ABSTRACT

DEVLIN, AMY ANN. Murine Host Response to Spirochetes and Their Ligands. (Under the direction of Dr. Scott M. Laster).

The order Spirochaetales includes three families: Leptospiraceae, Brachyspiraceae, and Spirochaetaceae. The family Leptospiraceae includes the genus *Leptospira*, which includes the pathogen *Leptospira interrogans*, the causative agent of leptospirosis. The family Spirochaetaceae includes the genus *Borrelia*, which includes the pathogen *Borrelia burgdorferi*, the causative agent of Lyme disease. The goals of these investigations were to further our understanding of the mechanisms of pathogenesis by these spirochetes and to better discern the host response to these organisms and their ligands. Understanding host defense is critical in the prevention and treatment of both *L. interrogans* and *B. burgdorferi*.

L. interrogans is known for its ability to establish chronic infection in the mammalian kidney, and our studies focused on understanding the mechanisms involved in persistent infection of this organ.We found that persistence of *L. interrogans* in the murine kidney is dependent on the anti-inflammatory cytokine, interleukin-10 (IL-10). IL-10 can indirectly suppress many pro-inflammatory cytokines, notably interferon γ (IFN-γ). Following *L. interrogans* infection, C57BL/6 (B6) mice rapidly and consistently produce *IL-10* transcripts. In the absence of IL-10, *L. interrogans*-infected mice produce increasing amounts of *IFN-γ* transcripts over the course of infection, as bacterial burden in the kidney decreases below the level of detection by 7 days post inoculation. Histological observations evidenced kidney damage in some B6 mice at 7 days post inoculation not seen in IL-10-deficient mice. We conclude that IL-10 is necessary for *L. interrogans* to persist in the mouse kidney and be chronically shed into the environment.

The majority of infections with *B. burgdorferi* are controlled by the host response; however, the *B. burgdorferi*-associated ligands and triggering pathways have not been fully elucidated. Previously, type I interferon (IFN) and IFN-stimulated genes (ISGs) were linked to the development of Lyme arthritis in mice following *B. burgdorferi* infection. *B. burgdorferi*-mediated induction of ISGs by murine bone marrow-derived macrophages (BMDMs) involved multiple ligands, including *B. burgdorferi* genomic RNA and non-nucleic acid ligands found in *B. burgdorferi* culture supernatant. We demonstrate that *B. burgdorferi*-mediated induction of ISGs by BMDMs is independent of TLR-7. *B. burgdorferi* genomic RNA-induced transcription of ISGs is TLR-7 independent but MyD88 dependent. Upon treatment with various RNases, *B. burgdorferi* genomic RNA is likely single-stranded. Cyclic-di-GMP, a second messenger molecule released by *B. burgdorferi*, does not enhance ISG transcription in stimulated BMDMs. The non-nucleic acid ligands present in ultracentrifuged *B. burgdorferi* culture supernatant are presumably proteinaceous in nature and are unlikely to include the lipoprotein OspA. The findings presented herein describe the composition of key *B. burgdorferi*-derived stimulatory ligands and host defense signaling pathways involved in the type I IFN and ISG induction associated with Lyme arthritis development in the mammalian host.

Murine Host Response to Spirochetes and Their Ligands

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

Microbiology

Raleigh, North Carolina

2015

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DEDICATION

This dissertation is dedicated to three strong women who have always supported me, encouraged me, and never let me give up on my goals. The first would be my Granny, Doris, who was always loving, inspiring, and full of grit. She was a true Missouri mule. You have been gone 23 years now, but you will always be in my memories. The second would be my Aunt Sandi. Thank you for your encouragement, dedication, and love. Your commitment to your students and Relay for Life is never ending. You are one of the hardest working people I know. And lastly, the final woman I must dedicate this dissertation to would be my mom, Teri. You are the most stubborn person I know. You never let me quit, ever. You have always pushed me to be a better person. I plan on always striving to be and do better in my life. Thank you for listening to my complaints and offering me proactive solutions. Thank you for loving me and never abandoning me.

BIOGRAPHY

Amy Ann Devlin was born June 10, 1986 in Bakersfield, CA. She attended Our Lady of Perpetual Help School and Garces Memorial High School in Bakersfield, CA. She graduated with a bachelor of science degree in biology, with minors in chemistry and sociology, from California State University, Bakersfield in 2008. She graduated with a master of public health degree in biomedical sciences from the State University of New York at Albany in 2010. After working for the New York State Department of Health, Amy realized that research focused on improving human health was her primary interest. She chose to pursue her doctoral research in the laboratory of Dr. Jennifer C. Miller at North Carolina State University. Upon her departure, Amy transferred to the laboratory of Dr. Scott M. Laster to finish her doctoral work at the same university. Following completion of her Ph.D., Amy will begin her post-doctoral work.

ACKNOWLEDGMENTS

There are many people I need to thank. First off, a big thank you goes to Dr. Jennifer C. Miller. Thank you for taking me into your lab, for pushing me to work harder, and for encouraging me. Thank you to Dr. Scott M. Laster for taking me into your lab. You have helped me tremendously. Thank you to my committee for your advice and encouragement. Thank you to my former labmates: Heather, Priya, Breanna, Karissa, Alex, and Michael. I learned many things from you all. Thank you to my current labmates: Travis, Nick, and Sarah. I appreciate your upbeat attitudes. Thank you to Lindsey and Farah from the Scholle lab for your conversation and friendship. To Farah, you are the extrovert to my introvert. You truly understand me, and I hope to always remain friends. Thank you to the people in the department who smile at me. I appreciate it. Thank you to my friends Annette and Jackie. Thank you to Cindy for your smile and warmth. To my three grandparents, enjoy heaven. I love you and I miss you. Denise and Bill, thank you for your support. To my Aunt Sandi and Uncle Mike, thank you so much for supporting my education. You helped me have shelter and a laptop on which to do my work, in addition to your constant emotional support. Thank you to my parents, Jim and Teri, for always encouraging me and helping me along the journey. I know you will always have my back. To the two people I cannot imagine my life without, my brother Justin and my love Bryan, thank you. Justin, you are one of the best human beings I know. Through every tough time in my life, you have been there for me. God blessed me with a great brother. And to Bryan, thank you for the last 4 wonderful years. I want to have 70 more with you. Your love is my source of strength.

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CHAPTER 1

Literature Review

1.1 *Leptospira interrogans*

1.1.1 *Leptospira interrogans* Origin and Phylogeny

In 1886, Adolph Weil (1) described a disease characterized by renal dysfunction, skin rashes, conjunctivitis, and jaundice accompanied by splenomegaly. Later, these combined symptoms were referred to as Weil's disease. The disease appeared to be infectious and was often associated with outdoor occupations in which workers came into contact with water. The spirochetes were first visualized in 1907 (2) and isolated in 1916 (3). Noguchi (4) proposed the genus name *Leptospira* in 1918 to distinguish the organism from other spirochetes known at the time. In 1982, two species of *Leptospira* were agreed upon: *Leptospira interrogans*, which would include pathogenic serovars, and *Leptospira biflexa*, which would consist of saprophytic serovars (5). As of 2015, there are 21 species of *Leptospira* (6). *Leptospira* are named, in order, by: species, serogroup, serovar, and strain. Species were later classified as pathogenic, intermediate, or saprophytic, as two classifications were insufficient for so-called intermediate *Leptospira*.

1.1.2 The Structure of *Leptospira interrogans*

L. interrogans is thin, highly motile, and spiral shaped. *Leptospira* have an average diameter of about 0.1 μm (7) and length range of 6-20 μm (8). Freshly isolated *L. interrogans* tends to be shorter and more tightly coiled than saprophytic strains and strains that have been repeatedly passaged in the lab (9).

L. interrogans' outer membrane is similar to that of Gram-negative bacteria in that lipopolysaccharide (LPS) is embedded in the outer leaflet. *L. interrogans* also has an inner membrane and a peptidoglycan layer in the periplasmic space like other Gram-negative organisms. Otherwise, *Leptospira* are unique among other Gram-negative bacteria and even

other spirochetes. *Borrelia* and *Treponema* do not possess LPS like *Leptospira*. Leptospiral LPS differences are the basis for the categorization of *Leptospira* into 24 serogroups and over 250 serovars (10). Of key importance is that LPS plays a critical role in leptospiral virulence (11-13). This area of research will be discussed further in the pathology section (1.1.4.1) of this review.

The peptidoglycan layer in the periplasmic space provides *Leptospira* rigidity, strength, and shape; unlike in other Gram-negative bacteria, the leptospiral peptidoglycan layer is found to be situated closer to the cytoplasmic membrane than to the outer membrane (14). *L. interrogans*, in comparison to *L. biflexa*, has a similar total peptidoglycan composition, but with less cross-linked peptidoglycan dimers. This adaptation may benefit *L. interrogans* when faced with the physiological osmolarity of hosts (15).

1.1.2.1 Motility

Additionally, within the periplasmic space, *L. interrogans* has endoflagella that are responsible for its distinctive corkscrew motility (16, 17). *L. interrogans* possesses two single flagella that are anchored about 0.18 nm from both ends of the cell (8, 18). These two flagella do not overlap or span the entirety of the cell length (19, 20). The combination of directional rotation, incomplete cell coverage by the flagella, and subterminal tethering give *L. interrogans* its characteristic hooks that appear at either one or both ends of the bacterium (8, 16, 18).

The two flagella of *L. interrogans* each have a motor that consists of the hook, rod, rod-associated L and P rings, MS-ring, stator ring, C ring, and export apparatus (21). The leptospiral flagellar filaments consist of a FlaB core surrounded by a flagellar sheath, postulated to be composed of FlaA (22). Four *flaB* genes and two *flaA* genes can be detected

within the *L. interrogans* genome (23), all of which are normally expressed (24). A *flaA1* mutant has decreased motility, but continues to express FlaA2 and maintains virulence, whereas a *flaA2* mutant expresses neither FlaA protein, losing both motility and virulence (25).

Similar to other spirochetes but different from externally flagellated bacteria, *Leptospira* display increased swimming speeds under high viscosity conditions (16, 26-28). Upon exposure to physiological osmolarity, ≈ 150 mM NaCl, pathogenic *L. interrogans* maintains motility, while the saprophyte *L. biflexa* loses motility under such conditions (28). This finding points to a critical link between motility and leptospiral virulence.

1.1.2.2 Physiology and Metabolism

Unlike *B. burgdorferi* and *T. pallidum*, *L. interrogans* has a genome that encodes complete pathways for amino acid and nucleic acid biosynthesis (8, 23). Different from many bacteria, *L. interrogans* derives its major energy and carbon sources via beta-oxidation of long chain (over 15 carbons) fatty acids (29). *L. interrogans* is not able to synthesize long chain fatty acids from pyruvate or acetate and must instead get these fatty acids from growth medium or from fatty acids at solid-liquid interfaces (30-32).

L. interrogans is an aerobic organism with complete gene sets encoding the tricarboxylic acid cycle and electron transport chain components. *Leptospira* display oxidase, cytochrome c, and catalase activity (33, 34), with catalase being required for in vivo survival, possibly by mediating protection from reactive oxygen species (35). *Leptospira* require a nitrogen source, often ammonia (8). Xue et al. (36) used microarray analyses to reveal that genes involved in the tricarboxylic acid cycle, oxidative phosphorylation, and fatty acid metabolism are down-regulated upon introduction of leptospires to macrophage cell lines of

both human and murine origin, whereas the gene encoding catalase and genes involved in nitrogen metabolism are up-regulated when interacting with these cell lines. Unlike *B. burgdorferi* and *T. pallidum*, *L. interrogans* has the genes needed for protoheme and vitamin B_{12} biosynthesis (37, 38).

Leptospira require carbon and nitrogen sources, vitamins, and nutritional supplements for growth. Detailed growth medium composition is discussed in the materials and methods section of chapter 2 (2.1). Of note, *L. interrogans* is only able to metabolize saturated fatty acids in the presence of unsaturated fatty acids (8, 39, 40). These necessary fatty acids are also toxic, so when growing *L. interrogans* in culture medium, detoxicants such as albumin or Tween need to be included (8, 41). The only recognized nitrogen source is ammonium ions, and they can be supplied as either ammonium salts or by deamination of amino acids (8, 42). Essential nutritional supplements include: phosphate, thiamin, calcium, magnesium, iron, copper, manganese, and sulfate (8, 42-45). Optimal growth of *L. interrogans* is achieved at 28-30°C, pH 7.2-7.6. Doubling time for *L. interrogans* in the animal model and in culture is estimated at 6-8 hours, while the doubling time for it from host into culture is about 14-18 hours with a lag time for initiation of growth from days to weeks (46). Transfer of a stationary phase seed culture composed of 1-10% of the total volume of fresh medium generally results in achievement of maximal growth of pathogenic leptospires in 4-7 days (8). Growth of cultures is best observed using a Petroff-Hauser counting chamber and dark field microscopy.

1.1.3 Genomics and Genetics

L. interrogans has one large circular chromosome and a smaller replicon. Within the last year, Zhu et al. (572, 573) presented evidence that some *L. interrogans* strains do have

extra-chromosomal plasmids. *L. interrogans* strains have a comparatively large genome at over 3.9 Mb relative to other spirochetes, such as *B. burgdorferi* at around 1.5 Mb (47) and *T. pallidum* with about a 1.1 Mb genome (48). Specifically, *L. interrogans* Fiocruz L1-130, utilized in our experiments, has a genome size of about 4.6 Mb (49). In many bacteria, the 16S, 23S, and 5S rRNA genes are clustered and co-transcribed; in *Leptospira*, these genes are widely dispersed on the large chromosome (50, 51).

As of 2015, there is no replicative plasmid vector available for *L. interrogans* (52). A phage, LE1, was used as the basis for the first *L. biflexa-E coli* plasmid shuttle vector (53). Kanamycin, spectinomycin, and gentamicin have been used as selective markers (53-56). Doxycycline and penicillin should not be used as markers because they are useful in the treatment of patients (57). Random mutagenesis using the *Himar1* mariner transposon has been developed in *L. biflexa* and *L. interrogans* (55, 58, 59). A suicide plasmid harboring the *Himar1* transposon carrying a kanamycin-resistant cassette, along with the C9 hyperactive transposase, is used to transfer the transposon, which can randomly insert into the chromosome (55, 58, 59). At least 1,000 different *L. interrogans* mutants have been generated using this technique (59).

1.1.4 *L. interrogans* Infection in Humans

Humans are accidental hosts, as leptospirosis is mainly a zoonosis. Transient leptospiral shedding does occur during human infection; human to human infection has been reported via sexual activity (60, 61) and through breast milk (62). If infection occurs during pregnancy, transplacental transmission may occur resulting in still birth (63, 63) or abortion (65).

Leptospira interrogans enters the body through cuts or mucous membranes, such as those of the eyes, mouth, or genital surfaces. Exposure often occurs through indirect contact with urine-contaminated soil or water or via direct contact with an infected animal. While working with potentially infected animals poses a risk, at greatest risk of infection with *Leptospira* on the job are agricultural workers. Rodents are attracted by the crops and shed their urine into the area where the workers come into contact with the bacteria (66, 67). Recreational sporting activities involving freshwater, including: canoeing (68), kayaking (69, 70), rafting (71), caving (72), and triathlons (73, 74), have been increasing in popularity, adding to exposure risk. Competitive events, such as the 2000 Eco-challenge competition (74) and 1998 Springfield triathlon (73), resulted in 80 and 98 leptospirosis cases, respectively.

People at greatest risk for contracting leptospirosis live in tropical areas. Inadequate housing and poor sanitation together exacerbate the risk of exposure to *L. interrogans* in both urban slum and rural communities (75-78). Sightings of rats and closeness to uncollected trash increased the risk of leptospirosis among inhabitants of urban slums (78). As a result of large outbreaks of leptospirosis following excessive rainfall (79-82), leptospirosis was labeled as an emerging infectious disease (83). The International Leptospirosis Society (84), through the use of surveys, estimates the incidence of severe leptospirosis at 350,000-500,000 cases per year. This estimate is thought to be low due to under reporting of cases.

1.1.4.1 Pathogenicity

After *L. interrogans* enters the body through a cut or mucous membrane, it disseminates through the blood. Blood taken from patients within the first eight days of fever is more likely to be positive for *L. interrogans* via quantitative PCR (qPCR) prior to antibody

formation and removal of organisms from the bloodstream (85). qPCR has detected leptospiremia levels as high as 10^6/ml of blood (85), with levels of greater than 10^4 leptospires/ml of blood associated with severe outcomes (86, 87).

High bacteremia seen in some patients with leptospirosis is partly explained by evasion of the human innate immune response. Whereas *E. coli* LPS is detected at low concentrations by human TLR-4, leptospiral LPS is not recognized (13). *L. interrogans* has a methylated phosphate residue unique to its lipid A component of LPS not found in any other form of lipid A (88). Mouse TLR-4 does recognize leptospiral LPS, unlike human TLR-4, implying that the murine innate immune response is adapted to *L. interrogans* infection (12). Mice are resistant to fatal infection and serve as reservoir hosts; humans are incidental hosts that can have fatal outcomes and infrequently transmit leptospires.

The pattern recognition receptors (PRRs) TLR-2, TLR-4, and TLR-5 have roles in leptospiral recognition. Young C3H/HeJ mice, with a non-functional TLR-4, are susceptible to lethal infection with *L. interrogans* (89). Murine and human TLR-2 can detect the polysaccharide or 2-keto-3-deoxyoctonic acid (KDO) part of leptospiral LPS (12, 90). Adult C57BL/6 J mice experience lethal leptospirosis only when both TLR-4 and TLR-2 are non-functional (12). TLR-2, TLR-4, and TLR-5 have been reported to be necessary for *L. interrogans* to induce expression of the cytokines TNF-α and IL-6 in whole human blood (91).

Sepsis-like syndrome or organ failure often results from high levels of leptospiremia during infection. People with severe leptospirosis have elevated levels of TNF-α, IL-6, IL-10, and other cytokines in comparison with patients exhibiting mild disease (92). Overproduction of IL-10 may block a protective Th1 immune response.

Major target organs of *L. interrogans* include: the liver, lungs, and kidneys. Congested sinusoids and distention of the space of Disse, found between sinusoids and hepatocytes, have been observed in the livers of cadavers infected with *Leptospira* (93). Using the hamster model, which mimics severe human pathology, *Leptospira* were found to infiltrate the space of Disse and invade and attach to the perijunctional region between hepatocytes (94). Hepatocyte apoptosis was seen following onset of leptospirosis in guinea pigs, another model used to mimic severe human leptospirosis (95). Disturbance of hepatocyte intercellular junctions and hepatocellular damage lead to leakage of bile from bile canaliculi into sinusoidal blood vessels, causing elevated levels of direct bilirubin noted in the icteric form of leptospirosis (96). Elevation of indirect bilirubin values may rise in the event of leptospirosis-induced hemolysis (97).

Pulmonary petechiae were found on pleural surfaces in 33/33 cases of fatal leptospirosis examined by Arean (93) in 1962. Twenty of the examined corpses had gross hemorrhage on the cut surfaces of their lungs, occurring in alveolar septa and intra-alveolar spaces (93). Guinea pigs with leptospirosis have pulmonary hemorrhage similar to that seen in humans. Seen in the lungs of infected guinea pigs is an extensive aggregation of complement and immunoglobulin along the alveolar basement membrane (98).

Minor renal dysfunction all the way to renal failure can result from leptospiral infection. Polyuria seen in mild leptospirosis is likely due to decreased expression of the sodium-hydrogen exchanger 3, resulting in reduced re-absorption of sodium and liquid by the proximal tubule (99). In mice and rats, the tubular lumen is a main site of colonization for *L. interrogans*, and immunohistochemistry often shows many leptospira attached to the brush border of the proximal tubular epithelium (100). Leptospiral lipoproteins, such as LipL32,

are recognized by TLR-2 on renal tubular epithelial cells, triggering release of monocyte chemoattractant protein-1 (MCP-1) and nitric oxide synthase (101). Infiltration of monocytes, lymphocytes, plasma cells, sporadically neutrophils, along with edema, define the interstitial nephritis that follows tubular inflammation (93, 102). During the first two weeks of leptospirosis, interstitial nephritis increases in range and intensity. Fortunately, the majority of patients with leptospirosis-related acute renal failure who survive infection regain their prior renal function. Few patients do end up with persistent renal dysfunction related to tubular atrophy and interstitial fibrosis (103).

1.1.4.2 Presentation of Leptospirosis

The incubation phase following exposure to *L. interrogans* until onset of symptoms is highly variable. A range from 3 days to as long as 30 days has been noted (100). After the incubation phase, symptoms often include: fever, chills, and headache. Other common symptoms include: abdominal pain, diarrhea, vomiting, and nausea.

Severe leptospirosis is defined by malfunction of multiple organs including: lungs, kidneys, the liver, and the brain. Weil's disease, the combination of renal failure and jaundice, is still one of the most clinically recognizable presentations of leptospirosis (1). Along with organ dysfunction, most patients with severe leptospirosis have minor internal bleeding. Some have gastrointestinal bleeding or pulmonary hemorrhage (100). A strong association was found between those infected with *L. interrogans* Icterohaemorrhagiae and jaundice and increased bilirubin levels (104). Kidney involvement is common, as elevated serum creatinine is often detected, as well as hematuria, pyuria, and increased urine protein levels via urinalysis (104). Pulmonary hemorrhage can be severe, often leading to poorer

patient outcomes. Altered mental status was found to be the strongest single predictor of a fatal outcome from leptospirosis in an active surveillance study in Salvador, Brazil (81).

1.1.4.3 Serological Diagnosis of Leptospirosis

Due to the limited ability to culture leptospires and perform PCR, the majority of leptospirosis cases are diagnosed by serology. In humans, IgM antibodies are detectable in patient blood 5-7 days following the onset of symptoms (100). The microscopic agglutination test (MAT) is the predominant method used to detect leptospires in both humans and animals. In order to perform the test, live leptospires of all serovars needed for use as antigens must be maintained. Varied antigens should be used in order to detect infections with less common serovars (105). Serum from infected patients is combined with live antigen cocktails of leptospiral serovars. Following incubation, combined serum and antigens are viewed under dark field microscopy, analyzed for agglutination, and titers are defined. The MAT is a serogroup-specific test and cannot accurately determine the infecting serovar (106-108). The MAT can also be difficult to perform, control, and interpret (109).

The MAT would usually distinguish titer by determining the highest dilution of serum in which fifty percent agglutination happens, but in this case, its endpoint is based on the presence of about fifty percent free leptospires in comparison with the control leptospiral suspension (110). Subjectivity is an issue with this detection method and must be reduced as much as possible. Cross-reactivity in acute phase leptospirosis is also a potential problem. Sera from any animal species can be used in the MAT. The extent of *L. interrogans* strains used can be increased or decreased as is necessary.

A single elevated titer found along with acute febrile illness indicates acute infection (66). In order to confirm a diagnosis, paired sera are needed. A positive test occurs when

there is at least a 4x increase in titer between paired sera (66). The length of time between serum samples does not impact the result; the 4x increase confirms diagnosis regardless of interval between collections of sera. Of note, some patients with severe leptospirosis may perish before the occurrence of seroconversion (111-113).

IgM antibodies can be detected within the first week of leptospirosis, aiding rapid diagnosis and quick antibiotic treatment. MAT is not as sensitive as IgM detection when serum is taken and analyzed early in acute illness (112-115). The specificity of IgM detection by enzyme-linked immunosorbent assay (ELISA) is affected by the presence of other diseases, previous exposure-related antibodies, and the antigen used to coat the wells (116).

1.1.4.4 Treatment

The majority of people infected with *L. interrogans* will have no symptoms or minor symptoms that will resolve over time. Adults with early disease not requiring hospitalization are recommended to receive either azithromycin 500 mg pills once per day or doxycycline 100 mg pills twice per day (100). Doxycycline is not recommended for pregnant women and children; amoxicillin or azithromycin is prescribed based on weight. Dosing recommendations are based on minimum inhibitory concentration studies (117,118), hamster studies (119, 120), and clinical studies. A randomized, placebo controlled, double-blinded study by McClain et al. (121) found that doxycycline treatment reduced the length of illness from leptospirosis by two days and reduced malaise, headache, fever, and myalgias, along with preventing shedding of *L. interrogans* in patients' urine. Patients with severe leptospirosis requiring hospitalization frequently are administered intravenous penicillin at a dose of 1.5 million units every 6 hours (122). Other antibiotics used intravenously for severe leptospirosis include: ceftriaxone, at 1 gram per 24 hours, cefotaxime, at 1 gram per 6 hours,

or ampicillin, at ½-1 gram per 6 hours, with ceftriaxone shown to be as effective as penicillin G in the treatment of severe leptospirosis (122). Ceftriaxone can also be administered intramuscularly, adding benefit to its use where hospitalization is not available.

Severe leptospirosis treatment often requires supportive therapy and antibiotics to help patients recover. Often found with the severe form of leptospirosis in patients is exacerbated potassium excretion (123, 124). Patients experiencing this symptom need intravenous hydration and potassium supplementation to prevent dehydration, renal failure, and hypokalemia (100). Critically ill patients with leptospirosis may require dialysis to improve their chances of survival (125).

1.1.4.5 Exposure Reduction

Awareness of leptospirosis is of greatest benefit to those attempting to avoid the pathogen. Poverty cannot easily be eliminated, nor can poor housing conditions. Rodent control may help, but bait must not endanger children or other wildlife. Control of flooding would likely lessen exposure among residents. People who work with animals or in contact with fresh-water sources can wear personal protective equipment, for example: boots, goggles, gloves, and coveralls. Immunization of pets and agricultural animals with killed whole-cell vaccines is an important method used to reduce the risk of transmission to humans (100).

1.1.5 Mechanisms of Leptospiral Pathogenesis

Currently, the molecular basis of leptospiral pathogenesis is not well understood. *L. interrogans* lacks classical virulence factors due to the wide phylogenetic distance between it and other well-studied, Gram-negative pathogens. *L. interrogans* likely has unique virulence

genes; 78% of its pathogen-specific genes have no known function, while 40% of its whole genome has no defined function (126).

Bioinformatics has identified few confirmed *L. interrogans* virulence factors. The few exceptions include: catalase, collagenase, heme oxygenase, and a mammalian cell entry protein (Mce) (35, 127-129). Murray et al. (127) characterized the virulence factor HemO, a heme oxygenase that degrades heme, likely involved in iron acquisition, utilizing the hamster model. Hamsters infected with the mutated *lb186* gene had significantly higher survival rates than those infected with a control mutant with an intergenic transposon insertion (127). Eshghi et al. (35) characterized KatE, the only annotated catalase found within *L. interrogans*. When exposed to hydrogen peroxide, pathogenic *L. interrogans* had a 50x higher survival rate than the saprophyte, *L. biflexa* (35). Recombinant KatE tested positive for specific catalase activity (35). KatE was found to be located in the bacterial periplasmic space using protein fractionation experiments (35). Transposon mutagenesis was used to generate an insertion that inactivated *katE* in *L. interrogans*, leading to greatly reduced viability when exposed to extracellular hydrogen peroxide and impaired virulence in the hamster model (35).

Zhang et al. (128) determined the function of the leptospiral mammalian cell entry protein (Mce), first confirming only pathogenic leptospira express the gene. Utilizing a mouse-mononuclear-macrophage-like cell line, the authors showed increased leptospiral *mce* mRNA and Mce protein levels following infection of the macrophages by *L. interrogans* (128). *L. interrogans* with a deletion in *mce* had impaired infectivity of macrophages, while complementation restored their ability to attach to and invade cells (128). Saprophytic *Leptospira* were able to infect macrophages when the *mce* gene was acquired (128). Mce-

deficient *L. interrogans* had decreased ability to infect hamsters versus wild-type *L. interrogans*; additionally, fewer mutant leptospires were found in the blood and urine from infected hamsters (128).

Kassegne et al. (129) characterized ColA, the collagenase of *L. interrogans* likely important in dissemination and tissue damage within the host. *colA* gene-deleted (ΔcolA) and *colA* gene-complemented (CΔcolA) mutants were generated to elucidate the roles of the collagenase in transcytosis in vitro and in vivo (129). Following infection of HUVEC or HEK293 cells by *L. interrogans*, *colA* mRNA and ColA proteins were found to be highly elevated relative to *L. interrogans* growing in culture medium (129). Like Mce-deficient *L. interrogans*, ColA-deficient *L. interrogans* had a decreased ability to infect hamsters compared to wild-type *L. interrogans* and fewer mutant *Leptospira* were detected in tissues and urine from infected hamsters (129). ΔcolA mutants were also found to be considerably less virulent than CΔcolA mutants and wild-type *L. interrogans*, which shared similar 50% lethal dose levels in the hamster infection model (129).

Upon examination of the genomes of two *L. interrogans* serovars, Nascimento et al. (49) found genes encoding phospholipase, sphingomyelinases, proteases, and orthologs of *Serpulina hyodysenteriae tlyABC* hemolysins. These are predicted genes, and as of late, their role(s) in virulence have not been further reported. Nascimento et al. (49) also found that *L. interrogans* lack type IV, type VI, and non-flagellar type III secretion systems. The genomes of *L. interrogans* strains encode many leucine-rich repeat proteins, harboring a motif frequently associated with pathogen-host interaction (49). The molecular mechanisms by which *L. interrogans* enters the body are not currently known.

1.1.5.1 Motility and Pathogenesis

The two periplasmic flagella of *L. interrogans* are each composed of a core covered by a sheath. The core contains FlaB, which is encoded by four *flaB* genes, a sheath made up of FlaA, encoded by two *flaA* genes, and potentially other unidentified proteins (130). *Leptospira* effectively traverse viscous substrates (131, 132), which may permit movement through tissue components, such as collagen and hyaluronic acid (16). Though not experimentally demonstrated, motility is likely required to breach mucous membranes or tissues of damaged skin. Motility inside the host has been shown to be essential for disease (133-135).

Liao et al. (136) used a suicide plasmid to inactivate the *fliY* gene, which encodes a flagella motor switch. Also found to be inactivated were flagellar genes *fliP* and *fliQ* (136). The *fliY* mutant (fliY-) exhibited reduced adhesion to macrophages and less induction of macrophage apoptosis (136). The fliY- mutant also was found to be attenuated in guinea pigs (136). Liao et al. (136) propose that these results may be due to reduced export of toxins and adhesions through the flagellar apparatus. Another possible explanation is that a deficiency in motility may decrease contact between leptospires and macrophages, lowering apoptosis levels; attenuation of the fliY- mutant in guinea pigs could be a result of impaired dissemination in the host (137).

Lambert et al. (130) used transposon mutagenesis to construct *flaA1* and *flaA2* mutants, both of which still produced periplasmic flagella. The *flaA1* mutant did not produce FlaA1 but still made FlaA2; the *flaA2* mutant did not express FlaA1 or FlaA2 (130). The *flaA2* mutant lost its helical shape, hooked ends, and lacked translational motility, similar to previously generated motility mutants (133). This mutant was greatly attenuated in hamsters, failing to cause disease, with no detectable kidney colonization 25 days following inoculation

(130). Unlike the high burdens found with wild-type *L. interrogans* infection, the *flaA2* mutant was nearly undetectable in kidneys and livers 5 days post injection (130). These data indicate that motility is required for the ubiquitous tissue distribution of *L. interrogans* observed in acute hosts (138).

Eshghi et al. (139) used transposon insertion to mutate *lb139*, a gene encoding a putative sensor protein. RNA sequencing showed down regulation of 115 genes and upregulation of 28 genes (139). Of the 115 down regulated genes, genes encoding putative secreted proteins, regulatory proteins, motility and chemotaxis proteins were most commonly suppressed (139). The *lb139* mutant was greatly attenuated in the hamster model when transmitted by both intraperitoneal and conjunctival routes (139). Attenuation may be related to the suppression of secreted proteins and/or reduced motility of the mutant (139).

1.1.5.2 Traversing Host Tissues

Barriers including: cell layers, extracellular matrix, and basement membranes present themselves to leptospires on their dissemination routes. While the mechanisms by which *L. interrogans* passes through tissue layers remains unknown, motility is most likely key (130). Marshall (140) inoculated mice intraperitoneally and observed leptospires crossing into the kidney lumen, traveling in between cells. Not found were viable *Leptospira* within proximal tubule cells (140). More recent studies utilizing polarized Madin-Darby canine kidney (MDCK) cell monolayers presented evidence of rapid transit through these cells (141, 142). *L. interrogans* quickly crossed through cell layers without considerable disruption of tight junctions unlike the intracellular pathogen *Salmonella enteritidis*, which greatly disrupted cell junction integrity (141). Some *Leptospira* were seen intracellularly (8% of counted cells), likely in transit across the cell layer (141). *L. interrogans* is an invasive organism, but

not a facultative intracellular organism (141). Vieira et al. (143) found that *L. interrogans* can trap plasminogen and generate plasmin on its surface. Following this observation, they next coated *L. interrogans* with plasminogen or plasmin and found enhanced migration ability through human umbilical vein endothelial cells (HUVEC) compared to untreated *L. interrogans*, suggesting a proteolytic mechanism (144). Collagenase may aid in dissemination of *L. interrogans*. Kassegne et al. (129) found that a collagenase mutant had decreased ability to cross HUVEC and human renal tubular epithelial cell line (HEK293) cell layers, along with reduced distribution in hamster tissues following inoculation.

1.1.5.3 Adhesion and Dissemination

Host extracellular matrix (ECM) provides structural and biochemical support to adjacent cells, with components including: laminin, several types of collagen, fibronectin, and proteoglycans (145). *L. interrogans* can bind to ECM components, including: hyaluronic acid, laminin, collagen, and fibronectin (146). Adhesion of *L. interrogans* to ECM molecules is increased following incubation at physiological osmolarity, which is approximately 300 mOsM in human and rat tissues (147). Although many leptospiral proteins have been observed to bind components of the ECM, not one has been shown to be essential for virulence. This may be a result of functional redundancy.

L. interrogans can bind fibrinogen (148) and inhibit fibrin formation (149). This may aid leptospiral dissemination and lead to hemorrhage. The fibrinogen-binding proteins Lsa33, Lig B, LIC12238, LIC11975 and OmpL1 inhibit thrombin-catalyzed fibrin formation in vitro (149-151). When *L. biflexa* was engineered to express LigA or LigB, bacterial adhesion to fibrinogen was enhanced (150). LigB was found to bind to the C-terminal αC domain of fibrinogen, inhibiting platelet adhesion and aggregation in vitro (151). Despite these data,

none of these proteins has a definitive role in pathogenesis since a *ligB* mutant retains full virulence (152).

In vitro, leptospires bind plasminogen, and in the presence of urokinase plasminogen activator (uPA), bound plasminogen can be converted to plasmin (143, 153). *Leptospira*-bound plasmin degrades fibronectin (143) and human fibrinogen (149) in vitro, potentially activating host matrix metalloproteases. Activation of matrix metalloproteases may lead to tissue destruction. Although the mechanism is unknown, plasmin-coated *L. interrogans* penetrated HUVEC monolayers more effectively than uncoated leptospires (144). Therefore, surface-bound plasmin may enhance crossing of ECM, tissue barriers, and destruction of fibrin clots by *L. interrogans*, permitting dissemination throughout the host. Surface-bound plasmin also impedes deposition of C3b and immunoglobulin on the leptospiral cell surface *in vitro* (154). Reduced binding of C3b to *L. interrogans* may decrease opsonization for phagocytosis and lower the activation of the complement cascade at the leptospiral cell surface by both classical and alternative complement pathways. Plasmin-coated *L. interrogans* displayed increased serum survival compared to uncoated *L. interrogans* (154).

1.1.5.4 Persistence in the Host

In humans, *L. interrogans* can be found in the blood for up to two weeks, requiring a high level of resistance to serum complement (8). Unlike *L. interrogans, L. biflexa* and other saprophytic species are highly susceptible to complement (155). Meri et al. (156) found C3 to be deposited on both serum sensitive and resistant strains of *Leptospira*, but terminal complement components were only found on the surface of non-pathogenic strains. Complement resistant and intermediate strains bound higher levels of factor H (156) and C4-binding protein (C4BP) (157) from human serum than complement sensitive strains. Surface-

bound C4BP enhances factor I-mediated cleavage of C4b; as a result, pathogenic *Leptospira* showed reduced deposition of the later complement components C5 to C9 upon exposure to serum (157). Inhibition of the complement cascade by *L. interrogans* results in: reduced bacterial cell lysis, reduced opsonophagocytosis, and likely decreased recruitment and activation of phagocytes (158).

Leptospira are not generally viewed as intracellular pathogens, but they may have intracellular phases that contribute to pathogenesis. *L. interrogans* appears to persist in macrophages (159, 160) and may be transiently intracellular when crossing through cell layers (141, 142). Internalization of *L. interrogans* occurred via receptor-mediated endocytosis rather than phagocytosis and induced programmed cell death of the murine macrophage-monocyte-like cells, J774A.1 (161). *L. interrogans* may use an Mce-like protein to adhere to and enter macrophage-like cells; when the *mce*-like gene is mutated, adhesion and entry ability is significantly reduced but restored upon complementation (128).

1.1.5.5 Renal Carrier State

A carrier host is a host in which infection is endemic, disease is asymptomatic or mild, and transmission occurs back to the same host species (162). For *L. interrogans*, rodents are the maintenance hosts. Humans, hosts that succumb to acute disease, are incidental hosts, unlikely to be sources of transmission. In the carrier host, the immune system likely clears *L. interrogans* from all tissues except the kidney. In the renal tubules, *Leptospira* survive, multiply, and are excreted in the urine at concentrations found to be as high as 10^7 leptospires/ml (163, 164). Carrier hosts may be life-long shedders of *L. interrogans*. Humans, acute hosts, have been found to shed leptospires in their urine for 2 weeks to one month (8).

Unlike humans, carrier host rats, when experimentally infected, display little to no tissue damage except for possible interstitial nephritis (164, 165).

The molecular basis for development of the carrier state is mostly unknown. A few mutants have been studied in carrier models of disease. LigB and LipL32 were dispensable for renal colonization of rats (152, 166). LPS and HtpG were required to cause disease in the hamster model, as well as colonize mouse kidneys (167).

Leptospiral LPS signals through TLR-2 and TLR-4 in murine cells, but does not signal via TLR-4 in human macrophages (12). The recognition of *L. interrogans* via TLR-4 is paramount for the resistance of mice to acute leptospirosis, as mice deficient in TLR-4 are vulnerable to acute disease (89, 168).

1.1.6 Lipopolysaccharide and Outer Membrane Proteins

1.1.6.1 Lipopolysaccharide (LPS)

The leptospiral outer membrane has LPS as a major component. In general, LPS is much more immunogenic in humans than in mice (574). Monoclonal antibodies to LPS mediate macrophage opsonization (169) and defend immunized hamsters against challenge with *L. interrogans* (170). LPS-specific immune responses are the foundation for immune memory provoked by whole cell vaccines (171). There is substantial selective pressure to alter LPS due to the sensitivity of *L. interrogans* to LPS-specific antibodies. There is great O-antigen variation across leptospiral serovars; addition of LPS-antiserum alone to *L. interrogans* in culture can result in mutants with altered LPS (172).

LPS appears to be indispensable for leptospiral survival both within and outside a host. Most LPS mutants are unable to grow in culture following random transposon mutagenesis

(59). The few mutants that did survive transposon insertion into the LPS locus were attenuated and rapidly cleared after challenge (11).

Leptospiral LPS has three components: lipid A, the core, and polysaccharide. Lipid A of *L. interrogans* is unique. Leptospiral fatty acids in its lipid A differ in length from those usually found in common Gram-negative lipid A, and some are unsaturated (172). Unlike *E. coli* lipid A, which has two phosphates, one on each end of the disaccharide, leptospiral lipid A has only one phosphate and it is methylated (172). Nahori et al. (12) found that this unique lipid A is recognized by murine TLR-4, but not by human TLR-4. This differential recognition of lipid A may be a critical component of host susceptibility to leptospirosis.

1.1.6.2 LipL32

LipL32 is the most abundant outer membrane lipoprotein in *L. interrogans* (173). Once thought to be a surface protein, it has now been identified as a subsurface lipoprotein (174). Proteinase K digestion of LipL32 only occurred if leptospires were disrupted (174). LipL32 appears to be localized to the periplasmic leaflet of the outer membrane (174), a site shared with LipL36 (175). It is estimated that LipL32 comprises one-fifth of the leptospiral outer membrane inner surface (172). A possible function of LipL32 is that of a calcium sink (172). LipL32 does not appear to be critical for outer membrane integrity, since a *L. interrogans* mutant lacking LipL32 had usual morphology and growth rate in comparison to wild-type *L. interrogans* (166). Beyond its possible role as a calcium sink, LipL32's function is unknown. While LipL32 is expressed during *L. interrogans* infection, LipL32 is not essential for infection since a *lipL32* transposon mutant was still able to cause acute, lethal infection in hamsters and chronic infection in rats no different from those caused by wild-type *L. interrogans* (166). Native, purified LipL32 stimulates an innate immune response via TLR-2

(13). LipL32 causes interstitial nephritis in kidney proximal tubule cells (176). Inflammation caused by LipL32 is mediated by TLR-2 (101). As a highly conserved outer membrane protein of pathogenic *Leptospira*, LipL32 arose as a potential vaccine target for induction of cross-protective immunity but attempts to vaccinate with various preparations of LipL32 have not had significantly positive results (172).

1.1.6.3 Other Outer Membrane Proteins

While LipL32 is the most abundant outer membrane protein, other outer membrane proteins play a role in *L. interrogans* survival and pathogenesis. The second most abundant outer membrane protein of *L. interrogans* is Loa22 (24). Loa22 is an OmpA-like protein (177). The role of Loa22 is unknown, but it appears to be tied to virulence; a transposon mutant lacking expression of Loa22 was unable to cause fatal disease in guinea pigs and hamsters, but was able to cause renal colonization and bacteremia (177). Complementation restored Loa22 expression and virulence in hamsters and guinea pigs (177). Loa22 was found to be expressed during host infection and surface-exposed (177).

The third most abundant outer membrane protein is LipL41 (24). LipL41 is useful as a control when examining the effects of growth conditions on gene expression and proteins. LipL41 protein and *lipL41*transcript levels are nearly unaffected by osmolarity, temperature, and other varied environmental factors (178-180). LipL41 may have a role as a hemin-binding protein (181), but this role has been disputed (182). King et al. (182) found that neither a *lipL41* nor a *lep* (LipL41 expression partner) mutant was impaired in causing acute leptospirosis in hamsters.

Lig (leptospiral immunoglobulin-like) proteins were identified in *L. interrogans*, including: Lig A, Lig B, and Lig C (183). Lig A and Lig B are likely expressed early in

mammalian host infection and are likely involved in critical bacterial-host interactions (172). Matsunaga et al. (183) confirmed that these Lig proteins are localized to the bacterial surface. Immunoblotting using patient sera identified Lig proteins as major antigens recognized during acute host infection (183). Because patients with acute leptospirosis have a powerful antibody response to Lig proteins, recombinant Lig repeats would likely be useful serodiagnostic antigens in confirming that Lig proteins are indeed expressed during infection (184). Lig A is also released from *L. interrogans* (185). Following osmotic induction of Lig expression, *L. interrogans* adhered more frequently to various extracellular matrix proteins, including: fibrinogen, fibronectin, and collagens I and IV (148). Lig B binds to complement components and Factor H; it also inhibits complement activity (186, 187). The likely role of LigB is to cover the leptospiral surface with various circulating host proteins, disguising the leptospires from the host's defenses (172). Lig A from *L. interrogans* serovar Copenhageni expressed in a lipidated form in *E. coli*, administered orally, and recombinant Lig A, when injected subcutaneously, effectively immunized hamsters (188). The hamsters survived at least 4 weeks after challenge, but still had renal leptospiral colonization and some kidney tissue damage (188). Therefore, Lig A provided the hamsters protection from fatal disease, but did not prevent the carrier state. The immunoprotective region of Lig A is variable, likely limiting cross-protective immunity (189). Lig A also does not provide sterilizing immunity. Immunization of hamsters with Lig A of serovars Manilae (190) or Canicola (172) does not offer protection from severe disease; it is unknown why Lig A seems to protect against challenge by serovar Copenhageni but not other serovars.

1.1.7 The Host Response to *Leptospira interrogans* Infection

L. interrogans inoculation results in either a chronic, mostly asymptomatic infection or an acute, severe disease. Acute leptospirosis is most common in accidental hosts, such as humans, with a wide range of symptoms and outcomes (8). Infection of a carrier host will generally result in chronic infection with minor outward signs of infection (8). The same *L. interrogans* strain can generally cause both acute and chronic infections, depending mostly on the mammal that is infected. Humoral and cell-mediated immunity are needed for protection against *L. interrogans*; simultaneously, immune responses may contribute to tissue damage during infection.

A majority of experimental infection studies have utilized the golden Syrian hamster due to its susceptibility to acute infection (191). Few well studied immunological reagents for use with hamster tissues have limited the characterization of the hamster immune response to *Leptospira* infection. Many well-characterized immunological reagents are available for studying the mouse immune response, but the majority of mouse strains are resistant to acute infection by *L. interrogans* after around 4 weeks of age (192).

L. interrogans expresses few proteins on its outer membrane surface (193). Outer membrane protein expression is likely varied in response to such factors as: iron, temperature, and osmolarity (185, 194, 195). Expression of few antigenic surface proteins gives few targets for recognition of *L. interrogans* and progression of a protective immune response by the host. The outer membranes of *Leptospira* are readily removed by mild detergent from the peptidoglycan layer (193, 196). The attachment of antibody to outer membrane proteins does not inhibit motility, allowing tissue penetration (199).

1.1.7.1 Animal Models

Guinea pigs were initially the preferred animal model for acute leptospirosis but hamsters, with general good health, rapid reproduction, and lower cost are now used more frequently as models for acute disease (198). Hamsters are susceptible to acute infection well into adulthood and mirror acute infections, sharing some commonalities with human clinical disease (199).

Mice and rats are only susceptible to developing acute leptospirosis within a short timeframe after birth (192) unless they have certain genetic deficiencies. Chronic infections limited to colonization of the kidney are common in adult mice and rats. Recently, rats have been used to study chronic leptospirosis (164, 165, 200). Genetically defined strains of mice and rats, along with well-defined immunological tools, are aiding the development of new knowledge on facets of the immune system that are crucial for protective immunity (199). While not a natural route of inoculation, most small mammals used in studies of *L. interrogans* pathogenesis are injected intraperitoneally with the bacteria (198). Three to four days after injection, enough leptospires can be found in the kidney that stained sections can be visually analyzed under a microscope (199). In susceptible hamsters, though, *L. interrogans* is often detected throughout the host, including in the blood (199).

1.1.7.2 Mammalian Host Detection of *L. interrogans*

Pathogen-associated molecular patterns (PAMPs) interact with pattern recognition receptors (PRRs) to initiate a cascade of intracellular signals that provoke the host response to infection (201, 202). Common bacterial PAMPs include: LPS, peptidoglycan, lipoproteins, flagella, and nucleic acids. The host receptors that interact with PAMPs include toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (201, 202). Related receptors that recognize damage associated molecular patterns (DAMPs) include receptors for advanced

glycosylation end products (RAGE) (203). PAMPs and DAMPs are recognized by nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (199).

The most studied PRRs are the toll-like receptors, which often initiate the innate immune response to infection (204). TLRs are transmembrane proteins with an extracellular pattern recognizing domain and a cytoplasmic domain used to transmit the signal from the extracellular domain to the subsequent host response (205). TLR cytoplasmic domains share a protein domain with the interleukin 1 receptor (IL-1R) (199). This common intracellular toll/interleukin 1 receptor (TIR) domain communicates with cytoplasmic proteins to trigger a signaling cascade that causes the host cell to react to the threat initiating the signal (199). The signaling cascade that follows the interaction between a PAMP and a PRR results in activation of many transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (206). Inactive NFκB is found in the cytosol, bound by the inhibitor IκB. After IκB is phosphorylated, it is degraded by proteosomes, releasing NFκB (205). NFκB then migrates to the nucleus where it binds chromosomal DNA at certain sites with help from accessory transcription factors that are also activated by phosphorylation (205). What follows is transcriptional activation of response genes allowing activated cells to react to infection. TNF-α and IL-1 via TRAF6, among other cytokines, can activate NFκB. NFκB activation often leads to induction of expression of inflammatory cytokines and chemokines, and aids in development of the acquired immune response (199).

Leptospiral LPS has low endotoxicity, but does stimulate a strong antibody response during infection (207). It is important to note that leptospiral LPS is predominantly recognized in humans by TLR-1/TLR-2, not TLR-4 (13). In murine cells, leptospiral LPS is

recognized mostly by TLR-2, but also by TLR-4 (12). TLR recognition of leptospiral LPS in other mammalian species is unknown.

Infection of C3H/HeJ mice, which have no functional TLR-4, with *L. interrogans* leads to severe disease and death (89, 208-210). From these studies, it can be deduced that TLR-4 is of major importance in controlling *L. interrogans* infection in mice. TLR-4 deficient mice on the C57BL/6 background infected with *L. interrogans* died, although not as rapidly as TLR-4, TLR-2 double knockout (DKO) mice (168). TLR-2-/- mice did not die from *L. interrogans* infection (168). Chassin et al. (168) found that infected DKO mice had high bacterial burdens in their livers, lungs, and kidneys, but infected TLR-2-/- mice and TLR-4-/- mice had greatly reduced bacterial burdens in comparison in their kidneys and lungs. Infected TLR-4-/- mice had elevated bacterial loads in their livers, while infected TLR-2-/- mice had few to no bacteria detected in their livers (168). Chassin et al. (168) concluded that TLR-2 and TLR-4 have overlapping roles in the kidney, but functional TLR-4 receptors are necessary for bacterial clearance in the liver. The result of few to no leptospires found in the livers of infected TLR-2-/- mice is consistent with data indicating that cytokine expression in murine macrophages involves the recognition of LPS by TLR-4 (89, 168).

NLRs recognize PAMPs and DAMPs from *L. interrogans*. TLR-2, TLR-4 interaction with LPS primes the NLRP3 inflammasome, and the inflammasome is activated by downregulation of the Na/K-ATPase pump by leptospiral glycolipoprotein (211, 212). Leptospiral glycolipoprotein activates peripheral blood mononuclear cells (PBMCs) (213) and is a potential cytotoxic component of the leptospiral outer membrane (214). Persistent inflammasome activation may lead to development of tissue lesions, especially in the kidney (215). Activation of inflammasomes has been associated with various chronic kidney

diseases (215). Specific activation of the NLRP3 inflammasome and induction of IL-18 and IL-1β secretion has been implicated in the development of chronic kidney disease following renal injury in mice (216).

The C-type lectin receptors DC-SIGN and mannose-binding protein have been identified as having potential roles in recognition of *L. interrogans*. Mannose-binding lectin (MBL) is elevated in human serum during leptospirosis, with greater serum levels of MBL detected in patients with the more severe Weil's disease as opposed to moderate disease (217). MBL may therefore be a useful tool to identify severe leptospirosis (217). *L. interrogans* detection by human dendritic cells through DC-SIGN induced the secretion of the pro-inflammatory cytokines TNF-α and IL-12, with limited induction of the anti-inflammatory cytokine, IL-10 (218). The virulent leptospiral strain used by Gaudart et al. (218) induced more TNF-α and IL-12 secretion than an avirulent strain. These data are consistent with other results indicating that elevated TNF-α in serum correlates with poor prognosis in leptospirosis patients (219-221). IL-10 is also elevated in serum during acute leptospirosis (199).

1.1.7.3 Cytokine Response

Chassin et al. (168) found that mRNA levels for pro-inflammatory cytokines were highest in infected TLR-2-TLR-4 DKO mice kidneys and livers in comparison to wild-type C57BL/6 J mice, TLR-2-/-, or TLR-4-/- mice. DKO mice may have more pro-inflammatory cytokines present in their lungs and kidneys due to higher bacterial burden in these tissues versus the other strains of mice used in the study (168). The induction of these pro-inflammatory cytokines is likely via a non-TLR based pathway (168). Infected MyD88-/- and TLR-2-TLR-4 DKO mice harbor similar levels of leptospires 3 days post-injection in their

livers and kidneys, with similarly elevated inflammatory cytokine levels as measured by mRNA analysis (168). The conclusion from these data is that TLRs other than TLR-2 and TLR-4 do not have major roles in sensing *L. interrogans*, or for inducing manufacture of IL-1β, IL-6, and chemokines (168). Unlike other known TLRs, TLR-3 signals via TRIF, not MyD88. Currently, there is no evidence of TLR-3 involvement in sensing *L. interrogans*. Similar to infected MyD88-/- and TLR-2-TLR-4 DKO mice, infected hamsters have high bacterial burdens in kidneys and livers and high transcript levels of inflammatory cytokines 3 days post-injection (222-226).

Key differences were seen when comparing *L. interrogans*-infected golden Syrian hamsters and OF1 outbred mice (224). No OF1 mice died from severe leptospirosis, while the majority of hamsters died following infection (224). Bacterial loads in hamster blood and lungs were consistently elevated over burdens in mouse blood and lungs (224). Between 4 and 5 days post-inoculation, *L. interrogans* was detected significantly less in OF1 mouse livers and kidneys in comparison to burdens observed in hamsters (224). Hamsters had severe histological lesions in their livers from day 1 following inoculation until their death 4-5 days post-injection (p.i.) (224). OF1 mice had few liver lesions from day 3 p.i. until day 15, the end of the experimentation (224). Hamsters had severe renal hemorrhaging beginning at 3 days p.i.; OF1 mice had some interstitial nephritis not observed in hamsters (224). Hamsters experienced alveolar hemorrhaging 2 days p.i., becoming severe at 3 days p.i. and beyond; mice had some moderate lung lesions 5 days p.i., but no lesions were detected in lungs at 15 days p.i. (224). TNF-α, IL-1β, and IL-6 were significantly elevated in mouse blood at 6 hours p.i., which was not the case in hamsters (224). IL-1β and IL-6 were not induced in hamster blood 6 hours p.i., and TNF-α was only slightly induced (224). As mRNA levels in mouse

blood of *IL-1β*, *IL-6*, and *TNF-α* decreased over time p.i., mRNA levels of *IL-1β*, *IL-6*, and *TNF-α* increased over time p.i. in hamster blood until their deaths (224). *IL-1β* was more highly elevated in hamster kidneys than in those of mice (224). *IL-6* and *TNF-α* transcript levels were much more elevated in mouse kidneys 6 hours to 3 days p.i. than in hamster kidneys, with a decline in *IL-6* and *TNF-α* mRNA at 4 days p.i. in the hamster kidneys (224). In hamster lungs, *IL-6* and *TNF-α* transcripts were more highly induced in comparison to levels seen in mouse lungs, which had induction of these transcripts as well, but not to as great of an extent (224).

The anti-inflammatory cytokine IL-10, though elevated, was induced later or present in lower levels in hamsters than in mice (224). In mice, *IL-10* mRNA induction was rapid and higher in the blood, lungs, and kidneys versus in these tissues of hamsters (224). IL-10 is an important regulator of innate immunity, and is grouped with Th2 cytokines; IL-10 can impede production of inflammatory mediators and upregulate expression of other anti-inflammatory compounds (227). Cytokine expression differences may be of great importance in determining outcome following *L. interrogans* infection, as hamsters are susceptible and mice are resistant and have very different cytokine expression profiles during infection. A clinical study on leptospirosis patients found that a low ratio of IL-10/TNF-α was correlated with poor patient outcomes (228). Matsui et al. (224) conclude that IL-10 over-expression may efficiently regulate the pro-inflammatory response with few lesions in mice and regulate B cells and the production of specific antibody, resulting in elimination of *L. interrogans* from mouse tissues.

Other studies note cytokines and chemokines induced at significant levels in both humans and mice during leptospirosis, including: IL-1β, IL-6, IL-10, MCP-1, and TNF-α

(229). Humans with leptospirosis had elevated GM-CSF and MCP-1 in sera (229). GM-CSF promotes granulocyte and monocyte production; MCP-1 recruits white blood cells to sites of inflammation. Human patients with leptospirosis also had increased levels of IL-11; IL-11 aids platelet replenishment due to thrombocytopenia and induction of acute phase molecules (229).

1.1.7.4 Cellular Response to Leptospirosis

Macrophages use PRR-mediated activation to provide innate immune defense to the host. Leptospiral hemolysins and LPS stimulate macrophages to produce TNF-α, IL-6, IL-1β, and interferons (229, 230). Leptospiral LPS triggers macrophages to have enhanced phagocytic activity (230). Phagocytosis leading to decreased leptospiral viability requires opsonization with homologous antibody (231-233).

Little is known about the role of polymorphonuclear leukocytes (PMNs) in protecting the host from *Leptospira* infection. The PMN group includes neutrophils, granulocytes, and eosinophils. Whole *Leptospira*, leptospiral LPS, or peptidoglycan induce PMN adherence to endothelial cells (234, 235). Resistance to PMN phagocytosis, however, has been postulated as a virulence factor for *L. interrogans* (236). Wang et al. (236) found that *L. interrogans* attached to but was not ingested by neutrophils in the presence of 10% normal serum and concluded that leptospiral virulence appears to be linked to their ability to resist serum attack, ingestion, and killing by neutrophils.

Platelets use TLRs to sense PAMPs and produce cytokines and antimicrobial peptides (237). Leptospiral LPS was found to induce platelet aggregation (238). Thrombocytopenia occurs in around half of human leptospirosis patients (239). *Leptospira* may be able to

subvert some of the innate immune response by contributing to platelet removal in the early stages of infection (199).

1.1.7.5 Humoral Immunity

Antibody-mediated immunity against *L. interrogans* plays a key role in providing protection against lethal infection in many mammalian hosts. Antibody to *L. interrogans* is necessary for protective immunity; mice treated with cyclophosphamide, which kills B cells, were susceptible to lethal *L. interrogans* infection (240, 241). Nude mice, which lack T cells but have B cells, were resistant to infection (241). When Rag-/- (no B or T cells), SCID (no B or T cells), μMT (no B cells), or nude mice are injected with cyclophosphamide then infected with *L. interrogans*, they succumb to lethal infection (168, 210, 241, 242). μMT mice treated using passive transfer of immune sera from infected wild-type mice at 20 days p.i. were protected from lethal challenge (168). Maternal antibodies were shown to protect mice from becoming chronic carriers of *L. interrogans* (243). T cells, on the other hand, do not seem to have a critical role in supplying protection from lethal challenge in the mouse model, as CD3-/- (no T cells) mice are resistant to lethal challenge (168).

Leptospiral LPS plays an important role in the development of immune protection in many host species. LPS-specific antibody passively transferred to hamsters, mice, guinea pigs, monkeys, and dogs prior to lethal infectious challenge protected these animals (244-246). Leptospiral LPS is a serovar-specific antigen; antibodies against LPS provide little cross-protection against other serovars and often provide only short-term immunity (199). Leptospiral LipL32 stimulates an early and sustained antibody response during infection (173, 193, 196). LipL32 is cleaved in vitro (173, 178, 196) and it is post-translationally modified (247), which may limit exposure of the protein's antigenic epitopes on the cell

surface (174). Antibodies to LipL32 are positive indicators of infection; however, development of antibody to LipL32 does not seem to be protective (190) and LipL32 is not required for successful infection (166).

Currently, the most successful *Leptospira* vaccines are composed of whole, killed bacteria, indicating that a complex grouping of antigens may be needed for protection (248).

1.1.7.6 Cell-Mediated Response

B cell mediated production of immunoglobulins in response to *L. interrogans* is critical to protective immunity, but both B and T cells have roles in promoting an immune response to the pathogen. Rag-/- mice do not make much IFN-γ in the kidney or liver, indicating that B and/or T cells are necessary for IFN-γ production during infection (168). Chassin et al. (168) found that B cells are likely primarily responsible for IFN-γ production and leptospiral clearance in the liver, whereas T cells are likely responsible for these roles in the kidney. Kidney tissue damage was greater in CD3-/- (no T cells) mice in comparison to wild-type or μMT (no B cells) mice (168). Serological markers of kidney damage were elevated in CD3-/- mice but not in infected wild-type or μMT mice (168). The Th1 response involving T cells is most likely an important component of the immune response to *L. interrogans* infection.
A much greater majority of studies have focused on protection against lethal challenge with *L. interrogans* over focusing on prevention of the chronic carrier state exhibited by rodent hosts. Elimination of chronic leptospirosis in carrier hosts is important so as to reduce the chances of disease transmission to accidental hosts, such as humans.

Studies have shown that exposure to leptospiral antigens causes a proliferative response in PBMCs. There is a preferential expansion of γδ T cells in leptospirosis patient blood samples treated with leptospiral antigens (249, 250). In contrast, Tuero et al. (251) did

not find evidence of a T cell memory response in patient blood following infection. Heat-killed *L. interrogans* induced the production of IFNγ, IL-12p40, and TNF-α in human whole blood, consistent with a prominent Th1 response to infection (252). Thai patients with, or suspected to have, leptospirosis had elevated plasma concentrations of granzyme B, IFNγ-inducible protein-10 (IP-10) and monokine induced by IFN-γ (Mig) over levels found in healthy blood donors, suggestive of cell-mediated immunity involvement in the early host response to leptospirosis (253).

1.1.7.7 Immune Pathology

LPS and potassium efflux trigger high-level activation of inflammasomes resulting in elevated production of IL-1β (254). *Leptospira* have LPS as an outer membrane component and leptospiral glycolipoprotein induces potassium efflux (212). Hyperstimulation of inflammasomes can lead to tissue damage in the lung (255) and kidney (215, 216). Robust production of pro-inflammatory cytokines by *Leptospira*-infected hamsters has been associated with lethal outcomes (223, 224, 226). Human leptospirosis patients with acute disease also have evidence of highly elevated inflammatory cytokines (221, 256). Increased expression of the PMN chemokines CXCL1 (257) and CXCL6 (258) can promote PMN migration, cell activation, and inflammation, the total of which are correlated with acute lung injury (259). Extracellular trap formation may result in release of neutrophil granules and tissue damage (260, 261).

L. interrogans interaction with endothelial cells causes cellular changes that are consistent with disturbance of endothelial barriers, supporting bacterial invasion (262). Production of TGF-β1 likely induces synthesis of type I and type IV collagen, causing acute tubulointerstitial nephritis to develop into tubulointerstitial fibrosis in the kidney if left

untreated (263). Transient expression of IL-1β contributes to tissue damage in acute lung injury and pulmonary fibrosis (264). iNOS production is important for host survival (265), but iNOS activity may also contribute to damage seen during *Leptospira*-induced pulmonary hemorrhage (266, 267). Autoimmunity induced by leptospirosis may contribute to uveitis in humans (268, 269).

Leptospiral infection, without proper treatment, is a constant battle between the host and leptospires, each with tools to attack and defend from the other. The mammalian host has a wide range of inflammatory cytokines and chemokines used to stimulate cells to resist infection and destroy the bacterial threat. *Leptospira interrogans* may trigger a destructive immune response in the host leading to sepsis and possibly death, or less severe chronic tissue damage as *Leptospira* establish persistent infection. Determination of leptospiral evasion techniques and significant antigens is necessary to develop effective and safe vaccines that work to clear *Leptospira* from the host. Future studies may examine genetic determinants of susceptibility, noting that genetic polymorphisms that may predispose humans to severe disease have already been identified (270). These genetic factors may be crucial for determining treatment regimens, especially in regions where people are chronic carriers of endemic *Leptospira* strains (199).

1.2 *Borrelia burgdorferi*

1.2.1 Identification of *Borrelia burgdorferi*

In the mid-1970s, Steere et al. (271) reported on a mysterious syndrome initially indicated to be juvenile rheumatoid arthritis. The authors believed the disease was transmitted by an arthropod vector (271). The condition was named Lyme arthritis after one

of the communities where the disease was prevalent. After the disease was given the name "Lyme arthritis," the quest to determine the pathogenic cause of illness began.

Ixodes ticks collected from Shelter Island, NY were found to have spirochetes present as visualized using dark-field microscopy (272). The organisms were then cultured using Kelly's medium, a medium initially used to cultivate *Borrelia hermsii* (272). Following growth of the spirochetes outside of a host, sera from a patient with Lyme disease reacted with the bacteria (272). Soon after, spirochetes were isolated from patients with Lyme disease (273, 274), followed by isolation of the bacteria from the skin of patients with the "bull's-eye" rash, erythema migrans (275).

Spirochetes were isolated from mammals other than humans. Bosler et al. (276) isolated spirochetes from the blood of white-footed mice inhabiting the same areas of Shelter Island, NY as the ticks collected in previous years found to harbor *Borrelia*. *Peromyscus leucopus*, the white-footed mouse, was found to be the main reservoir for these spirochetes in the northeastern United States. The mice were later found to be carrier hosts along the west coast (284) and northern United States (277) by Burgdorfer et al. (284, 277). Deer can become infected with *Borrelia*, but ticks feeding on deer very rarely acquire *Borrelia* from the bloodmeal, and therefore are unlikely to transmit *Borrelia* from deer to another host (278). Dogs on Long Island, NY with arthritis and tick-infestations were found to harbor *Borrelia*, which was cultured from their blood (279).

Transmission of *Borrelia* from infected rabbits to ticks and from infected ticks to rabbits was confirmed by Benach et al. (280) and Ribeiro et al. (281). Both studies found that the *Borrelia* preferentially inhabited the midgut until late-stage feeding, when the *Borrelia* were found in the saliva to be transmitted to a new host (281).

Bacteria isolated from *Ixodes* ticks collected on Shelter Island, NY were characterized by Johnson et al. (282). They determined these bacterial agents of Lyme disease had a guanine-plus-cytosine content of 29-30.5%, in line with other *Borrelia* species but different from other spirochetes, such as *Leptospira* and *Treponema* (282). The *Borrelia* were helical in shape, with dimensions of 0.18-0.25 by 4-30 μM (282). The authors noted rotational and translational motility, with an average of 7 periplasmic flagella located at the ends of the cell (282). The *Borrelia* are Gram-negative, with a multi-layered outer membrane covering the protoplasmic cylinder, which contains the peptidoglycan layer, cytoplasmic membrane, and enclosed cytoplasmic components (282). Barbour et al. (283) found the *Borrelia* to optimally grow at 34-37°C, with a generation time of 11-12 hours at 35°C in microaerophilic conditions. Unlike *Leptospira*, *Borrelia* will use glucose as a carbon source (282). This was the first publication to name the Lyme disease agent isolated from ticks found on Shelter Island, NY as *Borrelia burgdorferi* (282). More specifically, this species is known as *Borrelia burgdorferi* sensu stricto type strain B31, which was utilized by our laboratory for experiments.

1.2.2 *Borrelia burgdorferi* Genetics and Genomics

Borrelia burgdorferi strain B31 has a single linear chromosome of about 900 kbp in length, with 12 linear and 9 circular plasmids totaling about 612 kpb (285). The large chromosome harbors most genes encoding metabolic enzymes, and the smaller plasmids have genes encoding the majority of surface lipoproteins (285). While circular plasmid 26 (cp26) is required for growth in culture, the other small plasmids are dispensable (286, 287). These other small plasmids, though, have been found to be required for mouse infectivity or tick transmittability in laboratory studies (288-295). The small linear plasmid genes are not

compact, whereas the linear chromosome's genes are very dense; the small linear plasmids also appear to have a multitude of decaying pseudogenes (296, 297). In *B. burgdorferi*, the large chromosome appears evolutionarily stable, encoding products necessary for existence as a living cell; the many evolutionarily diverse plasmids encode the majority of proteins that interact with varied host environments, from vertebrates to arthropods (285).

Fraser et al. (47) published the complete genomic sequence of *Borrelia burgdorferi* strain B31 in *Nature* in 1997. The large linear chromosome was found to be 910,725 base pairs in length, with 28.6% guanine-plus-cytosine composition (47). *B. burgdorferi* has among the lowest percentage of mutationally inactivated chromosomal genes among described bacterial genomes (47). The genome of *B. burgdorferi* is around one-third the size of the genome of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. This is likely because *B. burgdorferi* does not exist freely in the environment like *L. interrogans* does; *B. burgdorferi* only exists in nature when associated with an exploitable host. Protein-encoding genes make up about 93% of the large chromosome (47). The chromosome has five rRNA genes: two 23S, one 16S, and two 5S grouped close to the middle of the chromosome, a tmRNA gene, and 31 tRNA genes (47). The tRNA genes are predicted to contain tRNAs specific for the twenty amino acids, and they are scattered along the large chromosome in 13 single genes and 7 clusters (47, 298).

Nearly sixty percent of the *B. burgdorferi* strain B31 chromosome's predicted genes have moderate similarity to a gene in another microbe which has a function or role that is somewhat understood (47). Ten percent of the predicted genes are similar to described genes in other organisms that have unknown roles; around 30 percent of predicted genes are unique to *Borrelia* and do not have known functions (47). The large chromosome appears to have

few genes necessary for replication and cell wall maintenance (285). It does have genes for cell wall biosynthesis, but not for phospholipids or lipopolysaccharide (LPS) (47). *B. burgdorferi* is a Gram-negative organism that lacks LPS, unlike *L. interrogans*. The chromosome contains genes for: DNA, RNA, and protein biosynthesis, protein secretion and lipidation, DNA repair, nucleotide metabolism (excluding *de novo* synthesis), glycolysis, and some enzymes that supply substrates for the glycolytic pathway, which is *B. burgdorferi*'s only method for generating ATP (47, 285). The complete set of genes known to be necessary for motility and chemotaxis are in the chromosome, as are many genes for transport of small molecules across the cell membrane (47). Therefore, the biosynthetic and intermediary metabolic capacity of *B. burgdorferi* is quite limited (285).

B. burgdorferi is a fastidious organism, limiting itself to either a vertebrate or arthropod host. *B. burgdorferi* nearly entirely lacks genes encoding enzymes important for: respiration, nucleotide synthesis, amino acid synthesis, lipid synthesis, and enzyme cofactor synthesis (285). Unlike *L. interrogans*, which utilizes iron, *B. burgdorferi* has no known need for iron nor for iron transporter genes (299, 300). For example, *B. burgdorferi* utilizes zinc as a metal cofactor, not iron, in peptide deformylase (300). *Borrelia* scavenge for nearly all their amino acids, nucleosides, cofactors, and lipids from the host (285). Solely two alternative sigma factors and two two-component systems have been determined by sequence homology (47, 285).

Borrelia burgdorferi species have many plasmids, linear and circular. *B. burgdorferi* strain B31 plasmids have one to two copies per chromosome (301, 302). They are named "lp" for linear plasmid and "cp" for circular plasmid, followed by a number used to indicate approximate size in kilobase pairs (kbp) by agarose gel electrophoresis. The plasmids are

mostly unrelated to known genes of other bacteria; about 6% of the linear plasmid genes are homologs of genes harbored by other microbes (285).

The 12 linear plasmids of *B. burgdorferi* strain B31 range in size from 5 to 54 kbp. Linear plasmid genes not involved in plasmid partition or replication have no homologs outside other *Borrelia* (285). Lp25 and lp56 are involved in restriction modification (303). Lp25 encodes PncA, which has nicotinamidase activity and is required for virulence in mice (304). Lp54 contains the gene *thyX*, which encodes thymidylate synthase in *B. burgdorferi*, but unlike its ortholog in *Borrelia hermsii*, it has weak to no activity in pyrimidine biosynthesis (305). Lp36 contains the gene *bbk17* (*adeC*), which encodes an adenine deaminase; an lp36- mutant retains infectivity in the tick, but is unable to infect mice (306). Lp54 also contains the gene *cspA*, encoding BbCRASP-1, which binds factor H and FHL-1, likely key to the complement resistance of *B. burgdorferi* (307). Lp28-3 harbors the gene *csp2*, which encodes BbCRASP-2, found to be well-conserved across Lyme disease-causing *Borrelia* and of high value in serological analysis (308, 309). Several linear plasmid genes encode surface lipoproteins, which will be discussed later.

Nine circular plasmids are frequently associated with *Borrelia burgdorferi* strain B31. Cp26 is the only plasmid shown to be necessary for growth in culture (310, 311). All *B. burgdorferi* strains possess cp26 (310). Cp26 encodes *ospC*, critical for mammalian infection (311-315). Cp26 encodes genes for chitobiose import (316), host integrin binding (317), oligopeptide import (318), telomere resolvase (285, 319), and proteins linked to GMP synthesis (320). Cp9 is rapidly lost after a few passages in culture and is likely not required for mouse infectivity (289, 321-322). There are 7 cp32 plasmids that are likely prophages (47). The cp32 plasmids harbor several genes with no homology to genes outside of the

Borrelia genus; they encode many surface expressed proteins, of which some bind factor H in the host and potentially act as adhesions (323-327). Examined *Borrelia burgdorferi* strains all have multiple versions of their cp32 plasmids, suggesting that together they have important functions (328-329). All 7 cp32 plasmids are highly similar, often with 99% sequence identity (287, 297).

1.2.3 Gene Regulation and Proteomics

Borrelia burgdorferi modifies expression of genes in an orchestrated manner to adapt to different host environments. As an *Ixodes* tick takes its bloodmeal, *B. burgdorferi* rapidly upregulate *ospC* as they travel from the tick midgut to the salivary glands to then be released into the mammalian host (313). Inactivation of *ospC* results in inability of the spirochetes to invade tick salivary glands; complementation restores *B. burgdorferi*'s ability to invade tick salivary glands (313). Once inside the mammalian host, *Borrelia* down-regulate *ospC* (330). Grimm et al. (330) found that OspC- mutants are unable to infect mice; complementation restores infectivity.

Borrelia burgdorferi lacking RpoS are avirulent in mice; complementation does restore virulence (331). Microarray analyses by Caimano et al. (332) demonstrated that mammalian host-specific signals are required for RpoS-mediated repression. Using dialysis membrane chambers, much of the *in vivo* RpoS regulon was specifically upregulated, supporting the role of host-derived environmental stimuli for varied gene expression in *B. burgdorferi* (332). Expression of *rpoS*, as determined using qRT-PCR, is induced during the bloodmeal of the nymphal tick but not within engorged larvae or unfed nymphs (332). Caimano et al. (332) propose that RpoS functions as a gatekeeper for the correlative

regulation of genes involved in the preservation of spirochetes within the tick vector and in the establishment of infection within the mammalian host.

Sigma 54 (σ54), also known as RpoN, was found to be required for vector transmission and mammalian infection with *B. burgdorferi*, but not for colonization of the tick vector (333). Microarray analyses of a σ54 mutant indicated that σ54 regulates 305 genes in *B. burgdorferi* (333). Sigma 54 deficient *B. burgdorferi* were unable to infect mammals, but complementation restored infectivity (333). Sigma 54 mutants did not migrate to the salivary glands during tick feeding; complementation restored entry of the spirochetes into tick salivary glands (333).

Yang et al. (334) generated a point mutation in *rrp2* of *B. burgdorferi* to assess the role of the gene. Following mutation of *rrp2*, OspC expression was lost, as verified by immunoblot, SDS/PAGE, and mRNA analysis (334). Using sequence analysis, Rrp2 is likely a bacterial enhancer-binding protein, important for RpoN-dependent gene activation that then influences expression of the alternative sigma factor, σ^s (334). The authors determined that Rrp2 modulates expression of OspC through σ^s, along with other lipoproteins, including DbpA and Mlp8 (334). Therefore, Yang et al. (334) conclude that Rrp2 is required for the expression of many *B. burgdorferi* outer membrane proteins (334).

Lp28-1 contains the *vls* antigenic variation locus, important for persistence in the host, as described by Bankhead and Chaconas (335). Deletion of *vlsE* and silent *vls* cassettes was achieved by insertion of a replicated telomere in lp28-1 (335). C3H/HeN-immunocompetent mice were injected with mutant, wild-type, or complemented-mutant *B. burgdorferi* (335). The deletion of *vls* resulted in a loss of persistence in the mice by *B. burgdorferi* (335). The *vls* locus of lp28-1 is the only encoded determinant necessary for

switching at *vlsE* and for persistent mouse infection (335). Bankhead and Chaconas (335) also proposed that *B. burgdorferi* differs from many other pathogens that have recombinatorial antigenic variation because its VlsE is unlikely to mask other surface antigens.

Lp54 harbors dbpBA, encoding the decorin-binding proteins DbpA and DbpB. These proteins bind glycosaminoglycans and decorin, which are important components of proteoglycans found in: connective tissues, the extracellular matrix (ECM), and on mammalian cell surfaces (336). Being an extracellular pathogen, *B. burgdorferi* frequently comes into contact with these tissues and surfaces during mammalian infection. Shi et al. (336) deleted the *dbpBA* locus, then complemented with the gene *dbpA*, *dbpB*, or both. BALB/c mice were inoculated with mutants, complemented-mutants, and wild-type *B. burgdorferi* (336). After 28 days, joints, skin, and heart samples were cultured. DbpA and DbpB were found to be pivotal for *B. burgdorferi* virulence, but not critical for mammalian infection (336).

Another DbpBA study by Weening et al. (337) expanded on the study by Shi et al. (336). Weening et al. (337) also deleted *dbpBA* in *B. burgdorferi*. C3H mice were infected with *dbpBA*-deleted *B. burgdorferi*, wild-type *B. burgdorferi*, or complemented-*dbpBA B. burgdorferi* (337). Deletion of *dbpBA* resulted in fewer *B. burgdorferi* isolates cultured from examined tissues (337). Differing from the Shi et al. (336) study, Weening et al. (337) concluded that DbpBA is important for *B. burgdorferi* infectivity in the mammalian host.

Whereas OspA is not expressed by *B. burgdorferi* in a mammalian host, it is the dominant lipoprotein expressed by the spirochetes in the tick vector. Adherence and colonization of *B. burgdorferi* to *Ixodes* midgut tissue is mediated mostly, if not only, by

OspA (338). OspA/B- mutants are unable to survive in *Ixodes* midguts, implying that adherence to tick midgut cells is vital to some aspect of *B. burgdorferi* survival (338).

Several genes are involved in the persistence of *B. burgdorferi* within ticks. The lp25-encoded gene *bptA* (formerly *bbe16*), so named "bpt" as an abbreviation for "borrelial persistence in ticks," was examined by Revel et al. (291). BptA was found to be widely conserved among *B. burgdorferi* strains, implicating BptA as a persistence factore used by the spirochetes in nature (291).

Li et al. (339) assessed the role of a Dps homolog encoded by *B. burgdorferi*, BB0690. Dps-deficient *B. burgdorferi* showed no defect in colonizing mice or ticks (339). However, four months after larval ticks acquired Dps-deficient *B. burgdorferi*, the then nymphs failed to transmit the mutants to mice when the nymphs were allowed to feed (339). Complementation of *dps* restored the ability of nymphs to transmit the *Borrelia* to mice (339). Li et al. (339) concluded that BB0690 is required for *B. burgdorferi* to persist in the tick midgut.

In a similar manner, Pal et al. (340) determined the role of BB0365 encoded by *B. burgdorferi*. Pal et al. (340) found by microarray analyses that *bb0365* transcripts increased the greatest of transcripts examined as *B. burgdorferi* entered *Ixodes* ticks. From this data, Pal et al. (340) sought to further assess the role of BB0365 in *B. burgdorferi* survival in the mouse-tick infection cycle. At only two weeks following inoculation with *bb0365- B. burgdorferi* mutants, feeding nymphs had significantly less bacteria present than in feeding ticks inoculated with wild-type *B. burgdorferi* (340). Like the conclusion of Li et al. (339), Pal et al. (340) found BB0365 to have a key role in *B. burgdorferi* persistence in the tick.

Early work on differential gene expression by *B. burgdorferi* in various environmental conditions led to the discovery of many genes that are upregulated or downregulated based on cues. The important work by Schwan et al. (341) found that OspA is abundantly produced by *B. burgdorferi* when in unfed ticks and in culture. The gene *ospC*, not *ospA* is upregulated by *B. burgdorferi* upon mammalian infection (341). OspC is produced by *B. burgdorferi* at 32-37°C but not at 24°C; 24°C would mimic the internal temperature of unfed ticks (341). With temperature increase, proteins such as OspE, OspF, Elp proteins, and several proteins encoded by lp54 are upregulated by *B. burgdorferi* (342-345). Changes in pH also alter the expression of several proteins, notably OspC, RevA, OspA, OspF, Mlp8, and RpoS (346-349). Certain proteins are only expressed during the mammalian phase of infection, including: EppA, Bbk2.10 (OspF homolog), P21, and pG (321, 350-352).

Microarray analyses have been crucial to understanding how *B. burgdorferi* modifies gene expression based on environmental conditions. The whole proteome of *B. burgdorferi* experiences dramatic changes during transmission of bacteria from the tick to a mammalian host. RNA and protein analyses are remarkably correlative, indicating that control of protein expression in varied environments is regulated mostly at transcription.

1.2.4 *Borrelia burgdorferi* Metabolism and Physiology

B. burgdorferi has a small genome with limited metabolic capabilities, making the organism very dependent on its arthropod vector or vertebrate host for survival. *B. burgdorferi* captures nucleotides, amino acids, and fatty acids from its host, conserving energy for synthesis of macromolecular components and to fuel motility, transport, and cell division (353).

As detailed by Fraser et al. (47), the genome of *B. burgdorferi* lacks genes encoding enzymes for amino acid, fatty acid, and *de novo* nucleotide biosynthesis. *Borrelia burgdorferi* also lacks genes encoding components of the TCA cycle, such as succinate dehydrogenase, aconitase, and fumarase; the genome lacks encoded genes for respiratory chain components, such as cytochromes, and lipopolysaccharide synthesis (47).

While *B. burgdorferi* lacks certain genes, it does have others. *B. burgdorferi* is a homofermentative organism that degrades few sugars, such as glucose and glucosamine, through the Embden-Myerhof pathway to lactate as the lone electron acceptor (353). Three carbon compounds such as glycerol-3-phosphate and glycerol, can possibly be used to supplement metabolism, in addition to lipid and lipoprotein biosynthetic pathways (299, 354).

Because *B. burgdorferi* are unable to perform *de novo* synthesis of nucleotides, amino acids, transport of these materials is essential for survival of *Borrelia* (47). Two outer membrane porins have been uncovered in the outer leaflet of *B. burgdorferi*. Oms28 is a 28-kDa outer membrane protein with a small, single channel (0.64nm) that could allow passage of low molecular weight molecules and ions (355), but this role has been disputed (356). Oms66 (P66) is a 66-kDa outer membrane protein with a solo channel conductance able to accommodate much larger solutes than Oms28 (357). P66 has also been shown to bind integrin $\alpha_3\beta_3$ (358). Deletion of *p66* abolished integrin binding activity, indicating that P66 likely functions not only as a porin, but as an integrin binding protein (359). Little else is known about solute transport across the outer membrane by *B. burgdorferi*.

B. burgdorferi's genome harbors very few predicted metal transporters (47). *B. burgdorferi* can transport detectable quantities of Zn^{2+} and Mn^{2+}, but does not accumulate Fe

(299). BmtA has been noted as a Mn specific transporter for *B. burgdorferi* (360). Zn^{2+} serves as a cofactor for necessary metalloproteins that are Fe^{2+}-dependent proteins in other prokaryotes (353). BosR, a ferric uptake homolog (361-363), and peptide deformylase (Pdf) (300) both depend on Zn^{2+} for DNA-binding activity (BosR) and enzymatic function (Pdf).

Biological targets for reactive oxygen species (ROS) are: DNA, RNA, lipids, and proteins (364). Boylan et al. (365) found that *B. burgdorferi* membrane lipids took the brunt of ROS damage, not DNA. When *B. burgdorferi* were grown in the presence of 5 mM hydrogen peroxide (H_2O_2), no effect on the DNA mutation rate was observed; when *B. burgdorferi* cells were exposed to 10 mM t-butyl hydroperoxide or 10 mM H_2O_2, there was no significant increase in DNA damage (365). The lack of damage could indicate that *B. burgdorferi* DNA is not exposed to the same oxidative threat from ROS as is *E. coli* DNA (353).

1.2.5 Pathology

Motility is a key factor enabling spread of *B. burgdorferi* from the initial site of inoculation (16). Videomicrography studies have shown the movement of *B. burgdorferi* within colonized mouse tissue and the interaction between the vascular endothelium and *B. burgdorferi* (366, 367). Translational speeds of up to 4 μm/s were observed (366, 367). The video observations document the critical role of motility in the spread of *B. burgdorferi* from a site of inoculation, as occurs during the erythema migrans stage of pathogenesis.

Previously, *B. burgdorferi* was found to bind endothelial cells and migrate through intercellular junctions or through endothelial cells (368-372). Spirochetes were frequently seen swimming up and down blood vessel surfaces, indicating an ability to travel against blood flow (366). Extravasation of the blood vessel wall was seen, requiring on average 10.8

minutes (366). BBK32, a fibronectin and glycosoaminoglycan binding protein, was shown to be important for adherence in the interactions of *B. burgdorferi* with vascular endothelial cells, and therefore, dissemination (367).

Fibronectin is around 440 kDa in size; it is a complex glycoprotein found in plasma and in the extracellular matrix (ECM), where it interacts with heparin, gelatin, collagen, integrins and other plasma and tissue components (373). Szczepanski et al. (371) found that antibody directed against fibronectin inhibited *B. burgdorferi* from adhering to the ECM. *B. burgdorferi* may encode more than one fibronectin binding protein (374), but BBK32 is the most studied fibronectin binding protein. BBK32 is a 47-kDa, surface-exposed lipoprotein encoded on lp36 (375). BBK32 mediates *B. burgdorferi* binding to GAGs through direct binding of heparin (376).

Infection with *B. burgdorferi* induces production of several matrix metalloproteinases (MMPs), including: MMP-1, MMP-3, MMP-9, MMP-13, and MMP-19 from various host cells: keratinocytes, fibroblasts, astrocytes, PBMCs, and chondrocytes in culture (377-381). MMP-9 has been shown to be upregulated in erythema migrans skin lesions from infected persons (381). MMPs have been linked with bone and cartilage degradation in other forms of arthritis besides Lyme arthritis (373). Synovial fluid of patients with Lyme arthritis, as well as cultured chondrocytes incubated with *B. burgdorferi*, have elevated levels of MMP-1 and MMP-3, MMP-13, and MMP-19 (379, 382). MMP-1 and MMP-13 are collagenases and MMP-3 and MMP-19 are stromelysins (373). While MMP-1, MMP-8, and MMP-13 are upregulated in human Lyme arthritis, they are not induced in the joints of C3H/HeN, Balb/c, or C57BL/6 mice infected with *B. burgdorferi* (377, 383).

1.2.5.1 Immune Evasion Strategies

Zhang et al. (384) reported on a 10-kb locus of lp28-1 in *B. burgdorferi* strain B31 that included a gene which expressed a 35-kDa surface lipoprotein (*vlsE*) and a group of 15 silent cassettes (*vls2*-*vls16*) with high sequence similarity to the central cassette region of the expression site. The gene *vlsE* experiences random, segmental gene conversion in which segments of any of the silent cassettes can be alternated for the current sequence within the cassette region of the expression site (385, 386). Recombination events can be determined as rapidly as 4 days post inoculation in mice and by 4 weeks after inoculation, each clone recovered from mice has a different sequence with around 8-13 separate recombination events (386). These events have not been detected when growing *B. burgdorferi* in culture (386), nor in infected ticks (387) pointing to yet unknown environmental signals in mammals that influence the *vls* antigenic variation system.

Active recombination of *vlsE* is likely required for *B. burgdorferi* evasion of host adaptive immunity, probably by altering variable region epitopes that are targets of host antibodies (373). VlsE has no known function other than immune evasion.

VlsE is a strongly antigenic protein. Lyme disease patients with disease beyond erythema migrans express anti-VlsE antibodies that can be easily detected by ELISA or immunoblot (388-390). IR6 (C6 peptide), a 23-amino acid segment that forms an α-helix, is part of an invariant region within VlsE; IR6 reacts strongly with sera of humans and animals infected with *B. burgdorferi* (391-393). VlsE and IR6 are utilized in diagnostic tests shown to have sensitivity and specificity comparable to the standard two-step testing system, which utilizes ELISA and immunoblot analyses with whole cell *Borrelia* proteins (388, 390).

Borrelia often display complement resistance. *B. burgdorferi* proteins that bind factor H include: factor H-like protein (FHL-1) and/or FH-related protein 1 (FHR-1) (394, 395).

Borrelia proteins that bind factor H-related proteins are grouped together and referred to as complement regulator-acquiring surface proteins (CRASPs). CRASPs are surface expressed, proposed to bind factor H-related proteins, and therby inhibit accumulation of active C3b on *Borrelia*'s surface. With less C3b on their surface, *B. burgdorferi* can likely evade opsonization and formation of the complement membrane attack complex (373).

B. burgdorferi can survive at least one year in many tissues of infected mice (386, 396). *B. burgdorferi* use their motility to penetrate deeply into connective tissue of the dermis and tendons (397, 398). *B. burgdorferi* has been detected binding to type I collagen (399). *Borrelia* may be inaccessible to antibody by residing in protective niches. These protective niches could shield *B. burgdorferi* against eradication by antimicrobials (400, 401).

1.2.6 Rodent Animal Models

As a natural reservoir host in North America, an early model and current model for Lyme borreliosis is *Peromyscus leucopus*, the white-footed mouse. These mice are highly susceptible to *B. burgdorferi* inoculation by the tick, which transmits bacteria in 48 to 72 hours following attachment (402). White-footed mice do not contract *B. burgdorferi* transplacentally from their mothers (403). Ear tissue can be sampled as a non-invasive way to determine infection status (404). *Peromyscus* have high levels of *B. burgdorferi* in the bladder if infected, which is why bladders are often tested to confirm disseminated infection in various animal model experiments (405, 406). Transfer of *B. burgdorferi* from a mammalian species to feeding ticks was first examined in this mouse model (407). Infant feeding *Peromyscus* mice infected with *B. burgdorferi* develop arthritis and carditis (408), while adult *Peromyscus* mice have no evidence of pathology (409).

1.2.6.1 Laboratory Mouse Models

In immunocompetent mice infected with *B. burgdorferi*, there are two phases of infection: pre-immune phase and immune phase (410). Prior to specific antibody production, *B. burgdorferi* can evade innate responses by the host, reproduce, and spread throughout the body, through tissues and blood. *Borrelia* in joint and heart tissue lead to an acute inflammatory response, but in other tissues, the response is minimal to non-existent (411).

Specific antibodies produced by the mammalian host clear most *B. burgdorferi* from the body, with resolution of acute inflammatory damage within the heart and joints (410). *B. burgdorferi* is cleared from most heart tissue and the synovium, but they remain within collagen of connective tissue of several areas, including: the aortic wall, tendons, ligaments, dermis, and joint capsules (396, 397, 411-414). Several months following inoculation with *B. burgdorferi*, mice endure sporadic exacerbations of acute carditis, acute oligoarticular arthritis, and bacteremia that follows a spike in spirochetes into the synovium (410). Also noted during this later stage of infection are: perineuritis, random foci of segmental perivasculitis, and additional chronic lesions (396, 411, 415).

Joint disease in mice is most evident at 2-3 weeks post inoculation (410). There is visible swelling of the tibiotarsal joint, exacerbated in immunodeficient mice (410). This visible swelling of joints, specifically ankle joints, is due to periarticular oedema (410). Oedema appears in the subcutis, with minor infiltration of macrophages and neutrophils in response to numerous *B. burgdorferi* in the subcutaneous tissue (396). Often found in infected mice is inflammation of attachment sites of tendons and ligaments to bone

(enthesopathy) correlated with *B. burgdorferi* in collagen fibers (410). Patellar ligaments, the tibial crest, and Achilles tendon are common sites for enthesopathy (410).

Carditis as a result of *B. burgdorferi* infection has been examined using susceptible C3H/He (C3H) mice. *Borrelia* were first detected in mouse heart tissue 7 days post inoculation, coinciding with the onset of inflammation (411). Macrophages dominate the inflammatory response to *B. burgdorferi* in cardiac tissues (416). *Borrelia* are found in all areas of the heart during the pre-immune stage of infection, but as the host response matures, *Borrelia* are predominantly cleared from heart base tissue and myocardium, while persisting in the aortic wall found at the base of the heart (397, 412, 417).

All tested outbred and inbred laboratory mouse strains have been found to be susceptible to *B. burgdorferi* infection (410). The C3H mouse has a disease-susceptible genotype and is most commonly used to study genetic susceptibility to development of Lyme arthritis, whereas BALB/c or C57BL/6 (B6) mice are the resistant genotype models most frequently utilized (410). Genetic resistance of BALB/c mice to *B. burgdorferi* pathogenesis can be overcome by dose, whereas B6 mice are resistant to disease at all doses (418, 419). B6 and C3H mice were found to have similar levels of spirochetes in joint tissues, but C3H mice had severe disease while B6 mice did not (420). Therefore, Lyme disease in the mouse model is influenced by varied and complex genetic control (410).

1.2.7 Human Lyme Disease

The incidence of Lyme disease has risen more than 30-fold since its identification in the 1970s and the disease is now the most common arthropod-borne infection in both Europe and the United States (421). *B. burgdorferi* sensu stricto is the sole species proven to be pathogenic for humans in North America (422). *Borrelia* have not been shown to produce

toxins; therefore, inflammatory disease likely results from the host's immune responses to the spirochetes. In North America, erythema migrans (EM) is the pathognomonic skin lesion that results from a local inflammatory response to *B. burgdorferi* in dermal tissue (422).

Lyme disease cases are most frequently reported among individuals residing in the northeast, north-central, and mid-Atlantic regions of the United States (423). Connecticut has the highest incidence of Lyme disease, while New York has the highest number of total cases (424). Lyme disease most frequently affects children 5-9 years of age and adults 55-59 years old (423). The majority of Lyme disease cases occur in the summer months when nymphs feed and humans are engaging in outdoor activities (423). Risk is relative to duration of time spent outdoors near or in tick-infested woods (421, 425).

1.2.7.1 Inoculation, Dissemination, and Disease

The progression to Lyme disease begins with a tick bite. The inoculum of spirochetes released by the tick has not been precisely measured, but estimation using qPCR analysis of *B. burgdorferi* DNA gave values of around 20 spirochetes per tick salivary gland at peak infectivity (426). Similar burden estimates were determined via immunofluorescence analysis (427). In mice, if the inoculation site is removed within the first few days of tick detachment, dissemination does not occur (428). The spirochete inoculum is antigenically heterogenous, with some *Borrelia* more likely to establish infection than others (427).

Within the first few days after inoculation, *B. burgdorferi* begin to replicate, reaching high enough numbers to detect via culture and by PCR (429-432). Light microscopy can be used to visualize *B. burgdorferi* in the superficial dermis, in contact with collagen and occasionally in the walls and lumens of blood vessels (433, 434). *Borrelia* then travel away from the feeding site and towards the dermal microvasculature in their journey to disseminate

through the bloodstream (435). The spirochetes trigger cell signaling, resulting in the accumulation of dendritic cells and macrophages, along with other circulating immune cells possessing skin-homing abilities (436). What follows is a local inflammatory response, where *B. burgdorferi*-specific T-cells are primed in draining lymph nodes, enter the bloodstream, and travel back to the inflamed site (422). Erythema migrans is the outwardly-visible manifestation of this inflammatory response. Unlike humans, mice do not develop EM (437), but rabbits (438, 439) and non-human primates (440) do develop EM.

B. burgdorferi has been known to disseminate through the host's bloodstream since it was first isolated (273, 274). Initially, it was believed that few EM patients became spirochaetaemic (431, 441-443). Later, spirochaetaemia was shown to occur in nearly 50% of EM patients but that the concentration of *Borrelia* in the blood, about 0.1 organism per ml of blood, was low (444). Bloodstream dissemination was not correlated with size or duration of erythema migrans, implying that spirochaetaemia is mainly dependent on borrelial, not host, immunological factors (445).

Carditis was documented in about 5% of Lyme disease patients prior to the widespread use of antibiotics for the condition (446). More recently, incidence rates of under 1% have been reported in the United States (425). While most laboratory mice have cardiac involvement (447), *B. burgdorferi* does not have a dominant tropism for human cardiac tissue (422).

In both mice and humans, *B. burgdorferi* do have a strong tropism for joints (448). Of 55 patients with EM who did not receive treatment over a 6 year timespan, 28 experienced intermittent episodes of oligoarticular and monoarticular arthritis, generally in large joints, with 6 patients developing chronic arthritis in one or more large joints (448). These

spirochetes are difficult to isolate from synovial fluid, though (449). *B. burgdorferi* have been detected by PCR in many untreated arthritis patients, providing evidence that joint inflammation may result from bacterial infection (450, 451). Some patients have arthritis symptoms after completion of antibiotic therapy. It is hypothesized that certain patients with a genetic predisposition develop an autoimmune response following *B. burgdorferi* infection and clearance, as confirmed by a negative PCR test, resulting in chronic arthritis symptoms (449). Patients testing positive by PCR for *B. burgdorferi* are likely having a persistent infection, requiring additional antibiotic therapy (452).

1.2.7.1.1 Skin Disorders

Erythema migrans is the most common manifestation of *B. burgdorferi* infection in the USA and in Europe (443, 453-455). Most patients present with a primary EM that develops at the site of inoculation within 7-14 days after tick detachment (456, 457). While a primary EM can be located anywhere on the body, in adults, it is most often found below the waist (458, 459). The Centers for Disease Control and Prevention (CDC) designated 5 cm as the minimum EM lesion diameter necessary for clinical diagnosis of Lyme disease, as to distinguish from other arthropod or insect bites (422). EM lesions can be under this diameter, though, leading to misdiagnoses. An EM is defined as an annular, expanding, erythematous skin lesion with central clearing, giving it the look of a "bull's eye" rash (446). The central clearing, though, is not very common in North American cases (458, 459). Rapid expansion, around 20 cm^2 per day, is a central characteristic of EM and is thought to mirror the speed at which *B. burgdorferi* are moving away from the tick bite location, being followed by the host's local inflammatory response (460). EM-associated symptoms vary from none to mild to moderate non-specific symptoms, including: malaise, fatigue, arthralgia, headache, chills,

and low-grade fever (455, 458, 459, 461). Even without an EM lesion, patients can be spirochaetaemic with low-grade fever, malaise, and arthralgias (456, 462). The most common physical finding correlated with EM in both North America and Europe is regional lymphadenopathy (422).

EM lesional infiltrates differ from those cells found in peripheral blood. The EM infiltrates consisted of more dendritic cells, T-cells, and monocytes/macrophages, with fewer neutrophils, few plasma cells, and minimal, if any, B-cells (436). The EM macrophages and dendritic cells had much greater expression of surface activation markers than those found in blood, as well as increased expression of TLR-1, TLR-2, and TLR-4 (436). CD4:CD8 cutaneous T-cell ratios were found to be comparable to those in peripheral blood, but T-cells found in the area of the EM mostly consisted of memory and memory-effector types of CD4+ T cells and effector subsets of CD8+ T-cells, in contrast to mostly naïve T-cells in circulating blood (436). Over 80% of T-cells from EM lesions expressed CXR3 and/or CCR5, indicative of a heightened Th1 response, which was verified by elevated IFN-γ found in cutaneous interstitial fluid (436).

IFN-γ was found to be the dominant cytokine made by peripheral blood leukocytes stimulated with *B. burgdorferi* lysates; PBMCs used in the study were drawn from patients with EM lesions found culture-positive for *Borrelia* (463). Jones et al. (464) found elevated transcript levels of IFN-γ and Th1-related chemokines in RNA extracted from patient EM lesions. Even though a strong Th1 response has been correlated with chronic inflammation in Lyme disease (465, 466), data from both humans (467) and murine models (468-470) has shown early IFN-γ to be beneficial to the mammalian host.

1.2.7.1.2 Neuroborreliosis

B. burgdorferi has the ability to invade both the peripheral and central nervous system, potentially causing neurological complications within weeks to years following infection (471, 472). Early studies of patients in North America with untreated EM showed that about 15% of these patients developed meningitis or cranial neuritis during the first 3 months following EM presentation (473, 474). In children, the most common neurological complications from infection are: sixth nerve palsy, facial nerve palsy, and lymphocytic meningitis (475-477). With rapid antibiotic treatment, neuroborreliosis is now rare in the United States.

1.2.7.1.3 Carditis

Early studies found that 4-10% of untreated patients presenting with EM developed carditis, generally manifested by an acute onset of varying intensity of atrioventricular heart block (478, 479). Later work of 233 patients who developed Lyme disease showed no cardiac involvement (462, 480). In the experimental mouse model, the predominance of heart block syndromes is associated with the propensity of *B. burgdorferi* to localize to the connective tissue at the base of the heart (411, 481). It is likely that reduced cardiac involvement with Lyme disease is due to early antibiotic treatment.

1.2.7.1.4 Lyme arthritis

The most common manifestation of late untreated infection with *B. burgdorferi* in North America is Lyme arthritis (446, 449). The number of individuals who develop arthritis as a complication of Lyme disease has decreased over time (462, 480). Lyme arthritis typically presents as a monoarticular or oligoarticular large joint arthritis accompanied by inflammation with swelling large effusions and minor pain, most commonly affecting a knee joint (446, 452). Antibiotic-refractory Lyme arthritis can be correlated with joint erosions, as

is noted with rheumatoid arthritis. Thirty-four of 55 patients with EM not treated with antibiotics developed arthritis; 28 of these 55 patients had episodic arthritis with periodic joint swelling lasting from days to several months before resolving on their own (448). Six patients did experience a year or more of unresolved arthritis. With antibiotic treatment, 3 of the 6 patients had resolution of arthritis; the other 3 of the 6 patients spontaneously had resolved arthritis after several years (422). With or without antibiotics, Lyme arthritis can resolve spontaneously.

1.2.8 Detection and Diagnosis of Lyme Disease

Berger et al. (482) cultured 4 mm skin biopsy specimens from patient EM lesions with 18/21 samples reported as culture-positive. *B. burgdorferi* can be isolated from both primary and secondary erythema migrans lesions (483, 484). The spirochetes generally cannot be recovered from EM lesions of patients who are already being treated with antibiotics (485). *B. burgdorferi* has not been recovered from the site of resolved erythema migrans of American patients who have completed prescribed antibiotic therapy (486, 487).

B. burgdorferi recovery from the blood of untreated patients with EM was minor, with 5% or less success, but the amount of blood cultured was low (273, 274, 442, 488). Later, using 9 ml of plasma from adult patients with EM in the USA inoculated into Barbour-Stonner-Kelly (BSK) II medium at a ratio of 20:1 medium to plasma, recovery of *B. burgdorferi* was frequently over 40% successful (444, 489). Using this much blood for culture is reasonable for most adult EM patients, but not for children.

Culture methods, though, are not routinely used in clinical settings to diagnose Lyme disease. BSK II medium used to grow *B. burgdorferi* has many components, several of which can vary lot to lot. Cultures require up to 12 weeks of incubation before being called negative

(490). Additionally, culturing methods are only useful for untreated patients. Even a few doses of recommended antibiotic treatment can prevent growth of *Borrelia* in culture (487, 491). Culture would be helpful in few cases in which a skin lesion is unusual or the patient did not have tick exposure in an area endemic for Lyme disease (490).

PCR detection of *B. burgdorferi* DNA can be used to confirm a clinical diagnosis, type strains from clinical specimens, and detect co-infection of *B. burgdorferi* and other tick-transmitted pathogens (490). PCR is a highly sensitive method to detect *B. burgdorferi* DNA in skin biopsies or synovial fluid samples from patients with Lyme disease, but is not a very sensitive method to detect the spirochetes in patient blood or cerebrospinal fluid (492).

The CDC recommends a two-step process when testing serum for antibodies against *B. burgdorferi* (493). Patient serum is first tested using an ELISA or IFA to detect the presence of IgM and IgG antibodies against *B. burgdorferi*. If samples test negative for *B. burgdorferi*-specific IgM and IgG, they are considered negative and are not further analyzed. If samples test positive or are inconclusive for specific antibody, they are further tested. If the patient sample tests positive or equivocal for specific antibodies and the patient has had signs or symptoms of Lyme disease for 30 days or less, the second test is an IgM and IgG western blot; if the patient sample tests positive or equivocal for specific antibodies and the patient has had signs or symptoms of Lyme disease for over 30 days, the second test is only an IgG western blot, as *B. burgdorferi*-specific IgM antibodies have likely subsided (493).

1.2.9 Host Response

B. burgdorferi has a predilection for connective tissue and extracellular matrix. Histopathological examination of infected tissues during acute disease reveals a strong inflammatory response that seems disproportionate to the few *B. burgdorferi* present (494).

Due to the lack of organisms detected in diseased tissues, pathology correlated with Lyme disease is likely due to the host immune response to the spirochetes rather than to direct tissue damage by *B. burgdorferi* (494).

Laboratory mice develop myocarditis and arthritis, two major clinical manifestations of human Lyme borreliosis. In mice, innate immune cells dominate in inflammatory lesions, with T-cells and B-cells infiltrating organs as disease subsides (494). In contrast, B-cells and T-cells are more prevalent in cellular infiltrates of all human tissues monitored by pathology (494). Additionally, mice experience chronic inflammation in tissues. This inflammation is characterized by segmental perivascular and perineural infiltrates of plasma cells, T-cells, and B-cells often in range of visible *B. burgdorferi* in adjacent tissues (494).

There is often a delay in *B. burgdorferi* dissemination from the site of injection or tick bite. *B. burgdorferi* introduced into mice via tick bite were found not to migrate for several days, as removal of a 0.6 mm area of skin around the tick bite or topical antibiotic treatment up to 48 hours after tick detachment prevented infection (428, 495). A delay in dissemination of *B. burgdorferi* exists even if a large inoculum is injected into the host (494). The regulated expression of lipoproteins by *B. burgdorferi* has led to the likely conclusion that the spirochetes spreading away from the site of inoculation are expressing different antigenic proteins than *B. burgdorferi* in culture (313) or in the tick (330, 341, 351, 496-498).

Expression of many outer surface proteins, especially OspA and OspC, has been examined in the tick, in the mouse, and in various culture conditions (427, 499-502). Montgomery et al. (503) utilized the mouse model to provide direct evidence that OspC is upregulated upon infection of the mammalian host while OspA is downregulated.

1.2.9.1 Innate Immunity

Both innate and adaptive immunity are crucial in the control of *B. burgdorferi* in the mammalian host. Extracellular *Borrelia* grow in the presence of serum, likely due to their ability to resist complement activation and complement-mediated lysis (504, 505). Mice lacking C3, which is key to complement pathway-mediated opsonization, had higher levels of *B. burgdorferi* in several tissues than did wild-type mice, indicating impaired clearance (506, 507). *B. burgdorferi* also binds factor H of human complement; five complement regulator-acquiring surface proteins (CRASPs) have been described, likely aiding *B. burgdorferi* survival in serum (307, 308, 508-510).

Several studies highlight phagocytic cells, including: macrophages, monocytes, neutrophils, and glial cells, which destroy *B. burgdorferi in vitro* (503, 511-519). Antibody dependent and independent phagocytosis and killing of *B. burgdorferi* by human PMNs and monocytes has been documented (511, 520). Phagocytosis and destruction by human PMNs requires IgG opsonization of *B. burgdorferi*; although, lysed PMNs' cytosolic components killed *B. burgdorferi in vitro* (515). Many phagocytic cell components have been shown to kill or retard growth of *B. burgdorferi*, including the granule components bactericidal/permeability-increasing protein, elastase, human neutrophil peptide-1, and LL37, the active fragment of calprotectin (515, 521, 522).

Many cell types produce inflammatory mediators when exposed to *B. burgdorferi* or *B. burgdorferi* components. Endothelial cells and macrophages exposed to *B. burgdorferi* showed increased expression of TNF-α, IL-6, IL-12, IL-1, IL-8, and ICAMs, products considered to be NF-κB dependent (522-527). Other products produced upon exposure to *B. burgdorferi* that are not NF-κB dependent include production of nitric oxide by macrophages and generation of reactive oxygen intermediates by PMNs (528, 529). *B. burgdorferi* have

many outer surface lipoproteins that contain tripalmitoyl-S-glycerol-cysteine (Pam_3Cys) modification and are strong stimuli of immune cells in cell culture (530).

Pam_3Cys-containing lipoproteins signal via TLR-1/TLR-2 (494). TLR-2 deficient cells poorly activated NF-κB signaling when exposed to *B. burgdorferi*, which was even more depressed in MyD88 deficient mice (531). TLR-2 and MyD88 deficient mice have a major defect in spirochete clearance not connected to defective antibody production (514, 531-533). Lack of IL-18 does not change host susceptibility to *B. burgdorferi* pathogenesis (532). The caspase 1-dependent inflammasome, which produces IL-1β, was found to be dispensible for host defense (534).

TLR-2 and MyD88 deficient mice infected with *B. burgdorferi* develop both carditis and arthritis (494). TLR-2 deficient mice on the B6 background develop more severe arthritis than the mild arthritis displayed by B6 mice and the severe arthritis of C3H mice following infection (533, 535, 536). TLR-2-/-/*scid* mice, though, displayed mild arthritis with greater burdens than TLR-2 deficient only mice (537). Therefore, arthritis severity is not solely dependent on elevated burden.

The anti-inflammatory cytokine IL-10 is produced by macrophages isolated from humans, mice, and rhesus monkeys incubated with *B. burgdorferi* and *B. burgdorferi* lipoproteins, with efficient signaling dependent on both CD14 and TLR-2 (538-542). Non-viable *B. burgdorferi* induce lower levels of IL-10 by murine macrophages in comparison to live *B. burgdorferi* (543). *B. burgdorferi* incubated with macrophages of mildly arthritic B6 mice induces higher IL-10 production than *B. burgdorferi* incubated with macrophages of severely arthritic C3H mice (539, 544). IL-10 deficient C3H and B6 mice have more severe

disease than their wild-type counterparts (539, 545). IL-10 deficiency does, though, reduce spirochetal burden (544).

γδ T-cells enhance production of IFN-γ and IgG2a following syringe-injection of *B. burgdorferi*, but tick transmission of *B. burgdorferi* does not enhance this response (546). IL-12 production is also enhanced by γδ T-cells in response to *B. burgdorferi* and its lipoproteins which induce maturation of dendritic cells that produce the cytokine (547-549).

NKT cells respond to *B. burgdorferi*. CD1d, a MHC-like restriction element, can bind the *B. burgdorferi* diacylglycerol BbGL-II to stimulate NKT cells *in vitro* (550). Loss of NKT cells results in elevated *B. burgdorferi* burden and more severe arthritis early during the course of infection in mice (551).

1.2.9.2 Acquired Immunity

B-cell activation and antibody production are important in the control of *B. burgdorferi*. Mice unable to produce antibodies have significantly elevated borrelial burden in tissues and blood that can be decreased by passive transfer of immune mouse serum or B-cells, whereas T-cells alone have no such effect (415, 469, 552, 553). Mice lacking both αβ and γδ T-cells produced IgM and IgG3 *B. burgdorferi*-specific antibodies that, when their serum was transferred, prevented infection and lowered *B. burgdorferi* burden in infected antibody-deficient mice (552, 554). Three B-cell populations produce such antibodies: marginal zone B-cells, B1 B-cells, and follicular B2 B-cells (494). Passive transfer of immune serum from infected mice has the ability to lower *B. burgdorferi* burden and attenuate carditis and arthritis in *rag*-/-, SCID, and B-cell deficient mice; serum from mice immunized with *B. burgdorferi* antigens can attenuate disease, but does not decrease bacterial burden (494).

Human *B. burgdorferi*-specific IgM is first detected by the third to sixth week of illness, while *B. burgdorferi*-specific IgG usually takes longer to be detected (274). Specific IgG titers rise and are greatest during late disease, when arthritis symptoms are present (451, 555). IgG titers can be elevated for years following treatment even with disease remission (494).

Adaptive immunity is important in disease resolution since *rag*-/- and SCID mice develop persistent myocarditis and arthritis. Whereas adoptive transfer experiments showed the necessity of B-cells and *B. burgdorferi*-specific antibody in pathogen clearance and disease resolution, αβ T-cells' role in host defense is not completely known (494). CD4+ T-cells in the absence of CD8+ T-cells have been shown to reduce pathogen burden, while CD8+ T-cells in the absence of CD4+ T-cells enhanced disease progression, possibly by impeding the generation of protective immunity (556). Studies to determine the effect of Th1 and Th2 cytokines associated IFN-γ with increased pathogen burden and enhanced arthritis and IL-4 with less severe disease (557-559). Blockage of IL-12p40 reduced arthritis in C3H mice, supporting a role for Th1 cells in arthritis severity (466). Later studies utilizing IFNγ-/- and IL-4-/- mice inoculated with *B. burgdorferi* found that mice developed arthritis of similar severity and duration to their wild-type counterparts (560-562). Therefore, resistance or susceptibility to development of Lyme arthritis in mice is not defined by the balance between Th1 and Th2 cells and their related cytokines (494).

1.2.9.3 Murine Lyme Arthritis

Laboratory mice are routinely used to analyze the host immune response in joints. Arthritis lesions were first identified in C3H mice, with features in common to arthritic joints of Lyme disease patients (437). PMNs dominate the population of early inflammatory

infiltrates in joints (437). *B. burgdorferi* can be detected in joint tissue by 1 week following intradermal injection, with PMN-dominated inflammatory cell infiltrate present by 2 weeks after inoculation (411). Histopathological changes peaked at 4 weeks post inoculation (411). Both the monocyte chemokine MCP1 and the PMN recruiting chemokine KC were elevated in joint tissue 2 weeks post inoculation and were greater in C3H mice, which are severely arthritic, versus B6 mice, which are mildly arthritic (563).

Genetic differences were examined to help explain why C3H mice develop severe arthritis following *B. burgdorferi* infection, while B6 mice only develop mild arthritis. Studies utilizing qPCR showed that mice with mild arthritis had similar burdens in ankle tissues as did mice with severe arthritis (418, 420). Quantitative trait loci (QTL) identified on five chromosomes of the mouse, chromosomes 1, 4, 5, 11, and 12, have been implicated in regulation of *B. burgdorferi*-induced arthritis (564, 565). The differences in Lyme arthritis severity in C3H and B6 mice are associated with genes encoded within the *Bbaa2Bbaa3* locus on chromosome 5 for C3H mice and within the *Bbaa4* locus on chromosome 11 for B6 mice (566, 567).

Induction of genes following *B. burgdorferi* infection of C3H and B6 joints was assessed over time by microarray analysis (383). Many interferon-inducible genes were upregulated in the joint tissue of C3H mice at 1 week post inoculation, but declined by two weeks post inoculation and stayed reduced at 4 weeks post inoculation, the peak of arthritis (383). Transcripts for interferons (IFNs) themselves were not upregulated (383). Brown and Reiner (560) reported that IFN-γ was not required for severe Lyme arthritis in C3H mice, hinting at a role for type I IFNs. IL-10-/- and TLR-2-/- mice on the B6 background displayed high upregulation of many IFN-inducible genes 2 weeks post inoculation (383, 536). Mice

injected with antibodies to block the type I IFN receptor prior to inoculation showed a significant reduction in arthritis severity (568). Therefore, the development of severe arthritis in mice is linked to upregulation of IFN-inducible genes in response to *B. burgdorferi B. burgdorferi*. Human PBMCs and murine bone marrow-derived macrophages generate an interferon response when *B. burgdorferi* is introduced, implying a cellular source of IFN-inducible genes during infection (568, 569). Murine macrophages with specific disruptions indicated that the IFN response to *B. burgdorferi* was not dependent of TLR-2, TLR-4, TLR-9, and MyD88, but completely dependent on feedback via the type I IFN receptor (568).

Miller et al. (570) expanded on their previous study (568) to uncover what borrelial ligands induced type I IFN-responsive genes in murine BMDMs. Miller et al. (570) found that the adaptor molecule TRIF and NOD2 are not required for type I IFN-responsive gene induction, but that STAT-1 and IFN regulatory factor 3 (IRF3) are required for this induction. Stimulatory ligands were found to include: *B. burgdorferi*-derived RNA, the lipoprotein OspA, and non-nucleic acid ligands released by *B. burgdorferi* in culture medium (570). OspA has been well characterized (571). Therefore, to expand on this work (570), our laboratory sought to characterize the *B. burgdorferi*-derived RNA and non-nucleic acid ligand(s) present in *B. burgdorferi* culture supernatant and to identify factors important to murine host defense when exposed to these ligands, as will be presented in chapter 3.

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CHAPTER 2

The anti-inflammatory cytokine IL-10 is required for *Leptospira interrogans* persistence in the mouse kidney

Abstract

Leptospira interrogans is the causative agent of leptospirosis, a widespread zoonosis with the potential to cause fatal infection in humans due to acute renal failure and pulmonary hemorrhage. Rodents are the natural reservoir hosts of *L. interrogans*, exhibiting asymptomatic infection with chronic carriage of the bacteria in the proximal renal tubules. The range of symptoms from none to multiple organ failure depends on variable host susceptibility toward *L. interrogans*. The goal of this work was to better understand the murine host response to *L. interrogans* in respect to the establishment of the chronic carrier state. We examined the role of IL-10 in establishment of the carrier state by comparing IL-10-deficient and C57BL/6 (B6) mice at various time points following infection with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. We found clearance of *L. interrogans* from the lungs of both strains of mice by 7 days post inoculation. In contrast, *L. interrogans* was cleared from the kidneys of IL-10-deficient mice by 7 days post inoculation, whereas B6 mice still harbored spirochetes in their kidneys at 7 days following inoculation. Histological observations evidenced kidney damage in some B6 mice at 7 days post inoculation not seen in IL-10-deficient mice. B6 mice showed systemically elevated *IL-10* transcripts during the course of infection. Unlike in *L. interrogans*-infected B6 mice, systemically elevated *IFN-γ* transcripts were detected in infected IL-10-deficient mice, which peaked at 7 days post inoculation, suggesting that IFN-γ is important for clearance of the bacteria and in regulating colonization of the kidney, including the chronic carrier state. We conclude that IL-10 is necessary for *L. interrogans* to persist in the mouse kidney and be chronically shed into the environment.

1 Introduction

Leptospira interrogans, the causative agent of leptospirosis, is contracted through direct or indirect contact with infected reservoir host animals, predominantly rodents, which harbor the pathogens in their renal tubules and shed *L. interrogans* in their urine (1). Leptospirosis is likely the most widespread and prevalent zoonotic disease in the world (2). The global burden of leptospirosis is predicted to increase with demographic shifts that favor rising numbers of urban poor in tropical regions exposed to severe storms and urban flooding related to climate change (1-3). For some humans, leptospirosis is asymptomatic or mild; for others, leptospirosis, without timely antibiotic treatment, can progress to multiple organ failure and death (3). High grade leptospiral bacteremia is associated with poor clinical outcomes (3, 4). Humans with severe leptospirosis undergo a cytokine storm defined by high levels of TNF-α, IL-6, and IL-10 (5, 6).

The majority of leptospirosis research thus far has focused on acute disease, while little data has been published on asymptomatic disease, such as that in the mouse or rat (7). During chronic infection of the rodent host, leptospirosis and renal colonization persist despite innate and adaptive immune responses by the host. Monahan et al. (8) found that *L. interrogans* within the renal tubules of rats were devoid of any immune response, indicating that leptospires in this location may be protected from the host immune response. *L. interrogans* has several mechanisms by which it can evade the host response. *L. interrogans* binds more factor H (9) and C4-binding protein (C4BP) (10) from serum than complement sensitive, non-pathogenic *Leptospira* strains. Leptospiral-bound C4BP enhances factor I-mediated cleavage of C4b, leading to a reduction in deposition of later complement components C5 to C9 upon serum exposure (10). Inhibition of the complement cascade by *L. interrogans*

decreases bacterial cell lysis and opsonophagocytosis (11). While *L. interrogans* complement evasion strategies have been described, mechanisms for establishing the chronic carrier state are mostly unknown.

Matsui et al. (20) found that rapid, sustained induction of interleukin-10 (IL-10) in the kidneys and lungs of resistant Oncins France 1 (OF1) mice protected them from the fatal leptospirosis experienced by susceptible golden Syrian hamsters. In addition, several cytokines were differentially expressed during the course of leptospiral infection in the OF1 mouse versus the hamster (20). Importantly, the hamster is a model of acute disease, not asymptomatic disease with chronic infection, as is the mouse. From these data, we sought to determine the role of IL-10 in *L. interrogans* infection of the mouse. We utilized C57BL/6 (B6) mice and B6 IL-10-deficient mice to expand on previously published data.

Little is known about the role of IL-10 in leptospiral infection of the mouse. A few studies have assessed the role of IL-10 in relation to infection with another spirochete, *B. burgdorferi* (12-15). Previous studies have shown that IL-10 can suppress a wide range of inflammatory mediators produced by macrophages in response to LPS; IL-10 is often referred to as a macrophage deactivating factor (16, 17). IL-10 can reverse the early stimulatory effects of IFN-γ on macrophages, halting production of reactive oxygen and reactive nitrogen intermediates, which could protect some pathogens (17-19).

IL-10-deficient mice are well-established models of chronic inflammation (21). IL-10-deficient mice exhibit exaggerated immune reactions in several tissues if stimulated with antigen (21). IL-10 inhibits the expression of B7 and class II molecules on macrophages, and IL-10 lowers macrophage production of IL-12 (22-24). As a result, macrophage-dependent stimulation of antigen reactive Th1 cells is impaired (24). Macrophages treated with IL-10

are not able to function as accessory cells for natural killer (NK) cells; the result is less IFN-γ production and cytotoxic activity by NK cells (25). IL-10-deficient mice develop normal numbers of B cells and T cells in their lymphoid organs and mount normal antibody responses to many T cell-dependent and -independent antigens (26). With age, though, IL-10-deficient mice succumb to chronic enterocolitis, which stems from routinely activated Th1 cells, macrophages, and NK cells (26). Therefore, short-term depletion of IL-10 may be beneficial, but chronic depletion of IL-10 can lead to early death in the mammalian host.

In this study, we sought to better understand the murine host response to *L. interrogans* by comparing wild-type C57BL/6 (B6) and IL-10-deficient tissues at various time points during infection. For this purpose, we determined cytokine and transcription factor mRNA expression profiles, in parallel with a histological analysis and the quantification of *L. interrogans* burden in kidneys and lungs.

2 Materials and Methods

2.1 Cultivation of *Leptospira interrogans*

Leptospira interrogans serogroup Icterohaemorrhagiae serovar Copenhageni strain Fiocruz L1-130, originally isolated from a patient in Brazil who succumbed to multiple organ failure (27), was grown in EMJH++ medium, as detailed by Zuerner (28). Complete medium consists of 880 ml of basal medium plus 100 ml of supplement, 10 ml of rabbit serum, and 10 ml of 5-fluorouracil. Basal medium consisted of 998 ml distilled water, 1 g disodium phosphate (Na_2HPO_4), 0.3 g monopotassium phosphate (KH_2PO_4), 1 g sodium chloride (NaCl), 1 ml of 10% v/v glycerol, and 1 ml of 25% ammonium chloride (NH_4Cl) solution, mixed, with the pH adjusted to 7.4 using filtered 10% sodium hydroxide (NaOH). The solution was then autoclaved in a glass flask loosely covered with foil. Supplement was made

by combining the following chemicals with 100 g of distilled water: 10 ml of 0.5% w/v thiamine chloride, 10 ml of 1% w/v calcium chloride ($CaCl_2 \cdot 2\ H_2O$), 10 ml of 1% w/v magnesium chloride ($MgCl_2 \cdot 6\ H_2O$), 10 ml of 0.4% zinc sulphate ($ZnSO_4 \cdot 7\ H_2O$), 1 ml of 0.3% w/v manganese sulphate ($MnSO_4 \cdot 4\ H_2O$), 100 ml of 0.5% ferrous sulphate ($FeSO_4 \cdot 7\ H_2O$), 10 ml of 0.02% w/v vitamin B12, and 125 ml of 10% Tween 80, then pH was adjusted to 7.4 using filtered 10% NaOH. Supplement was stored at -20°C until later use in EMJH++ preparation. Rabbit serum, stored at -20°C until use, was either purchased from Pel-Freez or Sigma-Aldrich, and was heat-inactivated for 30 minutes at 56°C prior to use. Stock solution of 5-fluorouracil was made using 1 g 5-fluorouracil dissolved in 50 ml filtered basal medium with 1.5 ml of filtered 2N NaOH. The solution was dissolved overnight at 56°C in a water bath, followed by the pH being lowered to 7.4 using filtered 1N HCl, after which the stock solution was brought up to a final volume of 100 ml using sterile basal medium, filtered, then stored at 4°C for future use. 2 g per liter of agar was added to basal medium to make it semi-solid.

L. interrogans stock cultures were housed in cryovials stored in liquid nitrogen. To grow bacteria for infections, one vial was removed from liquid nitrogen storage and thawed in lukewarm water. Upon thawing, the vial was opened in the biosafety cabinet, added to a 15 ml conical tube containing 8 ml of semi-solid EMJH++, and incubated at 30°C. Following the development of a Dinger's disc (about 10 days), the Dinger's disc (a white ring of heavy growth) was removed and added to 3 ml of fresh liquid EMJH++ and incubated for 7 days at 30°C. This culture was then transferred with 3 ml semi-solid EMJH++ into a 50 ml conical tube already containing 8 ml of liquid media. Of note, *L. interrogans* is an obligate aerobe, so caps on conical tubes during various passages are always loosely covering the cultures. After

the culture appeared turbid (around 5 days), 0.5 ml of the culture was added to 8 ml fresh liquid EMJH++ and became known as passage 1. This culture was allowed 5 days to grow. Following this, 0.5 ml cultures were taken from this passage 1 and added to 15 ml conical tubes containing 8 ml liquid EMJH++ to become passage 2 cultures, which again, were grown for 5 days. The same process was repeated to get passage 3 and passage 4 cultures. Only passage 4 cultures were used in experiments. *Leptospira* cultures for experiments were grown to a final density of 5×10^8 cells/ml.

2.2 Mouse Strains

All C57BL/6 (B6) mice used were purchased from The Jackson Laboratory (Bar Harbor, ME). Two B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME), with their progeny used in the following experiments. These mice have an IL-10 gene that has been inactivated by targeted mutation (26).

2.3 Mouse Infection and Euthanasia

Only female B6 mice were purchased for use in experiments. IL-10-/- mice were both male and female mice. 7-9 week old mice were injected intraperitoneally with either 500 µl of EMJH++ medium or 500 µl of 2×10^8 *L. interrogans* diluted in EMJH++ medium. Mice were provided water and chow *ad libitum*. Upon 1, 3, or 7 days post-inoculation, mice were euthanized via isoflurane narcosis. Blood was collected post-mortem via submandibular incision for serum and for RNA extraction. Corpses were doused in 70% ethanol and transferred for necropsy.

2.4 Necropsy

Mice were dissected. Livers were immediately excised with ½ tissue placed in 5 ml 10% formalin. Liver tissue was transferred into 70% ethanol approximately one year later in

preparation for histological analysis. Left lungs, left kidneys, and half hearts were placed in 10% formalin solution, with ethanol transfer about a year later. Right kidneys and right lungs were flash frozen inside 1.5 ml microcentrifuge tubes submerged in a dry ice-ethanol bath then stored at -80°C.

2.5 Homogenization of Lungs and Kidneys

Right lungs and right kidneys were quickly transferred from -80°C into round-bottom polypropylene tubes containing 1 ml ice-cold QIAzol. Samples were then homogenized using the ProScientific Bio-Gen Series PRO200 homogenizer at the maximum speed setting. Samples were visually checked for thorough homogenization, placed on ice then stored at -80°C. After each sample, the homogenizer was cleaned, in order, using 5 ml RNase away, 5 ml 70% ethanol, 5 ml diethylpyrocarbonate (DepC) water, and 5 ml QIAzol reagent.

2.6 RNA Extraction

RNA collection from homogenized samples was performed using phenol-chloroform extraction and ethanol purification, per the QIAzol Lysis Reagent manufacturer's instructions. Homogenized samples were placed on ice and allowed to thaw. Next, they were incubated at room temperature for 5 minutes, followed by addition of 200 µl fresh chloroform. Microcentrifuge tubes with samples in QIAzol and chloroform were well mixed by shaking for 15 seconds each, then allowed 3 minutes at room temperature before centrifugation. Samples were centrifuged for 20 minutes at 13,000 RPM at 4°C in the VWR Symphony 2417R refrigerated micro-centrifuge. Centrifugation partitioned samples into an aqueous and organic phase. Between 300 and 400 µl of aqueous phase were collected and transferred to a new 1.5 ml microcentrifuge tube, followed by addition of 500 µl isopropanol. Tubes were mixed by hand then incubated for at least 10 minutes at room temperature.

Samples were then centrifuged at 13,000 RPM for 10 minutes at 4°C. Centrifugation resulted in pellets at the bottom of the microcentrifuge tubes. Supernatant was removed, and 1 ml of ice-cold 95% ethanol was added to wash the pellet. Samples were centrifuged at 8,000 RPM for 6 minutes at 4°C. Ethanol was pipetted off the pellet, then the pellet was air-dried for 10 minutes. Pellets were resuspended in 150 µl of molecular grade water, then incubated at 37°C for 10 minutes in a heat block to aid solubility of the pellet. Nucleic acid content was estimated using the Thermo Scientific Nanodrop 1000 Spectrophotometer measuring optical density (OD) at 260 nanometers (nm).

2.7 cDNA Synthesis

Five micrograms of nucleic acid samples were added to 1.5 ml microcentrifuge tubes and brought up to 36 µl total volume using molecular grade water. Added to each sample was: 10 µl of 5x buffer (Affymetrix), 1 µl of dNTPs (Fermentas), 2 µl of random primers (Promega), and 1 µl of M-MLV reverse transcriptase (Affymetrix). 50 µl total volume samples were incubated for 1 hour at 37°C in a heat block. Following 1 hour of cDNA synthesis, 2 µl of 1 µg/µl RNase A (Roche) was incorporated into each sample, followed by incubation at 37°C for 5 minutes in the heat block.

2.8 cDNA Purification and Dilution

After cDNA was synthesized, it was purified. 250 µl of PB buffer (Qiagen) was added to cDNA. The total volume of 300 µl was added to a UPrep Universal Spin Filter Column (Genesee Scientific) and centrifuged for 1 minute at maximum speed to facilitate binding of cDNA. Filtrate was discarded. The spin column was centrifuged for an additional minute at maximum speed to ensure all wash buffer had been removed from the column. The column was removed and placed into a new microcentrifuge tube. 50 µl of elution buffer (7.5

mM Tris-HCl) was added to the column, followed by a one minute spin at maximum speed. cDNA was then diluted 1:10 by adding 90 µl molecular grade water to 10 µl of clean cDNA.

2.9 Quantitative Real-Time PCR (qRT-PCR)

PCR mastermix consisted of 5 µl of 10 µM equal parts forward and reverse primers (Table 1), 5 µl molecular grade water, and 12.5 µl of Bio-Rad iQ SYBR Green Supermix per sample. Added to the wells of a 96 well semi-skirted PCR plate (Genesee Scientific) were: 20 µl of PCR mastermix and 5 µl of diluted cDNA, to give a total volume per well of 25 µl. Molecular grade water was used as a no template control, *Leptospira*-infected kidney cDNA was used as a positive control, and uninfected kidney cDNA was used as a negative control. qRT-PCR was carried out using the Bio-Rad MyiQ2 Two Color Real-Time PCR Detection System connected to the Bio-Rad I-cycler. Bio-Rad.iQ5 software was used to program PCR reactions.

For the *β-actin*, *TNF-α*, *IL-4*, *FoxP3*, *TGF-β1*, *IL-10CD*, and *IFN-γ* transcripts, a 6 cycle program was used. The first cycle of 95°C occurred for 3 minutes, followed by a second cycle, which consisted of 2 steps, repeated 40 times. The first step of this second cycle was a 95°C denaturing step; the second step was a 65°C annealing step. The third cycle was a 1 minute 95°C denaturing step; the fourth cycle was a 1 minute 60°C annealing step. The fifth step determines the melting temperature of the amplified DNA via a stepwise increase of temperature from 60°C to 95°C with an elevation of 0.5°C increments over 10 seconds per increment. The sixth step is a 60°C hold indefinitely.

For the *Leptospira 16S rRNA* transcript, the same 6 cycle procedure was used as detailed above, with the only difference being to step 2 of cycle 2, using an annealing temperature of 63°C instead of 60°C. For *IL-6* transcripts, the same 6 cycles were used as

mentioned previously, with the only modification being to step 2 of cycle 2, with use of an annealing temperature of 65°C instead of 60°C.

2.10 Primers for qRT-PCR

The *Leptospira* 16S primer sequences utilized originated from the Goarant lab (20). *β-actin* and *IL-10CD* primer sequences were acquired from the Weis lab (30, 31).

Table 1. Primer sequences

Primers	Forward Sequence	Reverse Sequence
β-actin	5'-GTAACAATGCCATGTTCAAT-3'	5'-CTCCATCGTGGCCGCTCTAG-3'
Lepto-16S	5'-GGCGGCGCGTCTTAAACATG-3'	5'-TTCCCCCATTGAGCAAGATT-3'
IL-4	5'-CATCGGCATTTTGAACGAG-3'	5'-CGAGCTCACTCTCTGTGGTG-3'
IL-6	5'-ACCGCTATGAAGTTCCTCTCTGC-3'	5'-CCAGAAGACCAGAGGAAATTTTC-3'
IL-10CD	5'-GCTCTTACTGACTGGCATGA-3'	5'-TTCCGATAAGGCTTGGCAAC-3'
IFN-γ	5'-TCTTCAGCAACAGCAAGGCG-3'	5'-AATCTCTTCCCCACCCCGAATCAG-3'
FoxP3	5'-CCCATCCCCAGGAGTCTTG-3'	5'-GTAACGCCAGGAATTGT-3'
TGF-β1	5'-CGCCATCTATGAGAAAACC-3'	5'-GTAACGCCAGGAATTGT-3'
TNF-α	5'-ATGAGCACAGAAAGCATGATC-3'	5'-TACAGGCTTGTCACTGGAATT-3'

2.11 qRT-PCR Normalization

Copy number calculation for each transcript was done by standardizing each to the mouse housekeeping gene β-actin. A standard curve for β-actin was developed by Morrison et al. (29) by taking advantage of two molecular phenomena: the use of anti-Taq antibodies reduces the amount of non-specific products produced, and specific products have a higher melting temperature than non-specific products, thus double-stranded products will have a higher melting temperature. Utilizing various amounts of starting template, multiple polymerase chain reactions were run and the fluorescence versus cycle data was plotted. The

fractional cycle number at which the threshold fluorescence was surpassed could be determined by extending out the log-linear portions of the graphs. Plotting fractional cycle numbers versus the log of the starting amount of template copies gave linear standard curves of the purified PCR products. These plots enable approximation of unknown cDNA copy numbers over a 10^6-fold range (29).

2.12 Histological Analysis

Left lungs, left kidneys, ¼ livers, and half hearts from newly sacrificed mice were immediately removed and placed into 5 ml of 10% formalin. After about a year in 10% formalin, tissues were transferred into 70% ethanol in preparation for histological analysis. Using a razor blade, even slices were made to section lung, liver, kidney, and heart tissues, and the slices were placed into cassettes, which were labeled with sacrifice date and mouse ID number. These cassettes were placed into 70% ethanol then transferred to NCSU histology lab staff to be embedded in paraffin, cut, and stained using hematoxylin and eosin (H & E). Pathology scoring using a 0-5 scale was performed in a blinded fashion by John Cullen, VMD, PhD (North Carolina State University, Raleigh, NC).

2.13 Statistical Analyses

Statistical analyses were run comparing uninfected to infected groups at 1, 3, and 7 day time points. Additionally, statistical analyses were run comparing infected groups to one another based on length of infection. The statistical test used throughout was the non-parametric Mann-Whitney U test. The Mann-Whitney U test is an alternative to a *t* test, and it can be used when data are not normally distributed. A p-value of less than 0.05 was needed in order for differences between groups to be considered statistically significant. Statistical thresholds are displayed on figures and in figure legends as follows: * = $p < 0.05$, ** = $p <$

0.01, and *** = p-value < 0.001. All statistical analyses were conducted using GraphPad InStat3 for Windows, version 3.1a (GraphPad Software, San Diego, CA).

3 Results

3.1 IL-10-deficient mice clear *L. interrogans* by 7 days post inoculation

To determine whether IL-10-deficient mice exhibit differential clearance of *L. interrogans* from their kidneys and lungs in comparison to wild-type mice, 7-9 week old C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected intraperitoneally with either 500 µl of EMJH++ medium or 500 µl of 2 x 10^8 *L. interrogans* diluted in EMJH++ medium. For mouse injections, 2 x 10^8 is a conventional sublethal intraperitoneal dose of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (32). Mice were weighed daily, with no significant weight loss observed over the course of infection (data not shown). Leptospiral burden in mouse kidneys and lungs was assessed via qRT-PCR analysis. Copies of leptosprial *16S rRNA* transcripts per 1,000 mouse *β-actin* transcripts are shown for kidneys (Figure 1A) and lungs (Figure 1B) at 1, 3, and 7 days post inoculation. Representative experiments with 5-10 mice per group repeated two (1 and 3 day time points) or three times (7 day time point) with similar results are shown (Figure 1).

Leptospiral *16S rRNA* transcripts were detected in infected mouse lungs at 1 and 3 days post injection (Figure 1B). Leptospiral *16S rRNA* transcripts were not detected in mouse lungs 7 days post injection (Figure 1B). This result is as expected; *L. interrogans* is cleared from all tissues except the kidneys by 7 days post injection in the mouse (33). No significant difference in lung *16S rRNA* transcript levels was found between IL-10-/- infected (IL-10-/- I) and B6 infected (B6 I) mice at 3 days post injection; however, at 1 day post injection, a

significant difference was found between lung *16S rRNA* transcript levels from IL-10 I and B6 I mice ($P < 0.05$, Figure 1B).

Leptospiral *16S rRNA* transcripts were detected in infected mouse kidneys at 1 and 3 days post injection (Figure 1A). Leptospiral *16S rRNA* transcripts were detected in B6 I mouse kidneys 7 days post injection, whereas they were not detected in IL-10-/- I mouse kidneys 7 days post injection ($P < 0.001$, Figure 1A). From these data, IL-10 appears to be required for leptospiral persistence in the mouse kidney.

3.2 C57BL/6 mice exhibit kidney pathology not seen in IL-10-deficient mice following *L. interrogans* infection

We sought to determine whether pathological changes in the kidney accompany prolonged colonization by *L. interrogans* in B6 and IL-10-/- mice following injection with either EMJH++ medium or *L. interrogans*. While glomeruli appear normal in B6 uninfected, IL-10-/- uninfected, and IL-10-/- infected kidneys, altered structure is observed in B6 infected kidney glomeruli at 7 days post injection (Figure 2). Glomeruli were less organized and more difficult to identify in B6 infected kidney sections. At 7 days post inoculation with *L. interrogans*, wild-type mice can experience mild to moderate interstitial nephritis (33). Interestingly, kidney damage was not seen in IL-10-/- infected mice at 1, 3, or 7 days post inoculation (data not shown and Figure 2D). Therefore, *Leptospira* are cleared from the kidneys of IL-10-/- mice by 7 days post inoculation without any observed pathological damage.

Summarized histological data are presented in Table 2. Sectioned and stained hearts, lungs, livers, and kidneys from mice injected with either EMJH++ medium or *L. interrogans* were scored by a pathologist. Heart damage was not observed in any of the mice. Minimal

lung damage was observed in *L. interrogans*-infected mice, as was expected (20). Liver damage was observed in mice, but this damage was not limited to infected mice. The only kidney damage seen was in B6 infected mice at 7 days post inoculation (Table 2).

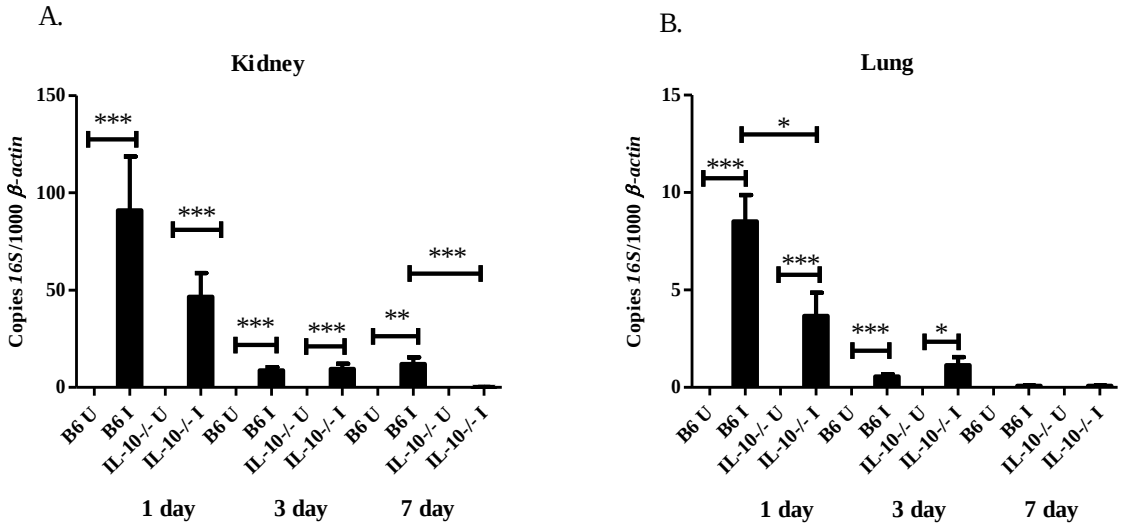

Figure 1. IL-10 is required for the persistence of *L. interrogans* in the mouse kidney. C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected with either 500 µl EMJH++ medium or 2x10^{8} *L. interrogans* diluted in 500 µl EMJH++ medium. Mice were sacrificed at 1, 3, or 7 days post inoculation. RT-PCR transcripts are displayed as the number of *16S rRNA* (16S) transcripts normalized to 1,000 copies of the mouse *β-actin* housekeeping gene. Transcript levels for kidneys (A) and lungs (B) from B6 uninfected (B6 U), B6 infected (B6 I), IL-10-/- uninfected (IL-10-/- U), and IL-10-/- infected (IL-10-/- I) mice are shown. Data are depicted as means ± SEM and are representative of 2 (1 and 3 day) or 3 (7 day) independent experiments (n = 5-10 mice). Statistical significance was assessed via the Mann-Whitney U test. ***, P < 0.001 for B6 I versus B6 U at 1 and 3 days post injection in the kidney and lung, for IL-10-/- I versus IL-10-/- U at 1 day post injection in the kidney and lung, for IL-10-/- I versus IL-10-/- U at 3 days post injection in the kidney, for B6 I versus IL-10-/- I at 7 days post injection in the kidney. **, P < 0.01 for B6 I versus B6 U at 7 days post injection in the kidney. *, P < 0.05 for IL-10-/- I versus IL-10-/- U at 3 days post injection in the lung, for B6 I versus IL-10-/- I at 1 day post injection in the lung.

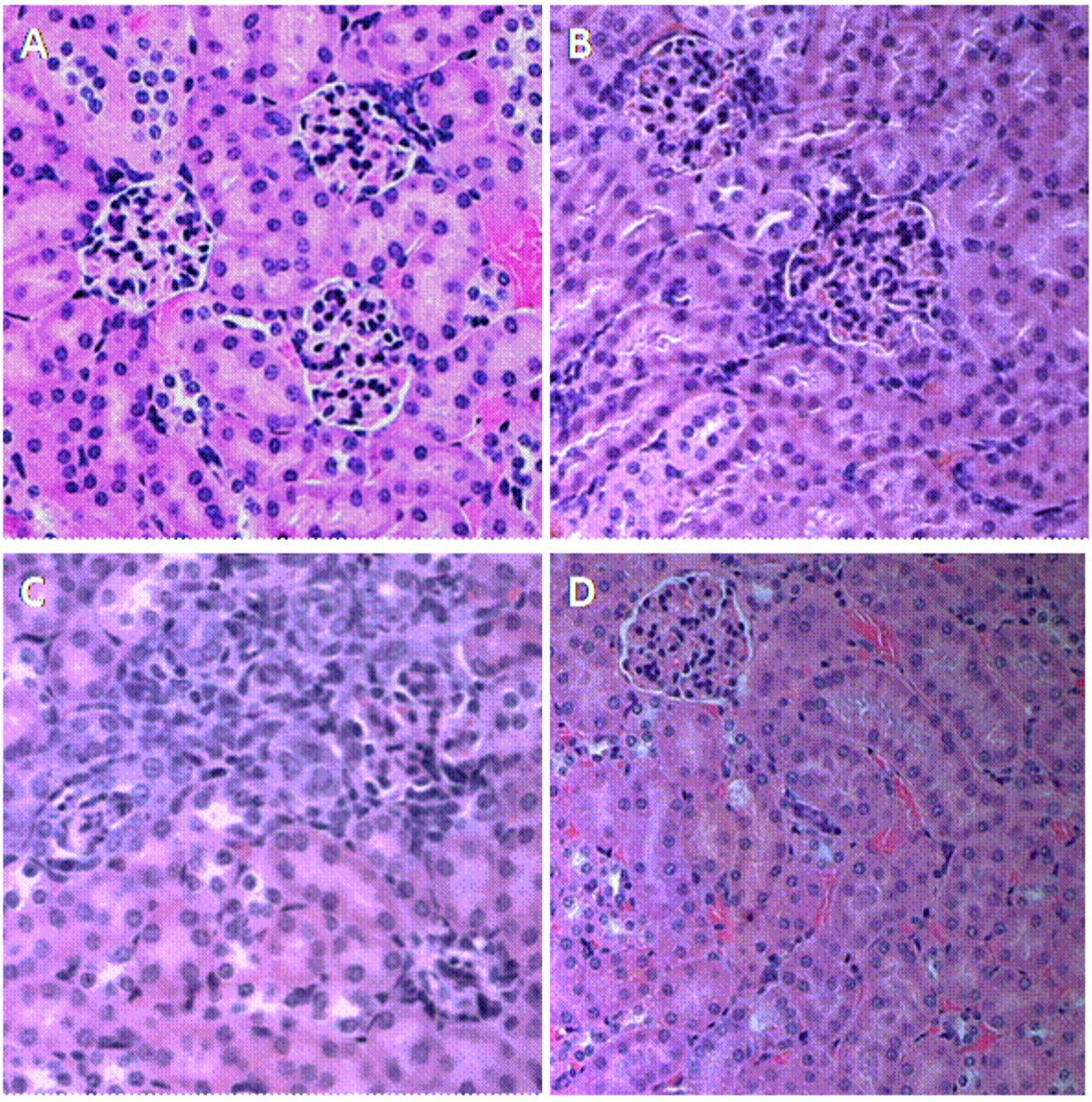

Figure 2. Infected C57BL/6 mice show kidney pathology at 7 days post injection with *L. interrogans*. C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected with either 500 µl EMJH++ medium (uninfected) or 2x10^{8} *L. interrogans* diluted in 500 µl EMJH++ medium (infected). Shown are hematoxylin and eosin (H & E) stained sections of mouse kidneys at 7 days post inoculation. While glomeruli appear normal in B6 uninfected (A), IL-10-/- uninfected (B), and IL-10-/- infected (D) kidney sections, altered structure is observed in B6 infected (C) kidney tissue. Magnification, X20 for all images shown.

Table 2. Summary of pathology observed in mice either injected with EMJH++ medium or with *L. interrogans*. C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected with either 500 µl EMJH++ medium (Uninfected) or 2x10^{8} *L. interrogans* diluted in 500 µl EMJH++ medium (Infected). Listed are the observed pathologies in examined tissues, which included mouse hearts, lungs, livers, and kidneys, at 1, 3, and 7 days post inoculation. Liver damage was observed in both infected and uninfected tissues. Heart damage was not observed. One IL-10-/- *L. interrogans*-infected mouse had a single lung focus observed at 3 days post injection. Kidney damage was only observed in 2 out of 6 *L. interrogans*-infected B6 mice at 7 days post inoculation.

Histology Results			
1 Day Infection			
Strain	Infected	Uninfected	Results
IL-10-/-	x		0/6 pathology
IL-10-/-		x	3/6 liver foci, 2/6 liver necrosis
B6	x		2/6 liver multifocal necrosis
B6		x	1/6 liver focus
3 Day Infection			
Strain	Infected	Uninfected	Results
IL-10-/-	x		3/6 liver foci, 1/6 lung focus
IL-10-/-		x	4/6 liver foci
B6	x		2/6 liver foci
B6		x	3/6 liver foci, minor
7 Day Infection			
Strain	Infected	Uninfected	Results
IL-10-/-	x		5/6 liver foci
IL-10-/-		x	4/6 liver foci
B6	x		5/6 liver foci, 3/6 kidney foci
B6		x	2/6 liver foci, minor

3.3 Cytokine and transcription factor expression vary between *L. interrogans*-infected C57BL/6 mice and IL-10-deficient mice

We next sought to determine if the cytokine IL-10 itself is involved in the elevated bacteremia seen in B6 mice. To test this, 7-9 week old C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected intraperitoneally with either 500 µl of EMJH++ medium or 500 µl of 2 x 10^8 *L. interrogans* diluted in EMJH++ medium. *IL-10* transcript levels in mouse kidneys and lungs were assessed via qRT-PCR analysis. Copies of *IL-10CD* transcripts per 1,000 mouse *β-actin* transcripts are shown for kidneys (Figures 3A and 3B) and lungs (Figures 3C and 3D) at 1, 3, and 7 days post inoculation. *IL-10CD* primers do not recognize the sterile transcripts of *IL-10* produced by IL-10-/- mice (30). Representative experiments with 5-10 mice per group repeated two (1 and 3 day time points) or three times (7 day time point) with similar results are shown (Figure 3).

As expected, *IL-10CD* transcripts were not detected in the tissues of IL-10-/- mice (Figures 3B and 3D); *IL-10CD* transcripts were detected in the kidneys and lungs of wild-type (B6) mice (Figures 3A and 3C). Significant elevation in *IL-10CD* transcripts in B6 infected (B6 I) kidneys above B6 uninfected kidneys (B6 U) was seen at 1 ($P < 0.01$), 3 ($P < 0.001$), and 7 ($P < 0.05$) days post injection (Figure 3A). Additionally, significant elevation in *IL-10CD* transcripts in B6 infected (B6 I) lungs above B6 uninfected lungs (B6 U) was seen at 1 ($P < 0.001$), 3 ($P < 0.05$), and 7 ($P < 0.01$) days post injection (Figure 3C). There was not a significant difference between *IL-10CD* transcript levels found in B6 I kidneys at 1, 3, and 7 day time points, suggesting sustained elevation of transcripts over time (Figure 3A). Also, there was not a significant difference between *IL-10CD* transcript levels found in B6 I lungs at 1, 3, and 7 day time points, also suggestive of sustained elevation of transcripts

over time (Figure 3C). Since the kidneys, not the lungs, are the sites of chronic colonization for *L. interrogans*, the elevation in *IL-10* transcripts appears to be a systemic response in the wild-type mouse in response to *L. interrogans* infection. Therefore, *L. interrogans* may be inducing *IL-10* transcript production in wild-type mice, allowing the bacteria to persist in the kidney and be chronically shed by the rodent reservoir host.

Accordingly, we set out to determine if other cytokine and transcription factor responses following *L. interrogans* infection are local or systemic and if these responses vary based on the genetic differences between wild-type and IL-10-deficient mice. We examined IFN-γ, TNF-α, and IL-6, three macrophage derived cytokines whose levels can be regulated by IL-10. We also examined IL-4, which promotes alternative activation of macrophages into M2 cells, which secrete IL-10 and TGF-β1. Next, we examined TGF-β1; IL-4 and TGF-β1 regulate each other in mice and humans (36). Finally, we examined FoxP3, as TGF-β1 can induce FoxP3+ t-regulatory cells, which can produce IL-10 (37).

We found that, indeed, *IFN-γ* transcript levels were greatly elevated in IL-10-/- mouse tissues compared to their wild-type counterparts (Figure 4). The induction of *IFN-γ* transcripts did not significantly vary between infected (I) and uninfected (U) B6 kidneys at any time point examined (Figure 4A). There was a significant increase in *IFN-γ* transcripts found in B6 infected lungs over B6 uninfected lungs at 1 day post injection ($P < 0.01$) not seen at later time points (Figure 4C). Unlike in B6 infected tissues, *IFN-γ* transcripts in IL-10-/- infected tissues increased over time following inoculation with *L. interrogans* (Figures 4B and 4D). The increase in *IFN-γ* transcripts in IL-10-/- infected tissues from 1 day post inoculation to 7 days post inoculation was significant ($P < 0.001$), suggestive of a systemic IFN-γ response in IL-10-/- mice to *L. interrogans* infection. From these data, it is evident that

IFN-γ transcripts are differentially regulated in *L. interrogans*-infected IL-10-deficient mice in comparison to *L. interrogans*-infected wild-type mice.

Trends in induction of *TNF-α* transcripts were found to be similar between IL-10-/- and B6 kidneys and between IL-10-/- and B6 lungs over the course of *L. interrogans* infection (Figure 5). Both IL-10-/- and B6 infected kidneys have significantly elevated *TNF-α* transcripts at 1 day post inoculation in comparison to 3 days post inoculation and to 7 days post inoculation ($P < 0.001$, Figures 5A and 5B). *TNF-α* transcripts decreased after a peak at 1 day post inoculation, but were not found to be significantly different among B6 infected and uninfected lungs at any time point examined (Figure 5C). IL-10-/- infected lungs have significantly elevated *TNF-α* transcripts at 1 day post inoculation in comparison to 7 days post inoculation ($P < 0.01$, Figure 5D). *TNF-α* transcripts peak early in *L. interrogans*-infected B6 and IL-10-/- mice then decline over time. *TNF-α* transcript copy numbers are higher in infected IL-10-/- tissues than in infected B6 tissues, but the kinetics of expression are similar.

Like *TNF-α*, early induction of *IL-6* transcripts in *L. interrogans*-infected tissues was common to both B6 and IL-10-/- mice (Figure 6). Both IL-10-/- and B6 infected kidneys have significantly elevated *IL-6* transcripts at 1 day post inoculation in comparison to 7 days post inoculation ($P < 0.001$, Figures 6A and 6B). B6 infected lungs have significantly higher *IL-6* transcript levels present at 1 day post inoculation in comparison to B6 uninfected lungs ($P < 0.05$, Figure 6C). Similarly, IL-10-/- infected lungs have significantly higher *IL-6* transcript levels present at 1 day post inoculation in comparison to IL-10-/- uninfected lungs ($P < 0.001$, Figure 6D). IL-10-/- infected lungs have significantly more *IL-6* transcripts present at 1 day post inoculation in comparison to 7 days post inoculation ($P < 0.001$, Figure

6D).Like *TNF-α, IL-6* transcripts are elevated early in infection of both B6 and IL-10-/- mice and decline over time.

IL-4 transcripts were not detected in the lungs of B6 or IL-10-/- mice (Figures 7C and 7D); they were, however, detected in the kidneys of B6 and IL-10-/- mice. Both B6 and IL-10-/- infected kidneys have significantly more *IL-4* transcripts detected at 1 day post inoculation in comparison to their uninfected counterparts ($P < 0.05$, Figures 7A and 7B). *IL-4* transcripts peaked early then declined over time in infected B6 and infected IL-10-/- kidneys, similar to both *IL-6* and *TNF-α* transcript expression patterns.

Due to the anti-inflammatory role IL-10 would normally play, we hypothesized that in its absence, other anti-inflammatory molecules would compensate upon *L. interrogans* infection. However, we found that *TGF-β1* transcripts were similarly elevated in IL-10-/- kidneys and lungs at 1, 3, and 7 days post injection (Figures 8B and 8D). B6 infected kidneys did have significantly more *TGF-β1* transcripts detected at 3 days post inoculation in comparison to B6 uninfected kidneys ($P < 0.05$, Figure 8A). B6 infected lungs did have significantly elevated *TGF-β1* transcripts at 7 days post inoculation in comparison to 1 day and 3 days post inoculation ($P < 0.01$, Figure 8C). Therefore, in the absence of IL-10, *TGF-β1* transcripts do not appear to be upregulated in response to *L. interrogans* infection.

FoxP3 transcript expression trends vary between kidneys and lungs (Figure 9). Interestingly, B6 mice injected with EMJH++ (B6 U) have higher levels of *FoxP3* transcripts detected early over later time points in tissues (Figures 9A and 9C). B6 infected and IL-10-/- infected kidneys have elevated levels of *FoxP3* transcripts detected at 7 days post inoculation in comparison to earlier time points (Figures 9A and 9B). Surprisingly, IL-10-/- uninfected lungs have significantly more *FoxP3* transcripts detected at 3 days post inoculation in

comparison to IL-10-/- infected lungs ($P < 0.01$, Figure 9D). In the absence of IL-10, *FoxP3* transcripts do not appear to be upregulated in response to *L. interrogans* infection.

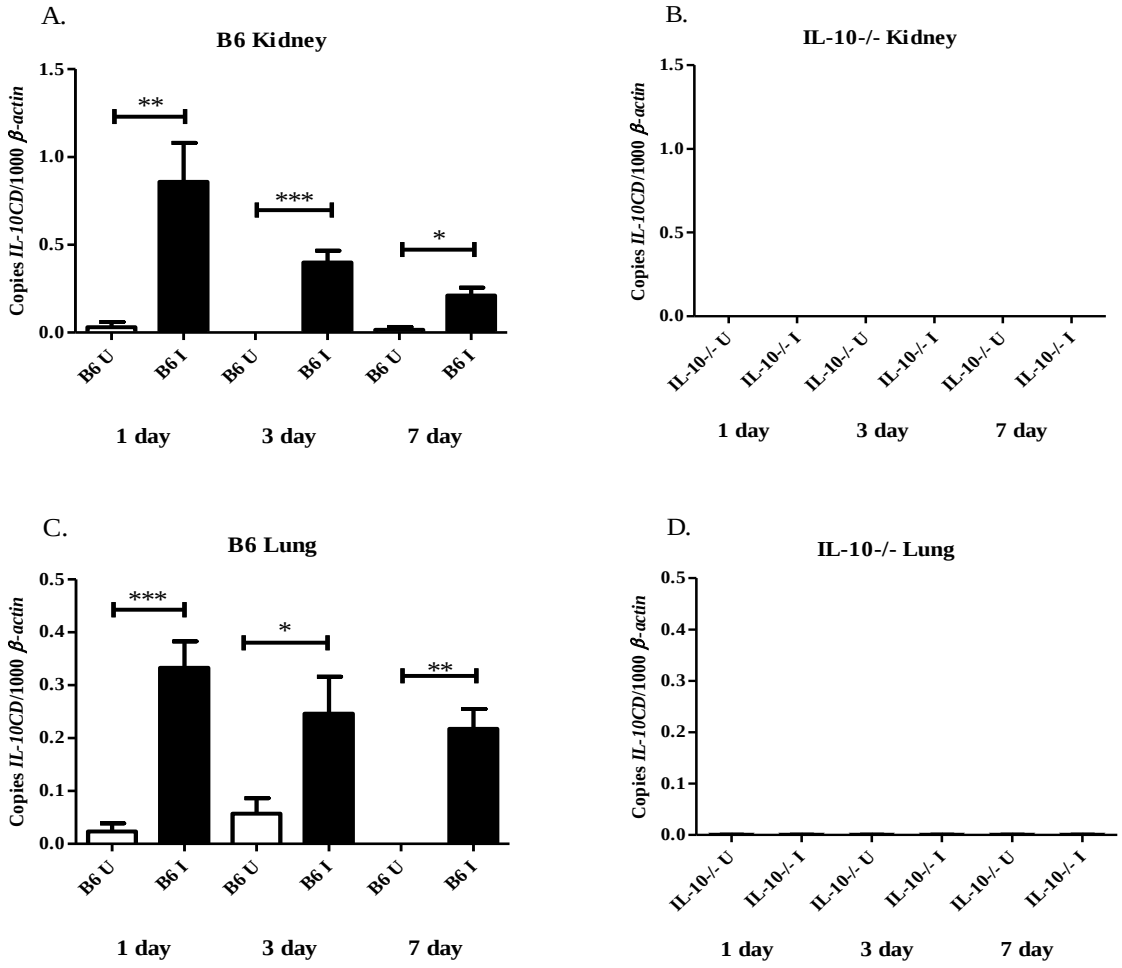

Figure 3. *IL-10* transcript induction correlates with the persistence of *L. interrogans* in the mouse kidney. C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected with either 500 µl EMJH++ medium (infected, I) or 2x10^{8} *L. interrogans* diluted in 500 µl EMJH++ medium (uninfected, U). Mice were sacrificed at 1, 3, or 7 days post inoculation (p. i.). RT-PCR transcripts are displayed as the number of *IL-10CD* transcripts normalized to 1,000 copies of the mouse *β-actin* housekeeping gene. Transcript levels for B6 kidneys (A), IL-10-/- kidneys (B), B6 lungs (C), and IL-10-/- lungs (D) are shown. Data are depicted as means ± SEM and are representative of 2 (1 and 3 day) or 3 (7 day) independent experiments (n = 5-10 mice). Statistical significance was assessed via the Mann-Whitney U test. ***, $P < 0.001$ for B6 I kidneys 3 days p. i. versus B6 U kidneys 3 days p. i., and for B6 I lungs 1 day p. i. versus B6 U lungs 1 day p. i. **, $P < 0.01$ for B6 I kidneys 1 day p. i. versus B6 U kidneys 1 day p. i., and for B6 I lungs 7 days p. i. versus B6 U lungs 7 days p. i. *, $P < 0.05$ for B6 I kidneys 1 day p. i. versus B6 U kidneys 1 day p. i., and for B6 I lungs 3 days p. i. versus B6 U lungs 3 days p. i. There was no significant difference between B6 I *IL-10CD* transcript levels in the kidneys at 1, 3, and 7 days p. i. There was also no significant difference between B6 I *IL-10CD* transcript levels in the lungs at 1, 3, and 7 days p. i.

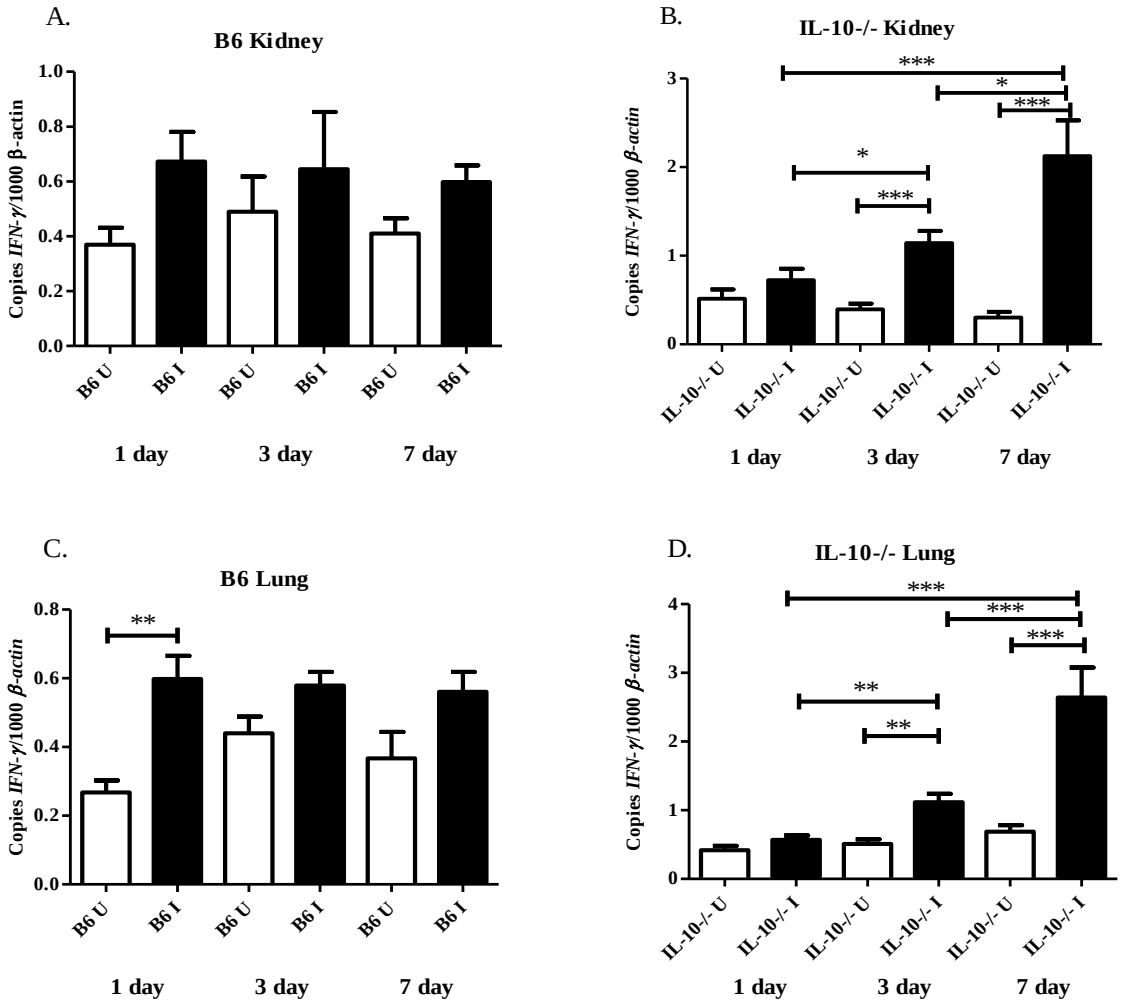

Figure 4. *IFN-γ* transcript induction correlates with the clearance of *L. interrogans* from the IL-10-deficient mouse kidney by 7 days post inoculation. C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected with either 500 µl EMJH++ medium (infected, I) or 2x10^{8} *L. interrogans* diluted in 500 µl EMJH++ medium (uninfected, U). Mice were sacrificed at 1, 3, or 7 days post inoculation (p. i.). RT-PCR transcripts are displayed as the number of *IFN-γ* transcripts normalized to 1,000 copies of the mouse *β-actin* housekeeping gene. Transcript levels for B6 kidneys (A), IL-10-/- kidneys (B), B6 lungs (C), and IL-10-/- lungs (D) are shown. Data are depicted as means ± SEM and are representative of 2 (1 and 3 day) or 3 (7 day) independent experiments (n = 5-10 mice). Statistical significance was assessed via the Mann-Whitney U test. ***, $P < 0.001$ for IL-10-/- I kidneys 3 days p. i. versus IL-10-/- U kidneys 3 days p.i., for IL-10-/- I kidneys 7 days p. i. versus IL-10-/- U kidneys 7 days p. i., for IL-10-/- I lungs 7 days p. i. versus IL-10-/- U lungs 7 days p. i., for IL-10-/- I kidneys 7 days p. i. versus IL-10-/- I kidneys 1 day p. i., for IL-10-/- I lungs 7 days p. i. versus IL-10-/- I lungs 1 day p.i., and for IL-10-/- I lungs 7 days p.i. versus IL-10-/- I lungs 3 days p. i. **, $P < 0.01$ for B6 I lungs at 1 day p. i. versus B6 U lungs 1 day p. i., for IL-10-/- I lungs 3 days p. i. versus IL-10-/- U lungs 3 days p. i., and for IL-10-/- I lungs 3 days p. i. versus IL-10-/- I lungs at 1 day p. i. *, $P < 0.05$ for IL-10-/- I kidneys at 7 days p.i. versus IL-10-/- I kidneys 3 days p. i., and for IL-10-/- I kidneys 3 days p.i. versus IL-10-/- I kidneys 1 day p. i.

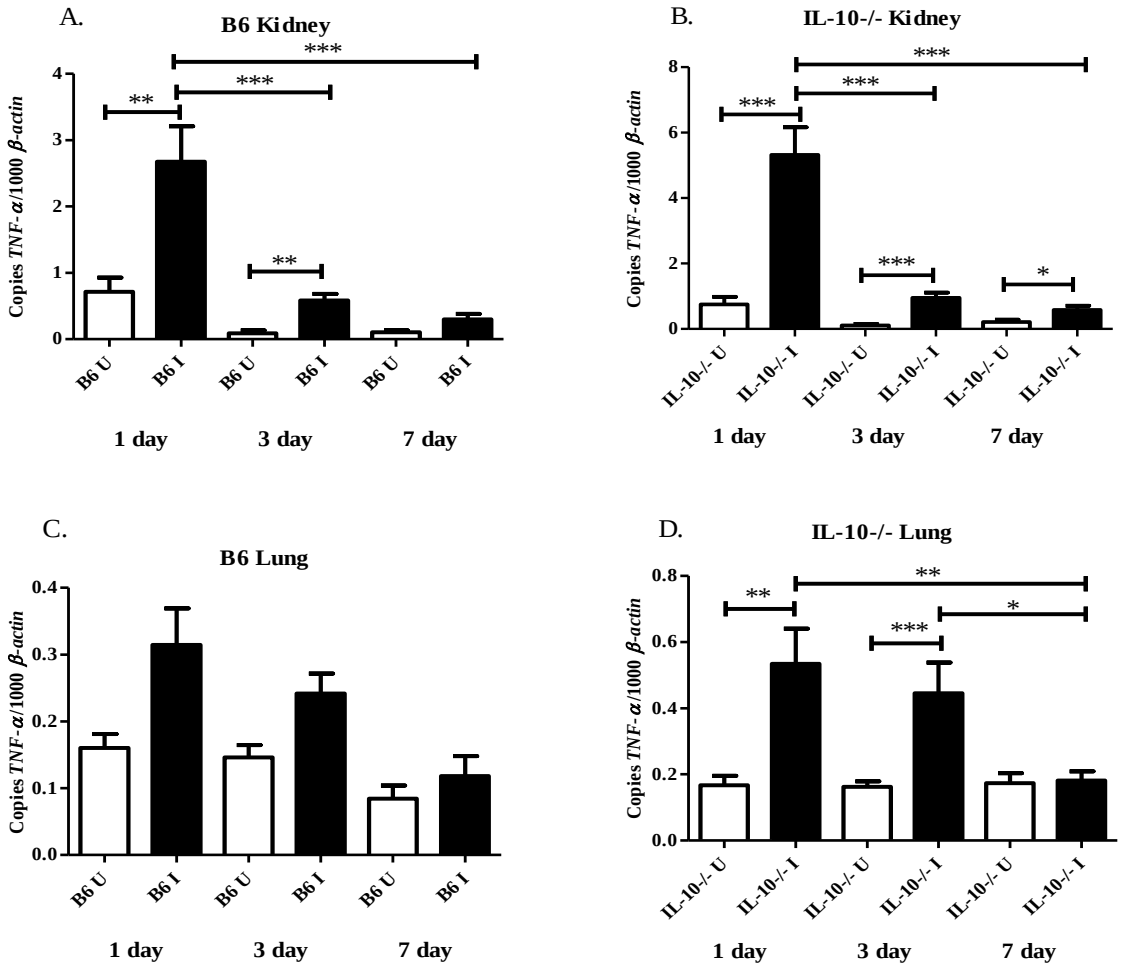

Figure 5. *TNF-α* transcript induction occurs early in *L. interrogans* infection of mice. C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected with either 500 µl EMJH++ medium (infected, I) or 2x10^{8} *L. interrogans* diluted in 500 µl EMJH++ medium (uninfected, U). Mice were sacrificed at 1, 3, or 7 days post inoculation (p. i.). RT-PCR transcripts are displayed as the number of *TNF-α* transcripts normalized to 1,000 copies of the mouse *β-actin* housekeeping gene. Transcript levels for B6 kidneys (A), IL-10-/- kidneys (B), B6 lungs (C), and IL-10-/- lungs (D) are shown. Data are depicted as means ± SEM and are representative of 2 (1 and 3 day) or 3 (7 day) independent experiments (*n* = 5-10 mice). Statistical significance was assessed via the Mann-Whitney U test. ***, P < 0.001 for B6 I kidneys 1 day p. i. versus B6 I kidneys 3 days p. i., for B6 I kidneys 1 day p. i. versus B6 I kidneys 7 days p. i., for IL-10-/- I kidneys 1 day p. i. versus IL-10-/- I kidneys 3 days p. i., for IL-10-/- I kidneys 1 day p. i. versus IL-10-/- I kidneys 7 days p. i., for IL-10-/- I kidneys 1 day p. i. versus IL-10-/- U kidneys 1 day p. i., for IL-10-/- I kidneys 3 days p. i. versus IL-10-/- U kidneys 3 days p. i., and for IL-10-/- I lungs 3 days p. i. versus IL-10-/- U lungs 3 days p. i. **, P < 0.01 for B6 I kidneys 1 day p. i. versus B6 U kidneys 1 day p. i., for B6 I kidneys 3 days p. i. versus B6 U kidneys 3 days p. i., for IL-10-/- I lungs 1 day p. i. versus IL-10-/- U lungs 1 day p. i., and for IL-10-/- I lungs 1 day p. i. versus IL-10-/- I lungs 7 days p. i. *, P < 0.05 for IL-10-/- I kidneys 7 days p. i. versus IL-10-/- U kidneys 7 days p. i., and for IL-10-/- I lungs 3 days p. i. versus IL-10-/- I lungs 7 days p. i.

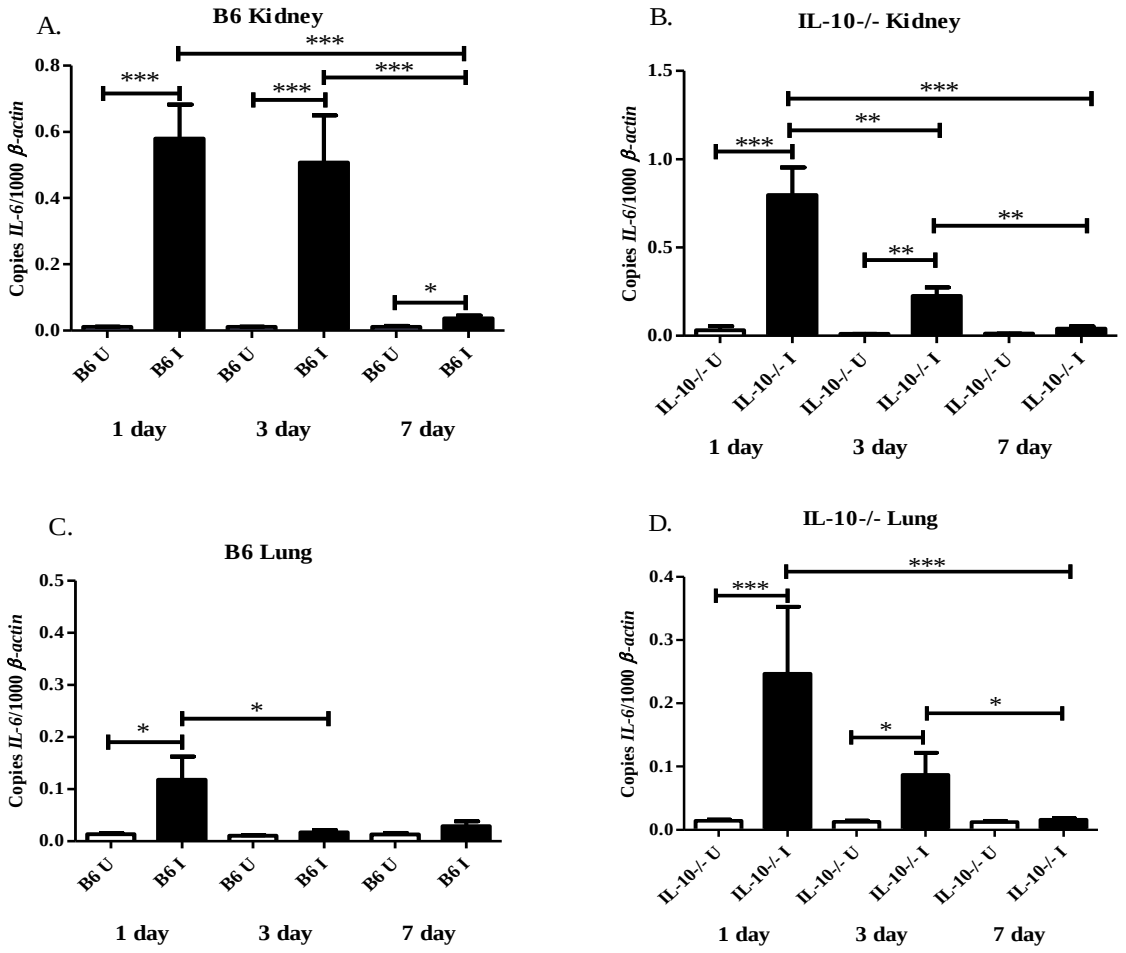

Figure 6. *IL-6* transcript induction occurs early in *L. interrogans* infection of mice. C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected with either 500 µl EMJH++ medium (infected, I) or 2x10^{8} *L. interrogans* diluted in 500 µl EMJH++ medium (uninfected, U). Mice were sacrificed at 1, 3, or 7 days post inoculation (p. i.). RT-PCR transcripts are displayed as the number of *IL-6* transcripts normalized to 1,000 copies of the mouse *β-actin* housekeeping gene. Transcript levels for B6 kidneys (A), IL-10-/- kidneys (B), B6 lungs (C), and IL-10-/- lungs (D) are shown. Data are depicted as means ± SEM and are representative of 2 (1 and 3 day) or 3 (7 day) independent experiments (*n* = 5-10 mice). Statistical significance was assessed via the Mann-Whitney U test. ***, P < 0.001 for B6 I kidneys 1 day p. i. versus B6 U kidneys 1 day p. i., for B6 I kidneys 3 days p. i. versus B6 U kidneys 3 days p. i., for IL-10-/- I kidneys 1 day p. i. versus IL-10-/- U kidneys 1 day p. i., for IL-10-/- I lungs 1 day p. i. versus IL-10-/- U lungs 1 day p. i., for B6 I kidneys 1 day p. i. versus B6 I kidneys 7 days p. i., for B6 I kidneys 3 days p. i. versus B6 I kidneys 7 days p. i., for IL-10-/- I kidneys 1 day p. i. versus IL-10-/- I kidneys 7 days p. i., and for IL-10-/- I lungs 1 day p. i. versus IL-10-/- I lungs 7 days p. i. **, P < 0.01 for IL-10-/- I kidneys 3 days p. i. versus IL-10-/- U kidneys 3 days p. i., for IL-10-/- I kidneys 1 day p. i. versus IL-10-/- I kidneys 3 days p. i., and for IL-10-/- I kidneys 3 days p.i. versus IL-10-/- I kidneys 7 days p. i. *, P < 0.05 for B6 I kidneys 7 days p. i. versus B6 U kidneys 7 days p. i., for B6 I lungs 1 day p. i. versus B6 U lungs 1 day p. i., for B6 I lungs 1 day p. i. versus B6 I lungs 3 days p. i., for IL-10-/- I lungs 3 days p. i. versus IL-10-/- U lungs 3 days p. i., and for IL-10-/- I lungs 3 days p. i. versus IL-10-/- I lungs 7 days p. i.

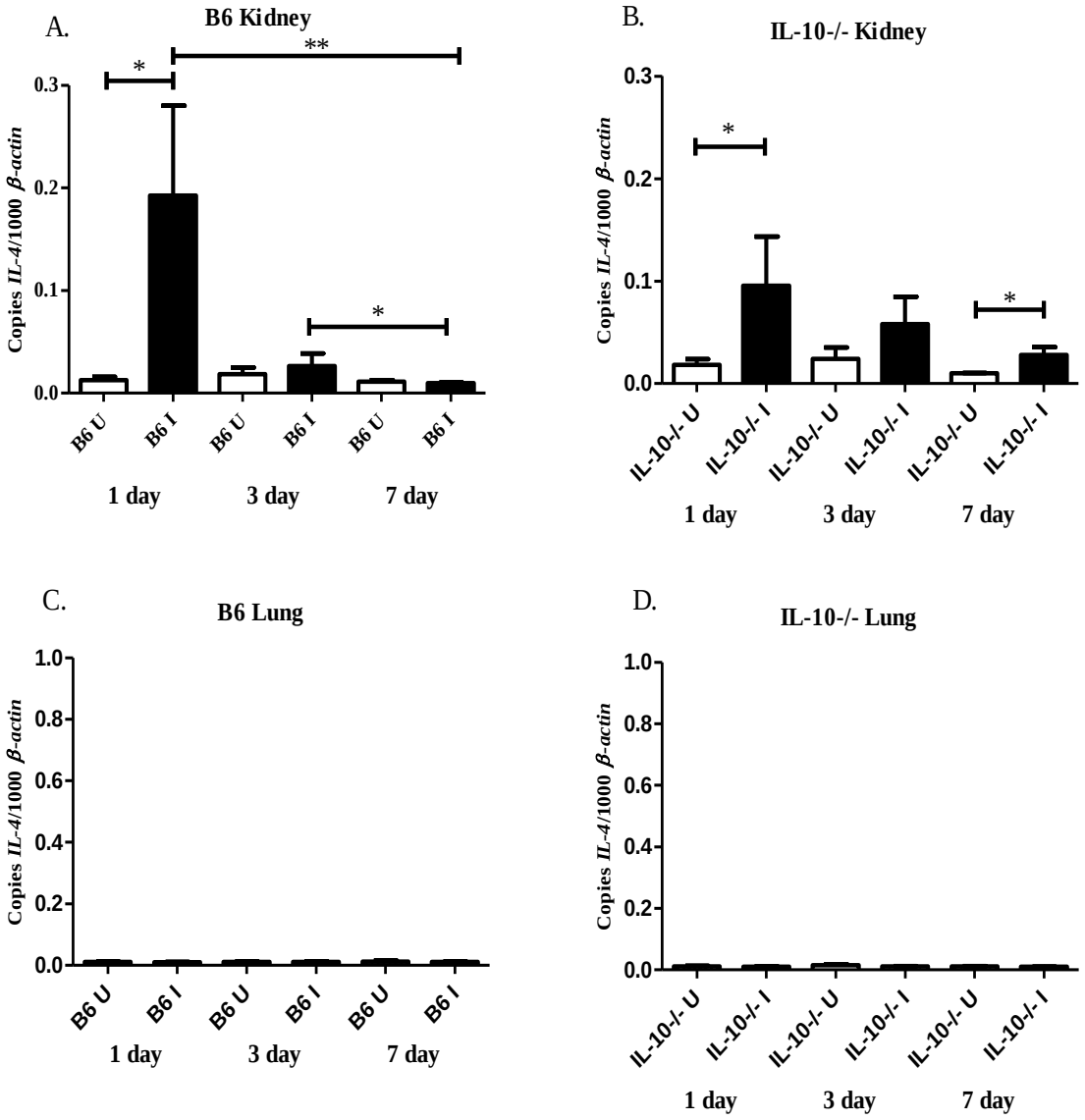

Figure 7. *IL-4* transcript induction in the kidney occurs early in *L. interrogans* infection of mice. C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected with either 500 µl EMJH++ medium (infected, I) or 2x10^{8} *L. interrogans* diluted in 500 µl EMJH++ medium (uninfected, U). Mice were sacrificed at 1, 3, or 7 days post inoculation (p. i.). RT-PCR transcripts are displayed as the number of *IL-4* transcripts normalized to 1,000 copies of the mouse *β-actin* housekeeping gene. Transcript levels for B6 kidneys (A), IL-10-/- kidneys (B), B6 lungs (C), and IL-10-/- lungs (D) are shown. Data are depicted as means ± SEM and are representative of 2 (1 and 3 day) or 3 (7 day) independent experiments (*n* = 5-10 mice). Statistical significance was assessed via the Mann-Whitney U test. **, P < 0.01 for B6 I kidneys 1 day p. i. versus B6 I kidneys 7 days p. i. *, P < 0.05 for B6 I kidneys 1 day p. i. versus B6 U kidneys 1 day p. i., for B6 I kidneys 3 days p. i. versus B6 I kidneys 7 days p. i., for IL-10-/- I kidneys 1 day p. i. versus IL-10-/- U kidneys 1 day p.i., and for IL-10-/- I kidneys 7 days p. i. versus IL-10-/- U kidneys 7 days p. i. *IL-4* transcripts were not detected in mouse lungs.

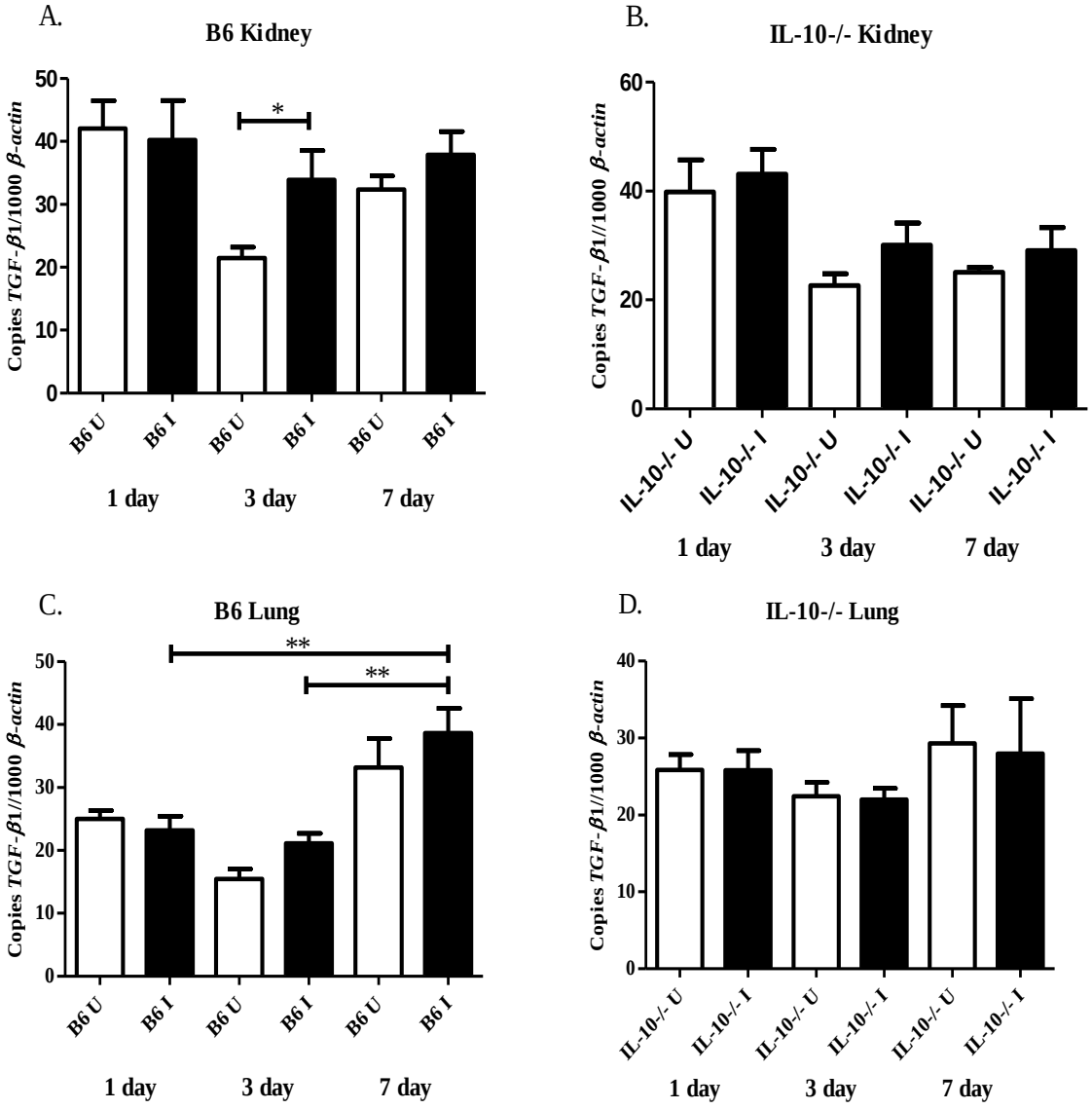

Figure 8. *TGF-β1* transcript induction in *L. interrogans*-infected and uninfected mouse tissues. C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected with either 500 µl EMJH++ medium (infected, I) or 2x10^{8} *L. interrogans* diluted in 500 µl EMJH++ medium (uninfected, U). Mice were sacrificed at 1, 3, or 7 days post inoculation (p. i.). RT-PCR transcripts are displayed as the number of *TGF-β1* transcripts normalized to 1,000 copies of the mouse *β-actin* housekeeping gene. Transcript levels for B6 (WT) kidneys (A), IL-10-/- kidneys (B), B6 (WT) lungs (C), and IL-10-/- lungs (D) are shown. Data are depicted as means ± SEM and are representative of 2 (1 and 3 day) or 3 (7 day) independent experiments (n = 5-10 mice). Statistical significance was assessed via the Mann-Whitney U test. **, $P < 0.01$ for B6 I lungs 7 days p. i. versus B6 I lungs 3 days p. i., and for B6 I lungs 7 days p. i. versus B6 I lungs 1 day p. i. *, $P < 0.05$ for B6 I kidneys 3 days p. i. versus B6 U kidneys 3 days p. i.

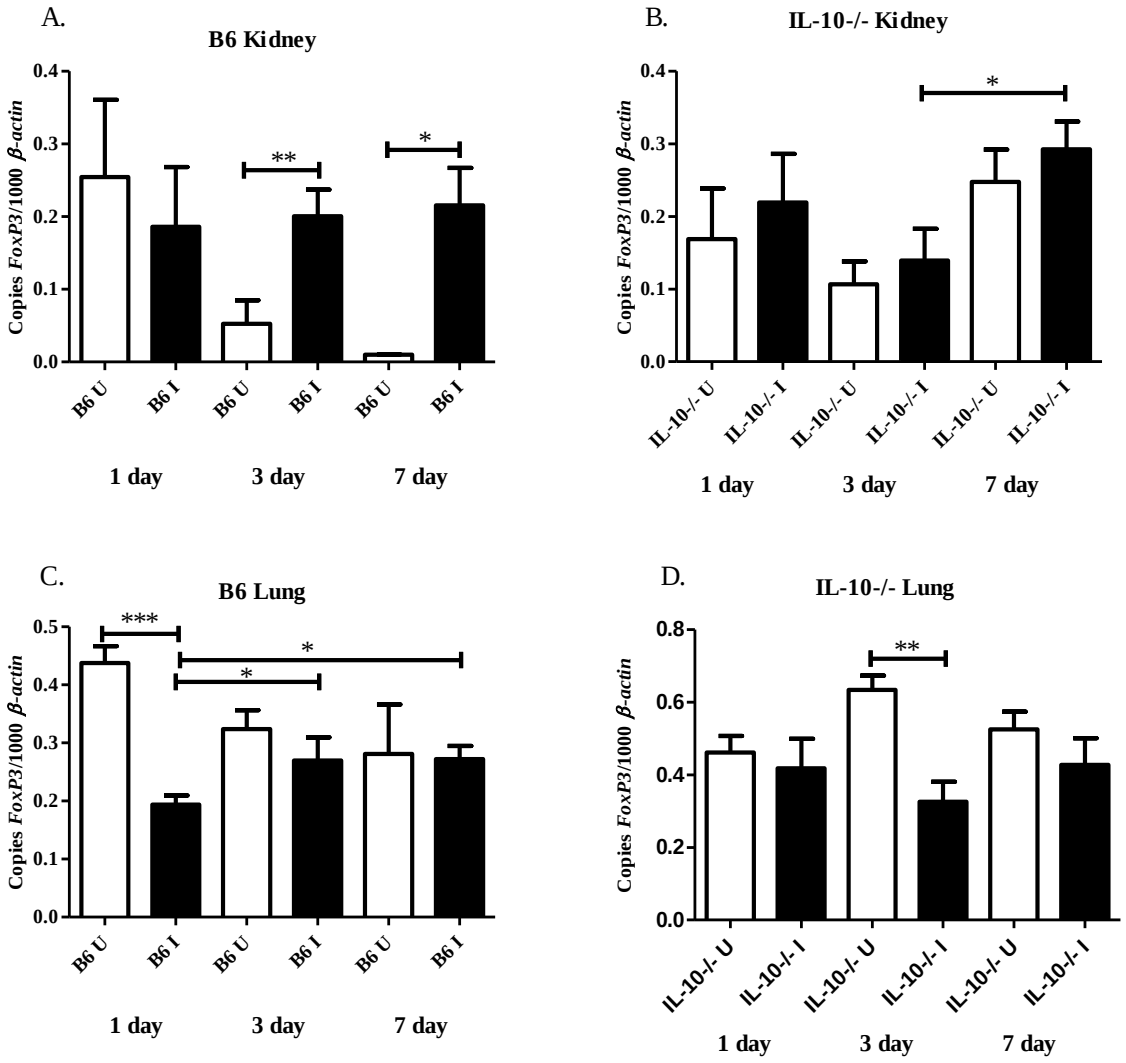

Figure 9. *FoxP3* transcript induction in *L. interrogans*-infected and uninfected mouse tissues. C57BL/6 (B6) and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10-/-) mice were injected with either 500 µl EMJH++ medium (infected, I) or 2x10^{8} *L. interrogans* diluted in 500 µl EMJH++ medium (uninfected, U). Mice were sacrificed at 1, 3, or 7 days post inoculation (p. i.). RT-PCR transcripts are displayed as the number of *FoxP3* transcripts normalized to 1,000 copies of the mouse *β-actin* housekeeping gene. Transcript levels for B6 kidneys (A), IL-10-/- kidneys (B), B6 lungs (C), and IL-10-/- lungs (D) are shown. Data are depicted as means ± SEM and are representative of 2 (1 and 3 day) or 3 (7 day) independent experiments (n = 5-10 mice). Statistical significance was assessed via the Mann-Whitney U test. ***, $P < 0.001$ for B6 U lungs 1 day p. i. versus B6 I lungs 1 day p. i. **, $P < 0.01$ for B6 I kidneys 3 days p. i. versus B6 U kidneys 3 days p. i., and for IL-10-/- U lungs 3 days p. i. versus IL-10-/- I lungs 3 days p. i. *, $P < 0.05$ for B6 I kidneys 7 days p. i. versus B6 U kidneys 7 days p. i., for IL-10-/- I kidneys 7 days p. i. versus IL-10-/- I kidneys 3 days p. i., for B6 I lungs 7 days p. i. versus B6 I lungs 1 day p. i., and for B6 I lungs 3 days p. i. versus B6 I lungs 1 day p. i.

4 Discussion

Similar to Matsui et al. (20) yet using inbred mice, we found sustained induction of IL-10 in the kidneys and lungs of B6 mice following infection with *L. interrogans*. Accordingly, this was not seen in IL-10-/- mice, but rather than exhibiting increased susceptibility to disease as do hamsters (20), IL-10-/- mice cleared *L. interrogans* from tissues examined, including the kidney, by 7 days post inoculation. B6 mice, however, did not clear the spirochetes by 7 days post inoculation, as was previously shown (34). Because IL-10 is known to possess anti-inflammatory properties and suppress several functions associated with immune cells, we sought to address whether enhanced immune responses in its absence might be responsible for the increased clearance of *L. interrogans*. A key finding was that *IFN-γ* transcripts were differentially elevated in the tissues of B6 and IL-10-/- mice. Infected B6 tissues exhibited minor early induction of IFN-γ that decreased slightly over time, whereas in infected IL-10-/- tissues, induction of IFN-γ post inoculation at 1 day was minor, with significant elevation at 3 days and 7 days that increased over time. IFN-γ induction following infection with *L. interrogans* appears to be a systemic response in IL-10-/- mice. Other pro-inflammatory cytokines, TNF-α and IL-6, were induced differently following infection in comparison to IFN-γ. These cytokines were expressed early following infection in both B6 and IL-10-/- mice and expression declined over time. IL-4 expression following infection followed a similar pattern to that seen with TNF-α and IL-6. TGF-β1 and FoxP3 expression did not indicate a significant role for these molecules following infection with *L. interrogans* in IL-10-/- mice.

The cellular source of IL-10 in *L. interrogans*-infected mice is unknown, but many cell types, including CD4+ and CD8+ T-cell subsets, macrophages, dendritic cells, B cells, and

keratinocytes, have been documented as sources of this cytokine (35). Upon examining the role of IL-10 during infection with the related spirochete *Borrelia burgdorferi*, Brown et al. (12) found that *B. burgdorferi* burdens in infected IL-10-/- mice were already reduced by 2 weeks post inoculation, at which time no differences in specific IgM or IgG levels were detected between B6 and IL-10-/- mice. A major target of IL-10 is the phagocytic cell and suppression leads to an inhibition of the innate ability of this cell type to fight microbial infections (38-42). Macrophage activity was indeed suppressed by IL-10 in B6 mice and there was more efficient killing of *B. burgdorferi* in the absence of IL-10 (13, 14). Lazarus et al. (13) found that increased clearance of *B. burgdorferi* in IL-10-/- mice is mostly due to enhanced innate immune mechanisms and that macrophage-mediated activities likely have a role in this outcome. While experiments utilizing IL-10-/- BMDMs were not performed in this work, the effect of *L. interrogans* inoculation with IL-10-/- BMDMs would be an important future study to better characterize the innate immune response in the absence of IL-10.

Recent studies have identified bacteria that can manipulate host IL-10 levels so as to enhance their virulence and/or long term persistence. Both pathogenic *Yersinia* species (43) and *Francisella tularensis* (44) can induce IL-10 production, leading to a severe reduction in IFN-γ. IFN-γ secretion is critical in the clearance of *F. tularensis* (45-48). Pathogenic *Mycobacteria* enhance IL-10 production by monocytes/macrophages in culture (49-51). Even though IL-10 modulating functions by several pathogens have been shown, the mechanism of immune suppression is presumed to be mostly mediated through elevated IL-10 production by macrophages, which in turn functions to suppress macrophages and other antigen-presenting cells via many different mechanism, including: prevention of inflammatory

clearance through suppression of pro-inflammatory cytokines, suppression of antigen presentation by down-regulating MHC class II expression, blockage of apoptotic pathways so as to allow persistence of intracellular pathogens, and suppression of phagocytosis and the resulting production of reactive oxygen and reactive nitrogen species (52). Similar mechanisms could also be involved in the inhibition of *L. interrogans* clearance seen in B6 kidneys permitting chronic, persistent infection.

While we did not determine the cellular sources of IFN-γ in infected IL-10-/- mice, we did show the critical importance of IFN-γ in the clearance of *L. interrogans* from the tissues of these mice. Lee et al. (53) infected NKT cell-deficient mice with a high-dose inoculum of *B. burgdorferi* and found these mice to harbor much greater numbers of spirochetes in joint tissue, a key colonization site, by 3 days post injection relative to their wild-type counterparts, suggesting that NKT cells may provide an early source of systemic antimicrobial IFN-γ. NKT cell-produced IFN-γ in the heart limits pathology and serves to activate nearby macrophages and induce killing of *B. burgdorferi* (54). Sonderegger et al. (30) found that CD4+ T-cells, but not NK/NKT cells were responsible for the high levels of IFN-γ in the serum of infected IL-10-/- mice, whereas both NK cells and CD4+ T-cells were the major sources of IFN-γ in the joint tissue of *B. burgdorferi*-infected IL-10-/- mice.

We found that IL-10 is required for the persistence of *L. interrogans* in the murine kidney and that IFN-γ is likely a key component in the clearance of *L. interrogans* from infected IL-10-/- tissues. Future studies would seek to determine the cellular source(s) of IFN-γ in IL-10-deficient mice to elucidate the mechanism of clearance of *L. interrogans* from the kidney. The cellular sources of IL-10 in the kidney of B6 mice should be examined to better understand how *L. interrogans* persists in the renal tubules to be chronically shed by

the rodent host. Administration of recombinant IFN-γ into the B6 mouse kidney may enhance clearance of the leptospires. While IL-10-deficient rats are not commercially available, antibody blocking of IL-10 in rats could provide insight into the mechansims of leptospiral persistence in this prominent reservoir host.

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CHAPTER 3

Borrelia burgdoferi-derived ligands: characterization and involvement in the murine host response

Abstract

Borrelia burgdorferi is the causative agent of Lyme disease. Previously, type I interferon (IFN) and IFN-stimulated genes (ISGs) were linked to the development of Lyme arthritis in mice. *B. burgdorferi*-mediated induction of ISGs by bone marrow-derived macrophages (BMDMs) involves multiple ligands, including *B. burgdorferi* genomic RNA and non-nucleic acid ligands present in *B. burgdorferi* culture supernatant. We now demonstrate that *B. burgdorferi*-mediated induction of IFN-responsive genes by BMDMs is independent of TLR-7. *B. burgdorferi* genomic RNA-induced transcription of ISGs is TLR-7 independent but MyD88 dependent. Upon treatment with various RNases, *B. burgdorferi* genomic RNA is likely single-stranded. Cyclic-di-GMP, a second messenger molecule released by *B. burgdorferi*, does not enhance ISG transcription in stimulated BMDMs. The non-nucleic acid ligands present in ultracentrifuged *B. burgdorferi* culture supernatant are presumably proteinaceous in nature and do not include the lipoprotein OspA. The findings presented herein describe the composition of key *B. burgdorferi*-derived stimulatory ligands and host defense signaling pathways involved in the type I IFN and ISG induction associated with Lyme arthritis development in the mammalian host.

1 Introduction

Borrelia burgdorferi, the causative agent of Lyme disease, is transmitted to humans and its reservoir animal hosts through the bites of infected *Ixodes* ticks (1-3). In the United States, Lyme disease is the most prevalent vector-borne illness (4), and recent studies conducted by the CDC indicate that the incidence of Lyme disease is ten times greater than originally reported, with revised estimates suggesting that the actual number of cases in the U. S. A. is closer to 300,000 per year (5-9). Patients afflicted with Lyme disease who do not receive timely antibiotic treatment may exhibit a myriad of clinical signs, including carditis and arthritis (10-12). Because the *B. burgdorferi* genome does not harbor genes encoding sophisticated secretion systems or exotoxins (13), the resultant inflammation and tissue damage that occur following infection are likely due to a pathological host response (4, 14).

The innate immune system is the first line of host defense against infectious agents. Bacterial pathogen-associated molecular patterns (PAMPs), such as RNA, DNA, LPS, lipoproteins, or flagellin, are recognized by their cognate pattern recognition receptors (PRRs), including TLRs, NLRs, and RLRs, that are located on the plasma membrane or within the endosome or cytosol of the host cell. All TLRs, except TLR-3, use MyD88 as an adaptor molecule. TLR-3 utilizes only TRIF, whereas TLR-4 can utilize both MyD88 and TRIF as adaptor molecules. PAMP ligation by TLRs activates MyD88- or TRIF-dependent signal transduction cascades that culminate with the transcription of NFκB-dependent pro-inflammatory cytokines and chemokines, and/or with IRF-3/7 homo- or heterodimerization and subsequent activation of Type I IFN gene transcripts (15-17).

B. burgdorferi lacks LPS (18); however, the multitude of lipoproteins that decorate its outer membrane elicit a robust pro-inflammatory cytokine response (19-24). Although

CD14-dependent ligation of cell surface TLR-2 (25-30) has been implicated in the lipoprotein-mediated pro-inflammatory cytokine response, phagocytosis (31, 32) and signaling through endosomally-located TLR-2 (33-36) is required to fully recapitulate cellular pro-inflammatory cytokine production triggered by live, intact *B. burgdorferi. B. burgdorferi* PAMP signaling through TLR-5, but not TLR-9, has also been implicated in pro-inflammatory cytokine production (33). It is well established that both TLR-2 and MyD88 assume critical roles in host defense to *Borrelia burgdorferi*. TLR-2-/- mice harbor hundreds more *B. burgdorferi*, whereas MyD88-/- mice contain thousands more spirochetes within their ankle joints, when compared with the bacterial burden present within wild-type mouse ankle joints (29, 37, 38). These data suggest that multiple TLR(s) may participate in the host defense against *B. burgdorferi*.

B. burgdorferi stimulation of various mouse or human cell types, including monocytes, macrophages, and endothelial cells, elicits the production of Type I IFNs and Type I IFN genes (ISGs) (34, 39-46). Multiple *B. burgdorferi* ligands have been reported to trigger cellular Type I IFN production (44, 47), and both MyD88-independent and –dependent signal transduction pathways have been implicated in the Type I IFN response to intact live spirochetes (36, 41, 43, 44, 46). Intriguingly, Miller et al. (41) discovered that *B. burgdorferi*-elicited Type I IFN enhanced the severity of murine Lyme arthritis without impacting host defense (41).

In a previous study, Miller et al. (44) demonstrated that *B. burgdorferi* genomic RNA and non-nucleic acid *B. burgdorferi*-associated products in culture supernatant are potent Type I IFN-stimulatory ligands for mouse bone marrow-derived macrophages (BMDMs). In addition, Cervantes et al. (47) recently reported that *B. burgdorferi* genomic RNA stimulated

TLR-8-dependent Type I IFN production by human monocytes. We sought to evaluate the role(s) of murine TLR-2, TLR-7, and MyD88 in Type I ISG expression when stimulated with *B. burgdorferi* or *B. burgdorferi* genomic RNA. Characterizations of non-nucleic acid *B. burgdorferi*-associated products in culture supernatant and of *B. burgdoferi* genomic RNA were performed. Additionally, DGC and PDE *B. burgdorferi* mutants were utilized to evaluate the role(s) of cyclic-di-GMP in the Type I IFN-stimulated response.

2 Materials and Methods

2.1 Bacterial culture, mice, and cell lines

C57BL/6 (B6), B6 MyD88-/-, and C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 TLR-7 -/- and B6 TLR-2-/- mice were generously provided by Frank Scholle (North Carolina State University, Raleigh, NC). Raw 264.7 macrophage-like cells were provided by Scott Laster (North Carolina State University, Raleigh, NC). The North Carolina State University Institutional Animal Care and Use Committee approved all mouse studies (Protocol numbers: 10-099-B, and 13-066-B). Mice were housed in the North Carolina State University Biological Resources Facility (Raleigh, NC) and utilized with strict adherence to all institutional and NIH policies governing the care and compassionate utilization of animals in biomedical research.

B. burgdorferi strain B31-MI-16 was utilized for all experiments. B31-MI-16 is a fully infectious clonal isolate that exhibits an identical plasmid profile with its parent strain B31-MI (55). Low passage isolates of B31-MI-16 were propagated at 32°C to late logarithmic phase ($6x10^7$ – $1x10^8$). Plasmid content was monitored by PCR, as previously described (56, 57) to ensure that plasmid loss did not occur during *in vitro* cultivation.

For cyclic-di-GMP mutant experiments, *B. burgdorferi* strain B31-A3, *B. burgdorferi* strain B31-K2 (kanamycin resistance cassette only), *B. burgdorferi* strain B31 Bb0363:PI-Kan/A3 (PdeA mutant), *B. burgdorferi* strain B31 Bb0363:PI-Kan-bb0374:S/A3 (PdeA and PdeB mutant), *B. burgdorferi* strain B31 Bb0374:S/K2 (PdeB mutant), *B. burgdorferi* strain B31-S9 (streptomycin resistance cassette only), and *B. burgdorferi* strain B31 Bb0419:PI-Kan/S9 (Rrp1 mutant) were kindly provided by M. D. Motaleb (East Carolina University, Greenville, NC) (58, 59). Low passage isolates were propagated at 32°C to late exponential phase ($6x10^7 – 1x10^8$). Strains were grown using appropriate antibiotics: kanamycin, streptomycin, or both, depending on resistance cassettes. Prior to use in BMDM experiments, all live *B. burgdorferi* were washed twice with phosphate-buffered saline (PBS) to remove BSK II contamination (44).

2.2 Reagents

R848, Pam3CSK4, and LPS-EB Ultrapure were purchased from InvivoGen (San Diego, CA). RNase A was obtained from Roche Applied Science (Indianapolis, IN). DNase I, RNase III, and RNase i_f were purchased from New England BioLabs (Ipswich, MA). Recombinant OspA was purchased from Merial (Duluth, GA). *B. burgdorferi* polyclonal membrane antibody was provided by Brian Stevenson (University of Kentucky, Lexington, KY). SYPRO Ruby was purchased from Bio-Rad Laboratories (Hercules, CA).

2.3 RNA extraction and quantitative reverse transcription PCR (q-RT-PCR) analysis

Total *B. burgdorferi* genomic RNA (Bb RNA) was extracted from late exponential phase B31-MI-16 cultures using Qiazol reagent, according to the manufacturer's protocol (Qiagen, Germantown, MD). The Nanodrop 1000 was utilized for quantification and initial quality control assessment of extracted RNA samples. All isolated genomic Bb RNA

exhibited 260/280 ratios of > 1.8 and 260/230 ratios of >1.3. Following extraction, the manufacturer's specifications were followed to remove genomic DNA from Bb RNA via treatment with DNase I. The integrity of the Bb RNA 23S and 16S ribosomal RNAs was assessed via 1% agarose gel electrophoresis, both before and following DNase I digestion, to ensure RNA degradation did not occur during experimental manipulation.

Cellular RNA was isolated from mouse bone marrow-derived macrophages (BMDMs) cultured in 12 well plates using 1 ml of Qiazol reagent (44). 1µg of RNA was reverse transcribed to cDNA using random primers (Promega, Madison, WI) and M-MLV reverse transcriptase (Affymetrix, Santa Clara, CA). q-PCR was conducted to amplify transcripts for genes of interest using the iQ SYBR Green Supermix and the MyiQ2 Two-color Real-time PCR Detection System (Bio-Rad, Hercules, CA). For each transcript of interest, copy number was calculated from the starting template sample and normalized to 1000 copies of the mouse housekeeping gene *β-actin*, as previously reported (60). The following gene transcripts were amplified by q-RT-PCR in this study: *β-actin* (40), *Cxcl10* (41), *Ifit1* and *Gbp2* (44). Only primer pairs that spanned introns were utilized to control for any potential effects of genomic DNA contamination.

2.4 RNase digestion

Extracted *B. burgdorferi* genomic RNA was subjected to treatment with various RNases according to the manufacturer's instructions. RNase A targets all species of RNA for degradation. RNase i_f preferentially targets single-stranded RNA for degradation. RNase III specifically degrades double-stranded RNA. In each instance, 1 µg of genomic RNA was treated with the various RNases then utilized in experiments.

2.5 Ultracentrifugation of *B. burgdorferi* supernatant and BSK II medium, and collection of ultracentrifuged supernatant, BSK II and membrane blebs

After *B. burgdorferi* cultures reached late exponential phase, cultures were centrifuged at 3,000 rpm for 10 minutes at 4°C. Culture supernatant was collected and filtered twice with 0.22 µm filters. Fresh BSK II medium was also filtered twice prior to ultracentrifugation. 11 milliliters each of filtered BSK II and culture supernatants were centrifuged at 40,000 x g for 4 hours at 4°C. Following ultracentrifugation, only the top 1 ml of supernatant and BSK II medium were used as stimuli in experiments at a concentration of 50 µl/ml (44). The bottom 1 ml from ultracentrifuged *B. burgdorferi* culture supernatant, consisting of lysed bacterial membrane blebs, was also collected for experiments.

2.6 Western blots and SYPRO Ruby staining

To confirm that ultracentrifugation completely removes *B. burgdorferi* membrane blebs, 5 µg of *B. burgdorferi* whole cell lysate (WCL), ultracentrifuged culture supernatant, and membrane blebs were subjected to one-dimensional (1D) SDS-PAGE. These gels were either stained with SYPRO Ruby to facilitate visualization of separated proteins or transferred to nitrocellulose membranes for Western blot analyses. The primary antibody used was rabbit-produced polyclonal antibody specific for *B. burgdorferi* membrane proteins. The secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit IgG. The dilution of the polyclonal *B. burgdorferi* membrane antibody used was 1:10,000. The dilution of the secondary antibody used was 1:5,000.

2.7 Heat inactivation and Proteinase K treatments

Ultracentrifuged *B. burgdorferi* culture supernatant and BSK II medium were heat inactivated for 1 hour at 56°C, cooled on ice for 30 minutes then warmed to 37°C for 5

minutes before being used in stimulation experiments. 20 µg/ml Proteinase K was used to treat ultracentrifuged supernatant and BSK II. After addition of Proteinase K, samples were heated for 1 hour at 56°C, followed by the addition of 2 ul of Proteinase K inhibitor, cooled on ice for 30 minutes then warmed to 37°C for 5 minutes before being used in stimulation experiments.

2.8 BMDM cell culture

Bone marrow was released from the femurs and tibias of 8-12 week old mice and BMDMs were differentiated by growth in RPMI medium (HyClone, Logan, UT) containing 30% L929 culture supernatant and 20% horse serum (Gemini BioProducts) at 37°C, 5% CO_2 (61). BMDMs were harvested and dispensed at 7.2 x 10^5 cells/ml in serum-free RPMI medium containing 1% Nutridoma (Roche) into triplicate wells of 12-well plates. Nutridoma was utilized in our studies, instead of other serum-containing medium formulations, because previous studies (41, 44) indicated that the BMDM Type I IFN response was optimal in serum-free medium. All cell culture reagents, including the L929 culture supernatants, utilized in this study were assayed by either Hoechst staining (Invitrogen, Carlsbad, CA) or with The MycoProbe Mycoplasma detection kit (R&D Systems, Minneapolis, MN) and confirmed to be negative for contaminating *Mycoplasma* species. Following an overnight incubation, medium was removed, and *B. burgdorferi* (MOI of 10) (44), or other stimuli (as indicated in the text of appropriate figure legends), were added to each set of triplicate wells. Plates were centrifuged at 300 x g for 10 minutes to facilitate *B. burgdorferi* contact with BMDMs (62) and incubated at 37°C, 5% CO_2 for 6 hours.

2.9 Statistical analysis

A paired, two-tailed Student t test was employed for two-group comparisons of

parametric data exhibiting no differences in standard deviation. An unpaired two-tailed Student t test was utilized for data where pairing was ineffective, and for categorical data exhibiting differences in standard deviation, the Welch correction was applied to that test. For all tests, $p < 0.05$ was considered significant. Statistical thresholds are displayed on figures and in figure legends as follows: * = $p < 0.05$, ** = $p < 0.01$, and *** = p-value < 0.001. All statistical analyses were conducted using GraphPad InStat3 for Windows, version 3.1a (GraphPad Software, San Diego, CA).

3 Results

3.1 Single-stranded *B. burgdorferi* RNA can induce IFN-responsive gene transcription

To determine the nature of the species of RNA derived from *B. burgdorferi* that stimulates BMDM induction of ISG transcripts (44), 1 µg of genomic RNA derived from *B. burgdorferi* was treated with either RNase A, RNase i_f, or RNase III. RNase A is a pyrimidine-specific endonuclease that targets single-stranded RNA (63). RNase i_f is an RNA endonuclease that cleaves all RNA dinucleotide bonds leaving mono-, di-, and trinucleotides (64). It preferentially cleaves single-stranded RNA (65). RNase III is an endonuclease that cleaves long double-stranded RNA into small fragments (66). As a control, 1 µg of *B. burgdorferi* (Bb) RNA was used for all BMDM stimulation experiments following dose-reponse studies indicating that 1 µg of Bb RNA stimulated equally as well as 2 µg of Bb RNA (data not shown). C3H/HeJ BMDMs were utilized, as they are unable to respond to LPS (67). C3H/HeJ BMDM stimulation allows one to conclude that IFN-responsive gene induction is not due to potential endotoxin contamination of cultured *B. burgdorferi* (44). Six- to eight-week old C3H/HeJ mice were sacrificed, BMDMs were matured and stimulated for 6 hours with live *B. burgdorferi* at an MOI of 10 and various RNA preparations (Figure

1). Selected ISG-transcripts (*Cxcl10, GBP2*, and *IFIT-1*) are shown from a representative experiment repeated twice with similar results (Figure 1A-1C).

Following treatment of Bb RNA with RNase A ($P < 0.01$) and RNase i_f ($P < 0.05$), there was a significant reduction in *Cxcl10* transcripts, as measured by qRT-PCR, produced by stimulated BMDMs after 6 hours incubation. No significant reduction in *Cxcl10* transcript production by BMDMs was observed between BMDMs stimulated with Bb RNA versus Bb RNA + RNase III (Figure 1A). While the same trend was observed with the transcripts *GBP2* (Figure 1B) and *IFIT-1* (Figure 1C), the changes in induction by RNase-treated Bb RNA were not significantly different from Bb RNA not treated with RNase.

3.2 Induction of IFN-responsive transcripts by BMDMs is not significantly affected by cyclic-di-GMP

From previous data indicating that Bb RNA is capable of inducing ISG transcript production by BMDMs (44), we decided to examine the inductive capacity of cyclic-di-GMP on BMDMs, as it is composed of RNA and produced by *B. burgdorferi*. Additionally, *B. burgdorferi* mutants unable to synthesize cyclic-di-GMP do not survive in the tick; *B. burgdorferi* mutants unable to degrade cyclic-di-GMP do not survive in the mammalian host (58, 59). This led to the hypothesis that cyclic-di-GMP can induce BMDMs to produce IFN-responsive transcripts.

Bis-(3'-5')-cyclic-dimeric guanosine monophosphate (cyclic-di-GMP) is a bacterial second messenger known to regulate many cellular processes. Cyclic-di-GMP signaling is likely limited to bacteria (48, 49), although this has recently been disputed (78). *B. burgdorferi* encodes one diguanylate cyclase gene (*rrp1*, *bb0419*) and two putative PDEs (*bb0363*, *pdeA* and *bb0374*, *pdeB*) (50-53). Rrp1 was shown to synthesize cyclic-di-GMP

when phosphorylated *in vitro* (54). BB0374 specifically binds cyclic-di-GMP (52). Microarray analyses of a DGC mutant in a non-infectious *B. burgdorferi* strain indicated that it regulated expression of several genes, including many virulence-associated genes, suggesting a role for cyclic-di-GMP in the infectious cycle of *B. burgdorferi* (53). Cyclic-di-GMP is produced from two molecules of GTP through the catalytic activity of diguanylate cyclase (DGC) and is degraded by certain phosphodiesterases (PDEs). Figure 2 is a schematic of this process.

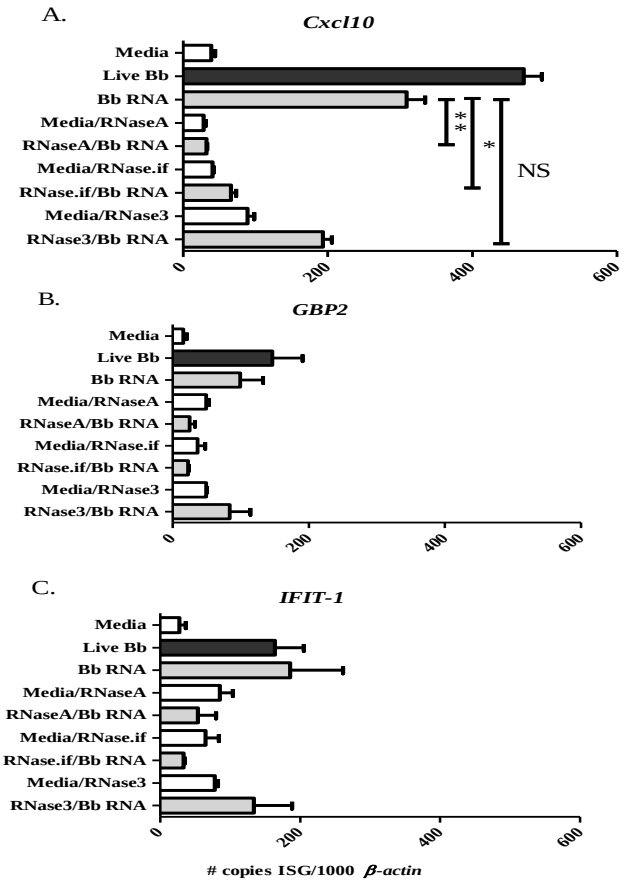

Figure 1. Single-stranded *B. burgdorferi*-derived RNA induces IFN profile gene transcription by BMDMs. C3H/HeJ BMDMs were treated for 6 hours with $7.2x10^6$/ml *B. burgdorferi*, 1 µg *B. burgdorferi* (Bb) RNA, RNase A-digested Bb RNA, RNase i_f-digested Bb RNA, RNase III (RNase 3)-digested Bb RNA, or RNases alone. RT-PCR transcripts are displayed as the number of copies of the gene of interest normalized to 1,000 copies of the mouse *β-actin* housekeeping gene. Transcript levels for (A) *Cxcl10*, (B) *GBP2* and (C) *IFIT-1* are shown. Data are depicted as the means ± SEM and are representative of two independent experiments ($n = 3$). Statistical significance was assessed via the two-tailed, unpaired Student *t* test with Welch correction. *, $P < 0.05$ for RNase i_f-digested Bb RNA versus Bb RNA. **, $P < 0.01$ for RNase A-digested Bb RNA versus Bb RNA. Brackets indicate no significant (NS) difference between RNase III (RNase 3)-digested Bb RNA versus Bb RNA.

B. burgdorferi strain B31-A3 is the wild-type strain from which the cyclic-di-GMP mutants were engineered (58, 59). *B. burgdorferi* strain B31-K2 has a kanamycin resistance cassette only. *B. burgdorferi* strain B31 Bb0363:PI-Kan/A3 lacks functional PdeA enzymes. *B. burgdorferi* strain B31 Bb0363:PI-Kan-bb0374:S/A3 lacks functional PdeA and PdeB enzymes. *B. burgdorferi* strain B31 Bb0374:S/K2 lacks functional PdeB enzymes. *B.*

burgdorferi strain B31-S9 has a streptomycin resistance cassette only. *B. burgdorferi* strain B31 Bb0419:PI-Kan/S9 lacks functional DGC enzymes.

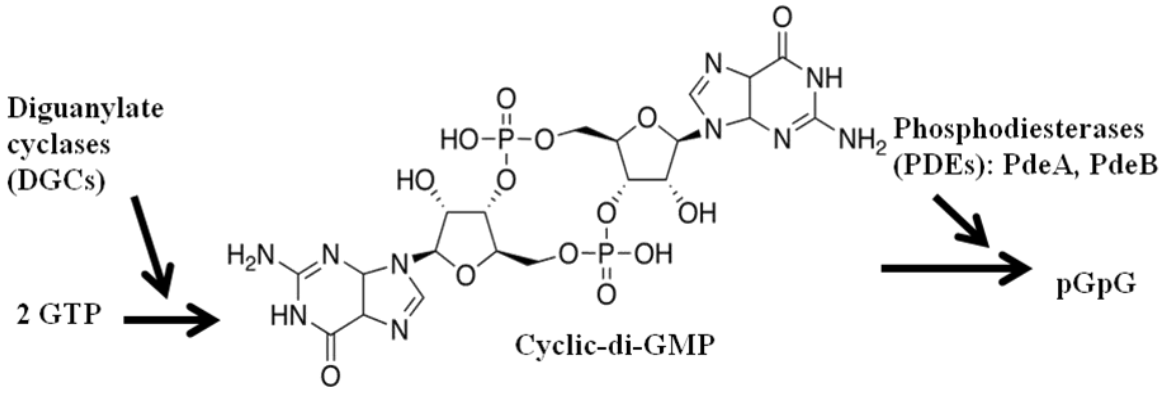

Figure 2. Schematic of the synthesis and degradation of cyclic-di-GMP. The conversion of two molecules of guanosine triphosphate (GTP) to cyclic-di-GMP is catalyzed by diguanylate cyclase (DGC). The phosphodiesterases PdeA and PdeB catalyze the breakdown of cyclic-di-GMP into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG).

Growth curves were calculated for the cyclic-di-GMP mutants in order to provide suitable time for all the strains to reach late exponential phase before being utilized in BMDM stimulation experiments (data not shown). Supernatants from *B. burgdorferi* wild-type and mutant strains were subjected to ultracentrifugation as detailed in section 2.5. Growth curves indicated that the doubling time for the individual strains was similar, except for the *pdeB* mutant, which required additional growth time to reach late exponential phase (data not shown).Wild-type *B. burgdoferi* doubling time varies from 6-12 hours (68). For use in BMDM stimulation experiments, all strains were grown to late exponential phase, with preparation of culture supernatants and late exponential bacteria immediately prior to stimulation.

To test the hypothesis that cyclic-di-GMP can trigger BMDMs to induce IFN profile gene transcription, C3H/HeJ BMDMs were stimulated for 6 hours with live wild-type and

mutant *B. burgdoferi*, ultracentrifuged culture supernatants, BSK II, or BSK II with antibiotics as appropriate.

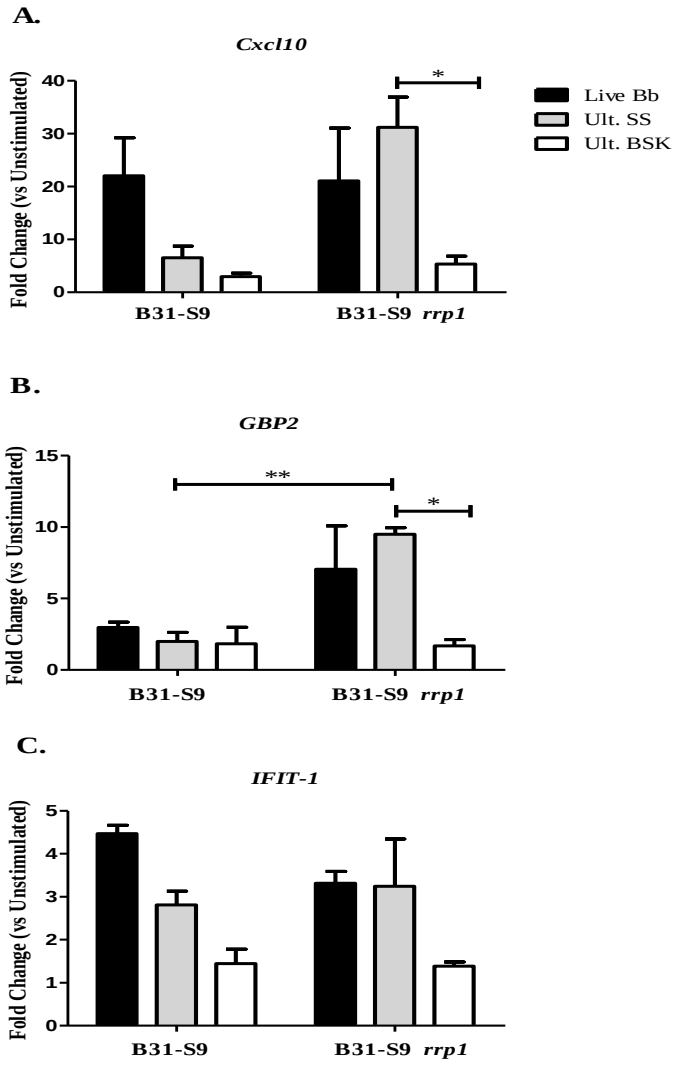

Figure 3. *B. burgdorferi* induction of IFN-responsive genes is independent of cyclic-di-GMP. C3H/HeJ BMDMs were treated for 6 hours with $7.2x10^6$/ml live B31-S9 (Live Bb) or live B31-S9 *rrp1*, 50 ul/ml ultracentrifuged supernatant (Ult. SS) of B31-S9 or ultracentrifuged supernatant of B31-S9 *rrp1*, 50 ul/ml ultracentrifuged BSK II (Ult. BSK) + 100 ug/ml streptomycin, or 50 ul/ml ultracentrifuged BSK II + 100 ug/ml streptomycin and 200 ug/ml kanamycin. Fold change shown for all transcripts is calculated by first normalizing all RT-PCR transcripts of the gene of interest to *β-actin*, then dividing by the average transcript value for the media control. BSK II without antibiotics is used as the control for calculating fold induction of transcripts produced by BMDMs upon stimulation with BSK II and antibiotics. Transcript levels for (A) *Cxcl10* (B), *GBP2* and (C) *IFIT-1* are shown. Data are depicted as the means ± SEM and are representative of two independent experiments ($n = 3$). Statistical significance was assessed via the two-tailed, unpaired Student *t* test with Welch correction. *, $P < 0.05$ for B31-S9 *rrp1* Ult. SS versus Ult. BSK for both *Cxcl10* and *GBP2* transcripts. **, $P < 0.01$ for B31-S9 *rrp1* Ult. SS versus B31-S9 Ult. SS for *GBP2*. Otherwise, no significant difference in induction of any examined transcript between strains of live Bb, strain-specific Ult. SS, or Ult. BSK II preparations was observed.

Significant induction of IFN-inducible genes by BMDMs treated with B31-S9 *rrp1* Ult. SS in comparison to Ult. BSK II with added streptomycin and kanamycin was observed for both *Cxcl10* and *GBP2* transcripts ($P < 0.05$, Figure 3A and 3B). Also, significant induction of *GBP2* by BMDMs treated with B31-S9 *rrp1* Ult. SS in comparison to B31-S9 Ult. SS was found ($P < 0.01$, Figure 3B). Otherwise, no significant difference in induction of any examined transcript between strains of live Bb, strain-specific Ult. SS, or BSK II preparations was observed (Figure 3A-C). The B31-S9 *rrp1* mutant lacks the ability to synthesize cyclic-di-GMP (53); therefore, the induction of *Cxcl10* and *GBP2* transcripts produced by BMDMs is not due to cyclic-di-GMP. Intriguingly, rather than a decline in ISG induction by BMDMs following stimulation with a DGC-lacking mutant, there is an increase in IFN profile gene transcripts.

From these data, the next logical step was to examine mutants lacking functional *pde* gene(s) encoded by *B. burgdorferi*. Phosphodiesterases are needed to degrade cyclic-di-GMP (69). To test the hypothesis that an abundance of cyclic-di-GMP can stimulate BMDMs to hyper-induce IFN profile gene transcription, C3H/HeJ BMDMs were stimulated for 6 hours with live wild-type and mutant *B. burgdoferi*, ultracentrifuged culture supernatants, BSK II, or BSK II with antibiotics as appropriate.

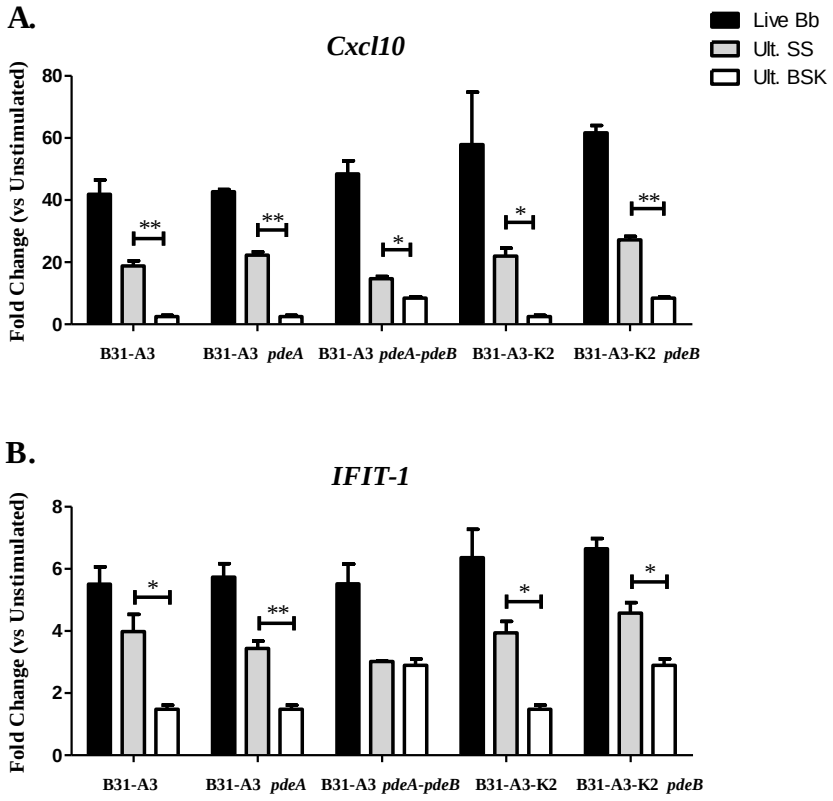

Figure 4. *B. burgdorferi* induction of IFN-responsive genes is not dependent of cyclic-di-GMP. C3H/HeJ BMDMs were treated for 6 hours with $7.2x10^6$/ml live B31-A3 (Live Bb), live B31-A3 pdeA, live B31-A3 pdeA-pdeB, live B31-A3-K2 or live B31-A3-K2 pdeB, 50 ul/ml ultracentrifuged supernatant (Ult. SS) of B31-A3, B31-A3 pdeA, B31-A3 pdeA-pdeB, B31-A3-K2, or B31-A3-K2 pdeB, 50 ul/ml ultracentrifuged BSK II (Ult. BSK), 50 ul/ml ultracentrifuged BSK II + 100 ug/ml streptomycin, 50 ul/ml ultracentrifuged BSK II + 200 ug/ml kanamycin, or 50 ul/ml ultracentrifuged BSK II + 100 ug/ml streptomycin and 200 ug/ml kanamycin. Fold change shown for all transcripts is calculated by first normalizing all RT-PCR transcripts of the gene of interest to *β-actin*, then dividing by the average transcript value for the media control. BSK II without antibiotics is used as the control for calculating fold induction of transcripts produced by BMDMs upon stimulation with BSK II and antibiotics. Transcript levels for (A) *Cxcl10* and (B) *IFIT-1* are shown. Data are depicted as the means ± SEM and are representative of two independent experiments ($n = 3$). Statistical significance was assessed via the two-tailed, unpaired Student *t* test with Welch correction. **, $P < 0.01$ for B31-A3 pdeA Ult. SS versus Ult. BSK for both *Cxcl10* and *IFIT-1* transcripts. **, $P < 0.01$ for B31-A3 Ult. SS versus Ult. BSK, B31-A3-K2 pdeB Ult. SS versus Ult. BSK for *Cxcl10* transcripts. *, $P < 0.05$ for B31-A3 pdeA-pdeB Ult. SS versus Ult. BSK, B31-A3-K2 versus Ult. BSK for *Cxcl10* transcripts, and for B31-A3 Ult. SS versus Ult. BSK, B31-A3-K2 Ult. SS versus Ult. BSK, B31-A3-K2 pdeB Ult. SS versus Ult. BSK for *IFIT-1* transcripts. No significant difference in induction of any examined transcript between strains of live Bb, strain-specific Ult. SS, or Ult. BSK II preparations was observed.

Significant induction of IFN-inducible genes by BMDMs treated with B31-A3 pdeA Ult. SS in comparison to Ult. BSK II with added kanamycin was observed for both *Cxcl10* and *IFIT-1* transcripts ($P < 0.01$, Figure 4A and 4B). Also, increased induction of an IFN-inducible gene by BMDMs treated with B31-A3 Ult. SS in comparison to Ult. BSK II or treated with B31-A3-K2 pdeB Ult. SS in comparison to Ult. BSK II with added streptomycin and kanamycin was observed for *Cxcl10* transcripts ($P < 0.01$, Figure 4A). Significant induction of IFN profile genes by BMDMs treated with B31-A3 pdeA-pdeB Ult. SS in comparison to Ult. BSK II with added kanamycin and streptomycin, B31-A3-K2 Ult. SS in comparison to Ult. BSK II with added kanamycin for *Cxcl10* transcripts, B31-A3 Ult. SS in comparison to Ult. BSK II, B31-A3-K2 Ult. SS in comparison to Ult. BSK II with added kanamycin, and B31-A3-K2 pdeB Ult. SS in comparison to Ult. BSK II with added kanamycin and streptomycin for *IFIT-1* was observed ($P < 0.05$, Figures 4A and 4B). No significant difference in induction of any examined transcript between strains of live Bb, strain-specific Ult. SS, or BSK II preparations was observed (Figure 4A and 4B).

The lack of degraded cyclic-di-GMP and presumable accumulation of cyclic-di-GMP did not enhance *pde* mutant live *B. burgdorferi* or *B. burgdorferi*-derived culture supernatant's ability to stimulate BMDMs over wild-type counterparts to produce more IFN-responsive transcripts. From data presented in figures 4 and 5, cyclic-di-GMP is unlikely to have a role in the stimulation of BMDMs to induce ISG transcript production.

3.3 Non-nucleic acid components present in *B. burgdorferi* culture supernatant can induce IFN-responsive gene transcription

As was previously published (44), *B. burgdorferi* culture supernatant treated with RNase A and DNase I did not lose IFN-stimulatory activity in comparison to untreated *B.*

burgdorferi culture supernatant. Additionally, *B. burgdorferi*-derived DNA was shown to not stimulate BMDMs to induce ISG transcription (44). To confirm that RNA is not a stimulatory ligand present in ultracentrifuged *B. burgdorferi* culture supernatant, we expanded on the evidence presented by Miller et al. (44). 16S rRNA was not detected in *B. burgdorferi* culture supernatant (Figure 5A). Every sample of *B. burgdorferi* culture supernatant tested and used in experiments was negative by RT-PCR for *B. burgdorferi* 16S rRNA. Additionally, treatment with RNase A, RNase i_f, and RNase III (RNase3) does not alter the IFN-stimulatory activity of *B. burgdorferi* culture supernatant (Figure 5B). RNase A, as expected, significantly reduced stimulation of C3H/HeJ BMDMs to induce ISGs by Bb RNA ($P < 0.01$), wherease the difference in induction between Bb RNA versus RNase A treated *B. burgdorferi* culture supernatant was not significant (Figure 5B).

Miller et al. (44) identified OspA, the predominant lipoprotein produced by *B. burgdorferi* in culture (70), as a ligand capable of stimulating BMDMs to induce ISG transcript production. To confirm that the non-nucleic acid stimulatory ligand(s) present in *B. burgdorferi* culture supernatant do not include OspA, SYPRO Ruby stains and Western blot analyses were performed of whole cell lysate (WCL), recombinant OspA, ultracentrifuged *B. burgdorferi* culture supernatant (SS), and ultracentrifuged *B. burgdorferi* membrane blebs (SS bleb) (Figure 6). The molecular weight of OspA is just under 30 kDa (71). When probed with polyclonal *B. burgdorferi* membrane antibodies, *B. burgdorferi* culture supernatant was negative for OspA protein, whereas WCL, recombinant OspA, and lysed *B. burgdorferi* membrane blebs were positive (Figure 6A). SYPRO Ruby staining of 5 µg total protein content of WCL, recombinant OspA, *B. burgdorferi* culture supernatant, and *B. burgdorferi* membrane blebs was performed. As expected, a band of nearly 30 kDa in size was detected

in WCL and in recombinant OspA (Figure 6B). A dominant protein band was also detected in *B. burgdorferi* culture supernatant and in *B. burgdorferi* membrane blebs, but this band was not near 30 kDa in size (Figure 6B). While it is possible that this band represents a dimerization of OspA, this is unlikely considering that the amino acid sequence of OspA includes only one cysteine residue (71) and the protein has been cited in the literature as a monomeric protein (81-83). The identity of the dominant band in *B. burgdorferi* culture supernatant and in *B. burgdorferi* membrane blebs remains unclear.

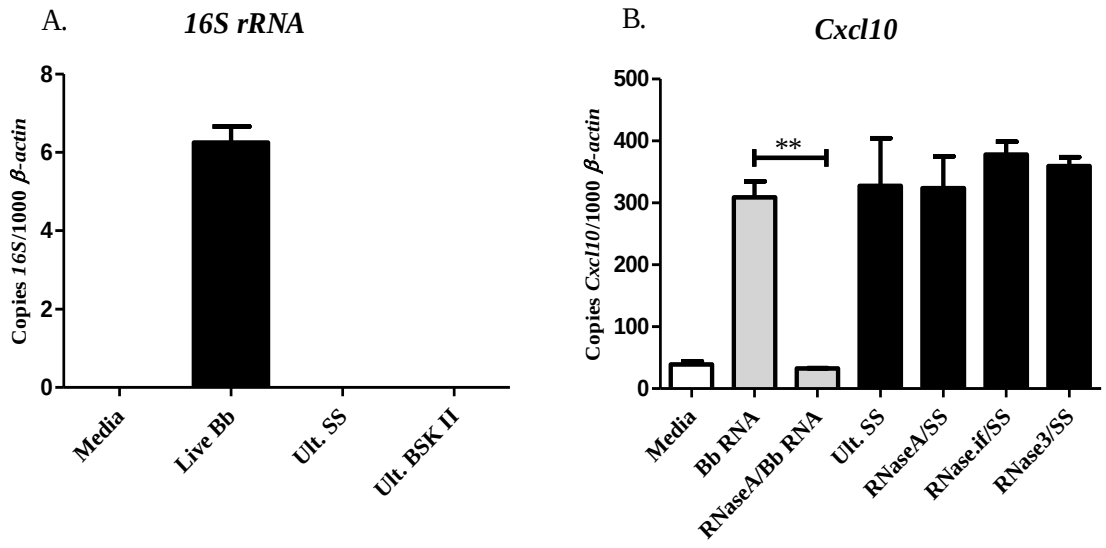

Figure 5. *B. burgdorferi* genomic RNA is not a stimulatory ligand present in ultracentrifuged *B. burgdorferi* culture supernatant. Cultured C3H/HeJ BMDMs were stimulated for 6 hours with $7.2x10^6$ live *B. burgdorferi* (Bb) (A), 1 µg Bb RNA (B), RNase A-treated Bb RNA (B), ultracentrifuged *B. burgdorferi* culture supernatant (Ult. SS) (A and B), ultracentrifuged BSK II (Ult. BSK II) (A), RNase-A + Ult. SS (B), RNase i_f + Ult. SS (B), or RNase III (RNase 3) + Ult. SS (B). RT-PCR data are shown as the number of copies of the gene of interest per 1,000 copies of the *β-actin* gene. Data are depicted as the means ± SEM and are representative of two independent experiments ($n = 3$). Statistical analysis was performed via a two-tailed, unpaired Student *t* test with the Welch correction. *B. burgdorferi* genomic RNA was not detected via RT-PCR for *16S rRNA* transcripts (A). Treatment with RNase A, RNase i_f, and RNase III (RNase3) did not alter the IFN-stimulatory activity of *B. burgdorferi* culture supernatant, nor was there a significant difference in *Cxcl10* induction from a triggering response via Bb RNA versus Ult. SS, RNase-treated or not (B). **, $P < 0.01$ for Bb RNA versus RNase A-treated Bb RNA stimulated induction of *Cxcl10* (B).

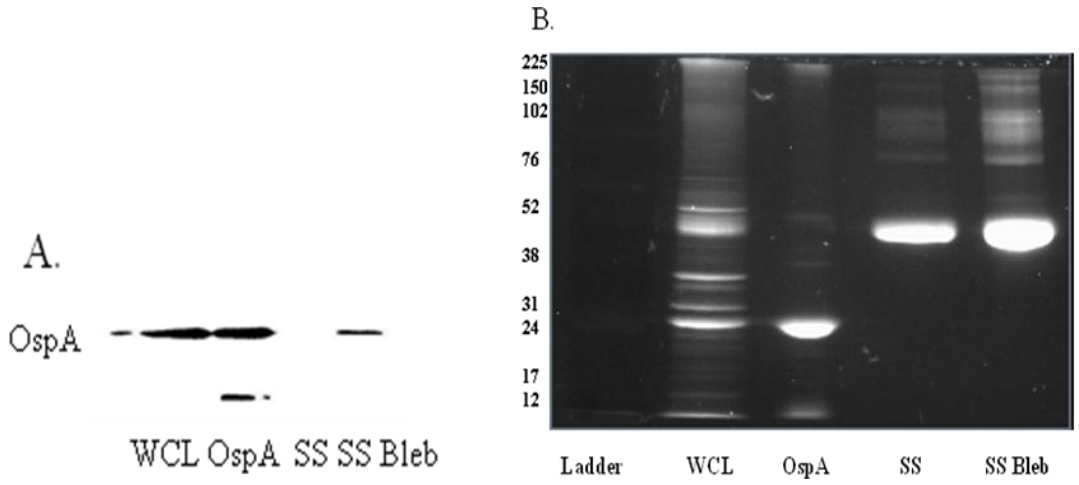

Figure 6. OspA is not detected in ultracentrifuged *B. burgdorferi* culture supernatant. *B. burgdorferi* polyclonal membrane antibody at a 1:10,000 dilution was used to probe 5 µg each of: whole cell lysate (WCL), recombinant OspA (OspA), ultracentrifuged *B. burgdorferi* culture supernatant (SS), and *B. burgdorferi*-associated membrane blebs (SS blebs) (A). 5 µg each of: WCL, OspA, SS, and SS Bleb were subjected to one-dimensional (1D) SDS-PAGE (B). Gels were stained with SYPRO Ruby to visualize separated proteins (B). The numbers to the left of the SYPRO Ruby-stained gel indicate molecular weight of protein bands in kDa (B).

In order to further delineate if the non-nucleic acid ligand(s) released by *B. burgdorferi* into culture supernatant include protein(s), we subjected *B. burgdorferi* culture supernatant to heat inactivation and Proteinase K treatment (Figure 7). *B. burgdorferi* culture supernatant and BSK II medium were either heat inactivated or treated with Proteinase K (20 µg/ml) for one hour at 56°C. Stimuli were added to both Raw 264.7 macrophage-like cells (Figure 7A) and C3H/HeJ BMDMs (Figure 7B) for 6 hours. A significant decrease in the induction of the ISG transcript *Cxcl10* by Raw 264.7 cells was seen with Proteinase K (PK) treatment and heat inactivation (HI) of ultracentrifuged *B. burgdorferi* culture supernatant ($P < 0.05$) in comparison to untreated *B. burgdorferi* culture supernatant (Figure 7A). While this reduction in *Cxcl10* transcripts produced by C3H/HeJ BMDMs was also observed, the decrease was not statistically significant (Figure 7B). These data indicate that non-nucleic acid ligand(s) produced during *B. burgdorferi* growth likely include protein(s) that contribute to ISG transcription.

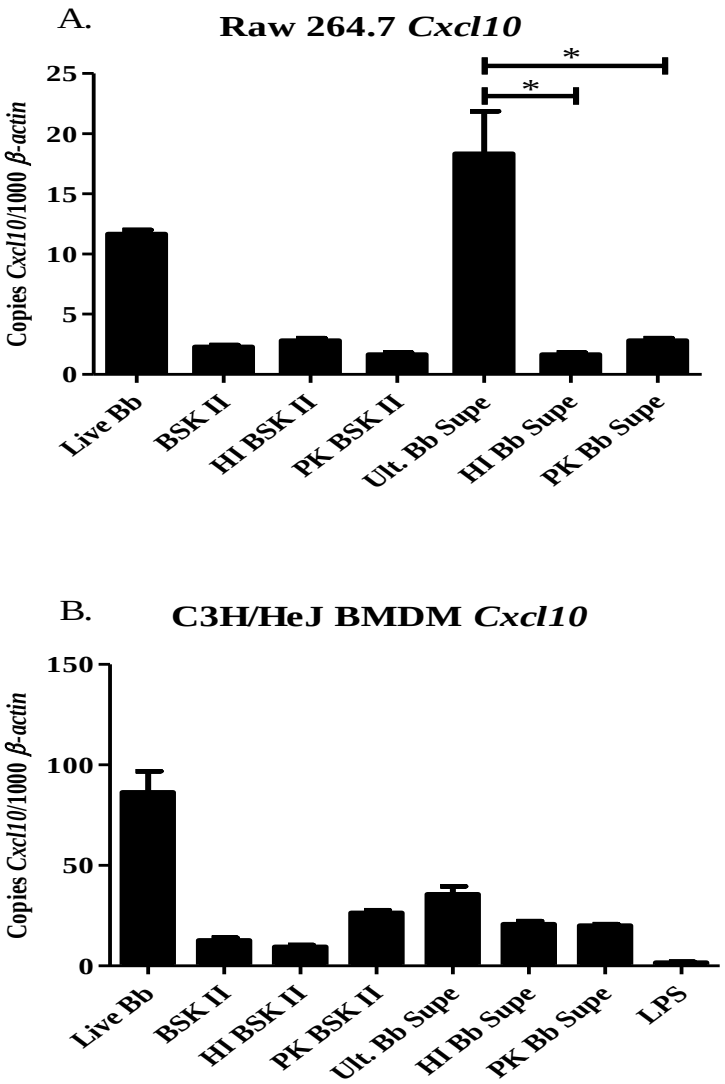

Figure 7. *B. burgdorferi*-released protein(s) contribute to the IFN-responsive gene transcription by BMDMs and Raw 264.7 macrophage-like cells. Raw 264.7 cells or C3H/HeJ BMDMs were treated for 6 hours with $7.2x10^6$/ml live *B. burgdorferi* (Bb) or 50 ul/ml of: BSK II, heat inactivated (HI) BSK II, Proteinase K (PK) treated BSK II, ultracentrifuged *B. burgdorferi* culture supernatant (Ult. Bb Supe), HI Ult. Bb Supe, or PK Ult. Bb Supe. C3H/HeJ BMDMs were also stimulated with 10 ng/ml LPS-EB Ultrapure (LPS) as a control. RT-PCR transcripts are displayed as the number of copies of *Cxcl10* normalized to 1,000 copies of the *β-actin* gene. Data are depicted as the means ± SEM and are representative of 3 independent experiments ($n = 3$). Statistical significance was assessed via the two-tailed, unpaired Student *t* test with Welch correction. *, $P < 0.05$ for HI and PK treatment of ult. Bb supernatant versus untreated ult. Bb supernatant (A). While reduced, no statistically significant difference in stimulation of BMDMs to produce *Cxcl10* transcripts was found between HI and PK treatment of ult. Bb supernatant versus untreated ult. Bb supernatant.

3.4 Induction of IFN-responsive transcripts by BMDMs in response to *B. burgdorferi* does not require TLR-7

B. burgdorferi stimulation of BMDMs to induce transcription of IFN-responsive genes has been found to be independent of TLR-2, TLR-4, TLR-9, and MyD88 (27, 41, 72). Our data agree with previously published results, showing that both TLR-2 and MyD88 are not required for induction of transcription of ISGs by BMDMs (Figures 8A, 8C and 9A, 9C). Others have published a role for TLR-7 in the induction of type I IFN and ISG transcripts but stimulated human-derived immune cells (43, 73). To determine the role of murine TLR-7 in Type I ISG expression when stimulated with *B. burgdorferi*, TLR-7-/- BMDMs were stimulated with live *B. burgdorferi* for 6 hours. No significant difference in induction of *Cxcl10* or *GBP2* transcripts was found between wild-type B6 BMDMs and TLR-7-/- BMDMs stimulated with live *B. burgdorferi* (Figure 8B, 9B).

3.5 Induction of IFN-responsive transcripts by BMDMs in response to *B. burgdorferi* genomic RNA is TLR-2 and TLR-7 independent, but is MyD88 dependent

We next sought to evaluate the role(s) of murine TLR-2, TLR-7, and MyD88 in Type I ISG expression when stimulated with *B. burgdorferi* genomic RNA. Miller et al. (44) stimulated C3H/HeJ BMDMs with *B. burgdorferi* genomic RNA, but did not report on the role(s) of murine TLR-2, TLR-7, and MyD88 in the induction of ISG transcripts upon stimulation with *B. burgdorferi* genomic RNA. To expand on the Miller et al. (44) data, TLR-2-/-, TLR-7-/-, and MyD88-/- BMDMs were stimulated with *B. burgdorferi* genomic RNA for 6 hours. TLR-2 deficiency should not affect induction of ISG transcripts since the role of TLR-2 is primarily to respond to *B. burgdorferi* lipoproteins, not RNA. TLR-7 is the endosomal receptor for single-stranded RNA, and therefore, could have an important role in

the detection of *B. burgdorferi* genomic RNA by BMDMs. MyD88 is the adaptor molecule for both TLR-2 and TLR-7, as well as all other known TLRs except TLR-3, so it also could have an important role in the detection of *B. burgdorferi* genomic RNA. While no significant difference in induction of *Cxcl10* and *GBP2* transcripts by TLR-2-/- and TLR-7-/- BMDMs stimulated with *B. burgdorferi* genomic RNA versus stimulated wild-type B6 BMDMs was observed (Figures 8A, 8B, and 9A, 9B), MyD88-/- BMDMs induced significantly less production of *Cxcl10* ($P < 0.01$) and *GBP2* ($P < 0.05$) transcripts upon stimulation with *B. burgdorferi* genomic RNA versus stimulated wild-type BMDMs (Figures 8C and 9C). Therefore, alternative TLR(s) are likely involved in the sensing of *B. burgdorferi* genomic RNA by murine BMDMs, inducing the production of ISG transcripts.

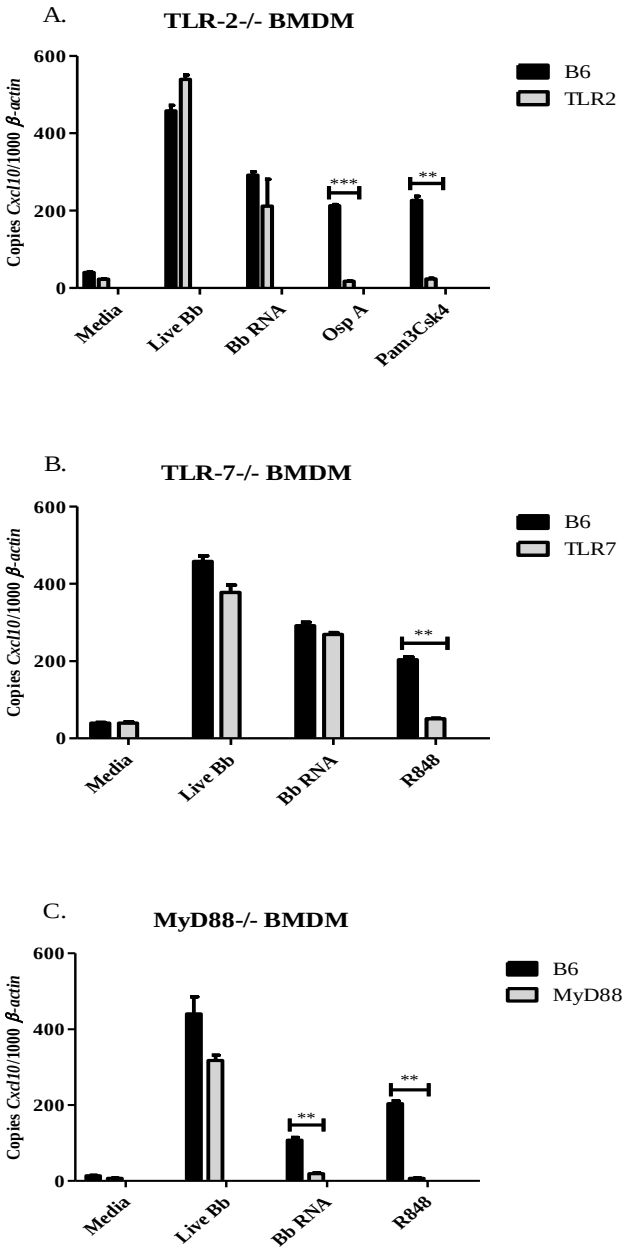

Figure 8. *B. burgdorferi* and *B. burgdorferi* genomic RNA-mediated induction of *Cxcl10* is independent of TLR-2 and TLR-7. *B. burgdorferi*-mediated induction of *Cxcl10* is independent of MyD88. *B. burgdorferi* genomic RNA-mediated induction of *Cxcl10* is dependent of MyD88. TLR-2-/-, TLR-7-/-, and MyD88-/- BMDMs were stimulated with $7.2x10^6$/ml *B. burgdorferi* (Live Bb) and 2 µg *B. burgdorferi* genomic RNA (Bb RNA). TLR-2-/- BMDMs were additionally stimulated with 100 ng/ml recombinant OspA and 200 ng/ml Pam3Csk4 controls. TLR-7-/- and MyD88-/- BMDMs were also stimulated with 1 µg/ml R848, which specifically signals via TLR-7 and MyD88. Data are depicted as the means ± SEM and are representative of four independent experiments ($n = 3$). Statistical significance was assessed via the two-tailed, unpaired Student *t* test with Welch correction. **, $P < 0.01$ for TLR-2-/- BMDMs stimulated with OspA and Pam3Csk4 versus wild-type (8A), for TLR-7-/- and MyD88-/- BMDMs stimulated with R848 versus wild-type (8B, 8C), and for MyD88-/- BMDMs stimulated with Bb RNA versus wild-type (8C).

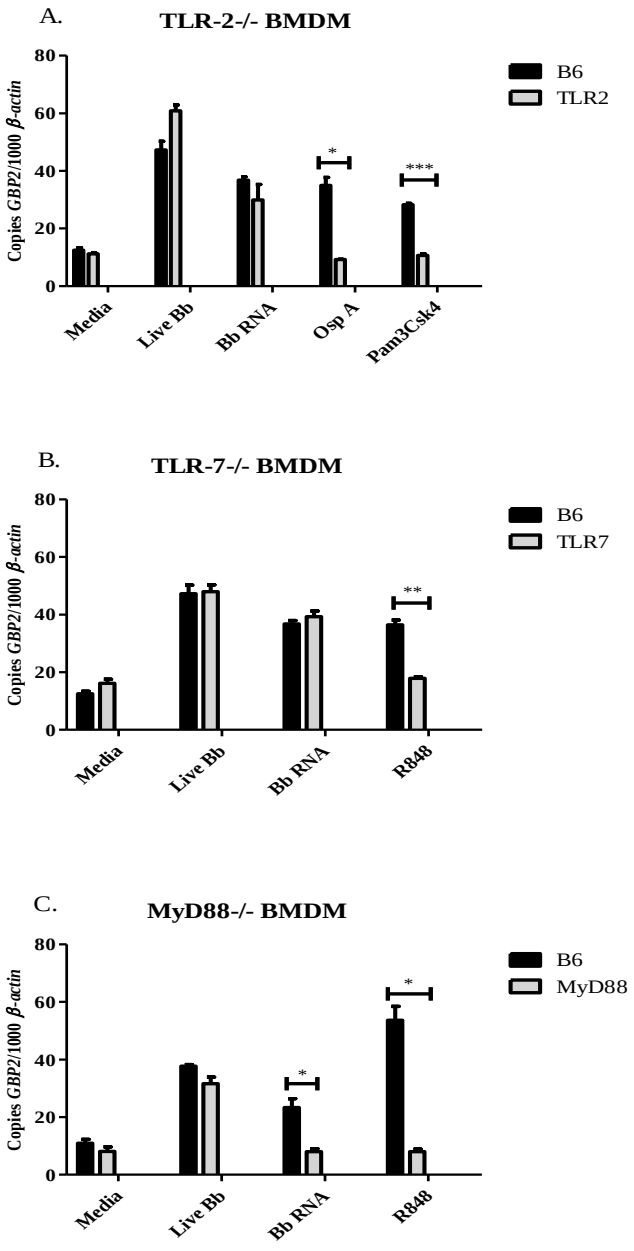

Figure 9. *B. burgdorferi* and *B. burgdorferi* genomic RNA-mediated induction of *GBP2* is independent of TLR-2 and TLR-7. *B. burgdorferi*-mediated induction of *GBP2* is independent of MyD88. *B. burgdorferi* genomic RNA-mediated induction of *GBP2* is dependent of MyD88. TLR-2-/-, TLR-7-/-, and MyD88-/- BMDMs were stimulated with $7.2x10^6$/ml *B. burgdorferi* and 2 µg *B. burgdorferi* genomic RNA. TLR-2-/- BMDMs were additionally stimulated with 100 ng/ml recombinant OspA and 200 ng/ml Pam3Csk4 controls. TLR-7-/- and MyD88-/- BMDMs were also stimulated with 1 µg/ml R848, which specifically signals via TLR-7 and MyD88. Data are depicted as the means ± SEM and are representative of four independent experiments (*n* = 3). Statistical significance was assessed via the two-tailed, unpaired Student *t* test with Welch correction. ***, $P < 0.001$ for TLR2-/- BMDMs stimulated with Pam3Csk4 versus wild-type (9A). **, $P < 0.01$ for TLR-7-/- BMDMs stimulated with R848 versus wild-type (9B). *, $P < 0.05$ for TLR-2-/- BMDMs stimulated with OspA versus wild-type (9A), and for MyD88-/- BMDMs stimulated with Bb RNA and R848 (9C).

4 Discussion

Miller et al. (44) concluded that at least four *B. burgdorferi*-derived ligands trigger IFN-responsive gene transcription by BMDMs. These ligands include: *B. burgdorferi* genomic RNA, a cell-associated, non-nucleic acid molecule found in sonicated *B. burgdorferi*, the lipoprotein OspA, and a non-nucleic acid component released by *B. burgdorferi* into culture supernatant (44). OspA is a well-characterized lipoprotein and has previously been shown to be a ligand that stimulates ISG transcription by BMDMs (21, 44). Therefore, OspA was not further characterized in our analyses. Preliminary studies using the non-nucleic acid molecule found in sonicated *B. burgdorferi* to stimulate BMDMs were discontinued, as data discrepancies occurred. Therefore, the focus of our work was to characterize *B. burgdorferi* genomic RNA and non-nucleic acid component(s) released by *B. burgdorferi* into culture supernatant. The other goals of the studies presented here were to determine the roles of TLR-2, TLR-7, and MyD88 in response to *B. burgdorferi* and *B. burgdorferi* genomic RNA, which stem from data published by Miller et al. (41, 44).

Petzke et al. (43) found that *B. burgdorferi* triggers TLR-9-dependent production of Type I IFN transcripts by human peripheral blood mononuclear cells (PBMCs). TLR-9 recognizes unmethylated CpG dinucleotides in bacterial DNA (74). In contrast, Miller et al. found that *B. burgdorferi* induction of IFN-responsive genes is independent of TLR-9 (41) and not influenced by regonition, cytosolic or otherwise, of *B. burgdorferi*-derived DNA (44). Petzke et al. (43) stimulated PBMCs, a mixed cell population, whereas Miller et al. (41, 44) stimulated a homogeneous cell population of BMDMs. Expanding on the data presented by Miller et al. (44), DNA was excluded as a *B. burgdorferi*-derived ligand of interest and RNA was further analyzed.

We found that RNase A and RNase i_f treatment of *B. burgdorferi* genomic RNA significantly reduced its ability to trigger BMDMs to produce ISG transcripts, whereas RNase III (RNase 3) had no such effect (Figure 1). Additionally, MyD88-/- BMDMs stimulated with *B. burgdorferi*-derived RNA were significantly impaired in ISG production in response to the ligand relative to wild-type BMDMs (Figures 8C and 9C). This reduced response was not observed in stimulated TLR-2-/- (as expected) and TLR-7-/- BMDMs (Figures 8A, 8B and 9A, 9B). MyD88 is the adaptor molecule for both TLR-2 and TLR-7, the latter which senses single-stranded RNA (75). TLR-3 senses only double-stranded RNA and does not signal via MyD88, but rather through the adaptor protein TRIF (76). Based on the MyD88-dependent response to *B. burgdorferi*-derived RNA in the induction of ISG transcripts, TLR-7 would be a likely receptor for the sensing of *B. burgdorferi*-derived RNA if it is indeed single-stranded, but the response was independent of TLR-7. Cervantes et al. (36, 47), though, found type I IFN and ISG transcript induction in human monocytes to be dependent of TLR-8. TLR-8 was long believed to be non-functional in mice, but there is now evidence to the contrary (77). TLR-8 may be the receptor responsible for sensing *B. burgdorferi*-derived RNA in humans and mice.

Cyclic-di-GMP is a small molecule of RNA released by *B. burgdorferi* important in signal transduction. As another potential *B. burgdorferi*-produced stimulant of BMDMs to induce transcription of ISGs, *B. burgdorferi* mutants either unable to produce cyclic-di-GMP (DGC/Rrp1) or unable/limited in their ability to degrade cyclic-di-GMP (Pde) were tested. When C3H/HeJ BMDMs were stimulated with either live *B. burgdorferi* or various live *B. burgdorferi* cyclic-di-GMP pathway mutants, no significant difference in ISG induction was found (Figures 3 and 4). No significant difference in ISG transcript induction was found from

BMDMs stimulated with ultracentrifuged supernatant from *pde* mutants versus ultracentrifuged supernatant from non-mutated *B. burgdorferi* (Figure 4). Surprisingly, induction of *Cxcl10* transcripts ($P = 0.0561$, Figure 3A) was nearly significant and induction of *GBP2* transcripts was significant ($P < 0.01$, Figure 3B) from BMDMs stimulated with the ultracentrifuged supernatant of a *B. burgdorferi* mutant unable to synthesize cyclic-di-GMP versus the ultracentrifuged supernatant of the unmutated parent strain of *B. burgdorferi*. The lack of cyclic-di-GMP signaling could be causing change(s) in *B. burgdorferi* production of stimulatory ligands, but to speculate exactly how this lack of cyclic-di-GMP affects ligand production would be premature, considering how many cellular processes the second messenger regulates (79). We were able to conclude, though, that cyclic-di-GMP itself is not required to stimulate BMDMs to produce ISG transcripts.

Miller et al. (44) found that DNase I and RNase A treatment of *B. burgdorferi* culture supernatant did not alter its IFN-stimulatory activity. We obtained similar results via treatment of *B. burgdorferi* culture supernatant with RNase A, RNase $i_{f,}$ and RNase III (RNase 3) (Figure 5B). Ultracentrifuged *B. burgdorferi* culture supernatant was also shown to be free of *B. burgdorferi* genomic RNA by RT-PCR (Figure 5A). Therefore, in agreement with Miller et al. (44), we found that the stimulatory molecule(s) present in *B. burgdorferi* culture supernatant do not include nucleic acids.

Miller et al. (44) also demonstrated that BMDM induced ISG transcript production can be triggered by *B. burgdorferi* OspA. Through SYPRO Ruby gel staining and Western blot analysis, we demonstrated that the lipoprotein OspA is not detected in ultracentrifuged *B. burgdorferi* culture supernatant (Figure 6). Heat inactivation and Proteinase K treatment of *B. burgdorferi* culture supernatant significantly reduced its ability to stimulate Raw 264.7

macrophage-like cells to induce *Cxcl10* transcript production ($P < 0.05$, Figure 7A), suggesting that non-nucleic acid ligand(s) in *B. burgdorferi* culture supernatant are protein(s). While this effect was muted upon stimulation of C3H/HeJ BMDMs, there was still some reduction in *Cxcl10* transcripts (Figure 7B). Small molecules or lipids may also be stimulatory ligands present in *B. burgdorferi* culture supernatant; protein ligand(s) may also not have accessible sites for cleavage by Proteinase K.

In some instances, more complete analyses should have been performed. *B. burgdorferi* genomic RNA could have been stained in a polyacrylamide gel with acridine orange, which stains differently depending on if the nucleic acids present are single-stranded or double-stranded (80). This could have strengthened the data presented on the nature of *B. burgdorferi* genomic RNA. Additionally, further characterization of the non-nucleic acid ligand(s) present in ultracentrifuged *B. burgdorferi* culture supernatant could have been aided by mass spectrometry analysis.

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CHAPTER 4

Summary

Summary

Leptospira interrogans and *Borrelia burgdorferi* are both extracellular pathogens. Their primary reservoir hosts are rodents. Both spirochetes have periplasmic flagella and a multitude of lipoproteins decorating their cellular surfaces. Otherwise, the two spirochetes cause vastly different diseases and have very different niches. *L. interrogans*, the causative agent of leptospirosis, exists both freely in the environment and within an animal host. Its genome is nearly 4 times as large as that of *B. burgdorferi* (1, 2). *B. burgdorferi*, the causative agent of Lyme disease, only exists in nature associated with either a vertebrate or arthropod host. This fastidious organism nearly completely lacks genes encoding enzymes important for: respiration, nucleotide synthesis, amino acid synthesis, lipid synthesis, and enzyme cofactor synthesis (3).

L. interrogans infection of humans can range from asymptomatic to mild disease to severe disease with multiple organ failure. In rats and mice, *L. interrogans* establishes a persistent infection in the proximal renal tubules, leading to chronic shedding of the organisms (4). The mechanisms through which *L. interrogans* establishes chronic infection in the kidneys is poorly understood. Utilizing IL-10-deficient mice, we sought to elucidate the role of the anti-inflammatory cytokine IL-10 in *L. interrogans* infection of mice. Whereas C57BL/6 (B6) mice harbored *L. interrogans* in their kidneys at 7 days post inoculation, IL-10-/- mice had undetectable levels of the spirochetes in their kidneys by 7 days post inoculation. This was coupled with a systemic, robust induction of IFN-γ in IL-10-/- tissues not seen in B6 tissues. In contrast, *L. interrogans* appeared to induce a rapid, sustained IL-10 response in B6 tissues. Other cytokines were not found to be drastically differentially expressed by infected IL-10-/- mice in comparison to infected B6 mice. IFN-γ appears to be a

key mediator in the clearance of *L. interrogans* from IL-10-/- tissues including the site of chronic infection in mice and rats, the kidney. We conclude that IL-10 is required for persistence of *L. interrogans* in the murine kidney.

Future experiments could examine the cellular sources of IL-10 in the tissues of *L. interrogans*-infected B6 mice and the cellular sources of IFN-γ in the tissues of *L. interrogans*-infected IL-10-/- mice. Antibody blocking studies could also be performed. IL-10-deficient rats are not commercially available, but one could antibody block IL-10 to help determine its role in leptospiral clearance of the rat kidney. The brown rat is the primary chronic shedder of leptospires into the environment (5). Further understanding of the chronic infection of the proximal renal tubules by *L. interrogans* could lead to improved prevention strategies to reduce the spread of the spirochetes in the environment and lower cases of human disease.

B. burgdorferi, unlike *L. interrogans*, does not establish a chronic infection in the mammalian kidney. *B. burgdorferi* are often associated with joint tissue, and infection with this pathogen can result in Lyme arthritis. Previously, type I interferon (IFN) and IFN-stimulated genes (ISGs) were linked to the development of Lyme arthritis in mice (6). *B. burgdorferi*-mediated induction of ISGs by bone marrow-derived macrophages (BMDMs) involves multiple ligands. At least four *B. burgdorferi*-derived ligands trigger IFN-responsive gene transcription by BMDMs (7). These ligands include: *B. burgdorferi* genomic RNA, a cell-associated, non-nucleic acid molecule found in sonicated *B. burgdorferi*, the lipoprotein OspA, and a non-nucleic acid component released by *B. burgdorferi* into culture supernatant (7). The focus of our work was to characterize *B. burgdorferi* genomic RNA and non-nucleic acid component(s) released by *B. burgdorferi* into culture supernatant. The other goals of the

studies presented were to determine the roles of TLR-2, TLR-7, and MyD88 in response to *B. burgdorferi* and *B. burgdorferi* genomic RNA.

B. burgdorferi was found to trigger TLR-9-dependent production of Type I IFN transcripts by human peripheral blood mononuclear cells (PBMCs) (8). TLR-9 recognizes unmethylated CpG dinucleotides in bacterial DNA (9). In contrast, *B. burgdorferi* induction of IFN-responsive genes was also found to be independent of TLR-9 (6) and not influenced by regonition, cytosolic or otherwise, of *B. burgdorferi*-derived DNA (7). Petzke et al. (8) stimulated PBMCs, a mixed cell population, whereas Miller et al. (6, 7) stimulated a homogeneous cell population of BMDMs. Expanding on the data presented by Miller et al. (7), DNA was excluded as a *B. burgdorferi*-derived ligand of interest and RNA was further analyzed.

We found that RNase A and RNase i_f treatment of *B. burgdorferi* genomic RNA significantly reduced its ability to trigger BMDMs to produce ISG transcripts, whereas RNase III (RNase 3) had no such effect. Additionally, MyD88-/- BMDMs stimulated with *B. burgdorferi*-derived RNA were significantly impaired in ISG production in response to the ligand relative to wild-type BMDMs. This reduced response was not observed in stimulated TLR-2-/- (as expected) and TLR-7-/- BMDMs. MyD88 is the adaptor molecule for both TLR-2 and TLR-7, the latter which senses single-stranded RNA (9). TLR-3 senses only double-stranded RNA and does not signal via MyD88, but rather through the adaptor protein TRIF (10). Based on the MyD88-dependent response to *B. burgdorferi*-derived RNA in the induction of ISG transcripts, TLR-7 would be a likely receptor for the sensing of *B. burgdorferi*-derived RNA if it is indeed single-stranded, but the response was independent of TLR-7. Cervantes et al. (11, 12), though, found type I IFN and ISG transcript induction in

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Cyclic-di-GMP is a small molecule of RNA released by *B. burgdorferi* important in signal transduction. As another potential *B. burgdorferi*-produced stimulant of BMDMs to induce transcription of ISGs, *B. burgdorferi* mutants either unable to produce cyclic-di-GMP or unable/limited in their ability to degrade cyclic-di-GMP were tested. When C3H/HeJ BMDMs were stimulated with either live *B. burgdorferi* or various live *B. burgdorferi* cyclic-di-GMP pathway mutants, no significant difference in ISG induction was found. No significant difference in ISG transcript induction was found from BMDMs stimulated with ultracentrifuged supernatant from degradation mutants versus ultracentrifuged supernatant from non-mutated *B. burgdorferi*. Induction of *GBP2* transcripts was significant from BMDMs stimulated with the ultracentrifuged supernatant of a *B. burgdorferi* mutant unable to synthesize cyclic-di-GMP versus the ultracentrifuged supernatant of the unmutated parent strain of *B. burgdorferi*. The lack of cyclic-di-GMP signaling could be causing change(s) in *B. burgdorferi* production of stimulatory ligands, but to speculate exactly how this lack of cyclic-di-GMP affects ligand production would be premature, considering how many cellular processes the second messenger regulates (14). We were able to conclude, though, that cyclic-di-GMP itself is not required to stimulate BMDMs to produce ISG transcripts.

Miller et al. (7) found that DNase I and RNase A treatment of *B. burgdorferi* culture supernatant did not alter its IFN-stimulatory activity. We obtained similar results via treatment of *B. burgdorferi* culture supernatant with RNase A, RNase $i_{f,}$ and RNase III (RNase 3). Ultracentrifuged *B. burgdorferi* culture supernatant was also shown to be free of

B. burgdorferi genomic RNA by RT-PCR. Therefore, in agreement with Miller et al. (7), we found that the stimulatory molecule(s) present in *B. burgdorferi* culture supernatant do not include nucleic acids.

Miller et al. (7) also demonstrated that BMDM induced ISG transcript production can be triggered by *B. burgdorferi* OspA. Through SYPRO Ruby gel staining and Western blot analysis, we demonstrated that the lipoprotein OspA is not detected in ultracentrifuged *B. burgdorferi* culture supernatant. Heat inactivation and Proteinase K treatment of *B. burgdorferi* culture supernatant significantly reduced its ability to stimulate Raw 264.7 macrophage-like cells to induce *Cxcl10* transcript production, suggesting that non-nucleic acid ligand(s) in *B. burgdorferi* culture supernatant are protein(s). While this effect was muted upon stimulation of C3H/HeJ BMDMs, there was still some reduction in *Cxcl10* transcripts. Small molecules or lipids may also be stimulatory ligands present in *B. burgdorferi* culture supernatant; protein ligand(s) may also not have accessible sites for cleavage by Proteinase K.

More complete analyses could have been performed. *B. burgdorferi* genomic RNA could have been stained in a polyacrylamide gel with acridine orange, which stains differently depending on if the nucleic acids present are single-stranded or double-stranded (15). This could have strengthened the data presented on the nature of *B. burgdorferi* genomic RNA. Additionally, further characterization of the non-nucleic acid ligand(s) present in ultracentrifuged *B. burgdorferi* culture supernatant could have been aided by mass spectrometry analysis.

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