

BALB/C MICE: A NOVEL MODEL  
FOR BORRELIA BURGDORFERI-  
INDUCED LYME  
ARTHRITIS

by

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## ABSTRACT

BALB/c mice have been used to study multiple diseases that cause inflammation such as Lyme arthritis, rheumatoid arthritis, and systemic lupus erythematosus. Lyme disease or, more specifically, Lyme arthritis is caused by *Borrelia burgdorferi* that has disseminated into joint tissues. A spectrum of Lyme disease severity and symptoms has been seen in Lyme disease patients, which suggests that genetics play a role in host response. Inbred mouse strains display the range of arthritis severity seen in patients from the mild arthritis phenotype in C57BL/6 mice to the severe arthritis phenotype in C3H mice. The C3H mouse model develops severe Lyme arthritis that can be attributed to 2 important inflammatory factors: a hypomorphic allele of *GusB* and hyper production of Type I IFN-induced transcripts. Using SNP-based assessment, the BALB/c mouse was found to be closely related to the C3H mouse; however, the BALB/c mouse does not have the hypomorphic *GusB* allele. It was hypothesized that the BALB/c mouse, which develops severe, dose-dependent arthritis, would also hyperproduce Type I IFN-related transcripts. This study found that the BALB/c mouse does not develop an IFN profile similar to the C3H mouse. Multiple inflammatory markers were analyzed to compare and contrast BALB/c and C3H mice, and TNF $\alpha$  was determined to be elevated in the BALB/c mouse above the levels in the C3H mouse both *in vivo* during infection and *in vitro* using bone marrow-derived macrophages. This suggests an important role of the innate immune response in these mice. Interestingly, peritoneal macrophages derived

from BALB/c were able to internalize *B. burgdorferi* much better than C3H-derived peritoneal macrophages. This suggests an elevated intrinsic response in controlling *B. burgdorferi* numbers. TNF $\alpha$  blockade has been shown to be beneficial in relieving symptoms in rheumatoid arthritis patients, so it was hypothesized that TNF $\alpha$  blockade in BALB/c mice would reduce Lyme arthritis symptoms. This study did not see any benefit to TNF $\alpha$  blockade in Lyme arthritis reduction. Overall, this study has shown that there are still mechanisms that are yet to be fully understood in the pathogenic relationship between the BALB/c mouse and the spirochete bacteria *B. burgdorferi*.

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## INTRODUCTION

### Lyme disease: from infection to fruition

Lyme disease is the most common tick-borne disease in the Northern Hemisphere (1). In the United States, Lyme disease is most typically seen in the Northeast and upper Midwest (2). Approximately 30,000 cases of Lyme disease each year are reported to the Center for Diseases Control; however, the CDC has acknowledged that there is underreporting of the actual cases and has recently published a projected incidence of approximately 300,000 cases/ year. This estimate is based on analysis of state health department records, diagnostic company records, and health insurance records for endemic regions (3). Lyme disease is caused by an infection with the spirochete *Borrelia burgdorferi* that is transferred from the *Ixodes* tick vector to the host (4). The most common types of *Ixodes* ticks that spread Lyme disease in the United States are the blacklegged tick (*Ixodes scapularis*) in the upper Midwest and Northeast, and the western blacklegged tick (*Ixodes pacificus*) along the west coast (1). *Ixodes* ticks are very small in comparison to ticks commonly associated with livestock and domesticated animals, so the presence of an attached *Ixodes* tick can easily be missed (2). The nymph, a juvenile stage in the tick life cycle that is much smaller than the adult tick, is responsible for most Lyme disease transmission to humans (3). The most common seasons for infection are during the spring and summer months when the nymph pursues a blood meal to fuel its transformation from a juvenile into an adult (1). Typically, the tick must be attached to

the host for 36 to 48 hours for Lyme disease to be transmitted (2, 5).

After an infected tick bite, the disease incubation period can range from 3 to 30 days (6). Upon infection, *B. burgdorferi* can proceed from an early localized infection to a late disseminated infection. During the early localized phase, the symptoms typically resemble the flu (6). These symptoms include: headache, fatigue, chills, muscle and joint pain, fever, and swollen lymph nodes (2). This is 1 of the reasons that Lyme disease cases are under-diagnosed (1). The most characteristic sign of an early localized infection is a circular red skin rash called erythema migrans, identified in 70% of individuals and considered to be diagnostic. This rash develops at the site of infection 1 to 30 days after the infected tick bite (4) and resembles a red, bull's eye target. If left untreated, the rash can become larger and can spread to additional skin lesions throughout the body and change in appearance and magnitude (2). In late disseminated infection, *B. burgdorferi* can be found throughout various body tissues and the nervous system (3). Regardless of whether antibiotic treatment is provided, 30-60% of patients will go on to develop late-stage Lyme disease symptoms that include encephalomyelitis, carditis, and arthritis (7, 8). These symptoms can also be seen in various other diseases, which makes Lyme disease difficult to diagnose properly. Patients can develop some or all of these signs and symptoms, and the severity and duration of the signs and symptoms are also highly variable. When Lyme disease is suspected, a physician can order antibody testing on the patient. Antibodies to *B. burgdorferi* are measured using a 2-step serological process (9). First, an enzyme immunoassay is run on a blood sample to screen for *B. burgdorferi* antibodies. If the blood sample test is positive for *B. burgdorferi* antibodies, a western immunoblot is performed to detect specific proteins related to the bacteria. In order for

these tests to be analyzed properly, it is best to know the approximate date of infection. Antibody levels vary depending on length and severity of infection, so a recently bitten patient may be infected but may not yet have detectable antibody levels in their blood (10, 11). The primary treatments for *B. burgdorferi* infection are various antibiotics. However, the age of the patient, the stage and severity of the infection, and the symptoms present will ultimately guide how the patient is treated (3).

Due to the wide range of symptom prevalence, severity of the symptoms, and severity of the disease, there is reason to believe that studying genetic differences in the bacteria and the host (12–15), as well as the host immune response (16, 17), will lead to a further understanding of the complete Lyme disease spectrum. It is estimated that 10% of patients treated with antibiotic will experience sustained symptoms, even though evidence of persisting active infection is lacking (18). This occurrence rate is of great interest for the study of Lyme arthritis and *B. burgdorferi* infection. Interestingly, not all isolates of *B. burgdorferi* are able to disseminate from the skin to other tissues. The ability of a *B. burgdorferi* isolate to cause disseminated disease is clearly dependent on its particular genetic composition, with at least 3 bacterial groups identified with distinct invasive potentials. Isolates were genetically grouped by genotyping outer surface protein C sequences, 16S and 23S rRNA intergenic spacer region sequence, and by additional multilocus sequence typing (8, 9, 19–21). In order for Lyme arthritis to develop, the isolates must invade the joint tissue. However, different individuals infected with the same genetically identical invasive isolate can display a spectrum of symptoms and severity, revealing that the individual's host immune responses are also responsible for some of the differences in infection, symptom severity, and symptom persistence. To

further assess these variances in bacteria and host, molecular markers are evaluated within the host's infected tissues, fluids, and systems.

### Studying the importance of genetics in Lyme arthritis

Animal models are used to study Lyme arthritis to evaluate *in vivo* effects of specific genes and gene products related to the bacterial infection and subsequent inflammation seen in Lyme arthritis. In a previous study, Barthold and colleagues demonstrated that a single low dose of *B. burgdorferi* given intradermally caused arthritis and carditis of varying severity in 5 different strains of laboratory mice, implicating genetics of the host as a determinant of disease severity (22). The arthritis severity of C57BL/6 mice and C3H mice are used to illustrate the wide spectrum of human Lyme arthritis. Over a range of inoculum doses, *B. burgdorferi* was found to cause mild arthritis in C57BL/6 mice and severe arthritis in C3H mice (23). BALB/c mice were seen to develop mild arthritis in these early studies, but later studies showed that larger infectious doses could cause the BALB/c mouse to develop severe arthritis (23–25). Due to the varying arthritis severity seen in the laboratory, the BALB/c mice has had limited use in the study of Lyme arthritis.

Both forward genetics and empirical approaches have been taken to assess differences in arthritis severity in the C57BL/6 and C3H mouse strains and these approaches have converged on the importance of the Type I interferon (IFN) response (26) and the hypomorphic *GusB* allele found in the C3H mouse (27). The *GusB* gene encodes the  $\beta$ -glucuronidase enzyme, which digests glycosaminoglycans (GAGs) in the tissues. C3H mice contain an allele that causes reduced  $\beta$ -glucuronidase activity, which

results in an increase in GAG accumulation in the tissues, which causes increased inflammation in the tissue (27, 28).

Previous studies comparing over 100 mouse strains using SNP-based analysis places C3H and BALB/c mice on the same branch of the SNP-based mouse family tree (29). Interestingly, the BALB/c mouse does not carry the hypomorphic *GusB* allele that is associated with Lyme arthritis development in the C3H mouse. So, due to their genetic relatedness (30) and the lack of the *GusB* hypomorphic Lyme arthritis marker, it was proposed that the BALB/c mouse might develop a similar exaggerated Type I IFN profile as seen in C3H mice during Lyme arthritis development.

Through gene expression profiling in joint tissues from C57BL/6 and C3H mouse strains, it was found that IFN-induced transcripts had a robust peak in C3H mice seen at 7 days post infection that did not appear in C57BL/6 mice (31). This was a preclinical response demonstrating that it may drive Lyme arthritis in these mice. Through forward genetic approaches, a quantitative trait locus (QTL) termed *Bbaa1* that spans the Type I IFN gene cluster on Chr4 was identified that regulated arthritis severity and the magnitude of the Type I IFN response (32). Congenic mice on the C57BL/6 background with the C3H *Bbaa1* allele (B6.C3-*Bbaa1*) revealed that *Bbaa1* regulates arthritis severity through intrinsic control of Type I IFN production (33). Using bone marrow-derived macrophages (BMDM) from C57BL/6, C3H, B6.C3-*Bbaa1*, and C3.B6-*Bbaa1* as a surrogate for myeloid cells, the laboratory saw that in the cells containing the C3H allele of *Bbaa1*, the Type I IFN profile was greater than in the mice that possessed the C57BL/6 allele for *Bbaa1* (the Type I IFN cluster) when co-incubated with *B. burgdorferi* or the unrelated IFN stimulant poly I:C (33). These studies suggest that the C3H alleles for the

Type I IFN gene cluster and flanking DNA within *Bbaa1* contribute to the severe Lyme arthritis seen in infected C3H mice.

### The importance of inflammatory cytokines and enzymes

When an infection event occurs, the immune system springs into action to fight the infection. To function efficiently, the immune system essentially has positive and negative regulatory cytokine production to control the magnitude of the response. Some major cytokines that work to increase the immune response are IFN, TNF, IL-6, and IL-12. Negative regulators include cytokines such as IL-10, TGF- $\beta$ , and cell surface markers such as PD-1. If the immune system fails to keep an accurate balance, these regulators can go from being helpful to pathogenic. For example, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are examples of inflammatory disorders caused by the immune system attacking itself called autoimmunity.

In mice, Type I IFNs are a group of interferon proteins that consist of 14 IFN- $\alpha$ 's and 1 IFN- $\beta$ . In response to *B. burgdorferi* infection, IFN $\beta$  is more highly produced than the IFN- $\alpha$ 's, so the laboratory has focused on determining what regulates IFN $\beta$  in the *Bbaa1* mouse locus, and how IFN $\beta$  relates to other genes within the locus. If IFN response is not regulated properly, it can become over-expressed, which will eventually lead to a pathogenic Type I IFN response causing severe inflammation in Lyme arthritis. In SLE, IFN $\alpha$  had been shown to be more important than IFN $\beta$  (34) in causing the inflammatory state, and blocking Type I IFNs has reduced symptoms severity (35).

Multiple cell types produce and respond to Type I IFNs. These include: fibroblasts, macrophages, endothelial cells, osteoblasts, B cells, T cells, and natural killer

cells. The laboratory found that the cells within the joint tissue that produce the largest amount of IFN-related transcripts related to Lyme arthritis are endothelial cells and synovial fibroblasts (36). In SLE, plasmacytoid dendritic cells (pDC) are a major source of IFN $\alpha$  and peripheral blood mononuclear cells (PBMCs) are used to measure Type I IFN-related gene products (37).

On the other side of the immune system, IL-10 has been found to be very important in reducing the immune response, and more specifically in Lyme arthritis, it reduces arthritis development. The laboratory found that macrophages and CD4<sup>+</sup> cells are the primary source of IL-10 in the infected joint tissue (38, 39). This study and others suggested that if IL-10 is produced early on in infection, the pathogenic IFN $\gamma$  profile could be reduced (38, 40). Other studies have shown that the origin of the macrophages (41, 42), as well as their polarization (43), can influence their ability to produce certain cytokines. The laboratory uses BMDM in many studies because they can be expanded as a pure primary cell culture that is genetically representative for each mouse strain (44).

Other cytokines that are directly related to Lyme arthritis include IL-1, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and matrix metalloproteinases (MMPs). In the synovial fluid of Lyme arthritis patients, and in *in vitro* studies, elevated levels of TNF $\alpha$  and IL-1 have been found (45–47). MMPs play a role in Lyme arthritis by participating in cartilage erosion, extracellular matrix degradation, and pathologic processes (45). These cytokines also play a role rheumatoid arthritis (RA) and osteoarthritis (OA) (48–54). BALB/c mice have been studied extensively with RA, but not with Lyme arthritis (55–58). In the model of systemic lupus erythematosus (SLE), BALB/c mice develop high levels of autoantibodies against dsDNA and RNA when injected with 2,6,10,14-



tetramethylpentadecane (more commonly known as pristine) (59). BALB/c mice also develop arthritis and nephritis in the lupus model and have an IFN gene signature. However, they develop a slower and less severe disease compared to some other mouse strains (37). BALB/c mice have been studied using multiple forms of induced arthritis (IA) which include: collagen (CIA), collagen antibody (CAIA), and proteoglycan (PGIA) (56, 57, 60, 61).

### BALB/cJ mice and inflammation

BALB/c mice became interesting to the laboratory as a possible new model for Type I IFN-driven Lyme arthritis. As previously stated, BALB/c mice have been studied in various forms of inflammatory diseases such as SLE and RA that result in arthritis among other symptoms. Research has also shown using SNP-based categorization that BALB/c mice and the hyper Type I IFN producing C3H mice are closely related. Interestingly, the BALB/c mouse lacks a hypomorphic *GusB* allele that is seen in the C3H mouse to be influential in more severe Lyme arthritis development. Therefore, we hypothesized that Lyme arthritis observed in BALB/c mice could be at least partially dependent on the *Bbaal* genetic locus. *Bbaal*, the locus that encodes Type I IFN, has been identified as important for severe Lyme arthritis in C3H mice, and might also be important for the BALB/c mouse to develop pathogenic hyper production of Type I IFN. Interestingly, widely used immortalized macrophage cell lines, the RAW 264.7 (ATCC® TIB-71™) cells, were originally derived from the BALB/c mouse. RAW 264.7 cells are suitable for transfection (62), have been used in the study of SLE (59), and could be a good, sustainable *in vitro* model to study IFN regulation important in Lyme arthritis.

Determining the IFN profile of the BALB/c mouse became a new direction that the laboratory could explore to further our understanding of *B. burgdorferi* and Lyme arthritis.

## MATERIALS AND METHODS

### Mice

BALB/cJ and C3H/HeJ mice were obtained from Jackson Laboratory at 6 weeks of age. All mice used in this study were housed in a pathogen-free University of Utah Animal Research Center (Salt Lake City, UT) and cared for following all institutional guidelines for the care and use of mice in biomedical research and in accordance with protocols approved by the University of Utah Institutional Animal Care and Use Committee (IACUC).

### Bacterial cultures for infection, co-incubation, and phagocytosis

For infection, a low-passage clonal derivative of *B. burgdorferi* strain N40 was stored at -80°C and then cultured 6–7 days in Barbour–Stoenner–Kelly II medium (BSK II) containing 6% rabbit serum (Sigma-Aldrich) before infection experiments. S. Barthold, University of California Davis, provided the N40 isolate. 6- to 7-day-old cultures of live *B. burgdorferi* were visualized using a dark field condenser and counted using a Petroff-Houser counting chamber. Spirochetes were diluted with BSK II when necessary to reach of concentration of  $2 \times 10^5$  or  $2 \times 10^6$  / 20 $\mu$ l for injection.

For co-incubation with bone marrow-derived macrophage (BMDM), *B. burgdorferi* was cultured for 12-14 days in BSK II medium containing 6% rabbit serum. After visualization and counting, aliquots of the *B. burgdorferi* were centrifuged at

5000xg for 10 minutes. The bacterial pellets were resuspended in room temperature PBS to wash the spirochetes, centrifuged again, and then resuspended in serum-replacement medium at  $3 \times 10^6$  or  $3 \times 10^7$ . Serum-replacement medium contains RPMI-1640 (Invitrogen), and 1% Nutridoma (Roche).

For phagocytosis by peritoneal-derived macrophages, GFP-*B. burgdorferi* were cultured for 7 days in BSK medium containing 6% rabbit serum (63, 64). The concentration of spirochetes was determined and aliquots of the bacteria were centrifuged at 5000xg for 10 minutes, washed, and resuspended in RPMI.B (75% [RPMI 1640 + 10% FBS] + 25% [BSK II + 6% rabbit serum]) at a concentration of  $2.5 \times 10^7$  /mL (65).

#### Cell culture

Bone marrow-derived macrophages (BMDMs) were isolated from the femurs and tibias of BALB/cJ and C3H/HeJ mice. BMDM were plated at  $7.5 \times 10^6$  / dish and cultured for 7 days in RPMI 1640 supplemented with 30% L929 conditioned medium and 20% horse serum (HyClone) (33, 44). Near confluency, macrophage cultures were collected and replated in 24-well dishes at a density of  $3 \times 10^5$  /well in medium containing 1% Nutridoma serum-replacement. The next day, the medium was removed and replaced with either medium alone or stimulated with a concentration of  $3 \times 10^6$  or  $3 \times 10^7$  *B. burgdorferi* /well which had been cultured and prepared as previously described (44). Stimulated macrophage cultures were incubated at 37°C, 5% CO<sub>2</sub>, and harvested either at 6 or 24 hours in TRIzol reagent (Invitrogen) for RNA extraction (66). At harvest, the cells were shaken for 7 minutes at 300 rpm using a plate shaker (Labnet Shaker 20) to homogenize the cells, then stored at -80°C.

Infection of mice, assessment of infection, and assessment  
of arthritis severity

Mice 6 to 7 weeks of age were infected with  $2 \times 10^5$  or  $2 \times 10^6$  of the clonal N40 *B. burgdorferi* spirochetes by intradermal injection into the skin of the back. This mode of infection is reported to require the fewest number of spirochetes and to most closely mimic a natural tick transmission (23). Mice were infected for a period of 7-35 days. Mice infected for 28-35 days had measurements of the thickest anteroposterior portion taken of the ankle with the joint extended using a metric caliper on the day of infection and at euthanization. The change in joint thickness was determined by the difference from the initial measurements and the final measurements. The most swollen joints were collected for histological assessment of arthritis severity and were fixed in 10% neutral-buffered formalin, and then decalcified prior to embedding, sectioning, and staining with H&E. The less swollen joints were stored in RNALater (Invitrogen) for RNA extraction.

To confirm infection in mice infected less than 14 days, bladder cultures were collected at the time of euthanization and observed using dark field condenser microscopy 14 to 21 days later for the presence of *B. burgdorferi*. ELISA quantification of *B. burgdorferi*-specific IgM and IgG concentrations was used to confirm infection in mice that were euthanized 14-35 days after infection. Blood was collected from mice at time of euthanization from submandibular puncture and heart puncture when necessary. Blood was allowed to clot and serum was collected after centrifugation. 16S RNA of *B. burgdorferi* was also assessed using qRT-PCR for mice infected 7-35 days.

### Phagocytosis assay

Peritoneal macrophages were harvested 4 days after intraperitoneal injection of 3 ml 3% sterile thioglycollate, which has been shown to recruit macrophages to the peritoneum (67). Macrophages were collected with ice-cold PBS, and RBCs were lysed with ACK lysis buffer. Cells in RPMI 1640 + 10% FBS were plated at  $5 \times 10^5$  /well in 12-well plates and allowed to adhere overnight. The next day, nonadherent cells were removed by washing with RPMI 1640 + 10% FBS. *B. burgdorferi* N40-expressing GFP were then added to the macrophages in RPMI.B (75% [RPMI 1640 + 10% FBS] + 25% [BSK II + 6% rabbit serum]) at a 50:1 ratio (65),(64). Plates were centrifuged at 5000x g for 5 minutes and incubated for 1 or 2 hours at 37°C and 5% CO<sub>2</sub>, conditions previously shown to capture midway and maximal phagocytosis (63). Plates were then washed and incubated with 0.25% Trypsin + EDTA (Sigma-Aldrich) for 5 minutes. After incubation, the cells were washed and resuspended in flow buffer (PBS + 2% FCS + 2.5% 1M HEPES). Cell suspensions were analyzed using a BD FACS Canto II flow cytometer FITC channel.

### ELISA assays

ELISAs were performed to quantify protein levels of: *B. burgdorferi*-specific IgG and IgM, Cxcl13, MMP3, MMP13, and TNF $\alpha$ . Determination of *B. burgdorferi*-specific IgM and IgG concentrations was performed as previously described (68). TNF $\alpha$  was quantified using methods previously described (63). Cxcl13 was analyzed using the Mouse BLC (Cxcl13) ELISA Kit (Thermo Fisher). MMP3 ELISA was performed using Mouse MMP3 ELISA Kit (Thermo Fisher). MMP13 was evaluated using the ELISA Kit

for Mouse MMP13 (Cloud-Clone Corp).

#### Isolation of RNA and quantification using qRT-PCR

Total RNA was recovered from homogenized tibiotarsal joint tissue or cell homogenate using TRIzol reagent (Invitrogen). The rear ankle joint tissue was obtained from sacrificing the BALB/cJ and C3H/HeJ mice, removing the skin, harvesting the joints, and storing the tissue in RNALater (Invitrogen) until homogenized. Joint tissue in TRIzol reagent was homogenized using a Benchmark BeadBug microtube homogenizer. 5 mg total RNA was reverse transcribed using random hexamer primers and Moloney murine leukemia virus RT (Invitrogen). Transcripts were quantified using LightCycler SYBR Plus master mix and a LC-480 PCR system (Roche). The copy number of the gene of interest was calculated from the starting template sample and normalized to 1,000 copies of the mouse  $\beta$ -actin housekeeping gene. The primers used for amplification are as follows:  *$\beta$ -actin*, *Iigp1*, *Stat1*, *MMP3*, *Cxcl13*, and *B. burgdorferi* 16S rRNA (31), *Cxcl9*, *Oasl2*, and *IFN $\gamma$*  (69), *Gbp2* (70), *TNF $\alpha$*  and *IFN $\beta$*  (66), with sequence information in the indicated citations. Primers for *MMP13* were: *MMP13* forward (5' TGCATATGAACATCCATCCC 3') and reverse (5' AGAAGAAGAGGGTCTTCCC 3').

#### $\alpha$ -TNF $\alpha$ infection experiment

Mice infected with  $2 \times 10^6$  *B. burgdorferi* received i.p injections of Rat IgG1 (TNP6A7) or anti-TNF $\alpha$  antibody (XT3.11) at 0.5mg/ mouse the day before infection and every other day after for 28 days. The antibodies used here for neutralization and isotype control were purchased from BioXCell, were aggregate and endotoxin free, and

sterile. The uninfected control group was injected every other day with PBS to mimic the treatment groups and once with BSK II to mimic the infection event.

#### Conjugated antibodies for flow cytometry

The following antibodies were purchased from either Biolegend or eBioscience and used for flow cytometry analysis: PE/Cy7-conjugated anti-CD11b (M1/70) and anti-CD11c (N418); FITC-conjugated anti-CD80 (16-10A1) and anti-TLR2 (T2.5); PerCP/Cy5.5- conjugated anti-F4/80 (BM8) and anti-CD4 (GK 1.5); PE-conjugated anti-CD3e (145-2C11) and anti-CD335/NKp46 (29A1.4); APC-conjugated anti-CD206/MMR (C068C2) and anti-CD44 (IM7); BV 605-conjugated anti-B220/CD45R (RA3-6B2); BV 421-conjugated anti-CD62L (MEL-14) and anti-MHC II (M5/114.15.2); and AF 700-conjugated anti-CD8a (53-6.7) and anti-CD86 (PO3).

#### Data and statistical analysis

All data represent mean  $\pm$  SEM. All statistical calculations were performed using GraphPad Prism 7. Statistical significance ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ) is indicated. Continuous variables and 2-sample data sets were analyzed by 2-tailed Student *t* test. Multiple-sample data sets were analyzed using 1-way ANOVA with Tukey's post hoc tests for pair-wise comparisons. Flow cytometry data were analyzed using FlowJo 10.1r7, then data were statistically analyzed using Prism 7.



## RESULTS

### Optimization of infection with *Borrelia burgdorferi* in the BALB/cJ mouse model

In trying to repeat previous infection protocols for BALB/c mice, we discovered through qRT-PCR, bladder cultures, histology, and ankle measurements that a dose of  $2 \times 10^5$  *B. burgdorferi* was not sufficient to establish infection. Failure of infection was observed at days 7, 8, 9, and 28 days after infection, and arthritis was not seen in mice sacrificed 28 days post infection.

A modified infection protocol was performed using a larger dose of  $2 \times 10^6$  *B. burgdorferi*. The infectious dose was now 100 times larger than the  $2 \times 10^4$  typically used in our C3H mouse model. Previous studies have shown that the C3H mouse will develop a severe arthritis phenotype regardless of increased dose (23). This is important for when C3H mice are used for comparison with BALB/c mice in this study, because the increased dose should not alter the arthritis development in the C3H mouse.

Arthritis severity was measured at 4 weeks post infection for the BALB/c mice and C3H mice, and there was evidence of moderate to severe ankle swelling in the infected groups. The arthritis was not as pronounced as the laboratory had seen previously in the BALB/c mice (23), so the experiment was allowed to run for an additional week. At 5 weeks post infection the animals were euthanized and their rear ankle joints were measured and harvested (Figure 1.A). The ankle swelling of the

BALB/c mice was considered an intermediate arthritis with an average change in swelling of the most swollen joint of 0.46mm. cDNA analysis of 16S *B. burgdorferi* RNA in the joint tissue homogenates of infected BALB/c mice (Figure 1.B) showed only a small increase of *B. burgdorferi* 16S RNA in infected BALB/c mice compared to the controls. To verify the qRT-PCR results and show a systemic presence of *B. burgdorferi* in the BALB/c mice, an ELISA was performed that was specific for IgG that reacts with *B. burgdorferi* in the serum of these animals. The results from the ELISA indicated that there was a significant humoral response to *B. burgdorferi* in the BALB/c mice (Figure 1.C). Additional experiments were performed with the  $2 \times 10^6$  infectious dose of *B. burgdorferi*, and at 2 weeks post infection, *B. burgdorferi*-specific IgM was tested. The *B. burgdorferi* IgM-specific ELISA showed a significant level of protein in the serum of BALB/c mice at 2 weeks post infection (Figure 1.D). There was now a working infectious model that resulted in intermediate arthritis in BALB/c mice when infected with *B. burgdorferi* and induced a strong adaptive immune response.

C3H mice were also included in the 5 week infection experiment as a control for arthritis severity. As seen in Figure 1.A, the C3H mice developed severe arthritis with an average change in joint thickness of the most swollen joint of 0.84mm, greater than seen with the BALB/c mice. This helped confirm that the *B. burgdorferi* stock being used was able to cause severe arthritis, and that the initial problem with the infection protocol was restricted to the infectious dose in BALB/c mice. It also showed a clear difference in the amount of 16S RNA of *B. burgdorferi* in the joint tissue of BALB/c and C3H mice (Figure 1.B), with C3H mice having close to 6 fold higher levels present than BALB/c mice at 5 weeks post infection. This result demonstrated that the *B. burgdorferi* were able

to infiltrate the joint tissue. The C3H mice were also tested for *B. burgdorferi*-specific IgG (Figure 1.C), and again that response was greater in the C3H mice than in the BALB/c mice. The results of testing the infected C3H mice confirmed the efficacy of the modified infection protocol and highlighted differences in arthritis severity and *B. burgdorferi* levels within the BALB/c and C3H mice.

Determining IFN responses in BALB/cJ mice infected  
with *Borrelia burgdorferi*

Using SNP-based assessment of genetic relatedness in >100 mouse strains, Petkov, et al. previously grouped BALB/c and C3H mice on genetically related branches of the mouse tree (29). Interestingly, BALB/c mice do not possess the hypomorphic allele for *GusB* previously associated with Lyme arthritis severity in C3H mice (27). This suggests that the second arthritis-promoting phenotype in C3H mice, the hyperproduction of Type I IFN, may be shared between the BALB/c and C3H mice, which could be important in arthritis development in the BALB/c mice. Furthermore, BALB/c and C3H mice share novel SNPs in the region flanking the Type I IFN genes (29), leading to the hypothesis that the BALB/c mouse would have a similar hyperproduction of Type I IFN responsive transcripts. To test the hypothesis, multiple mouse experiments were performed that directly compare the BALB/c mouse and the C3H mouse in response to infection with *B. burgdorferi*. We have already demonstrated that the BALB/c mouse and C3H mouse have different severities of arthritis (Figure 1.A) and levels of *B. burgdorferi* (Figure 1.B-C), so now we aimed to evaluate other differences and similarities between the 2 strains of inbred mice.

To explore the possibility of a hyper Type I IFN profile similar to what the laboratory has seen in the C3H mouse (31), a simple infection experiment was performed. BALB/c and C3H mice were infected with  $2 \times 10^6$  *B. burgdorferi* and sacrificed at 1 week post infection. Type I IFN responsive transcripts were analyzed along with 16S RNA of *B. burgdorferi* (Figure 2). As expected, the C3H mouse presented with elevated Type I IFN-related transcripts including: *Cxcl9*, *Oasl2*, and *iigp* (Figure 2.C-E).

In the BALB/c mouse, there was very little evidence of a Type I IFN response at 1 week post infection. The only IFN-related transcript that was elevated was *Cxcl9* (Figure 2.C). 16S RNA of *B. burgdorferi* (Figure 2.F) and bladder cultures demonstrated that *B. burgdorferi* had not fully disseminated in the BALB/c mice at 1 week post infection. These results indicate that there is not a 1 week post infection Type I IFN response detectable in joint tissue of BALB/c mice.

The lack of 16S RNA in joint tissue at 1 week of infection suggested that dissemination of *B. burgdorferi* in the BALB/c mouse was delayed, and that there might also be a delay in the appearance of the Type I IFN profile. Since the BALB/c mouse does develop arthritis in response to infection with *B. burgdorferi*, it was also important to evaluate if Type II IFN (IFN $\gamma$ ) plays a role in arthritis development. This is because Type II IFN response starting at 2 weeks post infection drives the development of Lyme arthritis in another model used in the lab, the C57BL/6 IL-10<sup>-/-</sup> mouse model (31). Thus, the hypothesis was developed that Type I or Type II IFN induced at 2 weeks of infection could contribute to arthritis development in BALB/c mice.

To assess the presence of any IFN response, BALB/c mice were infected with  $2 \times$

$10^6$  *B. burgdorferi* and euthanized at 2 weeks post infection. No C3H mice were included in this experiment, since it has already been shown that C3H mice have an IFN response at 1 week post infection that returns to baseline by 2 weeks of infection and do not display elevation greater than 2 fold in IFN $\gamma$  or IFN-inducible transcripts at 2 weeks of infection (31). Joint tissue was obtained and evaluated for *B. burgdorferi* infiltration and IFN-related transcripts as before (Figure 2.A-F). Upon analysis of 16S RNA of *B. burgdorferi*, it was confirmed that joint infiltration had occurred at 2 weeks post infection (Figure 2.F) in the BALB/c mice. However, analysis of IFN-related transcripts still demonstrated that there was not an IFN response present in the BALB/c mouse (Figure 2.A-E). After determining that there was no evidence of a robust IFN response similar to C3H in the BALB/c mouse, it was important to explore other inflammatory pathways in an attempt to find activated inflammation markers responsible for driving the arthritis phenotype in the BALB/c mouse.

Identification of inflammatory markers that are altered in response  
to *Borrelia burgdorferi* infection in BALB/cJ mice

After testing for the presence of a robust IFN profile in BALB/c mice, and not finding 1, there was a sufficient amount of samples remaining to assess other pathways. BALB/c and C3H samples at various time points were analyzed for 54 different inflammatory markers previously identified in tissues from *B. burgdorferi* infected mice. Of the 54 markers evaluated, 4 were deemed interesting: TNF $\alpha$ , Cxcl13, MMP3, and MMP13. TNF $\alpha$  is a member of the innate immune response, and has been seen to be important in disease progression and arthritis severity in rheumatoid arthritis (48, 71).

Cxcl13 is a B cell chemoattractant found primarily in germinal centers and considered a marker for neuroborreliosis. Matrix Metalloproteinases (MMPs) have been strongly linked to multiple forms of arthritis (45, 46, 48, 49, 72, 73) and are important in tissue remodeling. Each marker selected was considered to be of interest in the BALB/c mouse because it was elevated at least ten fold above the uninfected control animals in at least 1 of the time points collected (Figure 3.A, C, E, and G). ELISA assays were also performed for these markers to evaluate systemic levels in serum (Figure 3. B, D, F, and H). For the ELISAs, a group of C3H mice infected with  $2 \times 10^4$  *B. burgdorferi* that had been sacrificed at 4 weeks post infection were included to observe any systemic differences in comparison to the group infected with  $2 \times 10^6$  *B. burgdorferi* and sacrificed at 5 weeks post infection.

In the BALB/c mice,  $\text{TNF}\alpha$ , Cxcl13, MMP3, and MMP13 are all at their highest elevations at 2 weeks post infection in the joint transcripts (Figure 3.A, C, E, and G). This is also seen to be the peak in 16S RNA of *B. burgdorferi* in the BALB/c joint tissue (Figure 2.F) and in previous studies with C3H mice (31). However, in the serum at the 2 week post infection time point, Cxcl13 was the only marker elevated relative to uninfected mice (Figure 3.B, D, F, and H). The peak at 2 weeks post infection seems to correlate with the immune response being fully ramped up to fight the infection, and with the peak of *B. burgdorferi* in the joint tissue.

Since there were no C3H mice included in the 2 week post infection group, I used previous studies to compare to my findings with the 2 week post infection BALB/c mice (31). Using the previously published data, none of the transcripts upregulated in BALB/c mice at 2 weeks post infection were upregulated in C3H mice. However, 16S RNA of *B.*

*burgdorferi* was highest at 2 weeks post infection in both BALB/c and C3H mice. Thus, not only do BALB/c mice lack a robust IFN response, they also respond to *B. burgdorferi* infection and develop arthritis very differently from the C3H mice.

Evaluating differences in cytokine production between BALB/cJ  
and C3H/HeJ bone marrow-derived macrophages *in vitro*  
when challenged with *Borrelia burgdorferi*

It was hypothesized that in the BALB/c mouse, the innate immune response, mainly its ability to recruit immune cells to the site of infection, may be responsible for a heightened ability to control infection in the mouse by increasing cytokine production. This was hypothesized after discovering the heightened TNF $\alpha$  level, reduced *B. burgdorferi* numbers, and minimal IFN response following infection of BALB/c mice and relative to the C3H mice. To test this hypothesis, BALB/c and C3H bone marrow-derived macrophages (BMDM) were harvested from the mice to stimulate with *B. burgdorferi in vitro* and determine if 1 strain had a stronger cytokine response than the other. BMDM were chosen because they can be harvested and, using appropriate conditions, can be expanded as a pure primary cell culture that is genetically representative for each mouse strain (44). The macrophages were cultivated with *B. burgdorferi* as described in the materials and methods (44).

There were many differences between the BALB/c and C3H cytokine production and between the treatment groups. I will only discuss a few of the results that have meaning to this particular study. These results were significant in cultures tested with a 10:1 MOI for BMDMs and were significant between strains in the treated groups. Shown

in this experiment was evidence of robust induction of IFN responsive transcripts in the BALB/c macrophages (Figure 4.A-D) that was not seen in joint tissues collected from infected mice *in vivo* (Figure 2.). Interestingly, the magnitude of IFN-inducible response in the C3H macrophages was greater than in the BALB/c macrophages.

Importantly, transcriptional upregulation of TNF $\alpha$ , a gene downstream of NF $\kappa$ B and not considered IFN inducible, was more highly elevated in BALB/c macrophages than in the C3H macrophages treated with *B. burgdorferi* for 6 and 24 hours (Figure 4. E-F). This establishes that TNF $\alpha$  in BALB/c mice is elevated both *in vitro* and *in vivo* in response to *B. burgdorferi* challenge. These findings suggest that the innate immune system is influential in responding to infection from *B. burgdorferi*, and that TNF $\alpha$  is important in the early immune response. This led us to hypothesize that TNF $\alpha$  is an important cytokine for the control and development of arthritis in BALB/c mice.

#### Measuring the phagocytic ability of BALB/cJ and C3H/HeJ

##### peritoneal-derived macrophages to ingest

##### GFP-*B. burgdorferi*

We have shown that BALB/c mice are seemingly able to control levels of infection with *B. burgdorferi* better than C3H mice (Figure 1). The true mechanism for this difference is not known. It is hypothesized that macrophages in BALB/c mice are better at internalizing and presumably killing the *B. burgdorferi* than the C3H mouse. Phagocytic ability could be an important tool in controlling *B. burgdorferi* numbers and overall infection.

A phagocytosis assay using peritoneal derived macrophages was performed as



described in the Material and Methods (33, 63). GFP-*B. burgdorferi* is a *B. burgdorferi* strain N40 constitutively expressing GFP under the flaB promoter (64), which allows us to ascertain the number of *B. burgdorferi* that have been internalized by the macrophages using flow cytometry. After trypsin treatment, there was clear evidence that BALB/c-derived peritoneal macrophages were far better at internalizing *B. burgdorferi* at 1 and 2 hours post challenge in comparison to the C3H-derived peritoneal macrophages (Figure 5.A). Furthermore, not only did more macrophages from BALB/c mice ingest *B. burgdorferi*, but also each macrophage contained a greater number of *B. burgdorferi* within them as determined from the mean fluorescence intensity (MFI) (Figure 5.B). These findings suggest that BALB/c peritoneal macrophages are indeed better at internalizing *B. burgdorferi* in comparison to C3H macrophages.

Assessing the effects of blocking TNF $\alpha$  on control of *Borrelia burgdorferi* numbers in tissues and arthritis severity

After evaluating TNF $\alpha$  levels in joint transcripts, serum protein, and in bone marrow-derived macrophages, we next wanted to determine the effects of blocking TNF $\alpha$  in BALB/c mice. TNF $\alpha$  blockade has been shown to be beneficial in rheumatoid arthritis (RA) by both decreasing symptom severity and stopping disease progression (74, 75). Approximately 70% of the RA patients given the TNF $\alpha$  blocking treatment obtained these results. Of the 30% of RA patients that did not, many had a significant Type I IFN profile. The significant Type I IFN profile was not seen in the group of patients that benefited from the TNF $\alpha$  blockade (74, 75). This suggested that TNF $\alpha$  blockade might serve as a possible treatment for some Lyme arthritis patients lacking an IFN profile. To

test the effects of blocking TNF $\alpha$  on arthritis severity and to observe its effects on *B. burgdorferi* numbers in the joint tissue, an infectious experiment was designed using BALB/c mice. C3H mice were not used for this experiment since they develop a Type I IFN profile that drives arthritis and were not hypothesized to benefit from TNF $\alpha$  blocking treatment.

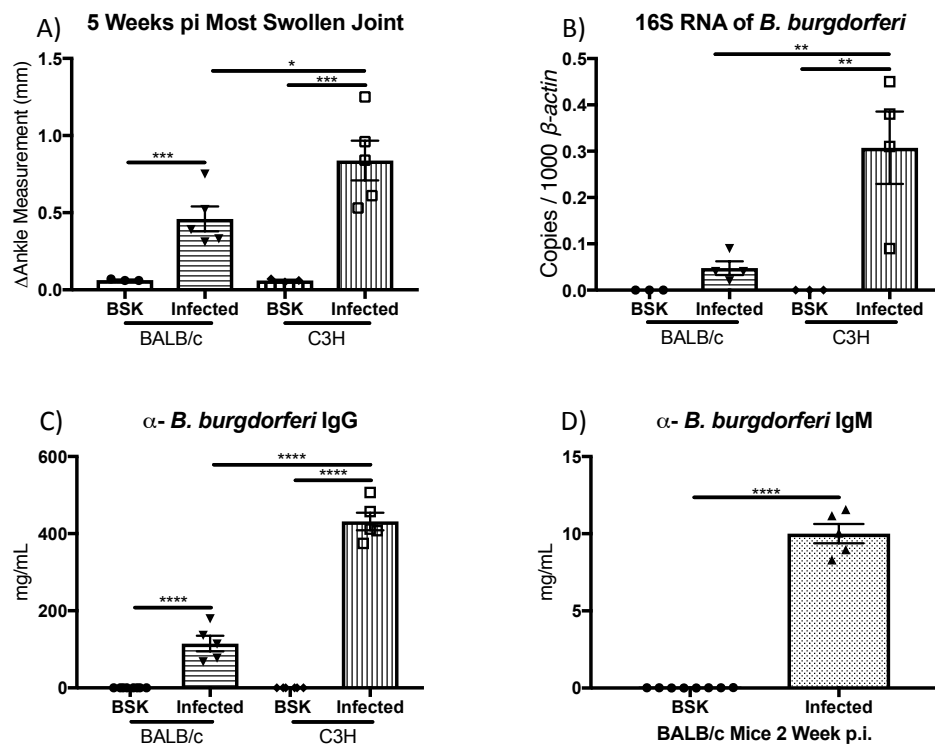
BALB/c mice were treated every other day with i.p injection of 1 of 3 conditions: PBS, rat IgG1, or anti-TNF $\alpha$ . The group receiving PBS treatment was an uninfected control group, and the other 2 groups were infected with  $2 \times 10^6$  *B. burgdorferi*. After 4 weeks, the mice were euthanized and their rear ankle joints were harvested. Draining lymph nodes were also harvested from these mice to compare leukocyte levels in the 3 groups. Joint measurements revealed that TNF $\alpha$  blockade had only a small effect, if any, on arthritis severity between infected groups (Figure 6.A). To confirm systemic dissemination, *B. burgdorferi*-specific IgG levels were assessed and found to be similar between infected groups (Figure 6.B), showing no difference obtained by TNF $\alpha$  blockade, but demonstrating an adaptive immune response. 16S RNA of *B. burgdorferi*, and transcripts for TNF $\alpha$ , Cxcl13, MMP3, and MMP13 were evaluated as well as various other inflammatory markers in the joint tissue. The 16S RNA of *B. burgdorferi* showed evidence of joint infiltration in the infected groups (Figure 6.C), but no effect of TNF $\alpha$  blockade on the level of bacteria in the joint tissue. Interestingly, transcripts for TNF $\alpha$  were not significantly different among any of the groups at 4 weeks post infection (Figure 6.D). Since TNF $\alpha$  is involved in early immune responses, it is possible that the lack of difference seen at 4 weeks is simply past the time that differences would be seen *in vivo*. The only marker analyzed of the joint transcripts that had a difference between

the 2 infected groups was MMP3 (Figure 6.E). MMP3 is a downstream product of TNF $\alpha$  through the NF $\kappa$ B pathway, so this demonstrated a downstream effect of blocking TNF $\alpha$ . Since there was no difference seen in joint swelling or in *B. burgdorferi* number, it is determined that blocking TNF $\alpha$  is not responsible for controlling *B. burgdorferi* infection in BALB/c mice.

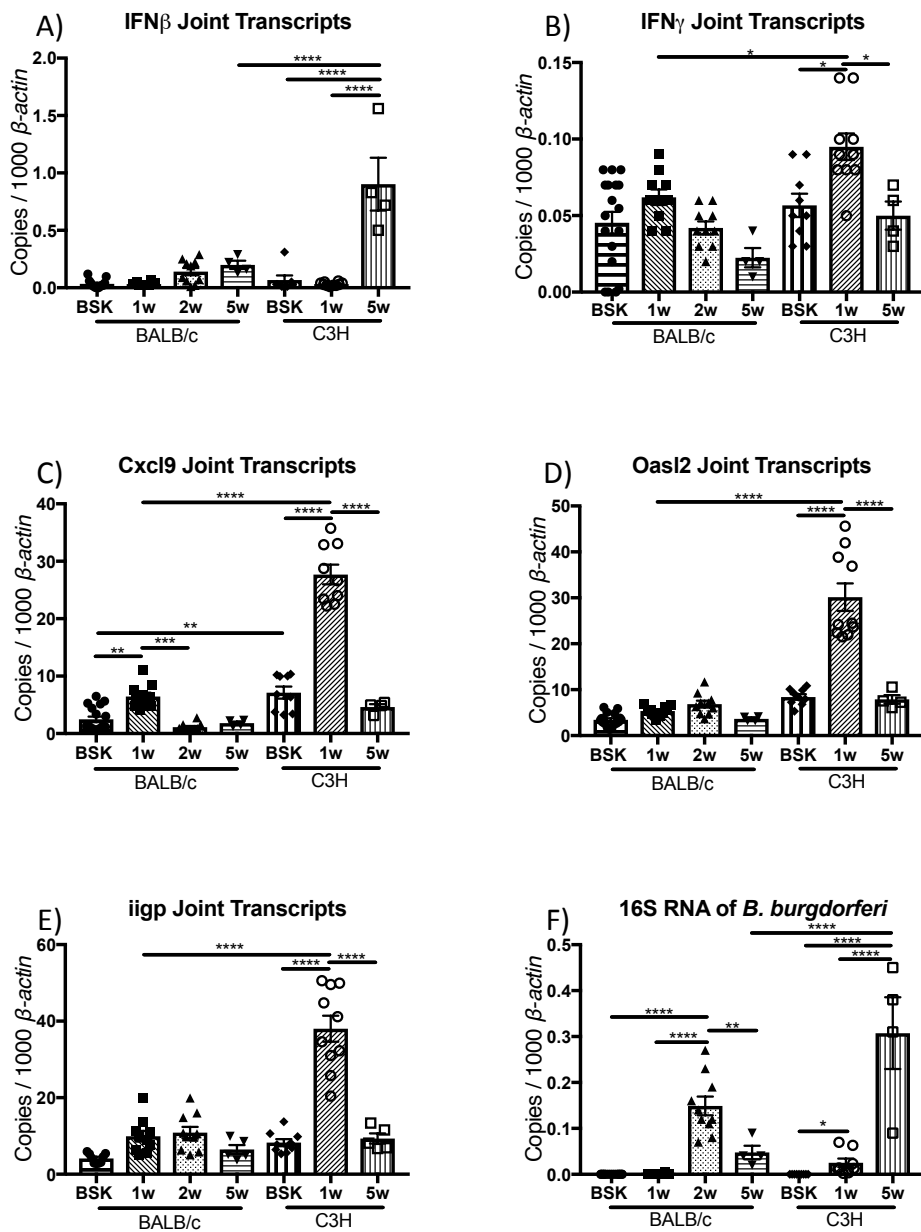
Draining lymph nodes were also harvested from these mice to evaluate cell populations. In the popliteal and inguinal lymph nodes, there were various differences between the control, infected isotype control, and infected TNF $\alpha$  blockade groups. The total number of cells in the lymph nodes of infected mice treated with TNF $\alpha$  blockade was greater than in those receiving infected isotype control (Figure 7.A). This increase could suggest that in absence of TNF $\alpha$ , the adaptive immune system has to elevate its attack on the infection. When looking at the percentages of B and T cells in the lymph nodes, (Figure 7.B-D) we see that there is a large increase in B cells and a concomitant decrease in T cells in the infected groups relative to mock-infected, which is a result that has been seen by our laboratory previously (76). After determining overall percent of B and T cells, the next step was to look at these cells for evidence of activation. Activated CD4<sup>+</sup> (CD44<sup>+</sup>, CD62L<sup>-</sup>) and B220<sup>+</sup> (B220<sup>+</sup>, GL7<sup>+</sup>) both showed an increase in number of cells in the infected TNF $\alpha$  blockade group in comparison to the infected isotype and uninfected control groups (Figure. E-H). This also suggests that TNF $\alpha$  blockade causes the adaptive immune system to increase its attack in *B. burgdorferi* infected BALB/c mice. The percentage of activated cells, however, was not significantly different between infected isotype control and infected TNF $\alpha$  blockade groups, which suggests that overall there was not a significant difference in how the adaptive immune system reacted to the

infection (Figure 7.E-H).

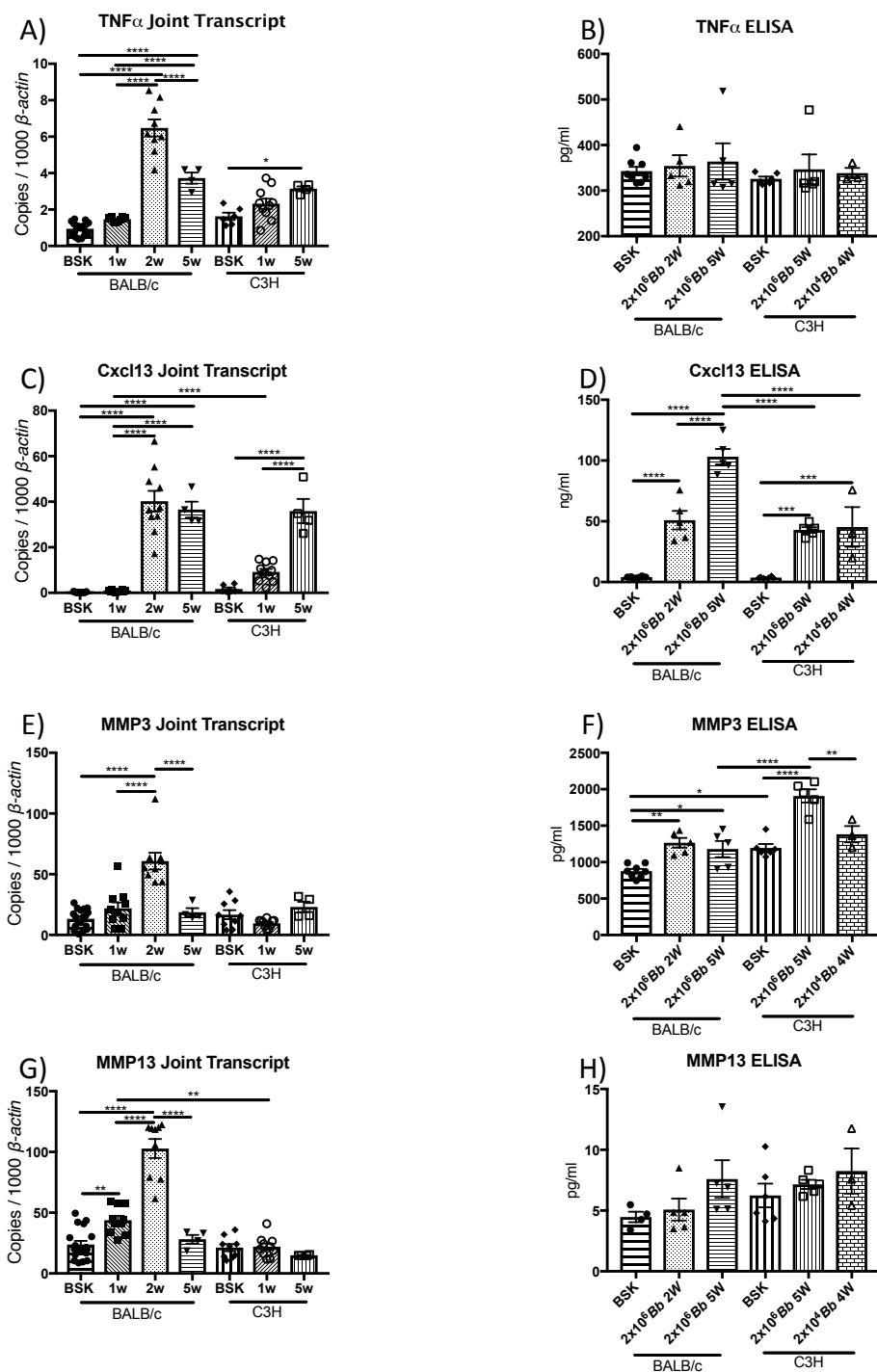
Also interesting was the increase in the number of natural killer cells, and the number of MHC II<sup>+</sup> cells in the TNF $\alpha$  blocked group (Figure 8.A-D), which indicates an increase in innate immune response. This does suggest that due to a lack of TNF $\alpha$  availability, the innate immune system was forced to fight the infection differently than if TNF $\alpha$  was available; however, as determined by *B. burgdorferi* 16S RNA in the joint tissue (Figure 6), the BALB/c mice were generally still able to control the infection when TNF $\alpha$  was blocked. Overall, TNF $\alpha$  blockade did not have any significant influence on controlling *B. burgdorferi* numbers or the severity of arthritis.



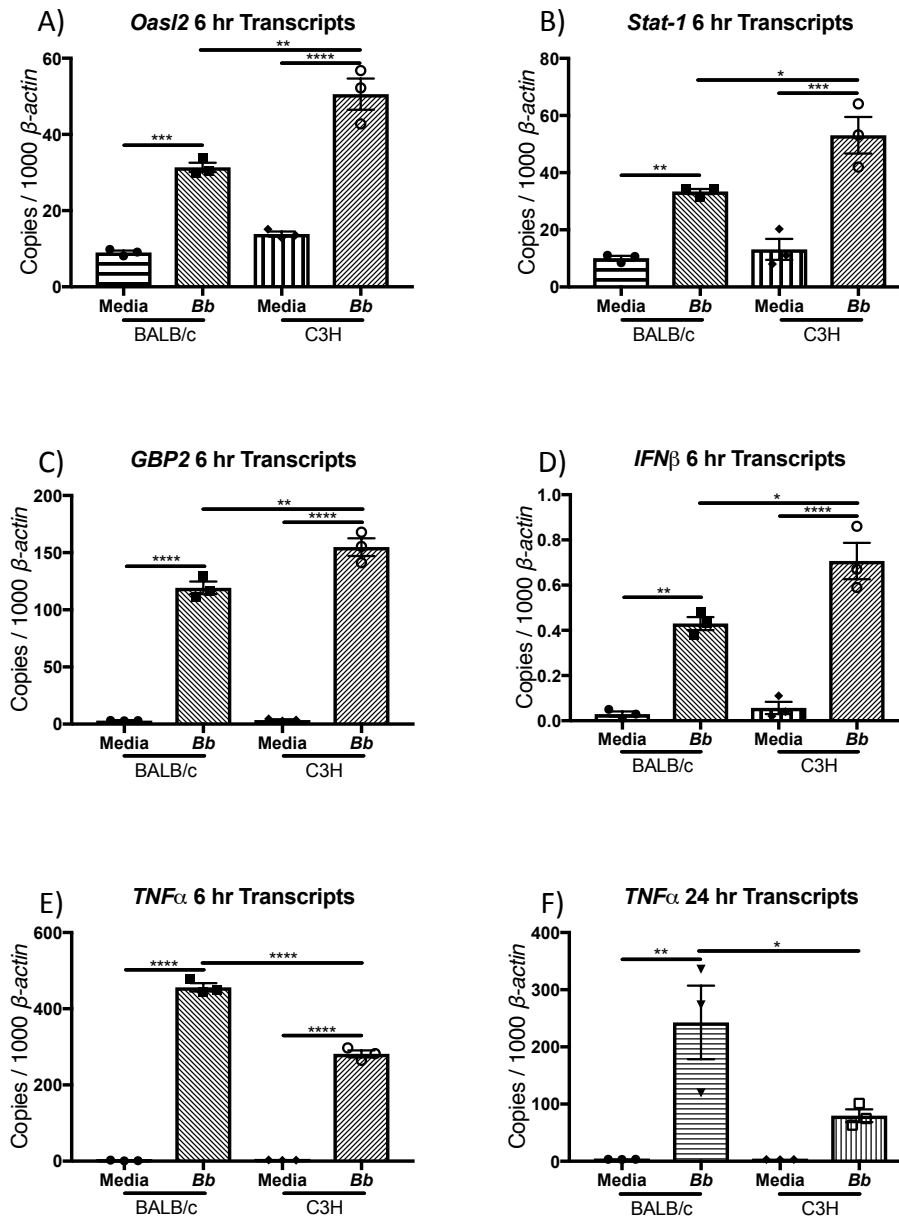
**Figure 1. Arthritis severity determination and infection verification in BALB/cJ and C3H/HeJ mice.** BALB/cJ and C3H/HeJ mice were infected for 5 weeks at a concentration of  $2 \times 10^6$  *B. burgdorferi*. A) Change in joint swelling, B) 16S RNA of *B. burgdorferi* from joint tissue normalized to  $\beta$  actin, and C) anti-*B. burgdorferi*-specific IgG levels in the serum. D) anti-*B. burgdorferi* IgM levels in the serum at 2 weeks of infection in BALB/cJ mice. Statistical significance was determined by ANOVA or Student *t* test for ankle swelling, joint transcripts, and ELISA Ig results.  $n = 3-10$  per group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .



**Figure 2. IFN-related transcripts and 16S RNA of *B. burgdorferi* in the joint tissue of BALB/cJ and C3H/HeJ mice.** BALB/cJ and C3H/HeJ mice were infected with  $2 \times 10^6$  *B. burgdorferi* for 1, 2, or 5 weeks or injected with BSK medium as a control. Joint transcript levels were normalized to  $\beta$  actin. **A-F)** Are joint transcripts levels of: *IFN $\beta$* , *IFN $\gamma$* , *Cxcl9*, *Oasl2*, *iigp*, and 16S RNA of *B. burgdorferi*, respectively. Significance was determined by ANOVA. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

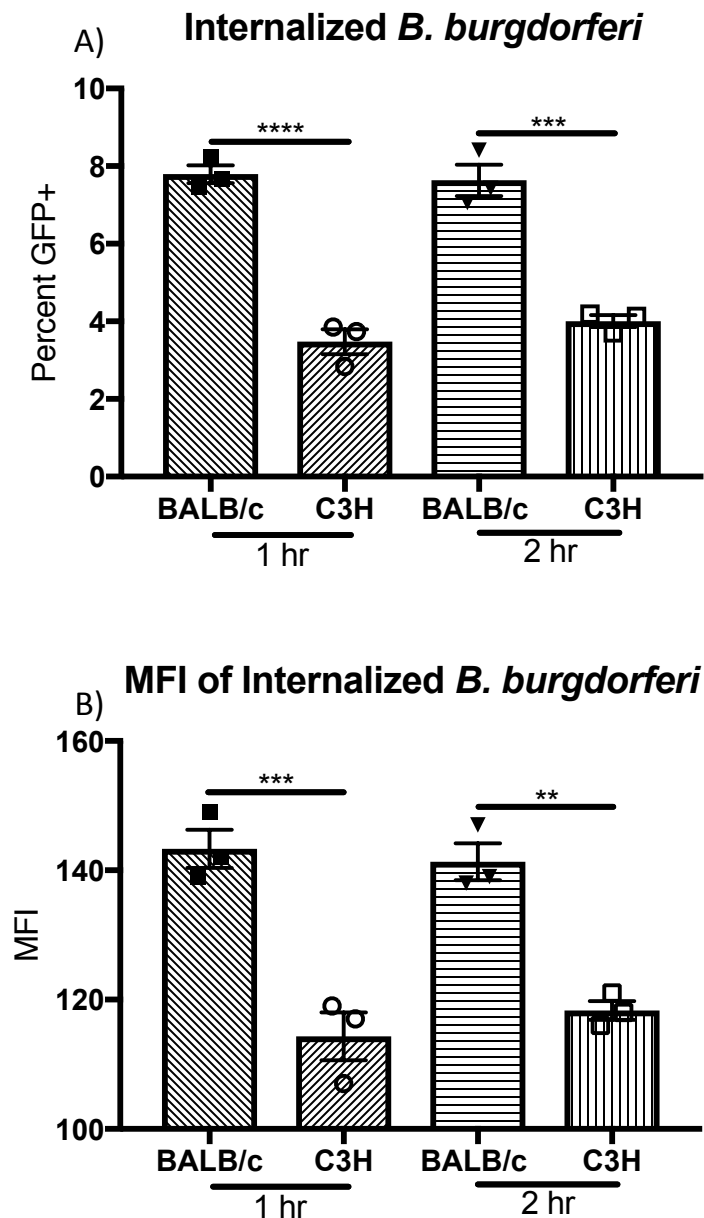


**Figure 3. Joint transcript levels of inflammation markers and related serum protein levels.** Joint transcript and serum protein levels of  $TNF\alpha$ ,  $Cxcl13$ ,  $MMP3$ , and  $MMP13$ , respectively seen at various time points throughout infection. Joint transcripts normalized to  $\beta$  actin. Serum Ig levels determined by ELISA assays at various time points in infection and infectious concentrations. Statistical significance was determined by ANOVA for joint transcripts and ELISA results. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$

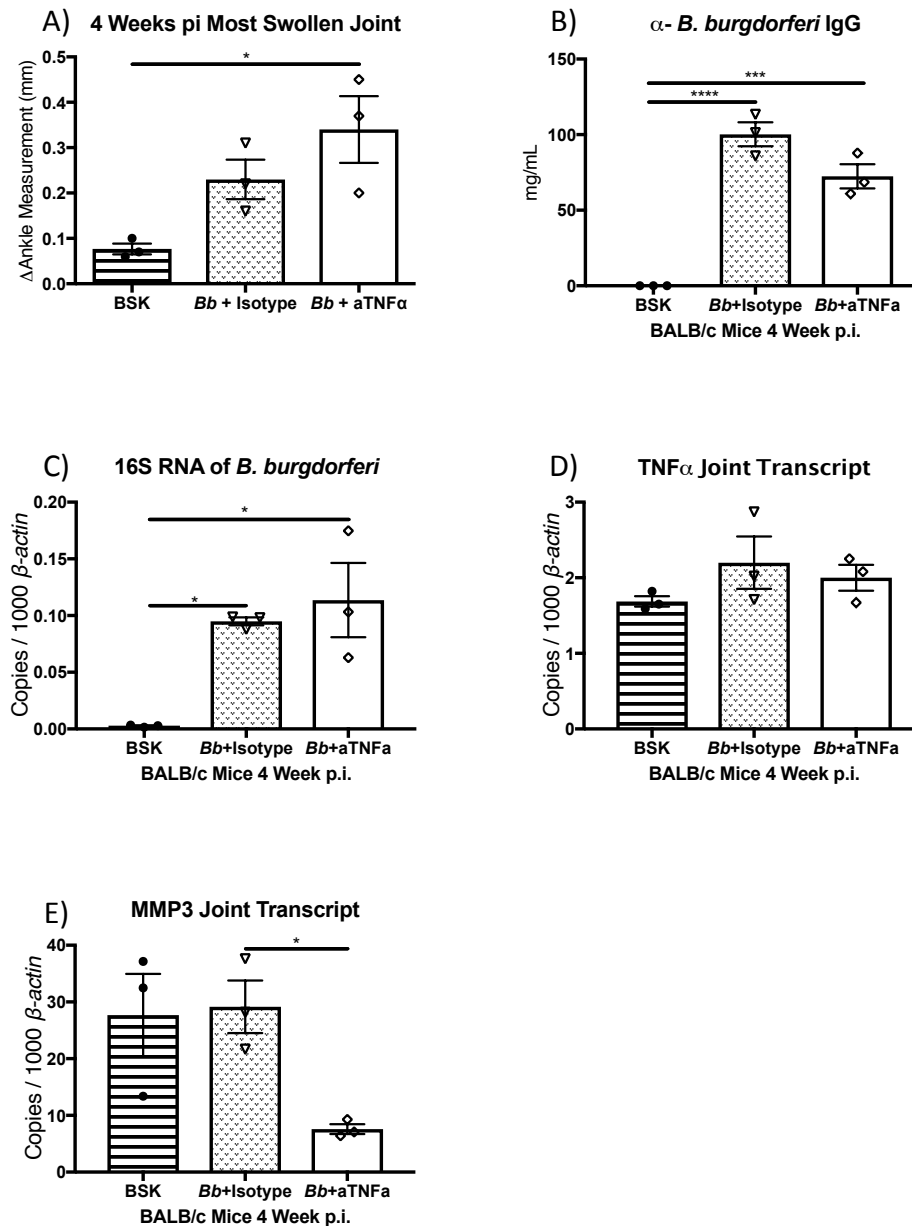


**Figure 4. Responses of BALB/cJ and C3H/HeJ BMDM to *B. burgdorferi*.** BMDM from BALB/cJ and C3H/HeJ mice were challenged with a 10:1 MOI of *B. burgdorferi*. Transcripts were analyzed using cDNA from cell lysate. **A-E)** Transcript levels from cells harvested at 6 hours of cultivation: *Oas12*, *Stat-1*, *GBP2*, *IFN $\beta$* , and *TNF $\alpha$* , respectively. **F)** *TNF $\alpha$*  transcript levels from cells harvested at 24 hours of cultivation. Statistical significance was determined by ANOVA and Student t test for cell lysate transcript results.  $n = 3$  per group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

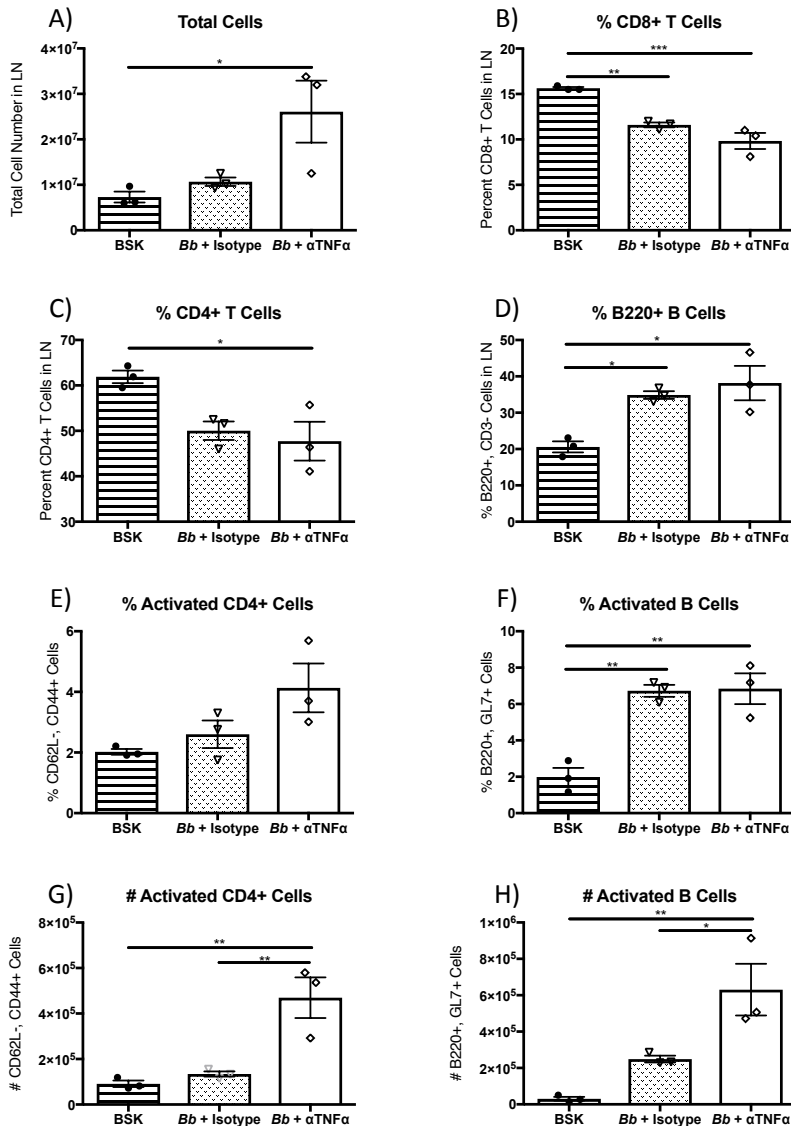




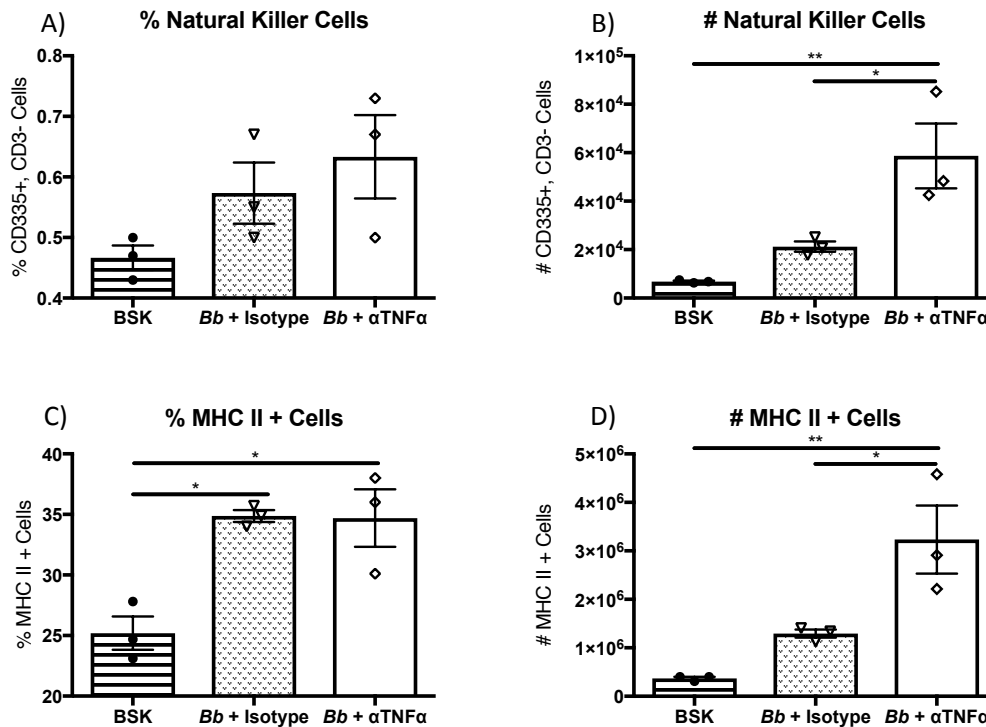
**Figure 5. Phagocytic ability of macrophages from BALB/cJ and C3H/HeJ mice.** BALB/cJ and C3H/HeJ peritoneal derived macrophages were stimulated with a 10:1 MOI GFP-*B. burgdorferi* for 1 or 2 hours. Upon completion, cells were trypsinized for 5 minutes to remove extracellular GFP-*B. burgdorferi*. **A)** Percent of macrophages with internalized GFP-*B. burgdorferi* ascertained using flow cytometry **B)** MFI of macrophages that internalized GFP-*B. burgdorferi*. Statistical significance was determined by Student t test for internalized GFP-*B. burgdorferi* results.  $n = 3$  per group.  $**p < 0.01$ , and  $***p < 0.001$ .



**Figure 6. Impact of TNF $\alpha$  blockade on Lyme arthritis in BALB/cJ mice.** BALB/cJ mice infected with  $2 \times 10^6$  *B. burgdorferi* and treated with isotype control or TNF $\alpha$  neutralizing antibody. **A)** Change in joint swelling at 4 weeks post infection. **B)** Serum levels of anti *B. burgdorferi* specific IgG. **C-E):** Joint transcript levels normalized to  $\beta$  actin. **C)** 16S RNA of *B. burgdorferi*, **D)** TNF $\alpha$ , and **E)** MMP3. Statistical significance was determined by ANOVA for ankle swelling, ELISA Ig, and joint transcript results. \* $p < 0.05$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .



**Figure 7. Analysis of lymphocyte populations and activated cell types in draining lymph nodes of BALB/cJ mice.** Popliteal and inguinal lymph nodes from BALB/cJ mice that were infected with  $2 \times 10^6$  *B. burgdorferi* and treated with PBS, isotype, or TNF $\alpha$  neutralizing antibody. Populations within the lymph nodes analyzed using flow cytometry for the presence of T cells (CD3<sup>+</sup>), then characterized to be either CD4<sup>+</sup> or CD8<sup>+</sup> by a (CD3<sup>+</sup>CD4<sup>+</sup>) or (CD3<sup>+</sup>CD8<sup>+</sup>) stain and evaluated for activation using CD62L and CD44 stains. Activated T cells (CD4<sup>+</sup>CD62L<sup>-</sup>CD44<sup>+</sup>) are distinguished from other T cell populations. B cells distinguished by (CD3<sup>-</sup>B220<sup>+</sup>) stain. Activated B cells (CD3<sup>-</sup>B220<sup>+</sup>GL7<sup>high</sup>) were distinguished from other B cell populations (77, 78). **A)** Total cells in the lymph nodes. **B-D)** Percent of total T and B cells. **E-G)** Percent and number of activated CD4<sup>+</sup> and B220<sup>+</sup> cells. Statistical significance was determined by ANOVA for percentage and total number results. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .



**Figure 8. Increased number of early immune response cell markers in BALB/cJ mice.** Popliteal and inguinal lymph nodes from BALB/cJ mice infected with  $2 \times 10^6$  *B. burgdorferi* and treated with PBS, isotype or TNF $\alpha$  neutralizing antibody. Natural killer cells (CD3<sup>-</sup>CD335<sup>+</sup>) and MHC II (MHC II<sup>+</sup>) populations were determined using flow cytometry. **A and B)** The percent and number of natural killer cells determined by CD335 (NKp46) positive stain (47). **C and D)** The percent and number of MHC II positive cells. Statistical significance was determined by ANOVA for percentage and total number results. \* $p < 0.05$  and \*\* $p < 0.01$ .

## DISCUSSION

### Summary

From this study, it seems that the BALB/c mouse is very different in regard to infection with *B. burgdorferi* when compared with the closely related C3H mouse. The laboratory had already found that the BALB/c mouse develops a dose-dependent arthritis that is not seen in C3H mice, and that the dose needed for BALB/c mice to develop arthritis is 100 times larger than that of the C3H mouse (23). This study performed roughly 20 years later now needed an infectious dose of  $2 \times 10^6$  *B. burgdorferi* to cause intermediate arthritis to develop in BALB/c mice, although low levels of *B. burgdorferi* still cause severe disease in C3H mice. This suggests that something in the laboratory's N40 strain or growth medium has again changed, which is responsible for the increased infectious dose needed to induce severe arthritis in the BALB/c mouse. Analysis from this study has also shown that there are multiple differences between BALB/c and C3H mice in response to *B. burgdorferi* infection aside from the dose needed for severe arthritis.

Previous studies have suggested that the reason for the dose-dependent nature of the BALB/c arthritis phenotype is that the BALB/c mouse is intrinsically better at controlling the infection from either the initial site of infection or through some mechanism of dissemination control (23). Through various analysis in this study, it was seen that the BALB/c mouse model has lower levels of 16S RNA of *B. burgdorferi* in

the joint tissue, lower levels of *B. burgdorferi*-specific IgG, and less severe arthritis at 5 weeks post infection than the C3H mouse (Figure 1). These findings suggest that the BALB/c mouse is better at controlling a *B. burgdorferi* infection both locally and systemically. In trying to determine what was responsible for the reduced Lyme arthritis phenotype, this study demonstrated that peritoneal-derived macrophages are more efficient at phagocytizing *B. burgdorferi*. Thus, enhanced phagocytic capacity of BALB/c mice throughout the animal may be 1 of the major contributors to the heightened ability to controlling an infection with *B. burgdorferi* (Figure 5). Another important factor in controlling infection may be increased cytokine production.

BALB/c mice sacrificed at 2 weeks post infection seem to have upregulated the largest quantity and magnitude of cytokines to fight the *B. burgdorferi* infection (Figure 3), but this upregulation seems to be a local reaction to infection, not a systemic 1. This could be due to the fact that at 2 weeks post infection, the BALB/c mouse has the highest number of *B. burgdorferi* 16S RNA in the joint tissue (Figure 2.F), so the immune system could be reacting in kind. At 2 weeks post infection, the adaptive immune system has not had time to develop mature IgG, so many innate systems are still functioning to control the infection. TNF $\alpha$ , being a major innate immune cytokine, could be influential in controlling the infection at this early stage.

The BALB/c mouse has elevated TNF $\alpha$  in the joint tissue collected from mice sacrificed at 2 weeks post infection when infected with  $2 \times 10^6$  *B. burgdorferi* *in vivo*, and BMDM at both 6 and 24 hours of cultivation with  $3 \times 10^6$  *B. burgdorferi* *in vitro*. Elevated TNF $\alpha$  is not seen in the Type I IFN-driven Lyme arthritis phenotype of C3H mice. BALB/c mice do not display a robust increase in IFN-related transcripts *in vivo*

(Figure 2). However, BALB/c BMDM's do display an elevated Type I IFN profile *in vitro*, but not to the magnitude of the C3H BMDM (Figure 4). This result suggests that the BALB/c macrophage has the ability to use an IFN pathway of inflammation *in vitro*, but elects to use another pathway, possibly involving TNF $\alpha$ , *in vivo*. As discussed previously, TNF $\alpha$  blockade has been very instrumental as a treatment for RA by both decreasing symptoms and stopping disease progression. Treatment outcome can vary depending on the disease duration, severity of disease, and IFN profile (74, 75).

1 way to test the importance of a cytokine in an infection is to deplete or neutralize it. To determine the influence that the elevated TNF $\alpha$  has on the BALB/c mouse, an experiment was performed that blocked it. BALB/c mice were infected with  $2 \times 10^6$  *B. burgdorferi* and the TNF $\alpha$  was neutralized *in vivo* using a neutralizing antibody. It was hypothesized that the arthritis severity would decrease similarly to how TNF $\alpha$  blockade reduces RA symptoms. The results, however, showed that blocking TNF $\alpha$  actually had very little influence on the *B. burgdorferi* infection in the BALB/c mouse at 4 weeks post infection. Compared to the infected isotype group, there was not a significant difference in the number of copies of *B. burgdorferi* 16S RNA in the joint, the concentration of *B. burgdorferi*-specific IgG, or in arthritis severity (Figure 6). This result was in contrast to the impact of TNF $\alpha$  blockade seen in RA.

To further analyze these data, joint transcript levels of various cytokines were evaluated to locate possible downstream effects of blocking TNF $\alpha$ , and of the 30 markers evaluated, only 1 marker was significantly different between the 2 infected groups. MMP3 was reduced in the infected group that was given the treatment of TNF $\alpha$  blockade compared with the infected isotype group and the uninfected control group (Figure 6.E).

This further justifies the conclusion that TNF $\alpha$  does not directly affect *B. burgdorferi* numbers or arthritis severity at 4 weeks post infection.

The only outcome found in this study that seemed to be influenced by TNF $\alpha$  blockade was draining lymph node populations. In the popliteal and inguinal lymph node populations, the number of activated CD4<sup>+</sup> and activated B cells was elevated above both the infected and uninfected control groups (Figure 7.G and H). This suggests that blocking TNF $\alpha$  caused the adaptive immune system to have a more robust response to the *B. burgdorferi* infection by elevating the number of these activated populations.

There was also evidence of increased innate immune responses in the draining lymph nodes (Figure 8). The number of MHC II positive and the number of natural killer cells increased in the infected TNF $\alpha$  blocked group. There were no markers used to differentiate B cells from other antigen presenting cells, so it is possible a majority of the MHC II cells are in fact B cells. The increase in natural killer cells, however, is a true increase due to the selective nature of the antibody used (79). TNF $\alpha$  blockade could be allowing more natural killer cells to proliferate and survive to attack the infection.

From the cumulative results found in this study, it is evident that there is still much that is not understood about the infectious relationship between BALB/c mice and *B. burgdorferi*. The BALB/c mice presumably possess an unknown mechanism that allows them to control *B. burgdorferi* infection better than C3H mice. This unknown mechanism could be a barrier of some kind or a combination of cell types in the tissues that work together more efficiently due to some other factor. The only results from this study that can address this mechanism are the enhanced ability of macrophages to phagocytize *B. burgdorferi* and produce more cytokines in response to *B. burgdorferi*



infection.

#### Future directions

With so many questions left unanswered, there are many directions in which this study could proceed. Most important to the laboratory would be to evaluate the change in virulence of the *B. burgdorferi*. The laboratory is currently using mouse models on the C3H and C57BL/6 backgrounds, which do not develop a dose-dependent arthritis, so the change in virulence might not be seen at  $2 \times 10^4$  *B. burgdorferi*. To evaluate the change, various stocks of *B. burgdorferi* N40 isolate in the laboratory would need to be tested in BALB/c mice at higher concentrations to determine if a particular stock is less virulent. Another possible reason for the change could be the BSK II growth medium. The stock could be fine, but the growth medium is no longer able to support the *B. burgdorferi* growth well enough to maintain virulence in the strain.

After determining the change in virulence, it would be informative to reevaluate the BALB/c mouse strain with an even larger dose of  $2 \times 10^7$  *B. burgdorferi* and determine if that dose will cause the BALB/c mice to develop severe arthritis. If the increased dose causes the arthritis severity to increase more than this study found, then the various results found in this study should be reevaluated to look for differences that may have arisen from the higher dose. If no changes are found in the cytokine or cellular makeup of the BALB/c mice infected with  $2 \times 10^7$  *B. burgdorferi*, then additional inflammatory pathways should be evaluated in an attempt to define the mechanism that causes BALB/c mouse to develop dose-dependent arthritis.

Regardless of increased dose, additional infection experiments should be

performed to evaluate draining lymph node populations. In this current study, lymph node populations were only assessed in the TNF $\alpha$  blockade experiment. That experiment showed differences in cell populations between the infected groups, but there were only minimal cellular markers evaluated. Further research into the lymph node populations and the cytokines they produce could help to develop a better picture of the immune response to *B. burgdorferi* in the BALB/c mouse.

Aside from *in vivo* experiments with the BALB/c mouse, additional *in vitro* experiments should be performed to evaluate the BALB/c macrophage abilities. This study demonstrated that the BMDM produced elevated TNF $\alpha$  *in vitro*, and the peritoneal-derived macrophages were highly efficient at phagocytizing *B. burgdorferi* *in vitro*. These 2 results suggest that macrophages would be a good cell type to further evaluate for their influence on the overall *B. burgdorferi* infection.

The first *in vitro* experiment that should be performed would be a repeat of the phagocytosis assay performed in this study, but include TNF $\alpha$  to prime the macrophages. This could tie together the upregulation of TNF $\alpha$  and phagocytic ability. Aside from the trypsin treated experiments, a nontreated phagocytosis experiment should also be performed to evaluate the total *B. burgdorferi* that is associated with the macrophages both internally and externally.

Another interesting *in vitro* experiment to perform with macrophages would be to determine if the BALB/c macrophage is better at internalizing other infectious agents than *B. burgdorferi*, or if it is a specific reaction to *B. burgdorferi* infection. There is some evidence that BALB/c macrophages are efficient at phagocytizing other bacterium

(80), but a direct comparison was not found. This could lead to other infectious experimental designs using the BALB/c mouse model.

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