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The Role of iNKT Cells in the Development of a Protective Immune Response to *Borrelia burgdorferi*, the Lyme Disease Agent

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ABSTRACT

Caused by the spirochete *Borrelia burgdorferi*, Lyme disease is the most common vector-borne infection in the United States today. With around 30,000 cases reported each year and most of them occurring in the northeastern states, this makes Lyme disease an important disease to study. In this particular study, we aim to determine the role of invariant natural killer T (iNKT) cells in developing a protective immune response to Lyme disease and against Lyme arthritis 4 weeks after infection, by studying CD1d-/- (iNKT-deficient) mice. ELISA, qPCR, and clinical rating guidelines to visually score arthritis in the joint were utilized to determine the role of iNKT cells in these mice versus wild-type mice 4 weeks post *B. burgdorferi* infection. Although NKT cells have been found to control infection 1-2 weeks post-infection in other studies, we found that after 4 weeks, iNKT cells do not play a significant role in controlling Lyme disease in the murine model used.

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INTRODUCTION

Other previous studies have shown that invariant natural killer T (iNKT) cells may be involved in lessening the pathology of Lyme infection when assayed at 1-2 weeks post-infection (Kumar et al., 2000). The objective of this study was to determine if indeed these iNKT cells are critical for developing a protective immune response to *Borrelia burgdorferi*, the Lyme disease agent. With the hypothesis that iNKT cells are essential for generating specific antibodies against *B. burgdorferi* and subsequent eradication of the bacteria, it was predicted that CD1d-/- mice (iNKT-deficient mice) would have lower anti-*B. burgdorferi* antibody titers compared to wild type mice, and have higher bacterial burden in the joints and heart. Lyme infection can also lead to arthritis, so it was predicted that the iNKT cells may have a role in controlling arthritis. Joint inflammation was monitored to determine if the predicted increase in bacterial loads in the joint correlate to a more severe (if any) arthritis.

Two different 4 week long *B. burgdorferi* infections were completed for this study; one where all of the mice were the BALB/c inbred strain and one where all of the mice were the C57BL/6J (B6) strain. Both of these strains are susceptible to Lyme infection. B6 mice develop severe heart and very mild joint disease, and BALB/c mice develop mild heart and mild to moderate joint disease (Moody and Barthold, 1998). At the end of the infection, tissue and sera from all the mice were collected. Enzyme-linked immunosorbent assays (ELISA) was utilized to determine the titers of specific antibody against *B. burgdorferi*. In addition, quantitative PCR (qPCR) was used to measure the total bacterial load in the joint and heart in each mouse. Joint inflammation was also visually monitored in the BALB/c experiment to determine if iNKT cells play a role in controlling arthritis.

The data from the two experiments in this project revealed that contrary to previous findings at 1-2 weeks post-infection, CD1d/ iNKT cells do not appear to play a significant role in developing a protective immune response to *B. burgdorferi* 4 weeks post infection. It was found, however, that they do play a role in controlling arthritis. Previous research has determined that iNKT cells do have a role in limiting *B. burgdorferi* burden 1-2 weeks post infection, so this study showed that iNKT cells may only have a role in eradicating Lyme infection early in the disease.

BACKGROUND

1.1 *Lyme Disease*

Lyme disease is the most common vector-borne infection in the United States, with 30,000 cases reported in 2010 (CDC, 2011). Most of these cases occurred in the northeastern and north-central states. Lyme disease is caused by the bacterial spirochete *Borrelia burgdorferi*, which is carried by *Ixodes scapularis* (also called *Ixodes dammini*) ticks (Burgdorfer et al., 1982). The spirochete is transmitted into the skin of the host through the salivary glands of the tick within 2-4 days of tick feeding (Piesman et al., 1987; Spielman et al., 1987).

After *B. burgdorferi* gets transmitted in to the host, a complex infection occurs. Lyme disease in humans occurs in 3 stages. Stage 1, early localized Lyme disease, is not yet widespread throughout the body (PubMed, 2011). In this stage, the “bull’s eye” rash, erythema migrans (EM), is present in most infected humans 3-32 days after the tick bite, and expands as the spirochetes begin to migrate from the infection site (Smith et al., 2002). Other symptoms such as headache and joint and muscle pain also may occur. After days or weeks, stage 2 may occur. In stage 2, early disseminated Lyme disease, the bacteria have begun to spread. The symptoms are the same as Stage 1, but with the addition of cardiac involvement. Stage 1 and stage 2 are both easily treated by antibiotics. If the disease continues to stage 3, called late disseminated Lyme disease, the spirochetes have spread throughout the body. This chronic manifestation of the disease can cause severe fatigue, speech problems, numbness and tingling. If left untreated, Lyme disease may lead to arthritis, which is severe inflammation and swelling of the joints (Marques, 2010; PubMed, 2011).

1.1.2 *Borrelia burgdorferi*

The agent for Lyme disease is the spirochete, *Borrelia burgdorferi*, a very motile corkscrew-shaped bacterium. The *Borrelia* cell wall consists of a cytoplasmic membrane surrounded by peptidoglycan and covered with flagella. The entire genome has been sequenced, and it is known that the spirochete depends on the host for its nutritional requirements because the *Borrelia* genome encodes few proteins with biosynthetic activity. It also causes infection by “migration through tissues, adhesion to host cells, and evasion of immune clearance”. (Steere et al., 2004)

Borrelia burgdorferi has a large number of known outer surface proteins (Osp) classified as A through F. *B. burgdorferi* had to adapt to survive living in a host; in fall, winter, and early spring the spirochete is in a dormant state in the nymphal tick midgut, where OspA is mostly expressed. In late spring/early summer, OspA gets down-regulated, and OspC is up-regulated when the tick feeds (Steere et al., 2004). Expression of OspC is required for infection of the mammalian host (Pal et al., 2004).

There are many strains of *Borrelia burgdorferi;* strain N40 was used in this project as it was previously used in research in our lab.

1.2 Mouse Model of *Borrelia* Infection

Mice are vital to the study of Lyme disease. Not only are they economical, but they are genetically defined and are susceptible to this disease at all ages (Moody and Barthold, 1998). Mouse infection during Lyme disease also parallels human Lyme disease. Once the mice are infected intradermally, *B. burgdorferi* colonizes at the site of the injection, then it eventually disseminates, as it would in humans. Within a few days, the bacteria migrate to distant sites such as the heart, joint, bladder, and ear. Arthritis can begin to develop after 4 days of infection, and

even carditis can develop after 7-10 days of infection. The level of bacteria in the tissue can be measured by quantitative PCR (qPCR). Various mouse strains do not infect equally with *Borrelia.* According to Barthold et al., mouse strain-dependent variation exists for the severity of the disease, but in general the level of *B. burgdorferi* is high from 2-4 weeks and then begins to resolve (Barthold et al., 1993).

As stated above, the severity of the disease is dependent upon the genetics of the mouse. C3H, C57BL/6, and BALB/c mice are the strains generally used to study Lyme disease. C3H mice are the most susceptible, followed by C57BL/6 and BALB/c mice. C3H mice develop severe heart and joint disease, C57BL/6 (B6) mice develop severe heart and mild joint disease, and BALB/c mice develop mild heart and mild joint disease (Moody and Barthold, 1998). Two different experiments were completed for this study; one using mice with a BALB/c background, and one using mice with a B6 background.

1.3 Mouse Immune Responses to *B. burgdorferi*

Lyme disease is an infection in which an immune response is necessary to limit *B. burgdorferi* colonization. Previous research has shown that lymphocytes are necessary to limit bacterial load and combat Lyme disease, because severe combined immunodeficient (SCID) mice, which are deficient in B and T cells, were unable to limit bacterial load (Barthold et al., 1992). Further research showed that T cells alone are not sufficient for preventing infection, because the adoptive transfer of T cells from infected mice did not protect uninfected mice from a *B. burgdorferi* infection (de Souza et al., 1993). Because T cells are not sufficient alone, B cells were found to be critical in controlling Lyme infection. McKisic et al found that B cell-deficient mice suffered from severe carditis and arthritis (McKisic et al., 2000). This shows that Lyme disease resolution is dependent upon the production of antibodies (the main role of B cells is to

make antibodies). Antibodies are crucial because they promote phagocytosis (Modolell et al., 1994). They can also bind to the surface of *Borrelia burgdorferi* to activate complement-mediated killing (Kochi et al., 1991). Interestingly, complement-independent anti-bactericidal activity was demonstrated for antibodies to *B. burgdorferi* (Munson et al., 2000).

Not only are antibodies necessary for the clearance of *B. burgdorferi*, but they are also needed for the resolution of arthritis. It was found that passive transfer of immune serum from previously infected mice to infected mice resulted in the resolution of arthritis, but not in carditis (Barthold et al., 1997).

1.3.1 Invariant Natural Killer T Cells (iNKT Cells) and *B. burgdorferi* Infection

Although T cells are not sufficient to combat Lyme disease, certain T cells are involved in helping control the infection. Invariant natural killer T (iNKT) cells are a subset of T cells that have a role in fighting infection. They have a unique property in that they express a T cell antigen receptor (TCR) and a natural killer (NK) receptor as well (Kronenburg, 2005). iNKT cells have an invariant Vα14-Jα18 TCR rearrangement in mice (Vα24-Jα18 in humans); this TCR is reactive to glycolipids when presented by the CD1d molecule on antigen-presenting cells (Kawano et al., 1997 and Godfrey and Rossjohn, 2011). This means that iNKT cells are CD1d-restricted T cells and cannot be activated without it (Lee et al., 2010 and Godfrey and Rossjohn, 2011). Constituting less than 1% of T cells in mice, and only 0.1% in humans, once stimulated these cells produce cytokines to boost an immune response to foreign antigens. However, they can also lead to the suppression of an immune response (Kronenburg, 2005).

These special T cells can act as innate and adaptive immune cells. They can be considered to be innate since they have a rapid response to foreign antigens, and are adaptive because they produce an antigen-specific response controlled by recognition via the TCR. Once they are activated they secrete cytokines such as IL-4 and IFN-γ, which then activate B cells and promote immune functions of B cells (Kronenburg, 2005).

Recently, iNKT cells have been found to directly recognize a *B. burgdorferi* diaglycerol glycolipid, BbGl-IIc (Kinjo et al., 2006). This glycolipid is recognized in humans because patients with Lyme arthritis have strong IgG antibody responses to BbGl-IIc (Jones et al., 2009). iNKT cells recognize BbGl-IIc while it is presented on CD1d. This causes their activation and then they secrete cytokines (Kinjo et al., 2006). **Note: BbGl-IIc was not tested in this study for non-scientific reasons. Getting this glycolipid highly purified is very expensive, so it was not used in this experiment.**

In addition to recognizing the specific BbGl-IIc glycolipid, studies have shown that CD1d-/- mice, which are deficient in iNKT cells, have significantly more *B. burgdorferi* in their joints compared to wild type mice (Kumar et al., 2000). Another strain of iNKT cell-deficient mice, Jα18-/-, did not clear *B. burgdorferi* efficiently and presented with more severe arthritis and carditis (Tupin et al., 2008).

1.3.2 Decorin Binding Protein A (DbpA)

Although lipid BbGl-IIc could not be used in this project, another *B. burgdorferi* antigen, called decorin binding protein A, was used in this study. Decorin binding protein A (DbpA) is a 20 kDa surface lipoprotein that binds to decorin, which is a collagen-associated proteoglycan (Guo et al., 1995). This protein aids *B. burgdorferi* in attaching to the extracellular matrix of the host (Guo et al., 1998). Research has shown that mice

deficient in decorin had fewer spirochetes in the joints and had less severe arthritis (Brown et al., 2001). Furthermore, immunization with DbpA was found to protect mice from Lyme infection (Hanson et al., 1998). When mice were infected using tick bites, however, they were not protected from Lyme infection after being immunized with DbpA (Hagman et al., 2000) because DbpA is not expressed by *B. burgdorferi* while in the tick (Hagman et al., 2000), but it is only up-regulated once it is in the host (Anguita et al., 2003).

PROJECT PURPOSE

Lyme disease, caused by the spirochete *Borrelia burgdorferi*, is the most common vector-borne infection in the United States with close to 30,000 confirmed cases each year. Many of these confirmed cases occur in Massachusetts, making it a relevant disease to study. The purpose of this study is to test whether iNKT cells in particular provide a vital function in combating Lyme disease by generating specific antibodies against *B. burgdorferi*, thus helping to eradicate it. These experiments will also begin to provide insight into the role of iNKT cells in the development of Lyme arthritis.

With the hypothesis that iNKT cells are critical for control of Lyme infection, it is predicted that anti-*B. burgdorferi* and anti-DbpA antibody titers will be lower in the iNKT-deficient mice when compared to wild type, and that the deficient mice will suffer greater bacterial loads in the joint and heart. Joint inflammation will also be visually monitored to determine if iNKT cells play a role in controlling Lyme arthritis, and to see if greater bacterial loads correlate with the presence of joint swelling.

MATERIALS AND METHODS

Borrelia burgdorferi

B. burgdorferi strain N40 clone D10/E9 (Coburn et al., 1993) was cultured at 33ºC in BSK-II medium supplemented with 6% rabbit serum (Sigma-Aldrich, St. Louis, MO) for approximately 2 days.

Mice and Infections

Mice were maintained in a pathogen-free facility of the Department of Animal Medicine at the University of Massachusetts Medical School in Worcester. Normal 10-12 week old BALB/c and C57BL/6J mice were purchased from The Jackson Laboratory or bred in the mouse facility. CD1d-/- mice and Rag1-/- mice (C57BL/6J background or Rag2-/- BALB/c background) were also purchased from the Jackson Laboratory.

To infect the mice, *B. burgdorferi* were thawed from frozen stocks. The spirochetes were cultured and counted by dark-field microscopy before the injections. Immediately prior to injection, Nair was used to remove the fur on the mouse along the midline above the hindquarter. Mice were injected intradermally with $2x10^4$ spirochetes in 50 µl of BSK-II medium along the midline above the hindquarter.

Measuring Joint Swelling During Infection

Mice were checked for signs of joint inflammation in the tibiotarsus on day 7, 13, 20 and 27 post-infection. A visual guide ranging in scores from 0-3, where 3 is the most severe, was used. Melissa Matzelle, an expert in murine modeling of arthritis (Ph.D. student in the lab of Dr. Ellen Gravellese, Chief, Division of Rheumatology, Department of Medicine at UMMS),

assisted in the scoring each week to give accurate scores. The guide for scoring arthritis can be found in **Appendix A**.

Quantitative Analysis of *B. burgdorferi* in Mouse Tissues (qPCR)

At 4 weeks post-infection, tissues were collected from the infected mice. The ears, bladder, right knee, right tibiotarsaral joint, injection site and the heart were all collected. The bladder, heart (apex and base), right knee and right tiobiotarsal joint were processed for qPCR. The tissues were frozen immediately after harvesting at -20ºC. The tissues were thawed and weighed, and DNA was extracted using DNeasy tissue kits according to the manufacturer's instructions for tissue (QIAGEN, Valencia, CA). DNA concentration was measured on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and diluted to 50 ng/µl.

qPCR was performed in a DNA engine Opticon^{TM} 2 (Bio-Rad, Hercules, CA) thermocycler in a 10 µl volume. Samples were loaded into Multiplate low-profile 96-well plates (Bio-Rad, Hercules, CA). The master mix contained dilution buffer with 3 mM $MgCl_2$ (Idaho Technology, Salt Lake City, UT), 0.015% SYBR Green I (Molecular Probes, Eugene, OR), 0.5 µM of each primer, 0.5 U of Taq polymerase (Invitrogen, Carlsbad, CA) 110 ng of Taqstart Antibody (Clonetech, Palo Alto, CA), and up to 200 ng of template DNA. The primers used to detect *B. burgdorferi* recA gene were Bb-RecA-F (5' GTGGATCTATTGTAT-TAGATGAGGCTCTCG 3') and Bb-RecA-R (5' GCCAAAGTTCTGCAACATTAA-CACCTAAAG 3'). Each of 45 amplification cycles comprised of heating at 20ºC per second to 94ºC with a 1-second hold, primer annealing at 66ºC for 15 seconds, primer extension at 70ºC for 20 seconds, and heating at 1ºC per second to 84ºC.

B. burgdorferi copy numbers were calculated by comparing the threshold PCR cycle values with the values of serial dilutions of known amounts of *B. burgdorferi* N40 genomic DNA in 50 ng/µl of mouse DNA. Serial dilutions were used as standards.

Enzyme-Linked Immunosorbent Assay (ELISA)

B. burgdorferi-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA). NUNC Maxisorp 96-well plates (Thermo Fisher Scientific, Wilmington, DE) were coated with *B. burgdorferi* N40 or recombinant DbpA (rDbpA). Frozen stocks of *in vitro* grown N40 were thawed, washed and heat–killed at 42ºC for 1 hour. $1x10^6$ spirochetes were added to each well in phosphate buffered saline (PBS). 100 ng of rDbpA in 50 mM Sodium Carbonate, 50 mM Sodium Bicarbonate buffer (at pH 9.6), was added to each well. The plates were then stored at 4ºC overnight.

The next day, antigens were discarded, the plates were washed, and blocking buffer (50 mM Tris, 140 mM NaCl, 1% BSA) was added to the plates for 1 hour at room temperature. After 1 hour, the plates were washed with wash buffer (50 mM Tris, 140 mM NaCl, 0.1% BSA, 0.025% Tween-20), then serial dilutions of serum in dilution buffer (50 mM Tris, 140 mM NaCl, 1% BSA, 0.025% Tween-20) were added to each well at 50 µl/well. The plates were incubated for 3 hours at room temperature with the serum dilutions, and then the plates were washed. Goat-anti-mouse AP-conjugated antibodies (Bethyl Laboratories, Montgomery, TX) were added and incubated for 1 hour at room temperature with anti-IgM (1:1000), anti-IgG (1:10,000), anti-IgG_1 (1:500), anti-IgG_{2a} (for BALC/c mice only), anti-IgG_{2b} (1:10,000), anti-IgG_{2c} (1:10,000; used for B6 mice only), or anti-IgG_3 (1:500). After washing the plates, 200 µl of 4-nitrophenol phosphate in glycine buffer (0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4) was added to

each well. Immediately after the substrate was added, the plates were read at room temperature in a SpectraMax 250 (Molecular Devices, Sunnyvale, CA) 96-well plate reader at OD 405 for 30 minutes with readings taken every 30 seconds. The Vmax of the AP enzymatic reaction was calculated for each sample with Softmax Pro Software v5.5 (Molecular Devices, Sunnyvale, CA). Dilutions of serum from pooled *B. burgdorferi* donor mice (stored as frozen aliquots) were run in parallel with every set of sample sera to serve as standards. The product of Vmax x the inverse serum dilution factor was calculated. The largest value within the dilution range was then expressed relative to the units of control pooled sera, which was set to 100 units.

RESULTS

To determine whether iNKTcells are important in combating Lyme disease, *B. burgdorferi* infection was compared in wild type mice, RAG-/- mice and CD1d-/- mice. Two separate experiments were completed; one where all of the mice had a BALB/c background and one where all of the mice had a B6 background.

During *B. burgdorferi* infection, the mice were visually scored for arthritis on day 7, 13, 20 and 27 for joint inflammation, using a scale from 0-3, where three is the most severe. To facilitate the assessment of the arthritis, Melissa Matzelle of University of Massachusetts Medical School assisted with the scoring. The results of the scores are shown below in **Table 1**. The results show that the BALB/c CD1d-/- mice have more severe arthritis compared to wild type mice.

Table 1: Results of Scoring the BALB/c Mice for Joint Inflammation

			Infection	Right Tibiotarsus (qPCR)			
Mouse	**Genotype**	**Sex**	**Date**	**Day 7**	**Day 13**	**Day 20**	**Day 27**
1	Cd1dko	F	9/8/2010	0	0.5	.5-1	0.5
2	Cd1dko	F	9/8/2010	0	0.5	.5-1	0.5
3	Cd1dko	F	9/8/2010	0	0.5	0.5	.5-1
4	Cd1dko	F	9/8/2010	0	0	0	0
5	Cd1dko	F	9/8/2010	0	0.5	0.5	0.5
6	Cd1dko	F	9/8/2010	0	0	0	0
7	WT	F	9/8/2010	0	0	0.5	0
8	WT	F	9/8/2010	0	0	0	0
9	WT	F	9/8/2010	0	0	0.5	0.5
10	WT	F	9/8/2010	0	0	0	0
11	WT	F	9/8/2010	0	0	0.5	0.5
12	RAG2-/-	M	9/8/2010	0	0	.5-1	0.5
13	RAG2-/-	M	9/8/2010	0	0	.5-1	1.5
14	RAG2-/-	F	9/9/2010	0	0	1	1
15	RAG2-/-	F	9/10/2010	0	0	1	1.5

Mouse	Genotype	Sex	Infection Date	Left Tibiotarsus Day 7	Day 13	Day 20	Day 27
1	Cd1dko	F	9/8/2010	0	0	0	0.5
2	Cd1dko	F	9/8/2010	0	0	0	0
3	Cd1dko	F	9/8/2010	0	0	0.5	.5-1
4	Cd1dko	F	9/8/2010	0	0.5	0.5	0.5
5	Cd1dko	F	9/8/2010	0	0	.5-1	0.5
6	Cd1dko	F	9/8/2010	0	0.5	.5-1	.5-1
7	WT	F	9/8/2010	0	0.5	0.5	.5-1
8	WT	F	9/8/2010	0	0	1	1
9	WT	F	9/8/2010	0	0	0	0
10	WT	F	9/8/2010	0	0	0	0
11	WT	F	9/8/2010	0	0	0.5	0.5
12	RAG2-/-	M	9/8/2010	0	0	.5-1	1
13	RAG2-/-	M	9/8/2010	0	0	.5-1	1.5
14	RAG2-/-	F	9/9/2010	0	0	1	1
15	RAG2-/-	F	9/10/2010	0	0	1	1

Four weeks after the mice were infected, sera and tissues were collected. The serum from each mouse was measured for antibody response to *Borrelia burgdorferi* and DbpA protein utilizing ELISAs. Total IgM, IgG, and the IgG isotypes titers were analyzed. Hyper-immune serum served as the positive control, which was defined as 100 antibody units. Serum from an uninfected mouse (Naïve) was also included as a control. This control was not displayed on graphs if the value was zero. All of the sera were normalized to the positive control. Significant differences ($p<0.05$) compared to the wild type mice were determined by one-way ANOVA and Turkey's post hoc multiple comparison test, and are indicated by brackets.

Figure 1 below shows the results of the ELISAs from the experiment of the mice with BALB/c background. Data points represent individual mice, and the red lines indicate the means. Overall, after 4 weeks of infection the CD1d-/- mice are not statistically different from wild type mice with respect to antibody titers. IgG1 anti-*B. burgdorferi,* and anti-DbpA and IgG3 anti-DbpA, were the only cases where CD1d-/- mice were statistically different from wild type mice.

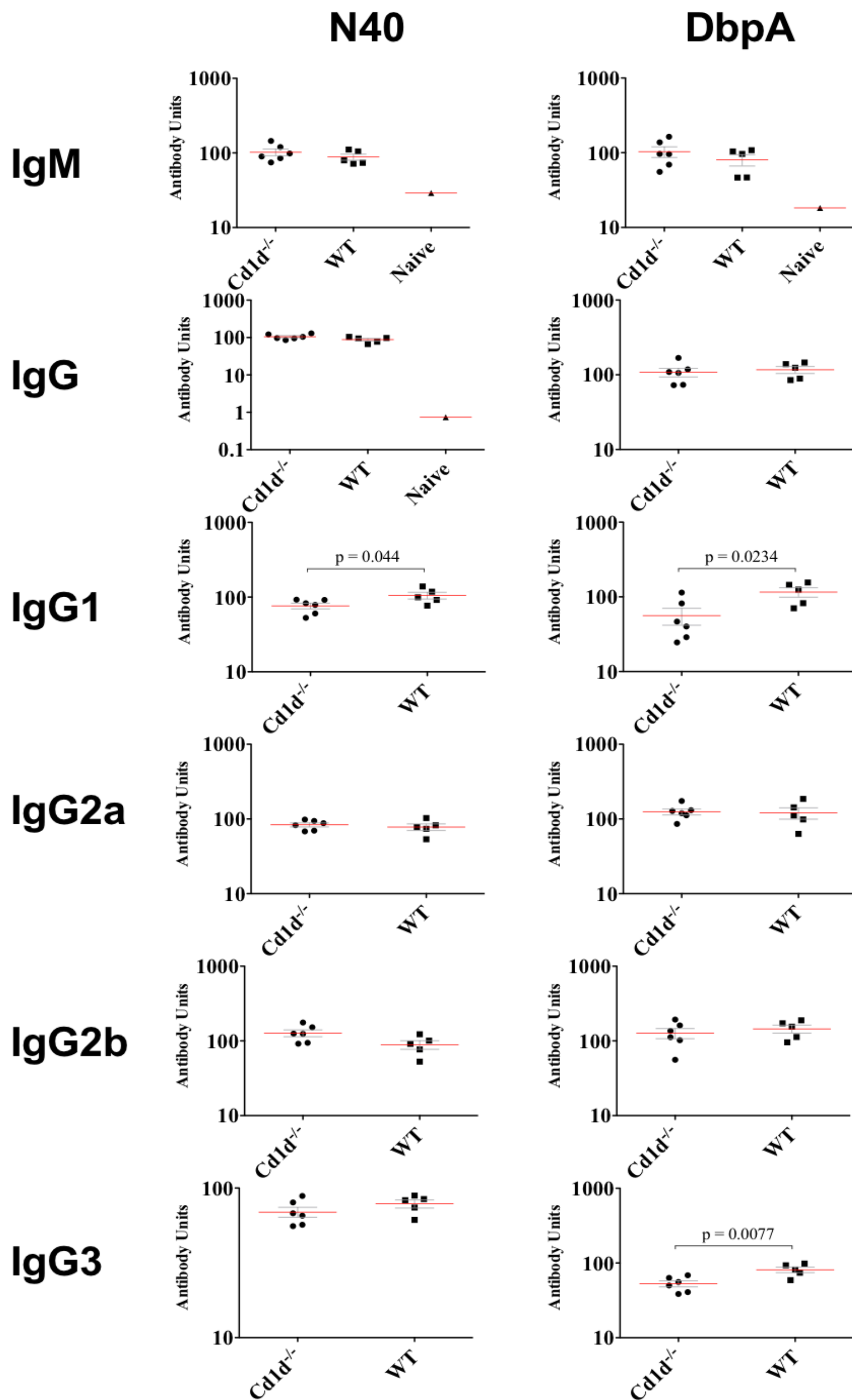

Figure 1: Anti-*B. burgdorferi* and Anti-DbpA Antibody Titers from the BALB/c CD1d-/- Experiment Utilizing ELISAs.

After the ELISAs were completed, the bacterial load was determined by quantitative PCR (qPCR) to determine whether if the antibody responses measured in the ELISAs are associated with immune protection. As stated above, after 4 weeks of *B. burgdorferi* infection, sera and tissues from the mice were collected. DNA was purified from the right knee, right tibiotarsus, and the heart using a DNeasy tissue kit according to the manufacturer's instructions for tissue (QIAGEN, Valencia, CA). The concentration of the DNA was measured by a NanoDrop 1000, and then diluted to 50 ng/µl. qPCR was performed, and the copy number of *B. burgdorferi* per 100 ng of mouse DNA was graphed. Significant differences compared to wild type mice were again determined by one-way ANOVA and Turkey's post hoc multiple comparison test and are indicated by brackets with a p value.

The results of the qPCR from the BALB/c CD1d-/- experiment are shown below in **Figure 2.** Each dot represents an individual mouse, and the red bars indicate the group mean. The results show that the iNKT cell-deficient mice yielded similar bacterial burden as the wild type mice; there was no significant difference.

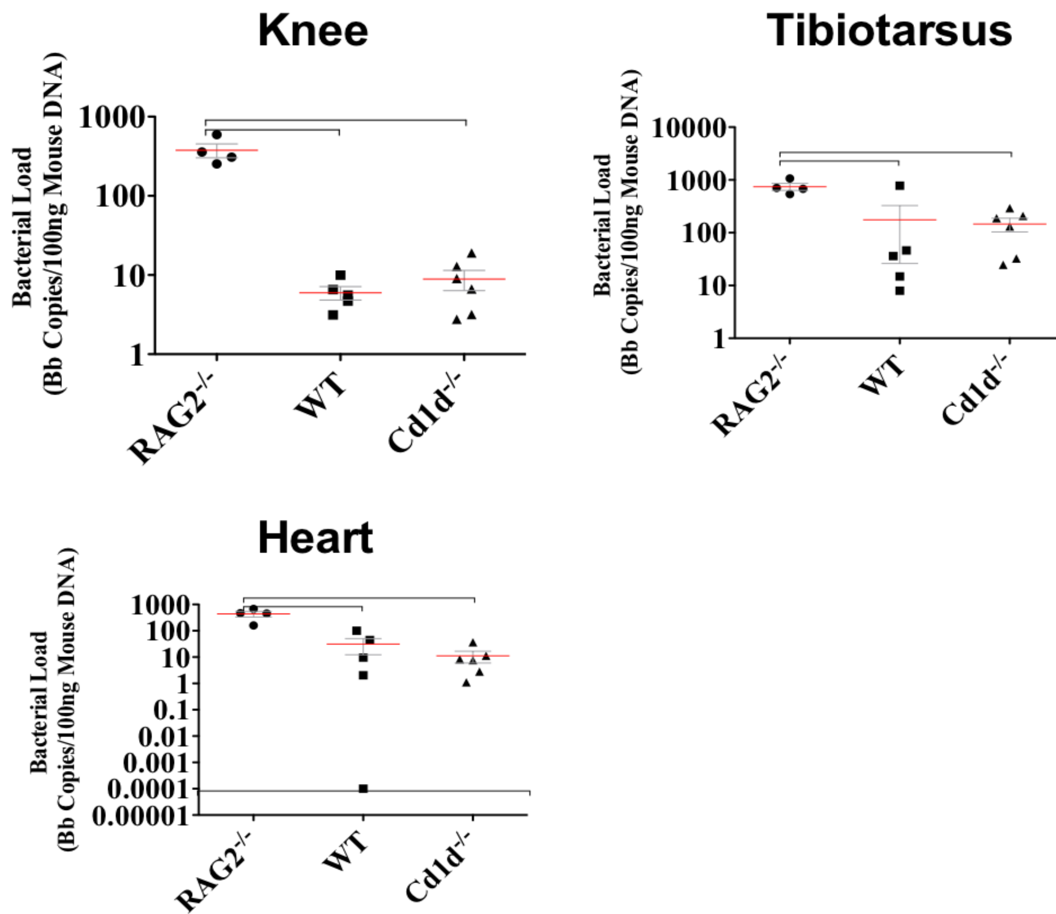

Figure 2: Bacterial Burden in the Joint (Knee and Tibiotarsus) and Heart as Determined by qPCR.

Because the CD1d-/- mice with a BALB/c background had the same response to *B. burgdorferi* infection as the wild type mice, another experiment was completed using CD1d-/- mice with B6 background. Previous research has shown that *B. burgdorferi* disease severity is strain-dependent; B6 mice generate severe heart disease and mild joint disease, while BALB/c mice generate mild heart and somewhat more joint disease (Moody and Barthold, 1998; Wooten and Weis, 2001).

The B6 CD1d-/- experiment was completed the same way as the previous experiment. After the 4 week *B. burgdorferi* infection, sera and tissues were collected from the mice. Anti-*B. burgdoferi* and anti-DbpA antibody titers of the sera were determined utilizing ELISAs. Total IgM, IgG, and IgG isotype titers were studied. Hyper-immune serum served as the positive control, which was defined as 100 antibody units. The negative control (Naïve) was serum from an uninfected mouse. All of the experimental sera were normalized to the positive control. Potential significant differences ($p<0.05$) compared to the wild type mice were determined by one-way ANOVA and Turkey's post hoc multiple comparison test and are indicated by brackets.

Figure 3 below shows the results of the ELISAs from the experiment of the mice with B6 background. Data points represent individual mice and the red lines indicate means. Like the first experiment, the deficient mice generated similar antibody response to the wild type mice. IgG3 anti- *B. burgdorferi* and anti-DbpA were the only cases where CD1d-/- mice were statistically different from wild type mice.

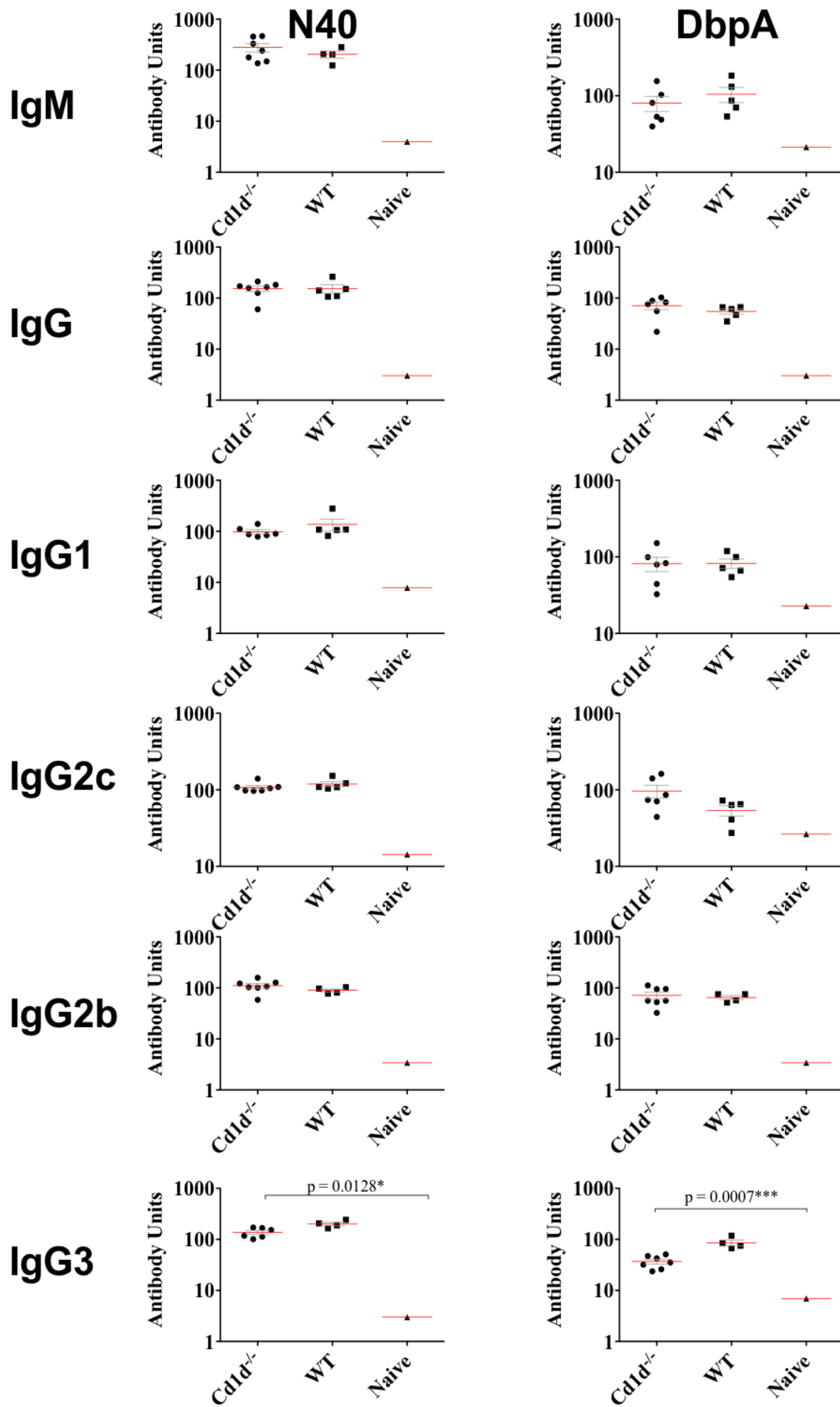

Figure 3: Anti-*B. burgdorferi* and Anti-DbpA Antibody Titers From the B6 CD1d-/- Experiment Utilizing ELISAs.

B. burgdorferi bacterial load was determined by quantitative PCR (qPCR) to study if the antibody responses seen in the ELISAs are associated with immune protection. Sera and tissues from the mice were collected after 4 weeks of infection. DNA was extracted from the right knee, right tibiotarsus, heart base, and heart apex using a DNeasy tissue kit according to the manufacturer's instructions for tissue (QIAGEN, Valencia, CA). The concentration of the DNA was determined using a NanoDrop 1000 spectrophotometer, and then the DNA was diluted to 50 ng/µl. The copy number of *B. burgdorferi* per100 ng of mouse DNA was determined by qPCR and then graphed. Potential significant differences compared to wild type mice were again determined by one-way ANOVA and Turkey's post hoc multiple comparison test, and are indicated by brackets with a p value.

The results of the qPCR from the B6 CD1d-/- experiment are shown in **Figure 4.** Each dot represents an individual mouse, and the red bars indicate the group mean. The results indicate that the CD1d-/- mice had a smaller, yet insignificant lower bacterial load than the wild type mice in the joint and heart.

Overall, the CD1d-/- mice (BALB/c and B6 backgrounds) did not have significantly lower immunological protection to *Borrelia burgdorferi* infection when compared to wild type mice.

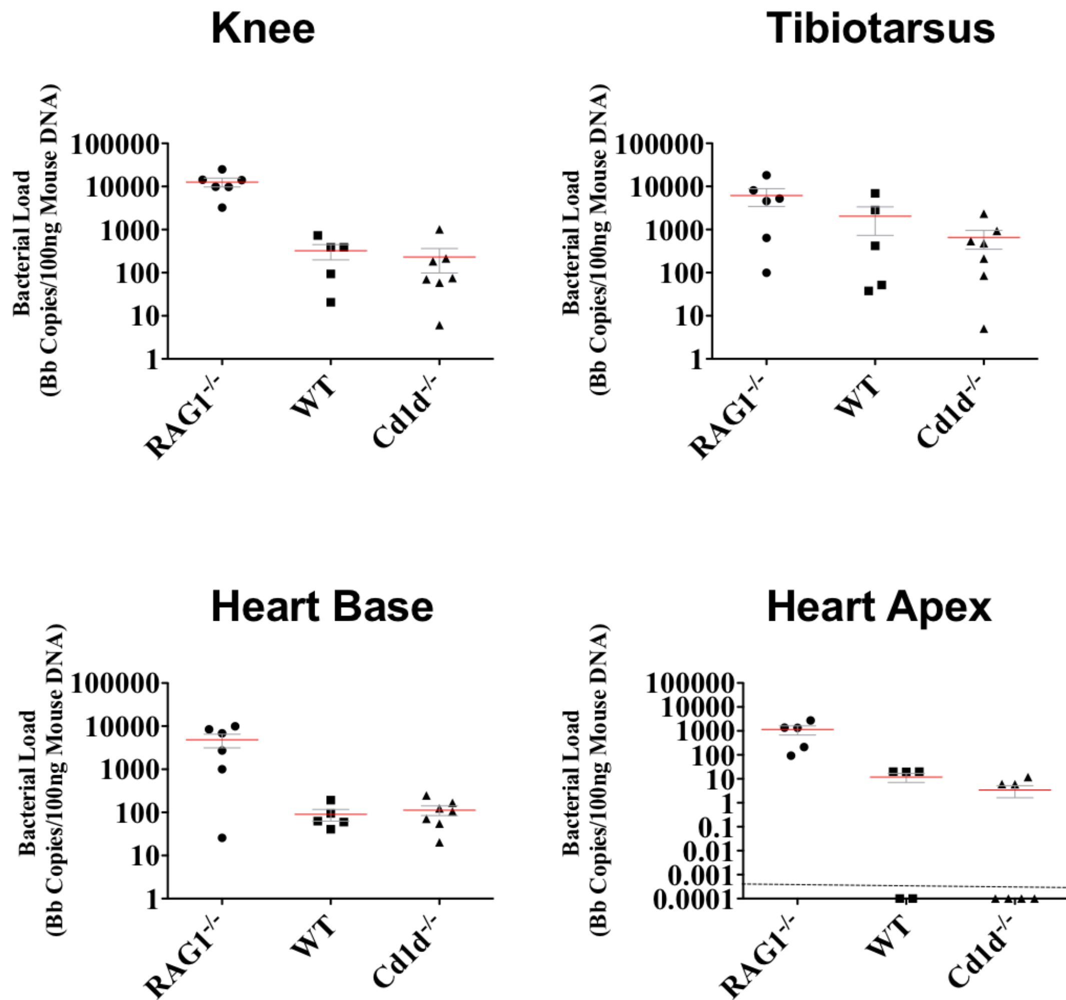

Figure 4: Bacterial Burden in the Joint (Knee and Tibiotarsus) and Heart (Base and Apex) as Determined by qPCR.

DISCUSSION

The objective of this study was to determine whether iNKT cells have a role in developing a protective immune response to *Borrelia burgdorferi*, the agent of Lyme disease. It was hypothesized that iNKT cells are critical for generating specific antibodies against *B. burgdorferi* and thus eradicating the bacteria since it has been shown that iNKT cells provide help for B cells, which produce antibodies (Kronenberg, 2005, Campos et al., 2006, and Leadbetter et al., 2007). The iNKT-deficient mice were predicted to have lower anti- *B. burgdorferi* and anti-DbpA titers compared to wild type mice, and suffer from higher bacterial loads in the joint and heart. Studying iNKT-mediated responses also provided insight into the role of these cells in developing Lyme arthritis, as these cells are implicated in lessening the pathology of Lyme infection in other studies (Kumar et al., 2000). To determine if the predicted increase in spirochetal colonization of the joint correlates with increased arthritis, joint inflammation was also monitored.

Previous research showed that at 1-2 weeks post-infection, CD1d deficiency resulted in less protection to infection, implying that iNKT cells are important in combating Lyme disease (Kumar et al., 2000). However, this does not seem to be the case with the data in this project, because at 4 weeks post infection, ELISAs to detect antibodies against N40 and DbpA antigens for the BALB/c NKT-deficient mice revealed no significant differences in IgM, total IgG, IgG2a, or IgG2b when compared to wild type mice **(Figure 1)**. There was a modest, but significant, decrease in IgG1 against both of the antigens and IgG3 for DbpA **(Figure 1)**. In addition, ELISAs for antibodies against N40 and DbpA for the B6 NKT-deficient mice showed no differences in IgM, total IgG, IgG1, IgG2c, or IgG2b when compared to wild type mice **(Figure 3)**. There was only a significant decrease in the levels of IgG3 against N40 and DbpA **(Figure**

3). These data suggest that iNKT cells do not play a significant role in limiting *Borrelia burgdorferi* pathogen burden at 4 weeks after infection. As shown by Kumar et al., iNKT cells may only play an important role in eradicating *B. burgdorferi* in the initial resistance to infection.

The decreased production of IgG1 antibody against N40 and DbpA in the BALB/c CD1d-/- mice **(seen in Figure 1)** provides insight that iNKT cells may be important in combating Lyme disease by providing the right cytokines to activate B cells. NKT cells are known to secrete IL-4 once activated (Kronenberg, 2005), which drives B cells to switch to IgG1 (Croft and Swain, 1991). iNKT cells are CD1d restricted, so in the CD1d-/- mice iNKT cells cannot be activated to secrete IL-4 to induce isotype switching to IgG1. This explains why the IgG1 titers are lower in the NKT-deficient mice when compared to wild type mice. Furthermore, the decreased production of IgG3 against N40 and DbpA in the B6 CD1d-/- mice **(seen in Figure 3)** also provides insight that iNKT cells may be important in limiting bacterial burden by providing the right cytokines to activate B cells. NKT cells secrete IFN-γ once activated (Kronenberg, 2005), which drives B cells to switch to IgG3 (Croft and Swain, 1991). In the NKT-deficient mice, the NKT cells are not secreting IFN-γ to activate B cells to produce IgG3. This explains why the IgG3 titers are lower in the NKT-deficient mice when compared to wild type mice. Research has already shown that the production of IFN-γ by iNKT cells modulates Lyme carditis (Olson et al., 2009), so this may be a mechanism by which the NKT cells help clear Lyme disease.

In addition to looking at the titers of *B. burgdorferi*- specific antibodies, the bacterial burden in the joint and heart was analyzed in the BALB/c and B6 mice. qPCR of the BALB/c CD1d-/- mice revealed no significant differences in colonization in the knee, tibiotarsus, or heart when compared to wild type mice **(Figure 2)**. There was a slight trend towards increased

colonization in the CD1d-/- mice, but one wild type mouse had very high levels of bacteria and two CD1d-/- mice had loads similar to wild type levels. qPCR of the B6 CD1d-/- mice also revealed no significant difference when compared to wild type mice; the bacterial burden of the CD1d-/- mice occurred within the range of the wild type mice **(Figure 4)**. These data refute the hypothesis; CD1d (iNKT cells) do not play a critical role in limiting *B. burgdorferi* infection or disease 4 weeks after infection.

Although iNKT cells do not seem to play an essential role in combating Lyme disease 4 weeks post infection, it is possible that they may still have a role in controlling arthritis. Severity and prevalence of arthritis in the knee and tibiotarsal joints in CD1d-/- mice 1-2 weeks post infection has been previously shown to increase compared to wild type mice (Kumar et al., 2000). As seen in **Table 1**, the CD1d-/- mice have more severe arthritis compared to wild type mice 4 weeks post infection. This suggests that CD1d also plays a role in controlling arthritis 4 weeks after infection. There does not seem to be a correlation between arthritis severity and bacterial loads, however, because mice with higher bacterial loads did not necessarily have more severe (or any) arthritis.

In summary, although previous research has shown that CD1d (iNKT cell) plays a critical role in limiting *B. burgdorferi* infection and controlling arthritis 1-2 weeks post infection, this study shows that iNKT cells do not play a vital role in limiting Lyme disease 4 weeks post-infection, but they do play a role in controlling arthritis.

BIBLIOGRAPHY

Anguita, J., Hedrick, M. N., & Fikrig, E. (2003). Adaptation of borrelia burgdorferi in the tick and the mammalian host. *FEMS Microbiology Reviews, 27*(4), 493-504.

Barthold, S. W., Feng, S., Bockenstedt, L. K., Fikrig, E., & Feen, K. (1997). Protective and arthritis-resolving activity in sera of mice infected with borrelia burgdorferi. *Clinical Infectious Diseases, 25*(Supplement 1), S9.

Barthold, S. W., Sidman, C. L., & Smith, A. L. (1992). Lyme borreliosis in genetically resistant and susceptible mice with severe combined immunodeficiency. *The American Journal of Tropical Medicine and Hygiene, 47*(5), 605-613.

Barthold, S., De Souza, M., Janotka, J., Smith, A., & Persing, D. (1993). Chronic lyme borreliosis in the laboratory mouse. *The American Journal of Pathology, 143*(3), 959.

Benach, J. L., Fleit, H. B., Habicht, G. S., Coleman, J. L., Bosler, E. M., & Lane, B. P. (1984). Interactions of phagocytes with the lyme disease spirochete: Role of the fc receptor. *Journal of Infectious Diseases, 150*(4), 497.

Brown, E. L., Wooten, R. M., Johnson, B. J. B., Iozzo, R. V., Smith, A., Dolan, M. C., et al. (2001). Resistance to lyme disease in decorin-deficient mice. *Journal of Clinical Investigation, 107*(7), 845-852.

Burgdorfer, W., Barbour, A. G., Hayes, S. F., Benach, J. L., Grunwaldt, E., & Davis, J. P. (1982). Lyme disease-a tick-borne spirochetosis? *Science, 216*(4552), 1317.

Campos, R. A., Szczepanik, M., Lisbonne, M., Itakura, A., Leite-de-Moraes, M., & Askenase, P. W. (2006). Invariant NKT cells rapidly activated via immunization with diverse contact antigens collaborate in vitro with B-1 cells to initiate contact sensitivity. *The Journal of Immunology, 177*(6), 3686.

CDC.com (2011) Centers for Disease Control and Prevention. http://www.cdc.gov/

Coburn, J., Leong, J. M., & Erban, J. K. (1993). Integrin alpha IIb beta 3 mediates binding of the lyme disease agent borrelia burgdorferi to human platelets. *Proceedings of the National Academy of Sciences, 90*(15), 7059.

Croft, M., & Swain, S. L. (1991). B cell response to T helper cell subsets. II. both the stage of T cell differentiation and the cytokines secreted determine the extent and nature of helper activity. *The Journal of Immunology, 147*(11), 3679.

De Souza, M., Smith, A., Beck, D., Terwilliger, G., Fikrig, E., & Barthold, S. (1993). Long-term study of cell-mediated responses to borrelia burgdorferi in the laboratory mouse. *Infection and Immunity, 61*(5), 1814.

Godfrey, D. I., & Rossjohn, J. (2011). New ways to turn on NKT cells. *The Journal of Experimental Medicine, 208*(6), 1121.

Guo, B. P., Brown, E. L., Dorward, D. W., Rosenberg, L. C., & Höök, M. (1998). Decorin-binding adhesins from borrelia burgdorferi. *Molecular Microbiology, 30*(4), 711-723.

Guo, B. P., Norris, S. J., Rosenberg, L. C., & Hook, M. (1995). Adherence of borrelia burgdorferi to the proteoglycan decorin. *Infection and Immunity, 63*(9), 3467.

Hagman, K. E., Yang, X., Wikel, S. K., Schoeler, G. B., Caimano, M. J., Radolf, J. D., et al. (2000). Decorin-binding protein A (DbpA) of borrelia burgdorferi is not protective when immunized mice are challenged via tick infestation and correlates with the lack of DbpA expression by B. burgdorferi in ticks. *Infection and Immunity, 68*(8), 4759.

Hanson, M. S., Cassatt, D. R., Guo, B. P., Patel, N. K., McCarthy, M. P., Dorward, D. W., et al. (1998). Active and passive immunity against borrelia burgdorferi decorin binding protein A (DbpA) protects against infection. *Infection and Immunity, 66*(5), 2143.

Jones, K. L., Seward, R. J., Ben-Menachem, G., Glickstein, L. J., Costello, C. E., & Steere, A. C. (2009). Strong IgG antibody responses to borrelia burgdorferi glycolipids in patients with lyme arthritis, a late manifestation of the infection. *Clinical Immunology, 132*(1), 93-102.

Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., et al. (1997). CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science, 278*(5343), 1626.

Kinjo, Y., Tupin, E., Wu, D., Fujio, M., Garcia-Navarro, R., Benhnia, M. R. E. I., et al. (2006). Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nature Immunology, 7*(9), 978-986.

Kochi, S. K., Johnson, R., & Dalmasso, A. (1991). Complement-mediated killing of the lyme disease spirochete borrelia burgdorferi. role of antibody in formation of an effective membrane attack complex. *The Journal of Immunology, 146*(11), 3964.

Kronenberg, M. (2005). Toward an understanding of NKT cell biology: Progress and paradoxes. *Annu.Rev.Immunol., 26*, 877-900.

Kumar, H., Belperron, A., Barthold, S. W., & Bockenstedt, L. K. (2000). Cutting edge: CD1d deficiency impairs murine host defense against the spirochete, borrelia burgdorferi. *The Journal of Immunology, 165*(9), 4797.

Leadbetter, E. A., Brigl, M., Illarionov, P., Cohen, N., Luteran, M. C., Pillai, S., et al. (2008). NK T cells provide lipid antigen-specific cognate help for B cells. *Proceedings of the National Academy of Sciences, 105*(24), 8339.

Lee, W. Y., Moriarty, T. J., Wong, C. H. Y., Zhou, H., Strieter, R. M., van Rooijen, N., et al. (2010). An intravascular immune response to borrelia burgdorferi involves kupffer cells and iNKT cells. *Nature Immunology, 11*(4), 295-302.

Marques, A. R. (2010). Lyme disease: A review. *Current Allergy and Asthma Reports, 10*(1), 13-20.

McKisic, M. D., Redmond, W. L., & Barthold, S. W. (2000). Cutting edge: T cell-mediated pathology in murine lyme borreliosis. *The Journal of Immunology, 164*(12), 6096.

Modolell, M., Schaible, U., Rittig, M., & Simon, M. (1994). Killing of borrelia burgdorferi by macrophages is dependent on oxygen radicals and nitric oxide and can be enhanced by antibodies to outer surface proteins of the spirochete. *Immunology Letters, 40*(2), 139-146.

Moody, K. D., & Barthold, S. W. (1998). Lyme borreliosis in laboratory mice. *Laboratory Animal Science, 48*, 168-171.

Olson, C. M., Jr, Bates, T. C., Izadi, H., Radolf, J. D., Huber, S. A., Boyson, J. E., et al. (2009). Local production of IFN-gamma by invariant NKT cells modulates acute lyme carditis. *Journal of Immunology (Baltimore, Md.: 1950), 182*(6), 3728-3734. doi:10.4049/jimmunol.0804111

Pal, U., Yang, X., Chen, M., Bockenstedt, L. K., Anderson, J. F., Flavell, R. A., et al. (2004). OspC facilitates borrelia burgdorferi invasion of ixodes scapularis salivary glands. *The Journal of Clinical Investigation, 113*(2), 220-230. doi:10.1172/JCI19894

Piesman, J., Mather, T. N., Sinsky, R., & Spielman, A. (1987). Duration of tick attachment and borrelia burgdorferi transmission. *Journal of Clinical Microbiology, 25*(3), 557.

PubMed (2011) http://www.ncbi.nlm.nih.gov/pubmed/

Smith, R. P., Schoen, R. T., Rahn, D. W., Sikand, V. K., Nowakowski, J., Parenti, D. L., et al. (2002). Clinical characteristics and treatment outcome of early lyme disease in patients with microbiologically confirmed erythema migrans. *Annals of Internal Medicine, 136*(6), 421-428.

Spielman, A., Ribeiro, J., Mather, T., & Piesman, J. (1987). Dissemination and salivary delivery of lyme disease spirochetes in vector ticks (acari: Ixodidae). *Journal of Medical Entomology, 24*(2), 201-205.

Steere, A. C., Coburn, J., & Glickstein, L. (2004). The emergence of lyme disease. *Journal of Clinical Investigation, 113*(8), 1093-1101.

Tupin, E., Benhnia, M. R., Kinjo, Y., Patsey, R., Lena, C. J., Haller, M. C., et al. (2008). NKT cells prevent chronic joint inflammation after infection with borrelia burgdorferi. *Proceedings of the National Academy of Sciences of the United States of America, 105*(50), 19863-19868. doi:10.1073/pnas.0810519105

Wooten, R. M., & Weis, J. J. (2001). Host-pathogen interactions promoting inflammatory lyme arthritis: Use of mouse models for dissection of disease processes. *Current Opinion in Microbiology, 4*(3), 274-279.

APPENDIX A

"Arthritis Scoring Guidelines:
0.5 – paw is visibly red with some swelling of the footpads, maybe slight swelling on top of the foot and around the heel
1 – paw is visibly swollen around footpads, on top of foot, swelling may be around heel but calcaneous bone which sticks out from rear of foot is still clearly visible
1.5 – is in-between observations of score 1 and 2
2 – Footpad swelling is obvious, calcaneous bone is obscured by thickening due to inflammation, there are clear "bumps" of swelling on top of the foot, toes are often swollen as well. The inflammation may be extending up limb from ankle – thickening obvious
2.5 – is in-between observations of 2 and 3
3 – footpad swelling is obvious, calcaneous is obscured by thickening due to inflammation around ankle, significant swelling on top of the foot, toes will often be swollen, inflammation, thickening extends along limb from ankle by ~5mm and this will often have look of "rolls of tissue" or bumps of swelling. Joint resists flexing and bending." (Nicole Walsh, Gravallese Laboratory, University of Massachusetts Medical School)