu} allele, served as susceptible controls. Wild-type B6 mice served as resistant controls. I measured parasitemia every day to every three days from day 7 through day 74 post infection (Figure 8B). As reported

in Figure 8A, *cd4*-/- mice experienced a modest but transient parasitemia. Interestingly, *cd4*-/- *cd8*-/- mice displayed the pattern of parasitemia seen in *cd4*-/- mice. Athymic mice, however, experienced an intense and sustained parasitemia. These results indicate that $CD8^+$ T cells are not required for early resistance to, and resolution of parasitemia in *cd4*-/- mice. Given that a T cell subset is required for resolution of parasitemia, as evidenced by the susceptibility of athymic mice, these results infer that $CD4^-CD8^-$ T cells, also known as double negative (DN) T cells, contribute to resolution of parasitemia in *cd4*-/- mice.

R2.1.2 – Depletion of mature B cells prevents resolution of parasitemia in cd4-deficient mice

Given that the phenotype in *igh6*-/- mice may result from a developmental effect of the targeted gene rather than its activity at the time of the experiment, we addressed the role of B cells in depletion experiments. B cells were depleted from *cd4*-/- mice by repeated administration

Figure 8 – The resolution of parasitemia in *cd4*-deficient mice requires B cells, but not CD8. At two to three months of age, B6 mice with single and double gene deficiencies were infected with 10^5 parasitized red blood cells (day 0). Wild type (wt) B6 mice served as controls. Parasitemia was measured every day to every three days from day 7 to day 74. Data are reported as mean ± SEM. The number of mice per group varied from four to nine (*rag1*-/-: n=6; *cd4*-/- *igh6*-/-: n=7; *cd4*-/-: n=9; *igh6*-/-: n=4; wt: n=5; *nu/nu*: n=6; *cd4*-/- *cd8*-/-: n=8; *cd8*-/-: n=5).

A) *Cd4*-/- mice experienced a modest but transient parasitemia. *Cd4*-/- *igh6*-/- mice, however, experienced intense and persistent parasitemia, indicating that B cells are required for resolution of parasitemia in *cd4*-/- mice. *Igh6*-/- mice were resistant, indicating that B cells are not required in wild type B6 mice.

B) *Cd4*-/- mice and *cd4*-/- *cd8*-/- mice experienced modest but transient parasitemia, indicating that $CD8^+$ T cells are not required in *cd4*-/- mice. *Cd8*-/- mice were resistant, indicating that $CD8^+$ T cells are not critical for resistance in wild type mice. Given that parasitemia is intense and sustained in athymic (nu/nu) mice, these results suggest a role for $CD4^-CD8^-$ T cells, also known as double negative (DN) T cells, in the resolution of parasitemia in *cd4*-/- mice.

of 18B12, an anti-mouse CD20 monoclonal antibody. 2B8, an anti-human CD20 monoclonal antibody, was used as the isotype control. Wild type and *cd4*-/- B6 mice were injected with 18B12, 2B8, or vehicle (PBS) every other week, starting two weeks prior to infection. Mice were infected with 10^5 parasitized red blood cells on day 0. I measured parasitemia every day to every three days from day 7 to day 70 post infection (Figure 9). The extent of B cell depletion was monitored every week. Wild-type B6 mice that were depleted of B cells were as resistant as wild-type mice that were not depleted of B cells. In *cd4*-/- mice, in agreement with our earlier observation (Figure 8), parasitemia sharply rose but quickly receded. In c*d4*-/- mice depleted of B cells, however, parasitemia sharply rose and oscillated between 30% and 35% throughout the remainder of the experiment (Figure 9). These results confirm that i) B cells are necessary for the resolution of parasitemia in *cd4*-/- mice, and ii) B cells are not critical for resistance of wild type B6 mice.

R2.1.3 – B cells contribute to resolution of parasitemia in young, but less so in aged DBA/2 mice

Like *cd4*-/- B6 mice, young D2 mice experience a modest but transient parasitemia.[20] To assess the contribution of B cells to resistance in young D2 mice, B cells were depleted by repeated administration of 18B12 every other week starting two weeks prior to infection. 2B8 was the isotype control. D2 mice were infected with 10^5 parasitized red blood cells at two to three months of age. Age-matched B6 mice were also infected. I measured parasitemia every day to every three days from day 7 to day 70 post infection. Consistent with our earlier observation (Figure 9), young B6 mice depleted of B cells were resistant to infection (Figure 10A). Young D2 mice depleted of B cells, however, experienced high, persistent parasitemia (Figure 10B).

Figure 9 – Depletion of B cells prevents resolution of parasitemia in *cd4*-deficient mice. At two to three months of age, wild type (wt) and *cd4*-/- B6 mice were infected with 10^5 parasitized red blood cells (day 0). 18B12, an anti-mouse monoclonal antibody that depletes B cells, and 2B8, the isotype control, were injected intraperitoneally every other week starting two weeks prior to infection. Parasitemia was monitored every day to every three days from day 7 to day 70. Data are reported as mean ± SEM (n = 7 per group, except for the wt group treated with 18B12 that consisted of 5 mice). Depletion of B cells did not alter the resistance of wild-type mice, but prevented the resolution of parasitemia in *cd4*-/- mice.

These results establish that i) B cells are critical for resolution of parasitemia in young D2 mice and ii) the B cell requirement for resolution of parasitemia is strain specific.

Unlike two-month-old ("young") D2 mice, 18-month-old ("old") D2 mice experience an intense parasitemia that only partially resolves.[20] To assess the contribution of B cells in old D2 mice, these mice were chronically depleted of B cells by administration of 18B12. Aged-matched D2 mice were injected with the isotype control 2B8. Age-matched B6 mice served as controls. Old B6 mice depleted of B cells were as resistant as old B6 mice that were not depleted of B cells (Figure 10C). Old D2 mice depleted of B cells experienced a high parasitemia that persisted between 25% and 30% whereas old D2 mice in which B cells were not depleted partially resolved parasitemia (Figure 10D). These results reveal that i) the partial resolution of parasitemia in old D2 mice is mediated by B cells, and ii) B cells are not required for resistance of B6 mice, regardless of age.

R2.2 – Impaired antibody response in susceptible hosts

Our observation that B cells are critical for resolution of *B. microti* parasitemia in *cd4*-/- mice points to secreted immunoglobulins as a possible effector mechanism for parasite clearance. A handful of studies have documented high levels of parasite-specific IgG in *B. microti* infected mice.[29, 39] The study by Chen and colleagues is particularly informative. In CBA mice that are highly susceptible to *B. microti* infection, the rise in IgM occurred concomitantly with the rise in parasitemia. Before parasitemia reached its peak (~60%), IgM levels had started to decline while IgG levels were rising. By the time parasitemia was completely resolved, IgM levels had receded to pre-infection levels whereas IgG levels had reached a plateau. IgG subisotypes, however, were not investigated. To understand the nature of the antibody response

Figure 10 – B cells contribute to the resolution of parasitemia in young, but less so in old DBA/2 mice. Young (Y) mice (i.e., two months of age) and old (O) mice (i.e., 18 months of age) from the D2 and B6 strains were infected with 10^5 parasitized red blood cells (day 0). 18B12 and 2B8 were injected intraperitoneally every other week started two weeks prior to infection. 18B12 is an anti-mouse CD20 monoclonal antibody that depletes B cells; 2B8 is the isotype control. Parasitemia was monitored every day to every three days from day 7 to day 70. Data are reported as mean ± SEM. Whether treated with 18B12 or 2B8, groups of young mice consisted of seven mice. The group of old mice treated with 18B12 consisted of at least seven mice (OB6+18B12: n=11; OD2+18B12: n=8). The OB6 group treated with 2B8 consisted of seven mice at time of infection. Given that two of these mice died during the course of the experiment, data from the five surviving mice are presented. The OD2 group treated with 2B8 consisted of seven mice at time of infection. Three mice survived until day 61, but only two until day 70. Death typically occurred within 24 hours of antibody administration. In some mice, health had already declined when death was noted. Although the group OD2 + 2B8 consisted of two mice from day 61 on, variance in this group is reported as SEM thereafter.

A) Young B6 mice depleted of B cells (YB6 + 18B12) were as resistant as young B6 mice that were not depleted of B cells (YB6 + 2B8).

B) Young D2 mice depleted of B cells (YD2 + 18B12) failed to resolve parasitemia whereas young D2 mice that were not depleted of B cells (YD2 + 2B8) experience intense but transient parasitemia.

C) Old B6 mice depleted of B cells (OB6 +18B12) were as resistant as old B6 mice that were not depleted of B cells (OB6 + 2B8).

D) Old D2 mice depleted of B cells (OD2 + 18B12) failed to resolve parasitemia whereas old D2 that were not depleted of B cells (OD2 + 2B8) partially but transiently resolved parasitemia.

in cd4-/- mice, we developed a flow cytometry based assay that utilizes red blood cells as the source of B. microti antigen. With this assay in hand, we tested the hypothesis that the antibody response in wild type mice differs from that in cd4-/- mice.

R2.2.1 – Optimization of a flow cytometry assay to determine antibody titers

Traditionally, antibodies produced during the course of B. microti infection in mice have been detected in indirect ELISA using a parasite extract.[39, 40] Parasite extract as a source of antigen is problematic as it is unlikely that antibodies in the bloodstream can access the whole array of parasite antigens.[9] Prior to my arrival, the laboratory had published that *B. microti* antigens exported to the surface of red blood cells can be recognized by polyclonal antibodies once red blood cells are fixed in a low concentration of glutaraldehyde, and exposed to the detergent Triton X-100.[33] Given the laboratory's expertise in flow cytometry, we aimed to develop an assay that detects the interaction of antibodies with parasite antigens expressed in, and presented by red blood cells.

R2.2.1.1 Endpoint titers

Blood was obtained 70 days after the infection of a wild type B6 mouse with *B. microti*. The plasma was serially diluted by a factor of three. Eight dilutions, ranging from 1/100 to 1/218,700, were prepared. Blood was also obtained from three *rag1*-deficient mouse that had been infected with *B. microti* for more than three months. Following fixation in glutaraldehyde, exposure to Triton X-100 and treatment with RNase, blood cells were incubated with the serial dilutions of plasma (ratio 1:1). *B. microti* infected red blood cells were revealed by YOYO-1

(Figure 11). As stated earlier, the intensity of YOYO-1 fluorescence is a function of parasite number.[33] The first vertical streaks (denoted by an arrow in the top right panel, Figure 11) consisted of cells containing one parasite. Uninfected cells resided to the left of the first streaks. Despite treatment with RNase, reticulocytes contain variable amount of residual RNA. As reticulocytes were not distinguished from mature red blood cells in this assay, uninfected mature red blood cells and uninfected reticulocytes appeared as a stretched horizontal cloud of cells.

The pattern of IgM binding was strikingly different from those of IgG subisotypes. As easily noted with the first dilution of plasma (Figure 11, top left panel), IgM bound uninfected red blood cells as strongly as infected red blood cells. Although the number of parasites per cell increased, the IgM staining stayed constant. On the other hand, regardless of the subisotype, the intensity of IgG staining on infected red blood cells was directly related to the number of parasites per cell. This positive, linear relationship began at cells infected by one parasite. IgG subisotypes also bound to uninfected cells, although not as strongly as to infected cells, including those infected with one parasite. No IgE was detected (data not shown). These observations suggest that i) IgM binds to red blood cell moieties that are not encoded by *B. microti*, and ii) IgG subisotypes bind to *B. microti* antigens but also recognize moieties of red blood cells.

In a clinical laboratory setting, endpoint antibody titers are used to identify patients that have been exposed to *B. microti*. An endpoint titer is defined by the last dilution at which IgM or IgG is detected. In our experiment, endpoint titers differed among isotypes and subisotypes (see inversed red diamonds in Figure 11). The reciprocal endpoint titer (i.e., the denominator of the titer) was 48,600 for IgG3 – 16,200 for IgG2b and IgG2c – 5,400 for IgG1 and 1,800 for IgM.

These results indicate that, at day 70 post-infection, IgG3 is the dominant isotype followed in decreasing order by IgG2b and IgG2c, IgG1 and IgM.

Figure 11 - Antibody endpoint titers. Blood was obtained from a wild type B6 mouse 70 days following infection with *B. microti* and plasma serially diluted by a factor of three. Eight dilutions were prepared. Blood was also obtained from a *rag1*-deficient mouse chronically infected with *B. microti*. Fixed and permeabilized blood cells were incubated the serial dilutions of plasma (ratio 1:1). Cells were stained for mouse IgM and IgGs (Y axis) and for parasite DNA (X axis). Cells infected with one parasite form the first vertical streak (denoted by the black arrow, top right panel). To the left of this streak are the uninfected cells. To the right of this streak, fluorescence is a function of parasite number.

IgM bound infected cells as strongly as uninfected cells (top row), indicating that IgM recognizes moieties on red blood cells that are not encoded by *B. microti*. IgG subisotype binding was proportional to the number of parasites per cell, indicating that IgG subisotypes recognize *B. microti* antigen. IgG subisotypes also bound uninfected cells.

Endpoint titer, defined as the last dilution at which antibody binding is detected (indicated by inversed red diamonds), differed among subisotypes and isotypes. The pattern of cells incubated with the eight dilution of plasma was the same as that of cells stained with YOYO-1 but not exposed to plasma (not shown). A threshold (red dotted line) was set to divide the cloud of infected cells exposed to the last dilution in half (far right column). This threshold was applied to all samples stained for a given isotype. IgG3 was the dominant isotype followed in decreasing order by IgG2b and IgG2c, IgG1 and IgM.

R2.2.1.2 Nuclear stains to detect B. microti infected red blood cells

In the laboratory, parasitemia is routinely assessed by staining *B. microti* DNA with YOYO-1 (Figure 1). YOYO-1 presents the advantage of a strong quantum yield (i.e., emission of fluorescence), but has an important limitation as it stains both DNA and RNA. The staining of RNA is minimized by pre-treatment with RNase, but this treatment leaves residual RNA. Furthermore, incubation of blood cells in RNase increases their stickiness to plastic. During the summer of 2010, the laboratory gained access to a LSRII flow cytometer, which excites DAPI, the classic DNA stain. Along with DAPI, we tested the SYBR Green I dye and the Vybrant DyeCycle Green stain. SYBR Green I is used to detect *Plasmodium* infected human red blood cells.[41, 42] Vybrant DyeCycle Green is a permeable live cell dye that preferentially binds DNA over RNA.[43, 44]

Susceptibility to Babesiosis, an Infectious Disease of Erythrocytes

Blood was obtained from a *rag1*-deficient mouse that had been infected with *B. microti* for more than three months. Once blood cells were fixed in glutaraldehyde and permeabilized in Triton X-100, one half was exposed to DNase-free RNase whereas the other half was left in staining buffer. Cells were exposed to dilutions (1/300, 1/1200 and 1/4800) of immune plasma. Cells were washed and exposed to a biotin goat-anti mouse IgG3 antibody followed by A647-coupled streptavidin. SYBR Green I, DAPI or Vybrant DyeCycle Green was added. As expected (see Figure 11), IgG3 binding to infected red blood cells was strong at the three dilutions tested (Figure 12). IgG3 binding to uninfected cells was weak, and even more so as the plasma became more dilute. Of the three stains, DAPI induced the strongest shift in fluorescence. The shifts by DAPI and Vybrant DyeCycle Green were minimally affected by RNase treatment. In contrast, the shift by SYBR Green I was dramatically decreased by RNase treatment. We conclude that DAPI is best at distinguishing reticulocytes from mature red blood cells. At this juncture, we opted for DAPI as our parasite DNA stain.

R2.2.2 – The antibody response is delayed in cd4-/- mice

Given that B cells are critical for resolution of parasitemia in *cd4*-/- mice (see sections R2.1.1 and R2.1.2), we quantified antibody isotypes during the course of infection with *B. microti* in wild type and *cd4*-/- B6 mice. From day 12 to day 45 post-infection, we obtained plasma every three to five days, i.e., at nine time points. Capitalizing on the capability of the LSRII flow cytometer to excite DAPI and to automatically sample 96 well plates, I modified our low-throughput assay to make it suitable for antibody isotype quantification on a large number of samples (see section M7.2). The high throughput format enabled us to detect in one day of work five isotypes and subisotypes in nine plasma samples. Plasma samples were serially diluted by a

factor of three. Seven dilutions were prepared. The first dilution was 1/100 whereas the seventh was 1/72,900. An eighth dilution was deemed unnecessary, as we had established that binding of IgM or IgG to infected red blood cells is not detected at and beyond the seventh dilution (see Figure 11). Blood was obtained from seven *rag1*-deficient mice. Once fixed in glutaraldehyde and permeabilized in Triton X-100, blood cells were exposed to the serial dilutions of plasma (ratio 1:1). Cells were stained for mouse immunoglobulins (IgM, IgG1, IgG2b, IgG2c, or IgG3) and for parasite DNA. Isotype staining was plotted vs. parasite DNA staining (see Figure 3 in section M8.2). A vertical threshold was set to distinguish uninfected cells (DAPI negative, left quadrants) from infected cells (DAPI positive, right quadrants). Cells that had been stained with DAPI but unexposed to plasma were used to set a horizontal threshold that divided the population of infected cells in half. This threshold was applied to all samples that had been

Figure 12 – DAPI is the preferred parasite DNA stain. Blood was obtained from a *B. microti* infected *rag1*-deficient mouse. Cells were fixed in glutaraldehyde, exposed to Triton X-100, and treated with RNase A or left in staining buffer. Cells were incubated with serial dilutions of immune plasma. Cells were stained for IgG3 binding (Y axis) and for parasite DNA (X axis) using one of the following fluorescent dyes: SYBR Green I dye (SybrGr), DAPI, or Vybrant DyeCycle Green stain (VybrGr). *B. microti* infected blood cells were strongly shifted by DAPI, but less so by SYBR Green or Vybrant DyeCycle Green. In agreement with the preferential binding of DAPI to DNA over RNA, DAPI fluorescence was not affected by RNase treatment.

stained with DAPI and that had been exposed to plasma dilutions (see Figure 3 in section M8.2).

> The fractions of antibody-bound infected cells and antibody-bound uninfected cells were calculated (see Section M8.2). Fractions of antibody-bound infected cells were plotted vs. time post-infection (Figure 13). For both wild type and cd4-/- mice, the fourth plasma dilution (P4; 1/5,400) allowed the greatest dynamic range for antibody binding to infected cells, regardless of isotype and time point (Figure 13, open green boxes). The fourth dilution was therefore chosen to compare the fraction of antibody-bound infected cells (Figure 14) to the fraction of antibody-bound uninfected cells (Figure 15).

Plasma samples had been obtained during the course of B. microti infection in the wild type B6 mice and cd4-/- B6 mice for which parasitemia values are presented in Figure 9. As plasma samples from one cd4-/- mouse were excluded from the antibody analysis for a technical reason, parasitemia values for the remaining mice are presented in Figures 14 and 15 (top left panels). On day 12 post-infection, when parasitemia was still marginal, levels of IgG subisotypes, as detected by their binding to infected red blood cells, were already higher in wild type mice than in *cd4*-/- mice (Figure 14). In wild type mice, IgG subisotype levels further increased to peak or plateau on day 28. In *cd4*-/- mice, IgG1 levels sharply increased from day 12 to day 28, and plateaued thereafter at levels seen in wild type mice. IgG2b and IgG2c levels also sharply increased until day 28, and more gradually from day 28 to day 36. From day 41 on, IgG2b and IgG2c levels did not differ between wild type and *cd4*-/- mice. In *cd4*-/- mice, IgG3 levels increased up to day 32, but failed to reach the levels seen in wild type mice. In contrast to IgGs, IgM modestly increased in wild type mice, and less so in *cd4*-/- mice. These observations

indicate that i) *B. microti* infected red blood cells can be bound by IgM and IgG isotypes, ii) the rise in IgG levels is delayed but concomitant with the resolution of parasitemia in *cd4*-/- mice, and iii) IgG3 levels in *cd4*-/- mice never reach the levels seen in wild type mice.

To determine whether antibody binding is specific for *B. microti* antigen, we analyzed all samples for the fraction of antibody-bound *uninfected* cells (Figure 15). IgM levels were higher in plasma samples obtained from wild type mice than from *cd4*-/- mice. For wild type and *cd4*-/- mice, IgM bound to uninfected cells (Figure 15) as strongly as to infected cells (Figure 14). IgG1 and IgG3 binding to uninfected cells was marginal throughout the infection, and did not differ between wild type and *cd4*-/- mice. IgG2b binding to uninfected cells was higher when plasma samples were obtained from wild type than from *cd4*-/- mice. For both strains, uninfected cells bound less IgG2b than did infected cells. The pattern of IgG2c binding was that of IgG2b binding. These observations indicate that i) uninfected cells can be bound by antibodies produced during the course of babesiosis, particularly by IgG2c, ii) uninfected cells do not bind as much IgG2b and IgG2c as infected cells do, and iii) IgM binds equally to uninfected and infected cells. We conclude that the binding to infected red blood cells is specific for *B. microti* in the case of IgG1 and IgG3, but less so in the case of IgG2b and IgG2c.

Figure 13 – Detection of antibodies by use of *B. microti* infected red blood cells requires plasma dilution. Blood cells obtained from seven *B. microti* infected *rag1*-deficient mice were fixed in glutaraldehyde and exposed to Triton X-100. Cells were exposed to serial dilutions of plasma samples obtained from wild type and *cd4*-/- mice. Cells were stained for mouse antibody isotypes and for parasite DNA. For each sample, the fraction of antibody-bound infected cells was calculated (see Figure 3 in section M8.2). Fractions of antibody-bound infected cells are reported as mean + SEM, and plotted vs. time post-infection for each dilution tested. Dilutions are 1/200 (P1, closed black diamonds) - 1/600 (P2, open blue squares) - 1/1,800 (P3, closed blue

diamonds) - 1/5,400 (P4, open green squares) - 1/16,200 (P5, closed green diamonds) - 1/48,600 (P6; open red squares) – 1/145,800 (P7; closed red diamonds). For each isotype and at each time point, the fourth dilution (1/5,400; open green squares) provided the greatest dynamic range to detect differences in antibody binding to infected cells between wild type and *cd4*-/- mice.

Figure 14 – The antibody response is delayed in *cd4*-/- mice infected with *B. microti*. Cells and plasma dilutions were prepared as outlined in Figure 13. Fractions of antibody-bound infected red blood cells are presented for the fourth dilution (1/5,400) of plasma obtained from wild type (wt) and *cd4*-/- mice. On day 12 following infection with *B. microti*, when parasitemia was still marginal, levels of IgM and IgG isotypes were already higher in plasma from wild type mice than in plasma from *cd4*-/- mice. In wild type mice, IgG levels reached peak or plateau by day 28. In *cd4*-/- mice, the rise of IgG levels was delayed, but concomitant with the resolution of parasitemia. By day 41, IgG levels did not differ between wild type mice and *cd4*-/- mice, with the exception of IgG3 levels that remained lower in *cd4*-/- mice. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 15 – Uninfected red blood cells are bound by antibodies produced during the course of babesiosis. Cells and plasma dilutions were prepared as outlined in Figure 13. Fractions of antibody-bound uninfected red blood cells are presented for the fourth dilution (1/5,400) of plasma obtained from wild type (wt) and *cd4*-/- mice. IgM bound uninfected cells (top right panel) as strongly as infected cells (see Figure 14). IgG1 and IgG3 binding to uninfected cells was marginal, and did not differ between wild type and *cd4*-/- mice. IgG2b and IgG2c binding to uninfected cells was greater for plasma samples obtained from wild type mice than for those obtained from *cd4*-/- mice. IgG2b and IgG2c binding to uninfected cells remained lower than those to infected cells (see Figure 14). * = $p < 0.05$, ** = $p < 0.01$.

DISCUSSION

Young, healthy individuals often experience asymptomatic *B. microti* infection or mild disease. On the other hand, the elderly and the immunocompromised individual are at risk for severe babesiosis, which requires hospitalization.[2, 7, 10, 13, 16, 20, 45, 46] Severe babesiosis is associated with life-threatening complications and may persist for months despite standard therapy.[9, 46] As the spectrum of clinical presentation is wide, and because age is a major risk factor for severe disease, babesiosis is an ideal disease model to study age-acquired susceptibility to infection. To date, our understanding of the pathogenesis of babesiosis is limited. The host immune response to babesiosis is poorly characterized, and the genetic determinants of host resistance remain to be identified. My thesis aimed to narrow the knowledge gap in the immunology and genetics of babesiosis.

The laboratory uses a well-established mouse model that is based on phenotypic differences in susceptibility to parasitemia; C57Bl/6 (B6) mice experience modest parasitemia that quickly resolves, whereas DBA/2 (D2) mice experience intense but transient parasitemia.[20] Using forward genetics, the laboratory mapped the main genetic determinant of resistance in young mice to the proximal region of chromosome 9. The laboratory created several B6.D2 congenic strains, and narrowed the main locus of resistance to a 1.5Mb interval that contains 18 genes. I participated in the creation of two additional congenic strains and narrowed this locus to a 0.9Mb interval that contains 14 genes. Two of the 14 genes contain non-synonymous single nucleotide polymorphisms (SNPs) between the B6 and D2 strains.[47] *Fam55b*, which stands for "family with sequence similarity 55, member b" is predicted to be a 558 amino acid protein with a short cytoplasmic tail but a long extracellular domain.[48] Five of the nine missense SNPs reside in the extracellular immunoglobulin domain, implying that allelic variation may affect its

interaction with a partner protein. *Fam55a* is predicted to be a protein encoding gene by The Jackson Laboratory, with two missense SNPs between the B6 and the D2 alleles.[47] The National Center for Biotechnology Information (NCBI), however, considers *fam55a* to be a pseudogene whereas Ensembl no longer maps *fam55a* to chromosome 9.[48, 49] *Fam55b* is expressed by early progenitor cells common to the erythroid and megakaryocytic lineages, but also by B cells and cells of the brush border of the gut epithelium.[50] *Fam55a* appears to be expressed ubiquitously.[50] Given its expression in erythroid cells, *fam55b* is the leading candidate determinant of resistance to *B. microti* infection in young mice.

The biology of the Fam55b protein is unknown. Given that *fam55b* is expressed by early erythroid cells and is predicted to have a large extracellular domain, it is reasonable to speculate that Fam55b plays a role in the invasion of red blood cells by *Babesia*. The process of invasion is thought to involve parasite proteins secreted at the interface between the parasite and the red blood cell. It also involves the binding of parasite surface antigens to red blood cell surface proteins.[10, 51-54] Sialoglycoproteins at the surface of red blood cells, including glycophorins, have been implicated in the invasion of red blood cells by *B. divergens*,[51] *B. rodhaini*,[52] and *B. bovis*.[53] Whether Fam55b favors or impedes the attachment of *B. microti* to sialoglycoproteins expressed on the surface of red blood cells is worthy of investigation. If proven a determinant of host resistance, Fam55b, a surface protein, would constitute a valuable therapeutic target. Modulation of Fam55b surface expression or activity may decrease invasion of red blood cells by *B. microti*.

In addition to red blood cells, immune cells are central to host resistance to babesiosis. Studies in mice have established the importance of $CD4^+$ T cells,[24, 26] but have failed to provide convincing evidence that B cells are required for host resistance to *B. microti* infection.[29, 30] Yet,

patients who have been or are treated with rituximab are at risk for persistent babesiosis.[8] Given that rituximab is a monoclonal antibody directed against the B cell marker CD20, this observation suggests that in certain hosts, B cells are essential for resolution of babesiosis. In the present study, I addressed the role of B cells by using two mouse strains for which parasitemia is intense but transient: the *cd4*-/- B6 strain and the D2 strain.

CD4 is a membrane glycoprotein that functions as a co-receptor on T cells.[55] $CD4^+$ T cells bind antigens presented by major histocompatibility complex (MHC) class II molecules. In the case of *B. microti* infection, $CD4^+$ T cells are central to host resistance,[21, 26] but are unable to recognize parasite antigens on infected red blood cells as these cells do not express MHCII molecules. $CD4^+$ T cells most likely interact with babesial antigens presented by MHCII on macrophages and dendritic cells (APCs) once they have engulfed and digested free parasites or infected red blood cells. In support of this scenario, depletion of $CD4^+$ T cells confers susceptibility to babesiosis, i.e., peak parasitemia is higher or parasitemia persists longer.[26, 31] The laboratory examined the role of CD4 by using *cd4*-/- mice, and observed an intense but transient *B. microti* parasitemia. Using mice that lack *cd4* and *igh6*, the gene that encodes the heavy chain of IgM, the laboratory established that B cells are critical for resolution of parasitemia in *cd4*-/- mice.[21] The present study confirmed this observation. Repeated administration of the anti-mouse CD20 antibody 18B12 prevented the resolution of parasitemia in *cd4*-/- mice. I also extended this observation to D2 mice. Like *cd4*-/- mice, young D2 mice experience intense and transient parasitemia. In old D2 mice, parasitemia is intense but persists.[20] I established that B cells are critical for the complete resolution of parasitemia in young D2 mice, and for the partial resolution of parasitemia in old D2 mice.

$CD8^+$ T cells and $CD4^-CD8^-$ T cells expand during the course of *B. microti* infection in *cd4*-/- mice (Vannier, unpublished data). In c*d4*-/- mice, half of $CD8^+$ T cells are MHCII restricted.[56] To investigate the role of $CD8^+$ T cells, I infected *cd4*-/- *cd8*-/- mice that the laboratory had created. The time course of parasitemia in these mice did not differ from that in *cd4*-/- mice. Keeping in mind that athymic mice (lacking T cells) experienced high, sustained parasitemia, I concluded that $CD8^+$ T cells are not necessary for resolution of parasitemia in *cd4*-/- mice. These results also suggested that $CD4^-CD8^-$ T cells, known as double-negative (DN) T cells, play a role in resolution of parasitemia in *cd4*-/- mice. Whether DN T cells promote resolution of parasitemia by direct activation of B cells remains to be established.

Antibody production is the quintessential function of B cells. Given that *Babesia* parasites quickly invade red blood cells, antibodies most likely are ineffective at neutralizing extracellular sporozoites.[9] Antibodies most likely bind babesial antigens presented on infected red blood cells.[10, 33, 57] The characterization of the antibody response that may be associated with parasite clearance could aid in the design of a vaccine. In the present study, I characterized the antibody response in *cd4*-/- mice infected with *B. microti*.

I developed a high-throughput assay to detect and quantify immunoglobulins in plasma samples from *B. microti* infected mice. Our assay is based on the exposure of infected red blood cells to plasma dilutions; antibody binding is detected by flow cytometry. Our assay has the advantages of precise quantification and automated detection of fluorescence conferred by use of the LSRII flow cytometer. Our assay differs from the indirect immunofluorescent assay (IFA), the method used in clinical diagnosis.[58] IFA typically uses infected red blood cells from hamsters (source of antigen) to test serial dilutions of plasma from the patient (source of antibodies);

antibody binding is detected by fluorescence microscopy. IFA is somewhat subjective as the experimentalist uses the naked eye to assess whether the colorimetric reaction is positive. Furthermore, IFA does not distinguish infected from uninfected red blood cells present in the blood sample obtained from the infected hamster. Our flow cytometry based assay removes the subjectivity from the experimentalist, and distinguishes infected from uninfected red blood cells.

The rise in IgM levels was concomitant with the rise in IgG subisotypes (IgG1, IgG2b, IgG2c, and IgG3). This rise was less pronounced in *cd4*-/- mice. In a study of CBA mice, IgM peaked concomitantly with the rise in parasitemia whereas IgG rose during the resolution of parasitemia.[39] In that study, IgM levels were reported to be *B. microti* specific. In our assay, IgM bound infected red blood cells as strongly as uninfected red blood cells. The apparent discrepancy between the two studies may be explained by the unspecific nature of the IgM binding detected by our assay. This discrepancy also implies that we may have missed an early wave of IgM. IgM may favor parasite survival and invasion as IgM-deficient mice are resistant to *B. microti* infection.[32, 59] Low IgM levels may therefore contribute to protect *cd4*-/- mice. Higher IgM levels in wild type mice may be irrelevant since B cells are dispensable for host resistance in these hosts. Further research is needed to determine the role of IgM in babesiosis.

The rise in IgG levels was sharp in wild type mice, but delayed in *cd4*-/- mice. In *cd4*-/- mice, IgGs rose concomitantly with the resolution of parasitemia and remained elevated through the remainder of the infection. This latter observation is consistent with reports of human cases. Clinically, IgG titers greater than 1:1024 are indicative of acute infection. In our experiment, IgG titers were detected at the dilution of 1:5400. In patients, IgG titers remain elevated (>1:64) for

eight to 12 months.[4, 9, 10, 60, 61] In our experiment, we also observed that IgG levels remained elevated beyond the time of parasitemia resolution.

B. microti specificity was evaluated by comparing the fractions of antibody-bound infected cells and antibody-bound uninfected cells. As stated earlier, IgM bound infected cells and uninfected cells to the same extent, indicating that IgM recognizes moieties not encoded by *B. microti*. This finding is in agreement with a previous report on the non-specific nature of IgM in *B. bigemina* infection,[59] but contradicts another study that detected *B. microti* specific IgM.[39] IgG1 and IgG3 had a similar time course in wild type mice; the strong binding to infected cells contrasted the weak binding to uninfected cells. When IgG2b and IgG2c were considered, the fractions bound to infected cells were greater than the fractions bound to uninfected cells. We conclude that IgG1 and IgG3 are highly specific for *B. microti* whereas IgG2b and IgG2c recognize *B. microti* antigens, but also moieties of red blood cells.

Neoantigens are present on host cell proteins, but are not accessible to naturally occurring antibodies. Due to changes in the intracellular environment induced by intracellular parasites, neoantigens become exposed on the outer surface of the membrane. This phenomenon is well documented in malaria.[62] Band 3 proteins become exposed at the surface of *Plasmodium* infected red blood cells, thereby promoting cell adhesiveness.[63-65] Antibodies directed against neoantigens exposed on band 3 help reduce cytoadherence of infected red blood cells, and therefore mitigate anemia.[65] I propose that, in addition to *B. microti* antigens, IgG2b and IgG2c bind neoantigens exposed on infected and uninfected red blood cells in infected hosts.

Antibodies may be active as a metaphorical “back-up plan” to enable resolution of *Babesia* infection when T cell-mediated responses are deficient or diminished. In dogs, the

antibody response is key during chronic *Babesia* infection but not during the acute infection that quickly resolves.[57] In the present study, we have established that the antibody response is delayed in *cd4*-/- mice. This delayed antibody response could result from a poorly effective activation of B cells in the absence of help from $CD4^+$ T cells.

Antibody constant regions are recognized by Fc receptors on macrophages and other immune cells. Phagocytosis of infected red blood cells through opsonization by IgG could be a mechanism of resolution of parasitemia in *cd4*-/- mice. Antibodies could also promote host resistance to *B. microti* infection through activation of the complement system. Complement-mediated lysis of *Babesia* parasites has never been reported.[9] Complement, however, can bind antigen-antibody complexes, resulting in increased phagocytosis of infected cells.[55] Additionally, complement could assist in the resolution of parasitemia by competing with *Babesia* for shared receptors (for example, CR1) expressed on the red blood cell membrane.

SUMMARY

My research has fine mapped the main determinant of host resistance in young mice to a genomic interval containing 14 genes. Based on the observation that patients who experience persistent babesiosis often have been treated with a monoclonal therapy that depletes B cells, I examined the role of B cells in controlling *B. microti* infection in susceptible strains of mice. I established that B cells are required for resolution of parasitemia in *cd4*-/- B6 mice and D2 mice, both of which experience intense but transient parasitemia. I modified an assay to quantify antibody isotypes during *B. microti* infection in wild type and *cd4*-/- B6 mice. I established that the antibody response is delayed but concomitant with resolution of parasitemia in *cd4*-/- mice. I

observed that IgG1 and IgG3 are highly specific for *B. microti*, whereas IgG2b and IgG2c also recognize moieties of red blood cells. Overall, my research has helped narrow the knowledge gap pertaining to the genetic and immunologic bases of resistance to babesiosis, once a rare infectious disease which has been emerging in the United States over the last two decades.

FUTURE DIRECTIONS

F1 – Importance of Fam55b in host resistance to *B. microti* infection

The present study points to *fam55b* as the main genetic determinant of resistance to *B. microti* infection in young mice. To assess the importance of *fam55b*, the laboratory aimed to create and infect *fam55b* deficient mice. Embryonic stem cells carrying the targeted allele were injected into B6 blastocysts. Four chimeric males were obtained. Unfortunately, the targeted *fam55b* allele did not get transmitted into the germ line. Recently, *fam55b* deficient mice created on a 129:B6 background have become available for purchase. These *fam55b* deficient mice will be infected with *B. microti*. If the D2 allele is non-functional or poorly functional, we expect *fam55b* deficient mice to be highly susceptible. If the B6 allele were to be non-functional or poorly functional, *fam55b* deficient mice would be backcrossed onto the D2 background, and infected with *B. microti*.

F2 – Implications for *Plasmodium*, the causative agent of malaria

Babesia belongs to the phylum *Apicomplexa*, of which the most infamous member is the malaria-causing parasite *Plasmodium*. Malaria sickens half a billion people around the world, and causes approximately one million deaths each year.[66] *Plasmodium* has been extensively studied, but determinants of host resistance to malaria remain elusive. As members of the same phylum, *Babesia* and *Plasmodium* are thought to invade red blood cells by a similar invagination process.[53, 54] The laboratory will test the hypothesis that *fam55b* is a determinant of host

resistance to infection with *Plasmodium*. If true, the congenic strains used in the present study will display similar differences in susceptibility to *Plasmodium* as they did to *Babesia*.

F3 – Identification of a dominant *Babesia* antigen for vaccine development

Universal vaccination against babesiosis is not necessary as the majority of the human population neither resides in *Babesia*-endemic areas nor is highly susceptible to severe infection. Elderly and immunocompromised individuals are at high-risk for severe babesiosis, and could benefit from vaccination, particularly if residing in endemic areas, such as the Northeast or the Upper Midwest.

Vaccine development has focused on *Babesia* species that infect cattle.[9] Many countries rely on live attenuated vaccines despite their potential dangers.[67] Recombinant vaccines using sporozoite surface antigens have yet to achieve complete protection in cattle.[53] One challenge is the antigenic diversity during the course of infection, and among *Babesia* strains that infect cattle. To date, there is no evidence to support the notion of antigenic diversity among clinical isolates of *B. microti*, although *B. microti* is a species complex.[68] The laboratory will perform western blot analysis, probing for babesial antigens using *B. microti* specific IgG1 and IgG3. Identification of the dominant epitopes on these babesial antigens should eventually guide the design of a human vaccine.

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