SPIROCHETES AND IMMUNE EVASION: INFECTION, PERSISTENCE AND CLEARANCE

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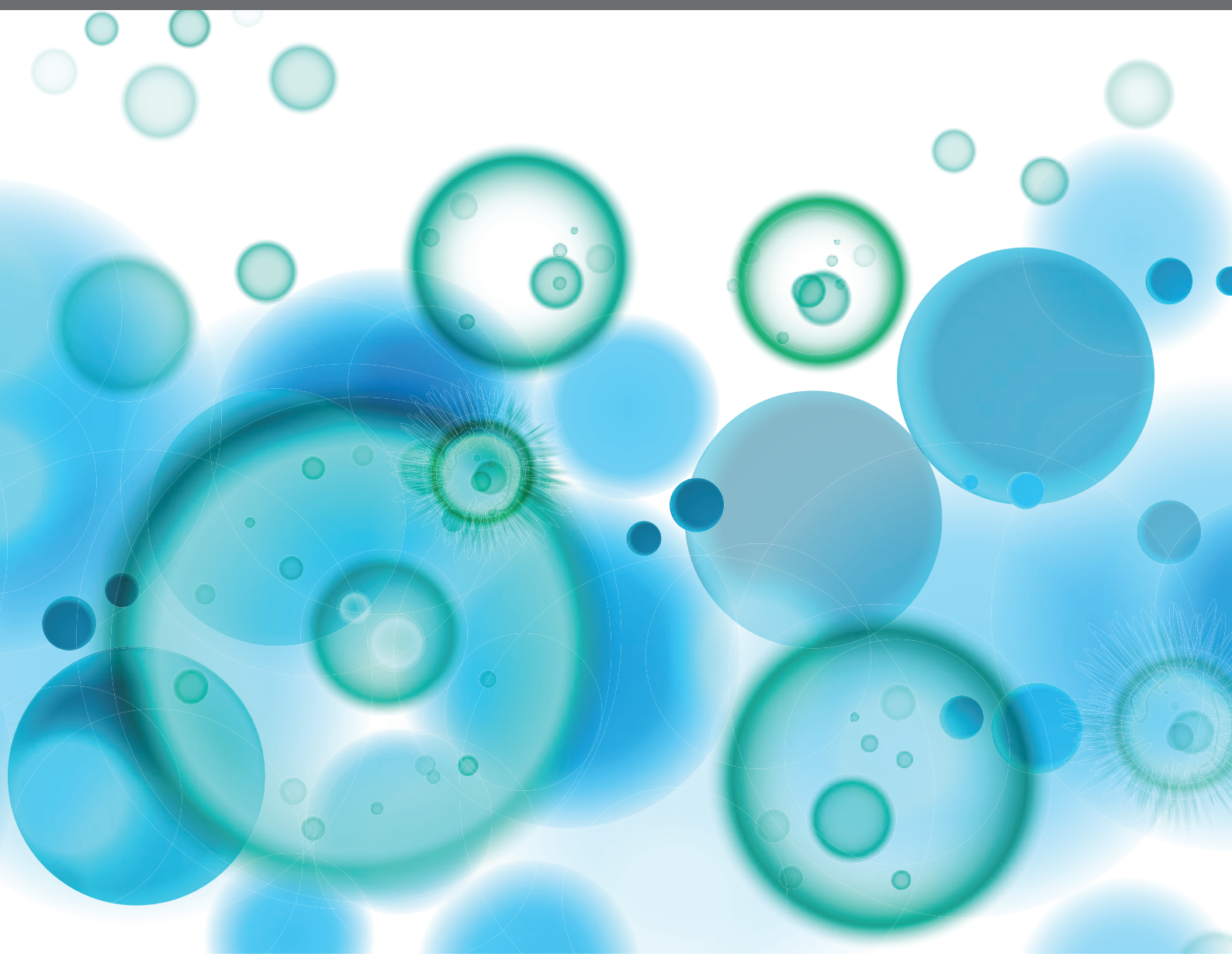

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SPIROCHETES AND IMMUNE EVASION: INFECTION, PERSISTENCE AND CLEARANCE

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The interface between spirochetes and the immune response is of significant importance to their pathogenesis and persistence. Evasion from the immune system leads to infections that present as Leptospirosis, Syphilis, Lyme Disease and Relapsing Fever and may lead to putative persistence and latency. Understanding the mechanisms involved in immune evasion will shed light not only on the host-pathogen factors involved in the process but also on how resistance to infection leads to protection.

Broad examples include spirochetal interaction with the immune system, spirochetal molecules involved in immune evasion and in immune activation, innate immune responses in the skin and other compartments, factors involved in spirochetal adhesion to the extracellular matrix, interaction of spirochetes with antigen presenting cells, in vitro, ex vivo or in vivo, spirochetal lipoproteins and immunity.

Specific examples include innate immunity to pathogenic spirochetes (T. pallidum, B. burgdorferi and Leptospira spp.), invasion and pathogenesis by L. interrogans, subversion and suppression of B cell responses by B. burgdorferi, role of antibody in clearance versus persistence of relapsing fever Borreliae, evasion of the complement system by B. burgdorferi, immune suppression by Ixodes tick saliva for effective transmission, adhesins and enzymes involved in dissemination of T. pallidum, spirochetal variable surface proteins in immune evasion, intravital imaging of pathogenic spirochetes (Borreliae and Leptospira) in host tissues, spirochete-host surface interactions.

Additional specific examples for B. burgdorferi include novel approaches to control infection within the vector and/or in mammal; tick innate immune defenses and interaction of Ixodes scapularis salivary immunomodulatory molecules with human immune cells, tick-innate immune defenses (from the perspective of the tick midgut), mouse models of infection and genetic basis for pathogenicity, diverse roles of outer surface protein C.

Additional specific examples for Leptospirosis include animal models of acute, sub-lethal and persistent infection; neutrophils and innate immune response; Toll-like receptor mediated B cell responses; markers of endothelial cell activation for disease severity in human leptospirosis, corticosteroid treatment of advanced human leptospirosis, and urinary biomarkers of chronic Leptospirosis.

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Interaction of Bovine Peripheral Blood Polymorphonuclear Cells and *Leptospira* Species; Innate Responses in the Natural Bovine Reservoir Host

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Cattle are the reservoir hosts of *Leptospira borgpetersenii* serovar Hardjo, and can also be reservoir hosts of other *Leptospira* species such as *L. kirschneri*, and *Leptospira interrogans*. As a reservoir host, cattle shed *Leptospira*, infecting other animals, including humans. Previous studies with human and murine neutrophils have shown activation of neutrophil extracellular trap or NET formation, and upregulation of inflammatory mediators by neutrophils in the presence of *Leptospira*. Humans, companion animals and most widely studied models of Leptospirosis are of acute infection, hallmarked by systemic inflammatory response, neutrophilia, and septicemia. In contrast, cattle exhibit chronic infection with few outward clinical signs aside from reproductive failure. Taking into consideration that there is host species variation in innate immunity, especially in pathogen recognition and response, the interaction of bovine peripheral blood polymorphonuclear cells (PMNs) and several *Leptospira* strains was evaluated. Studies including bovine-adapted strains, human pathogen strains, a saprophyte and inactivated organisms. Incubation of PMNs with *Leptospira* did induce slight activation of neutrophil NETs, greater than unstimulated cells but less than the quantity from *E. coli* P4 stimulated PMNs. Very low but significant from non-stimulated, levels of reactive oxygen peroxides were produced in the presence of all *Leptospira* strains and *E. coli* P4. Similarly, significant levels of reactive nitrogen intermediaries (NO_2) was produced from PMNs when incubated with the *Leptospira* strains and greater quantities in the presence of *E. coli* P4. PMNs incubated with *Leptospira* induced RNA transcripts of IL-1β, MIP-1α, and TNF-α, with greater amounts induced by live organisms when compared to heat-inactivated leptospires. Transcript for inflammatory cytokine IL-8 was also induced, at similar levels regardless of *Leptospira* strain or viability. However, incubation of *Leptospira* strains with bovine PMNs did not affect *Leptospira* viability as measured by limiting dilution culture. This is in contrast to previously reported results of innate inflammatory activation by *Leptospira* in human and other animal models, or the activation and interaction of bovine PMNs with *Escherichia coli* and other bacterial pathogens. While it could be hypothesized that variations in innate receptor recognition, specifically variance in toll-like receptor 2, could underlie the observed reduction of activation in bovine PMNs,

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additional studies would be needed to explore this possibility. Reduction in neutrophil responses may help to establish nearly asymptomatic chronic *Leptospira* infection of cattle. This study emphasizes the importance of studying host-pathogen relationships in the appropriate species as extrapolation from other animal models may be incorrect and confounded by differences in the host responses.

Keywords: *Leptospira*, serovar Hardjo, neutrophils, bovine host

INTRODUCTION

Leptospirosis is a disease caused by pathogenic spirochetes in the genus *Leptospira*, incorporating numerous species and over 200 serovars. A globally important zoonosis, leptospirosis is transmitted through contact with contaminated soil or water, and urine from infected mammals. Disease ranges from mild febrile illness with flu-like symptoms to acute severe disease with pulmonary, renal and hepatic complications, and may result in death in incidental hosts. However, chronically infected, reservoir hosts are generally asymptomatic with intermittent shedding of bacteria in urine. Worldwide, cattle are most commonly infected with *Leptospira borgpetersenii* serovar Hardjo. Leptospirosis infection is the leading cause of reproductive failure in cattle, and can result in weak/stillborn calves, reduced growth rates, and reduced milk production, all contributing to considerable economic loss to the cattle producer. Some cattle develop a chronic infection/shedding state and serve as a reservoir of infection for cattle and other incidental hosts including humans. Serovar Hardjo infection in incidental hosts, like humans or dogs, can result in acute disease (Blackmore and Schollum, 1982; Ryan et al., 1982; Zuerner et al., 2012).

Neutrophilia has been observed in acute leptospirosis infections in dogs, hamsters and humans (Ryan et al., 1982; Kobayashi, 2001; Libraty et al., 2007; Ganoza et al., 2010; Chow et al., 2012; De Silva et al., 2014) (D. Alt, unpublished observations). It is currently unknown if neutrophilia occurs in newly infected cattle before or during the onset of chronic disease. The impact of *Leptospira* infection on circulating neutrophils during infection in cattle remains uncharacterized.

Neutrophils are the first-line of defense for the innate immune system. Released as mature cells from the bone marrow, neutrophils are the most numerous leukocyte in blood (Nauseef and Borregaard, 2014). Circulating in an active state, neutrophils have the ability to quickly localize to specific tissues to combat infection (Nauseef and Borregaard, 2014). Neutrophils can participate in pathogen clearance or neutralization in a variety of ways including: production of azurophilic granules (which contain a number of proteolytic enzymes, elastase, antimicrobial defensins, and myeloperoxidase), production of antimicrobial peptides, formation of reactive oxygen and nitrogen species, production of cytokines which interact with other immune cells, phagocytosis of the pathogen, and formation of Neutrophil Extracellular Traps (NETs) (Amulic and Hayes, 2011). Previous reports have also suggested that *Leptospira* spp. are sensitive to bacterial killing by reactive oxygen intermediates such as H_2O_2 and low molecular weight primary granule components released from neutrophils (Murgia et al., 2002). Recently, it was shown that *Borrelia burgdorferi*, a spirochete that is the causative agent of Lyme's Disease, was ensnared and killed by human neutrophil NETs (Menten-Dedoyart et al., 2012). Furthermore, human neutrophils in culture with pathogenic *Leptospira interrogans* serovar Copenhageni produced NETs, reducing leptospiral viability (Scharrig et al., 2015). Humans, mice and hamsters all exhibit acute disease when infected with pathogenic *Leptospira* serovars. These authors also indicated that mice had nucleosomes, hallmarks of NET formation, circulating in blood after infection, hypothesizing that NET formation may play a role in prevention of bacterial dissemination (Scharrig et al., 2015). Thus, *Leptospira* should be significantly impaired by the innate abilities of neutrophils and other classical innate immune cells. The effect of bovine neutrophils or other innate immune cells on *Leptospira* has not been studied.

Neutrophils are recognized as an important contributor of cytokines and chemokines at the site of an inflammatory response. IL-8 has a potent effect on neutrophils themselves, as well as being the primary chemokine produced by neutrophils after contact with a foreign particle (Scapini et al., 2000; Walter and Morck, 2002). Depending on the stimulus, neutrophil derived cytokines can impact the magnitude and the duration of inflammation via production of IL-10, activation of T-helper cells, and by recruitment of other phagocytes to the inflammatory foci (Scapini et al., 2000; Thomas and Schroder, 2013). Bovine neutrophils have been shown to express IL-1β, IL-8, IL-10, IFN-γ, TNF-α, and MIP-1α in response to bacterial pathogens *in vitro* (Hassfurther et al., 1994; Scapini et al., 2000; Worku and Morris, 2009). During acute infection with *L. interrogans* serovar Icterohaemorrhagiae, hamsters upregulate gene expression of TNF-α, TGF-β, IP-10 (CXCL10), and IL-10 at the site of infection (kidney) and peripheral blood mononuclear cells upregulate expression of TNF-α, IFN-γ, IL-12 (Vernel-Pauillac and Merien, 2006; Lowanitchapat et al., 2010).

Attempts to understand the innate immune responses in cattle to *Leptospira* infection through extrapolation of results from human studies or those employing laboratory animal models using *Leptospira* strains typically associated with acute disease in humans is less than optimal (Vernel-Pauillac and Merien, 2006; Lowanitchapat et al., 2010; Fraga et al., 2011; Goris et al., 2011). Infection in cattle is commonly caused by highly host-adapted strains of *Leptospira* that primarily establish chronic infections in the kidneys and reproductive tract. While it has been previously reported that natural killer (NK) T cells and T helper type 1 (Th1) cells may play a role in adaptive immune responses to vaccination, studies evaluating the innate responses to leptospiral organisms during active infection in cattle are lacking (Naiman et al., 2002; Zuerner et al., 2011).

Recent studies have highlighted differences in innate recognition of *Leptospira* between mouse and human cells (Fraga et al., 2011) and even greater differences exist between mice and cattle innate immune recognition (Werling et al., 2009; Bryant and Monie, 2012). It is unknown if bovine neutrophils are activated by *Leptospira* bacteria, or participate in the killing of host-adapted *Leptospira* strains in cattle. Therefore our studies were designed to characterize naïve bovine neutrophil activation after incubation various *Leptospira* strains. In contrast to studies in human and laboratory animal models, bovine neutrophils exhibited only modest or slight activation in response to incubation with *Leptospira* bacteria and showed no reduction in *Leptospira* viability. These results illustrate the complicated *Leptospira*-host relationships and underscore the necessity of studying both reservoir and acute hosts.

MATERIALS AND METHODS

Neutrophil Preparation

All procedures involving the use of cattle were approved by the Animal Care and Use Committee at National Animal Disease Center Ames, IA under protocol numbers 2677 and 2357. Whole blood from adult female Jersey and Holstein cattle in the blood donor pool free of disease or discernable health problems were used for isolation of peripheral blood polymorphonuclear cells (PMNs). Animals were sero-negative by microscopic agglutinating antibody test (MAT) for all *Leptospira* serovars used in this study. Peripheral whole blood was collected by venipuncture in acid-citrate dextrose anticoagulant and processed within 1 h of collection. Whole blood was diluted 1:2 with sterile phosphate buffered saline (PBS, pH 7.2) and centrifuged at 1000 × g for 40 min at 25°C. Plasma and buffy coat (lymphocyte layer) were removed. Red blood cells (RBCs) were lysed by dilution of the red blood cell pellet 1:5 in 150 mM ammonium chloride 10 mM Tris lysis buffer for 1.5 min while rotating. Remaining cells were washed 3-4 times by centrifugation with PBS and resuspended in phenol red-free RPMI 1640 (GIBCO) supplemented with 10% heat inactivated to 70°C fetal bovine serum and 1 mM HEPES (hereafter referred to as cRPMI). Heat inactivation at 70°C for 1 h inactivates endogenous DNases that can interfere with NETosis assays (von Kockritz-Blickwede et al., 2009). Live cells were counted by trypan blue exclusion on a hemacytometer. To determine cell morphology and purity, 1×10^5 cells were washed once by centrifugation, resuspended in PBS with 0.5% fetal bovine serum and applied to a glass slide using a Shandon Cytocentrifuge and Cytospin apparatus (Shandon Inc.). Slides were methanol-fixed, stained with Giemsa Stain and visually inspected for neutrophil percentage using a light microscope.

Bacterial Preparation

L. borgpetersenii serovar Hardjo strains 203 and JB197 were previously isolated from beef steers during slaughter (Miller et al., 1991). *L. interrogans* serovar Pomona type kennewicki isolate RM211 was originally isolated from a swine fetus (Thiermann et al., 1985). *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was a gift from Dr. David Haake (Los Angles Veteran Health Care System). All pathogenic *Leptospira* strains used in the current study were pathogenic in hamsters and used at no more than 3 *in vitro* passages from hamster infection. Non-pathogenic *Leptospira biflexa* strain Patoc (ATCC® 23582™) was purchased from American Type Culture Collection. All *Leptospira* strains were propagated in modified T80/40/LH media with 5-fluorouracil (100 μg/ml) at 29°C as described previously (Zuerner, 2005; Zuerner et al., 2011, 2012). In some assays, *L. borgpetersenii* serovar Hardjo strain JB197 was inactivated by incubation at 56°C for 1 h with bacterial inactivation (killing/no growth) confirmed by culture. *Escherichia coli* strain P4 was a gift from Dr. John Lippolis (Ruminant Diseases and Immunology Research Unit, National Animal Disease Center, Ames IA). *E. coli* P4 was grown overnight in tryptic soy broth with 1% fetal bovine serum. Log phase bacteria were quantified by direct counting under dark field using a Petroff-Hauser counting chamber, washed twice by centrifugation in PBS and resuspended in cRPMI. If needed, *E. coli* were held on ice until incubation with neutrophils.

Observation of Neutrophil Extracellular Traps (NETs)

Following methods similar to previously published studies (Lippolis et al., 2006; Fuchs et al., 2007; Aulik et al., 2010; Wardini et al., 2010), bovine PMNs were seeded at 1×10^6 cells per ml onto 8 well chamber slides (Lab-Tek). Cells were allowed to settle for 1 h at 37°C under 5% CO_2, before bacteria or other stimulants were added. *Leptospira* and *E. coli* strain P4 were added at a multiplicity of infection (MOI) of 10 bacterial cells per PMN. Positive control stimulant consisted of Phorbol 12-myristate 13-acetate (PMA) (Sigma) at a final concentration of 25 ng/ml. After addition of bacteria or stimulant, slides were incubated at 37°C for 2 h. To visualize NETS, cells were centrifuged gently at 400 × g for 5 min at 25°C, and supernatant aspirated. LIVE/DEAD® *Bac*Light™ Bacterial Viability and Counting Kit (Molecular Probes) stain was used per manufacturer's recommendation with the following modification: Red:Green dye ratios were mixed in 2:1 proportion of component A to component B, diluted 1:50 in PBS. 100 μl was applied to each well of the slide and incubated in the dark for 15 min at room temperature. Stain was aspirated and slides rinsed with 500 μl PBS by careful application and removal using a pipette. Chambers were removed and coverslips were mounted with included oil component from *Bac*Light kit and visualized within 48 h. Slides were visualized using a Nikon E8400 microscope, 40x and 60x objectives, triple fluorescence filter and digital camera attachment. Three representative fields were imaged per slide.

NET Quantitation

Following method published in Chuammitri et al. (2009), isolated bovine PMNs were plated at 1×10^6 in quadruplicate in 96 well plates for NET stimulation and quantitation. Cells were allowed to settle in plates for 1 h at 37°C, 5% CO_2, at which time bacteria MOI 10 or stimulant was added to quadruplicate wells and incubated at 37°C for 2 h. Replicate wells were set up in the same manner, with the addition of 100 U/ml DNase I (RQ1 RNase free-DNase, Promega). After incubation, micrococcal nuclease

(Worthington Biochemical Corporation) was added to a final concentration of 0.1 U/ml and incubated for an additional 30 min at 37°C. 5 mM EDTA was added to stop the reaction. Plates were centrifuged at 400 × g for 2.5 min at 25°C, and 100 µl of supernatant removed. DNA concentration of the supernatant was quantified (PicoGreen DNA quantification kit, Molecular Probes) and read in a fluorescence plate reader (SpectraMAX GeminiXS, Molecular Devices, excitation 492 nm, emission 520 nm; with SOFTMax PRO software). Data is expressed as mean of replicate wells ng/ml DNA in supernatant. Data was analyzed from three independent experiments using cells isolated from six animals. Data was analyzed using Graphpad Prism 6 Software and one-way ANOVA with Tukey's multiple comparisons post-test.

NET Confirmation

To confirm that the extracellular structures observed visually and by quantification of DNA in cellular supernatant were indeed NETs, bovine PMNs were seeded at 1×10^6 cells per ml onto 8 well chamber slides (Lab-Tek). Cells were allowed to settle for 1 h at 37°C under 5% CO_2, before bacteria or other stimulants were added. *Leptospira* and *E. coli* strain P4 were added at a multiplicity of infection (MOI) of 10 bacterial cells per PMN. Positive control stimulant consisted of PMA at a final concentration of 25 ng/ml. After addition of bacteria or stimulant, slides were incubated at 37°C for 2 h. After incubation, slides were centrifuged at 400 × g for 5 min at 25°C and supernatant carefully aspirated. Slides were fixed by adding 0.5 ml of 4% formalin in PBS to each chamber for 10 min at room temperature. Fixative was carefully aspirated, chamber well rinsed with PBS, and 0.5 ml PBS containing 1% normal goat serum was added (Equitech-Bio Inc.) and 10 µl azide-free Fc Receptor Blocker (INNOVEX Biosciences). After overnight incubation in humidified chamber at 4°C, chamber portion of the slide was removed and slides were stained with anti-bovine H2A antibody (AbCam rabbit polyclonal to Histone H2A, ab13923, 1:200 dilution), followed by Alexa Fluor 594 F(ab)'2 goat anti-rabbit (1:2000 dilution) and DAPI nuclear counterstain (Molecular Probes, 1:3000 dilution). Slides were visualized using a Nikon E8400 microscope at 40x magnification with triple fluorescence filter and digital camera attachment. The total number of cells and number of cells with H2A staining were hand counted for five random fields. Data is presented as the mean percentage of H2A positive PMN cells from 4 different cows assayed in two independent experiments. Data was analyzed using Graphpad Prism 6 Software and one-way ANOVA with Tukey's multiple comparisons post-test.

Production of Reactive Oxygen Species (ROS) by PMNs

As a surrogate reactive oxygen intermediate activity in the inflammatory process, ROS in the form of H_2O_2 was measured in cells and cell supernatants after stimulation with *Leptospira*. Cells and supernatants from NET quantitation experiments described for NET quantitation were stored at −80°C. Cells were lysed by freeze-thaw fracture and lysis confirmed by visual inspection. Peroxide concentration was determined using Amplex Red Reagent Kit (Molecular Probes) following manufacturer's recommendations. Data presented is the mean results from quadruplicate wells of cells isolated from six individual cows on two separate occasions. Data was analyzed using Graphpad Prism 6 Software and one-way ANOVA with Tukey's multiple comparisons post-test.

Production of Reactive Nitrogen Species (RNS) by PMNs

Cells and supernatants from NET quantitation experiments described above were stored at −80°C. Cells were lysed by freeze-thaw fracture and lysis confirmed by visual inspection. Nitrate concentration was determined using Griess Reagent Kit (Molecular Probes) following manufacturer's recommendations. Data presented is the mean results from quadruplicate wells of cells isolated from six individual cows on two separate occasions. Data was analyzed using Graphpad Prism 6 Software and one-way ANOVA with Tukey's multiple comparisons post-test.

Cytokine Gene Transcription by Activated PMNs

Isolated PMNs were plated in 24-well plates at a concentration of 2.5×10^6/ml. Cells were allowed to settle in plates for 30 min at 37°C, 5% CO_2, at which time bacteria (MOI 10) or stimulant were added to triplicate wells. Plates were incubated for 1.5 h at 37°C. After incubation, plates were centrifuged for 7 min at 500 × g at 25°C, and supernatants removed. RNA was extracted from the cell pellet (RNeasy + Mini Column Kit, Qiagen) according to manufacturer's protocol. RNA quality was assessed using Agilent 2100 Bioanalyzer. cDNA was synthesized using Invitrogen Superscript III polymerase, oligo dT, dNTPs, 0.1M DTT, and RNAse H. Real-Time PCR was performed using Qiagen 2X SYBR Green QuantiFast kit on an ABI 7900 384-well platform. 6 µl of cDNA template in a 20 µl reaction using primers listed in **Table 1** with the following amplification conditions: 2 min at 50°, 10 min at 95°, 40 cycles of 15 s at 95° and 1 min at 60°, and a dissociation step (15 s at 95°, 1 min at 60°, 15 s at 95°, 15 s at 60°). Data was analyzed by $2^{(-\Delta\Delta CT)}$ method as outlined in Livak and Schmittgen (2001) with no stimulation being the control treatment and normalized to housekeeping gene RPS9. All statistical calculations were performed on $\log_2$ transformed data and presented as means of 4 animals tested in two independent experiments.

Bacterial Killing by PMNs

PMNs were seeded at 1×10^6 in triplicate in 96 well plates and allowed to settle for 1 h at 37°C, 5% CO_2. *Leptospira* or *E. coli* P4 were added at an MOI of 10 and plates were centrifuged at 400 × g for 5 min at 25°C. After incubation for 4 h at 37°C, PMNs were lysed by addition of sterile filtered Saponin (0.05% final concentration). *Leptospira* samples were cultured by limiting dilution in modified T80/40/LH (Tween 80/Tween 40/ lactalbumin hydrolysate) with 5-fluorouracil (100 µg/ml) and incubated at 29°C for up to 4 weeks (Goris et al.,

TABLE 1 | Bovine RT-PCR Primers Used.

Gene	Primer	Direction	References
RPS9	CGC CTC GAC CAA GAG CTG AAG	Forward	McGill et al., 2013
	CCT CCA GAC CTC ACG TTT GTT CC	Reverse	
IL-1β	GTG ACG AGA ATG AGC TGT TAT TTG	Forward	Zuerner et al., 2007
	TGT TGT AGA ACT GGT GAG AAA TCT G	Reverse	
MIP-1α	AAG CCT GGT GTC ATC TTC C	Forward	McGill et al., 2013
	CTC CAG GTC GGT GAT GTA TTC	Reverse	
TNF-α	TCT ACC AGG GAG GAG TCT TCC A	Forward	Zuerner et al., 2007
	GTC CGG CAG GTT GAT CTC A	Reverse	
TGF-β	CTG AGC CAG AGG CGG ACT AC	Forward	McGill et al., 2013
	TGC CGT ATT CCA CCA TTA GCA	Reverse	
IL-17	CAC AGC ATG TGA GGG TCA AC	Forward	Vordermeier et al., 2009
	GGT GGA GCG CTT GTG ATA AT	Reverse	
IFN-γ	AGA ATC TCT TTC GAG GCC GGA G	Forward	McGill et al., 2013
	TAT TGC AGG CAG GAG GAC CAT TAC	Reverse	
IL-8	GTG TGA AGC TGC AGT TCT GTC	Forward	Zuerner et al., 2007
	GGT GGA AAG GTG TGG AAT GTG	Reverse	

2011). Tubes were inspected visually for evidence of growth by dark field microscopy. Samples from PMNs incubated with *E. coli* P4 were serially diluted in PBS and plated on tryptic soy agar supplemented with 1% yeast extract and 5% sheep blood. Bacterial numbers were determined by plate counts after incubation at 37°C for 48 h. Data presented as percent survival (bacterial survival after incubations with PMNs divided by bacteria counts after incubation in the absence of PMNs) (Goris et al., 2011). Data presented is representative of duplicate independent experiments utilizing cells isolated from six animals.

Effect of Immune Serum on PMN Activation

After counting, cells were divided equally into four portions and resuspended in cRPMI supplemented with 20% bovine serum archived from a previous experiment (Zuerner et al., 2011). The study used a commercial vaccine containing *L. borgpetersenii* serovar Hardjo (type hardjo-bovis) (http://pfizerah.com) with 2 doses given 4 weeks apart and animals were challenged (1 year after vaccination) with *L. borgpetersenii* serovar Hardjo strain 203. Serum was selected to provide the following groups: (1) naïve cattle, (2) cattle receiving a commercial leptospirosis vaccine preparation 12 weeks post-vaccination (Vaccinated), (3) naïve cattle 8 weeks post-infection with *L. borgpetersenii* serovar Hardjo strain 203 (Challenged), and (4) cattle receiving a commercial leptospirosis vaccine and challenged with *L. borgpetersenii* serovar Hardjo strain 203, 8 weeks post-challenge (Vaccinated + Challenged) (Zuerner et al., 2011). Serum was heat inactivated to destroy endogenous nucleases and pooled serum titer was verified by MAT performed as previously reported (Zuerner et al., 2011). Resulting MAT titers to all *Leptospira* strains in this study are given in Table S1. Net quantitation and bacterial survival/killing studies were conducted with immune serum incubated PMNs as described above.

RESULTS

Visualization, Quantification, and Confirmation of Bovine PMN Net Formation Following Incubation with Leptospira

After visualization with cell permeable and impermeable nucleic acid dyes (**Figure 1**), extracellular cloud-like formations consistent with previous reports of NETS could be observed (Brinkmann et al., 2004; Lippolis et al., 2006; Aulik et al., 2010; Wardini et al., 2010). *E. coli* strain P4 had been shown previously to induce bovine neutrophils to undergo NETosis and was included in all assays as a positive control (Lippolis et al., 2006). While there appeared to be an increase in the numbers of cells stained with the permeable dye when PMNs were incubated with *Leptospira* (**Figures 1C–E**), *E. coli* P4 (**Figure 1B**) or PMA (**Figure 1F**) over the unstimulated PMNs per field of view (**Figure 1A**), these results could not be quantitated due to uneven distribution of cells on the slide, variability across individual slides and variability from experimental day to day. While *E. coli* cells could be visualized in association with the PMNs (using 60x objective, not shown), we were unable to distinguish the thin, spirochetal shaped *Leptospira* from NETs or cellular membranes.

Extracellular DNA extruded as part of NETosis was quantified in accordance with previous reports by quantifying DNA in culture supernatants (Lippolis et al., 2006; Aulik et al., 2010; Wardini et al., 2010). PMNs incubated with *E. coli* P4, demonstrated significant increases ($p < 0.0001$) of 97.5 ng/ml DNA in culture supernatant over unstimulated cells (10 ng/ml DNA in culture supernatant) as did the PMA stimulant (733.6 ng/ml DNA in culture supernatant) (**Figure 2**). Incubation with *Leptospira* strains induced approximately a two-fold increase in supernatant DNA concentration compared to non-stimulated PMNs resulting in strain 203.27.3 ng/ml, JB197 26.6 ng/ml, RM211 26.8 ng/ml, Fiocruz 28.3 ng/ml, Patoc 24.8 ng/ml, heat-killed JB197 24.3 ng/ml which were all statistically different from no stimulant, *E. coli* P4 and PMA ($p < 0.0001$) but not among the *Leptospira* strains ($p < 0.05$). These increases were eliminated by the inclusion of DNase in the assay (+DNase range 7 to 14.5 ng/ml).

To confirm that the extracellular structures visualized and quantified were indeed NETs, structures were labeled using an anti-Histone antibody (Gunderson and Seifert, 2015). Examples of positive and negative stained images are shown in **Figures 3A,B**, respectively. Of the *Leptospira* co-cultured cells, strain 203 17.0%, JB197 17.6%, RM211 18.7%, Fiocruz 19.8% Patoc 16.8%, heat-killed JB197 18.0% of total cells expressed NETs and were positive for H2A antibody staining (**Figure 3C**). 27.2% of the total cells cultured with *E. coli* P4 and 42.1% of the cells cultured with PMA were positive for NET-like formations and H2A antibody staining as described in Material and Methods.

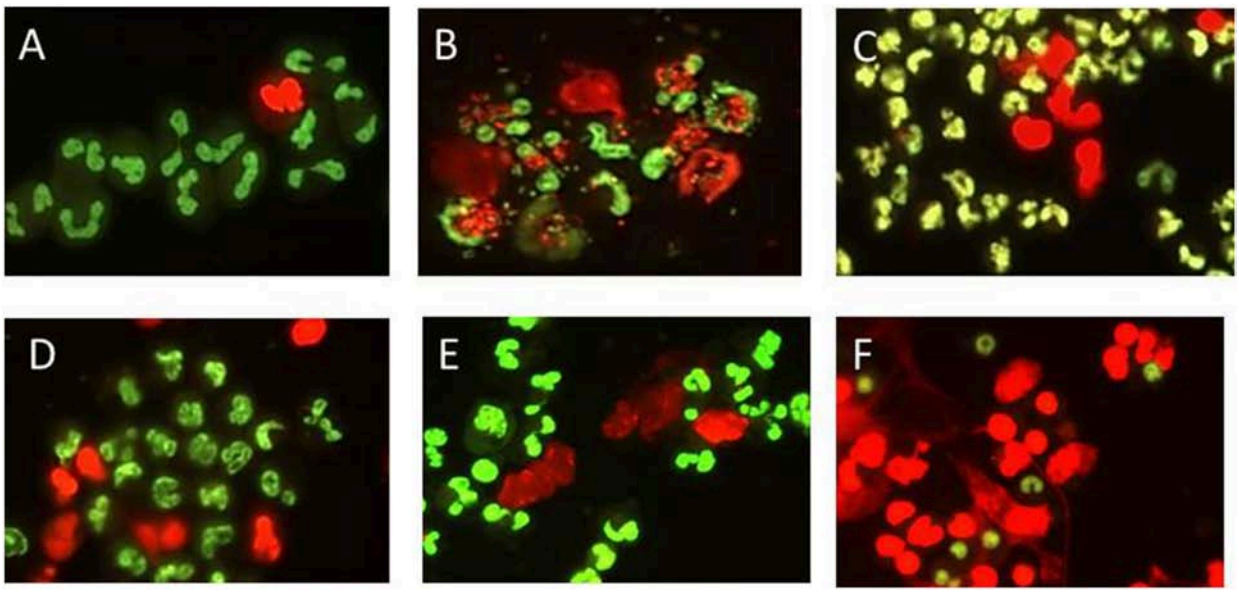

FIGURE 1 | Representative visualization of bovine PMN NET formation using DNA binding dye that is either cell permeable (green) or impermeable (red) indicating extrusion of cellular DNA or otherwise compromised membrane. PMNs were incubated in chamber-slides with MOI 10 for 2 h in supplemented RPMI 1640 with 10% FCS, stained, mounted, and visualized using Nikon E8400 microscope 40x objective and digital camera attachment. **(A)** No Stimulant, **(B)** *E. coli* P4, **(C)** *L. borgpetersenii* serovar Hardjo strain JB197, **(D)** *L. borgpetersenii* serovar Hardjo strain 203, **(E)** *L. interrogans* serovar Pomona strain RM211, **(F)** PMA at a final concentration of 25 ng/ml.

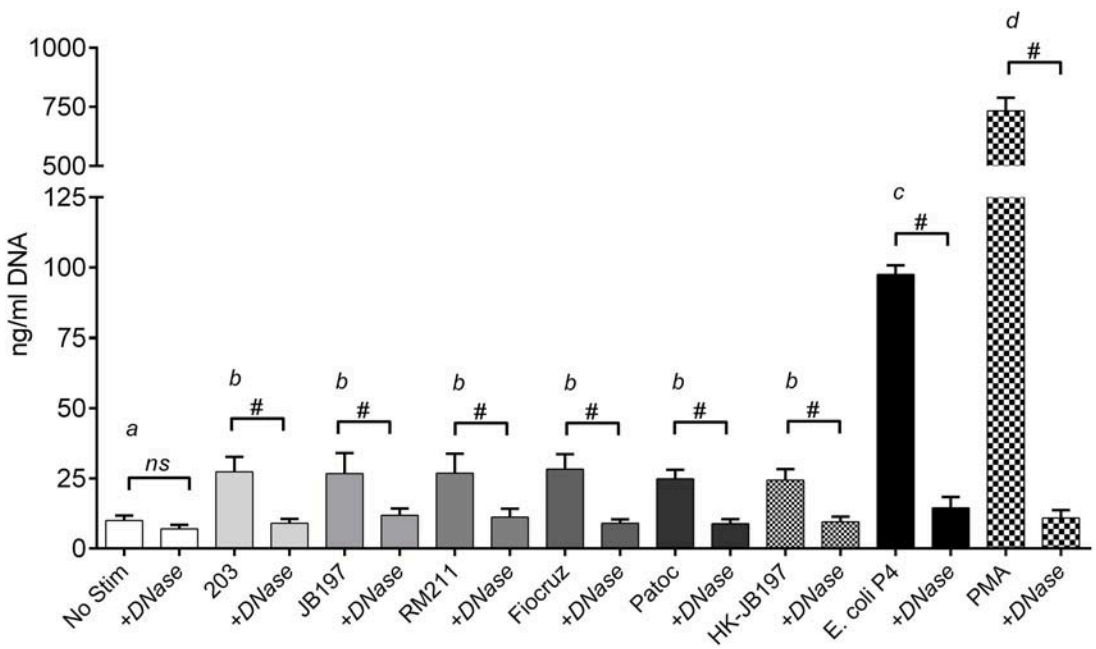

FIGURE 2 | Quantitation of extracellular DNA as an indication of NET formation. Following incubation of isolated bovine PMNs in supplemented RPMI 1640 with 10% FCS, with media only (No Stimulant), *L. borgpetersenii* serovar Hardjo strain 203, *L. borgpetersenii* serovar Hardjo strain JB197, *L. interrogans* serovar Pomona strain RM211, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. biflexa* strain Patoc, heat killed (HK) *L. borgpetersenii* serovar Hardjo strain JB197, or *E. coli* P4 in 96 well plates, extracellular DNA was released by digestion with Staphylococcal Endonuclease and supernatants assayed using fluormetric DNA quantitation assay (PicoGreen). Results depicted are mean and standard deviation (error bars) of six individual animals assayed on at least three different days without and with 100 U/ml DNase I (+DNase). Capped bars (without and with DNase) were compared to each other using students *T*-test # = $p < 0.0001$. Treatment bars (without +DNase) were compared to each other by one-way ANOVA with Tukey's multiple comparisons post-test. Treatments with different letters (*a, b, c, d*) are statistically different from each other but not from treatments with the same letter ($p < 0.05$).

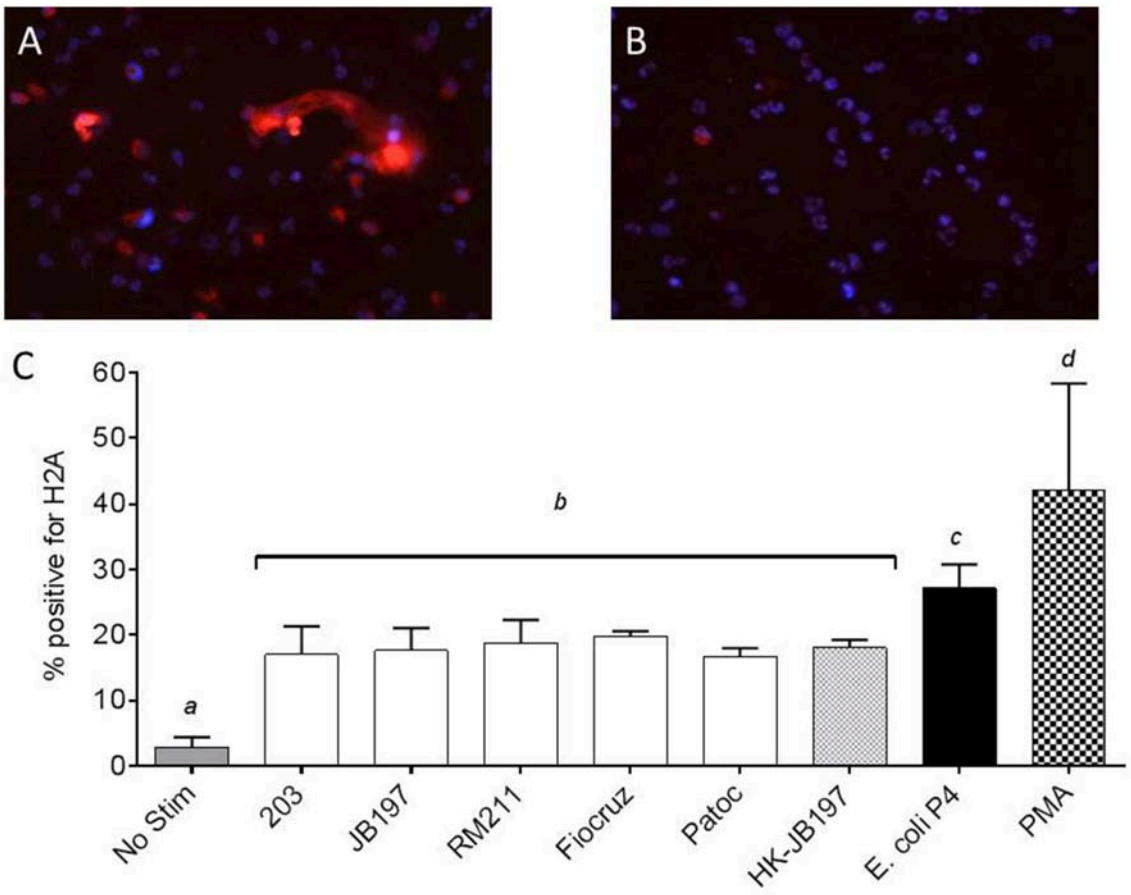

FIGURE 3 | Extracellular DNA has histone proteins consistent with NETosis of neutrophils. PMNs were incubated in chamber-slides with media only (No Stimulant), *L. borgpetersenii* serovar Hardjo strain 203, *L. borgpetersenii* serovar Hardjo strain JB197, *L. interrogans* serovar Pomona strain RM211, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. biflexa* strain Patoc, heat killed (HK) *L. borgpetersenii* serovar Hardjo strain JB197, *E. coli* P4, each at MOI 10, and PMA at a final concentration of 25 ng/ml for 2 h, stained with anti-bovine H2A antibody and DAPI nuclear counterstain, mounted and visualized using Nikon E8400 microscope using 40x objective. **(A)** Example of PMA stimulated bovine PMNs with positive extracellular staining for H2A. **(B)** Example of unstimulated bovine PMNs with negative extracellular staining for H2A **(C)** Mean and standard error of the mean (error bars) of four individual cows and two independent experiments as percentage of total number of cells in three random microscope fields staining positive for H2A. Means were compared to each other by one-way ANOVA with Tukey's multiple comparisons post-test. Treatments with different letters (*a, b, c, d*) are statistically different from each other but not from treatments with the same letter ($p < 0.05$).

Incubation of Bovine PMN with *Leptospira* Resulted in Production of Reactive Oxygen Species (ROS) or Reactive Nitrogen Species (RNS)

In an effort to determine if ROS and RNS were generated, PMNs and culture supernatants were tested for levels of peroxide and nitrates. Bovine PMNs produced similar quantities of H_20_2 when stimulated with *Leptospira* strains (strain 203 0.6 μM H_20_2, JB197 0.5 μM H_20_2, RM211 0.5 μM H_20_2, Fiocruz 0.5 μM H_20_2, Patoc 0.5 μM H_20_2, heat killed JB197 0.4 μM H_20_2) or *E. coli* P4 (0.6 μM H_20_2,) (**Figure 4A**). While all were statictically significant ($p < 0.05$) from No Stimulant (0.2 μM H_20_2,) it is unknown if the low levels of H_20_2 observed (0.4–0.6 μM) has any biological relevance. In contrast, bovine PMNs incubated with *E. coli* P4 induced significantly ($p < 0.05$) higher levels of nitrates (82.1 μM NO_2) in comparison to cells stimulated with *Leptospira* strains (**Figure 4B**). While there was no statistical difference between the cattle-adapted (strain 203 26.2 μM NO_2, JB197 27.2 μM NO_2), other pathogenic *Leptospira* strains (RM211 27.7 μM NO_2, Fiocruz 32.3 μM NO_2) or non-pathogenic *Leptospira* (Patoc 25.5 μM NO2, or heat-killed JB197 25.7 μM NO_2), all were greater and significant ($p < 0.05$) than cells receiving no stimulation (8.4 μM NO_2) (**Figure 4B**).

Induction of Inflammatory Cytokine Gene Transcripts

Neutrophils can influence local inflammation by production of pro-inflammatory cytokines. RNA was extracted from bovine PMNs incubated for 1.5 h with *Leptospira* strains, heat killed *Leptospira* cells or *E. coli* P4. Cytokine gene expression for each *Leptospira* strain or *E. coli* strain P4 was compared to No Stimulant and normalized to housekeeping gene RSP9 by

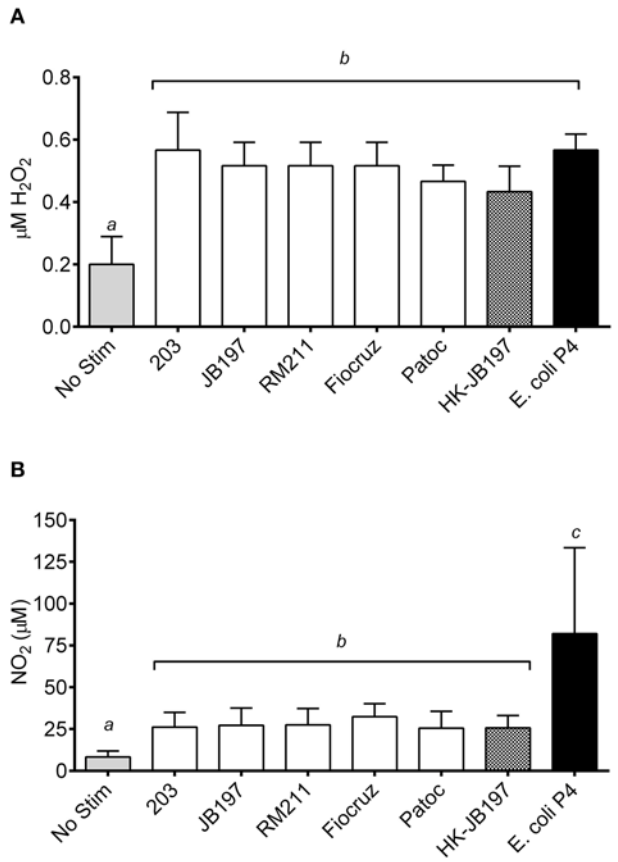

FIGURE 4 | Quantification of cellular (A) reactive oxygen species in the form of hydrogen peroxides and (B) reactive nitrogen species in the form of nitrates. Following incubation of isolated bovine PMNs with media only (No Stim), *L. borgpetersenii* serovar Hardjo strain 203, *L. borgpetersenii* serovar Hardjo strain JB197, *L. interrogans* serovar Pomona strain RM211, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. biflexa* strain Patoc, heat killed (HK) *L. borgpetersenii* serovar Hardjo strain JB197, or *E. coli* P4 in 96 well plates, cells and supernatant were stored at –20°C until assayed. **(A)** H_2O_2 and **(B)** NO_2 production in cells and supernatant was determined by incubation with Amplex Red or Griess Reagent and optical density compared to standard following manufacturer's protocols. Results depicted are mean and standard error of the mean (error bars) of PMNs isolated from four individual cows assayed in three independent experiments. Means were compared to each other by one-way ANOVA with Tukey's multiple comparisons post-test. Treatments with different letters (*a, b, c*) are statistically different from each other but not from treatments with the same letter ($p < 0.05$).

qRT-PCR. For IL-1β, IL-8, MIP-1α, and TNF-α, gene expression was significantly increased for all treatments above no stimulant or background levels, with *E. coli* P4 being greater than any of the *Leptospira* strains tested (**Figure 5**). Interestingly, for IL-1β, MIP-1α, and TNF-α heat-killed JB197 induced relatively less expression than cattle adapted (203, JB197), pathogenic (RM211, Fiocruz), or the live saprophyte (Patoc) strain, but was still significantly above no stimulant ($p < 0.05$). $2^{(-\Delta\Delta CT)}$ values for IL-1β relative gene expression were 1, 32.8, 27.8 29.7, 31.4, 24.7, 15.8, and 46.4 for No Stim, 203, JB197, RM211, Fiocruz, Patoc, heat-killed JB197, and *E. coli* P4 respectively. $2^{(-\Delta\Delta CT)}$ values for IL-8 relative gene expression were 1, 11.2, 8.7, 10.7, 9.8, 8.8, 8.9, and 25.5 for No Stim, 203, JB197, RM211, Fiocruz, Patoc, heat-killed JB197, and *E. coli* P4 respectively. $2^{(-\Delta\Delta CT)}$ values for MIP-1α relative gene expression were 1, 8.0, 7.7, 7.2, 7.5, 7.2, 3.6, and 17.3 for No Stim, 203, JB197, RM211, Fiocruz, Patoc, heat-killed JB197, and *E. coli* P4 respectively. $2^{(-\Delta\Delta CT)}$ values for TNF-α relative gene expression were 1, 20.1, 17.5 19.9, 18.7, 14.8, 6.9, and 34.8 for No Stim, 203, JB197, RM211, Fiocruz, Patoc, heat-killed JB197, and *E. coli* P4, respectively. Cytokine gene expression showed no increase in gene expression of TGF-β, IL-17 or IFN-γ above no stimulation or background levels, with all means being at or below 2.

Incubation of Bovine PMNs with Leptospira Did Not Reduce Bacterial Viability

Leptospira strains and *E.coli* P4 (MOI 10) were incubated for 4 h with bovine PMNs. Following this incubation, PMNs were lysed with saponin, and bacterial viability determined by either limiting dilution culture in semi-solid media tubes for *Leptospira* or dilution and plating to obtain CFU for *E. coli* P4. Bacteria incubated under the same conditions but in the absence of PMNs were set as 100% survival. Incubation of the *Leptospira* with bovine PMNs had no effect on *Leptospira* viability ($p < 0.05$), however PMNs had a negative impact on *E. coli* P4 viability, resulting in 64% survival (**Figure 6**).

Effect of Immune Serum on PMN NETosis

Serum banked and pooled from a previously published study was used as the source for immune serum (Zuerner et al., 2011). The sera pools used in this assay [naïve, vaccinated, vaccinated, and infected (vaccinated + challenged), or naïve-infected (challenged)] had MAT titers to *L. borgpetersenii* serovar Hardjo strains 203 and JB197 that were negative (<1:12), 1:200, 1:800 and 1:100 for the four groups respectively (Table S1). All serum pools were MAT negative for other *Leptospira* strains. NETosis assay (measurement of extracellular DNA) was performed with the presence of bovine serum from naïve, vaccinated, vaccinated, and infected (vaccinated + challenged), or naïve-infected (challenged) cattle which contained *Leptospira* specific antibody (Zuerner et al., 2011). Addition of bovine anti-leptospiral serum (naïve, Vaccinated, Vaccinated + Challenged, or Challenged) at 20% of the final cell culture media did not alter the response (NET formation) to any individual *Leptospira* strain (**Figure 7**). Within a PMN treatment with *Leptospira*, there was no significant difference when different immune serum was added ($p < 0.05$) (**Figure 7**), nor did the overall results of the assay change from the trend depicted in **Figure 2**, where all *Leptospira* strains induced similar level of NETosis (22.3 to 29.8 ng/ml DNA in culture supernatants) and were significantly different from *E. coli* P4 (with 77.0–86.1 ng/ml DNA in culture supernatant) and all greater than no stimulant (No Stim 10.5–12.25 ng/ml DNA).

Effect of Immune Serum on PMN *Leptospira* Killing

PMN and *Leptospira* incubation was performed as above with the addition of bovine anti-leptospiral serum, at 20% of media. No effect was observed on *Leptospira* survival (Table S2).

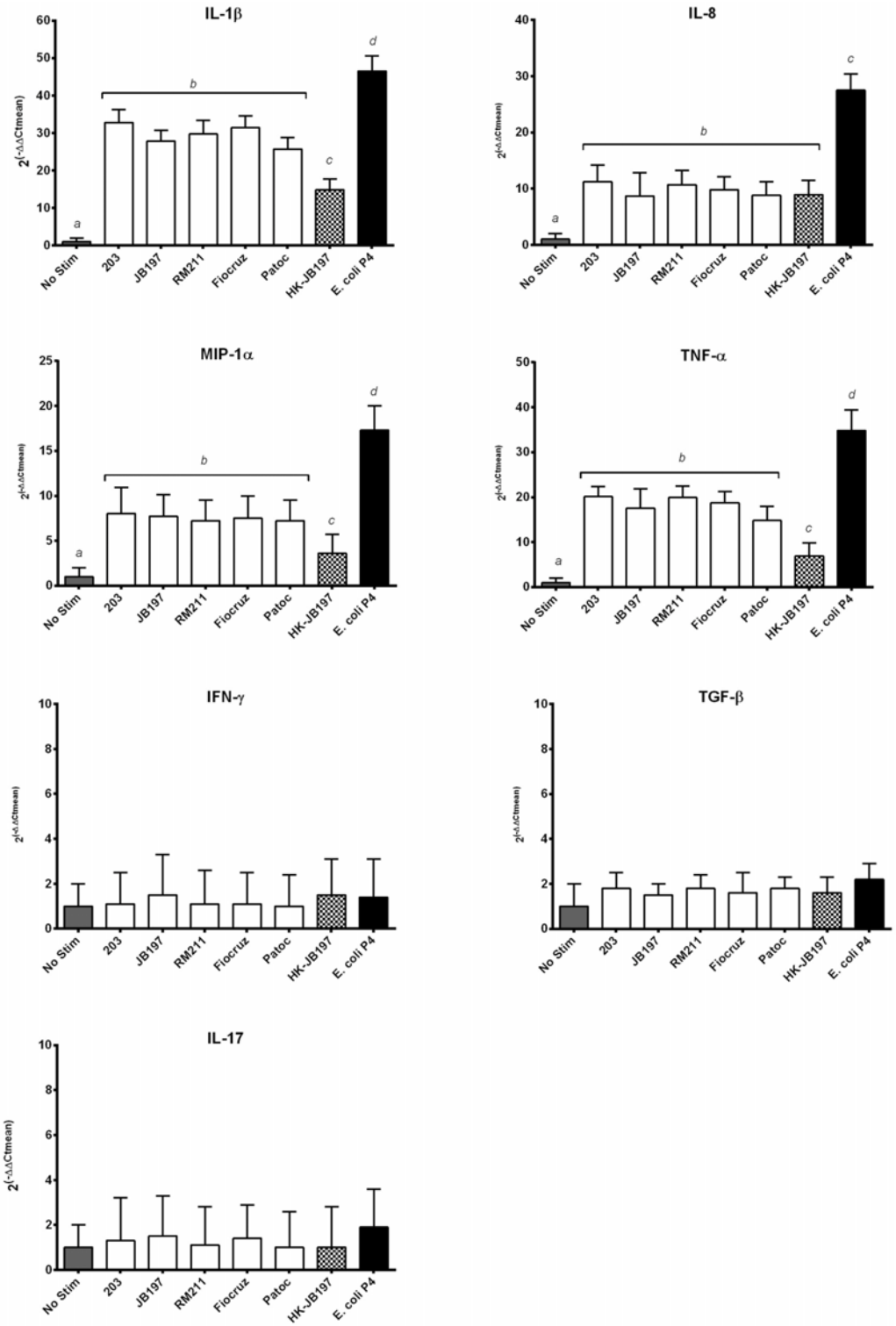

FIGURE 5 | *Leptospira* induce inflammatory cytokine mRNA transcript expression. Bovine PMNs were incubated with media only (No Stim), *L. borgpetersenii* serovar Hardjo strain 203, *L. borgpetersenii* serovar Hardjo strain JB197, *L. interrogans* serovar Pomona strain RM211, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. biflexa* strain Patoc, heat killed (HK) *L. borgpetersenii* serovar Hardjo strain JB197, or *E. coli* strain P4 for 1.5 h at MOI 10. The relative mRNA expression of cytokines IL-1β, IL-8, TNF-α, MIP-1α, TNF-α, IFN-γ, TGF-β, and IL-17 were assayed by quantitative RT-PCR. Data reported as means and standard error of the mean (error bars) for PMNs isolated from 4 individual naive cows assayed in two independent experiments. Data is depicted as $2^{(-\Delta\Delta Ct)}$ where cytokine expression is relative increase over No Stim normalized to housekeeping gene RPS9. Statistical calculations were performed on $\log_2$ transformed data. Treatments with different letters (*a, b, c, d*) are statistically different from each other but not from treatments with the same letter ($p < 0.05$).

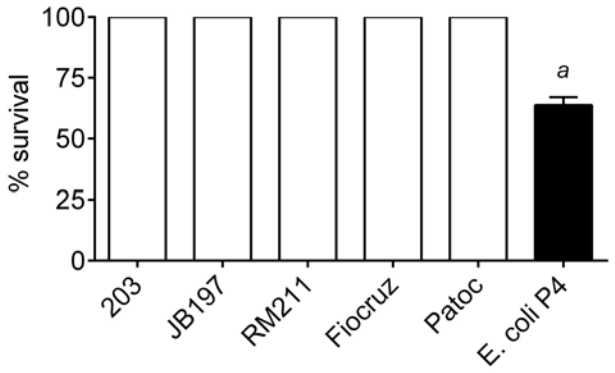

FIGURE 6 | Bacterial survival after incubation with bovine PMNs. *L. borgpetersenii* serovar Hardjo strain 203, *L. borgpetersenii* serovar Hardjo strain JB197, *L. interrogans* serovar Pomona strain RM211, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. biflexa* strain Patoc, or *E. coli* strain P4 were incubated with freshly isolated bovine peripheral blood polymorphonunclear cells (PMNs) at MOI 10 for 4 h. At the end of the incubation, PMNs were lysed by the addition of 0.05% Saponin. *Leptospira* were cultured for up to 4 weeks in serial dilution series tubes for estimation of number. *E. coli* P4 was diluted by serial dilution and spread on sheep blood supplemented tryptic soy agar plates and incubated overnight. Results depicted as percentage survival calculated from lowest dilution tube (or plate CFU count for *E. coli* P4) with positive growth compared to bacterial cells under the same incubation conditions (culture media, time, saponin, etc.) without the addition of bovine PMNs. Each bacterial strain was incubated with PMNs from four individual cows in duplicate sampled in two independent experiments. *a*: *E. coli* P4 was statically different from all *Leptospira* (63 vs. 100% survival; $p < 0.05$).

Growth was consistently observed out to the –6 dilution, same as for *Leptospira* manipulated and incubated under the same conditions, without the presence of bovine PMNs or immune serum.

DISCUSSION

Recent reports demonstrated human and mouse neutrophils produced NETs in response to pathogenic *Leptospira*, resulting in killing of the *Leptospira* and reduced tissue burdens (Scharrig et al., 2015). Older studies documented *Leptospira* interactions with human and guinea-pig PMNs (McGrath et al., 1984; Wang et al., 1984a). However, infections in these species are more representative of acute leptospirosis, hallmarked by an overt inflammatory reaction and systemic septicemia-like condition. Cattle are a reservoir host of *Leptospira*, demonstrating chronic infection, with colonization limited to a few tissues (reproductive and urinary tracts) and intermittently shed *Leptospira*, infecting herd-mates, humans and other animals (Gamage et al., 2014; Samir et al., 2015). Therefore, it is important to understand the complex interactions of host/*Leptospira* in both types of infection, the acute and chronic/reservoir disease in the proper host. These studies were attempted to evaluate if and how various strains of *Leptospira* would activate bovine PMNs.

One very visual determination of neutrophil or PMN activation is induction of NETosis. Previously NETosis has been demonstrated with *E. coli* P4 and other bacterial pathogens (Lippolis et al., 2006; Aulik et al., 2010). Incubation of bovine PMNs with *Leptospira* strains did induce some NET activation, and while greater than background, it was much less than that with the *E. coli* P4 pathogen or PMA. In contrast to previous reports using *B. burgdorferi*, confirmation of leptospiral structures within NET formations was not observable (Menten-Dedoyart et al., 2012), most likely due size difference, *Leptospira* being half the length and width of *Borrelia* species. Using an assay to measure DNA extruded into the cell culture supernatant, we observed a roughly two-fold increase over background when bovine PMNs were incubated with leptospiral strains. This increase was diminished with the addition of DNase enzyme, further indicating that NETosis was occurring. Again, in contrast to recent publication using human neutrophils (Scharrig et al., 2015), we did not observe a difference in magnitude of response in regards to virulence; the same response was seen for pathogenic and saprophytic strains, and for heat-inactivated organisms. We have confidence overall in the execution of our assays because in our studies, percentages of *E. coli* P4 or PMA stimulated cells undergoing NETosis were consistent with previous reports (Lippolis et al., 2006; Aulik et al., 2010; Wardini et al., 2010). Previously published studies have demonstrated that NETs contain both nucleic acids and histone proteins (Brinkmann et al., 2004; Aulik et al., 2010; Gunderson and Seifert, 2015). Using histone antibody staining as a secondary indication of NET formation, we again observed an increase in the number of PMNs with NETs as compared to background/no stimulant, but less than *E. coli* P4 or PWM mitogen. Taken together, *Leptospira* did induce NETosis in bovine PMNs, just not as robustly as *E. coli* P4 or PMA mitogen.

Stimulation of pattern recognition receptors (PRRs) along with pro-inflammatory cytokines (IFN-γ, IL-1β, and TNF-α) can trigger signaling cascades leading to iNOS production including NF-kB and MAPK (Fang, 2004). Production of nitric oxide (NO) and superoxide ions represent a mechanism of pathogen destruction in activated neutrophils, and production or expression of iNOS has been associated with many inflammatory associated diseases (Mariano et al., 2012). During the "respiratory burst," superoxide can react with many potential complexes, including nitric oxide to form the reactive nitrogen species (RNS) peroxynitrite (Amulic et al., 2012). Usually produced more from macrophages than neutrophils, RNSs can control as well as contribute to local tissue and vascular inflammation through signaling and other non-antimicrobial functions (Fang, 2004). In the current study, H_2O_2 produced by PMN in response to incubation with *Leptospira* strains was increased over no stimulation, but the overall levels were low (0.4–0.6 μM H_2O_2) calling into question the biological significance. In a cell free assay, Murgia et al. observed that pathogenic *L. interrogans* strain Hardjoprajitno showed a 2 Log reduction in the presence of 0.06 mM H_2O_2 and complete inhibition at 0.34 mM H_2O_2 (Troxell et al., 2014). These levels are at least 100 times greater in H_2O_2 concentration than was measured in the present study. In contrast, bovine PMNs produced higher levels of NO_2 in the presence of *E. coli* P4 and significant amounts in the presence

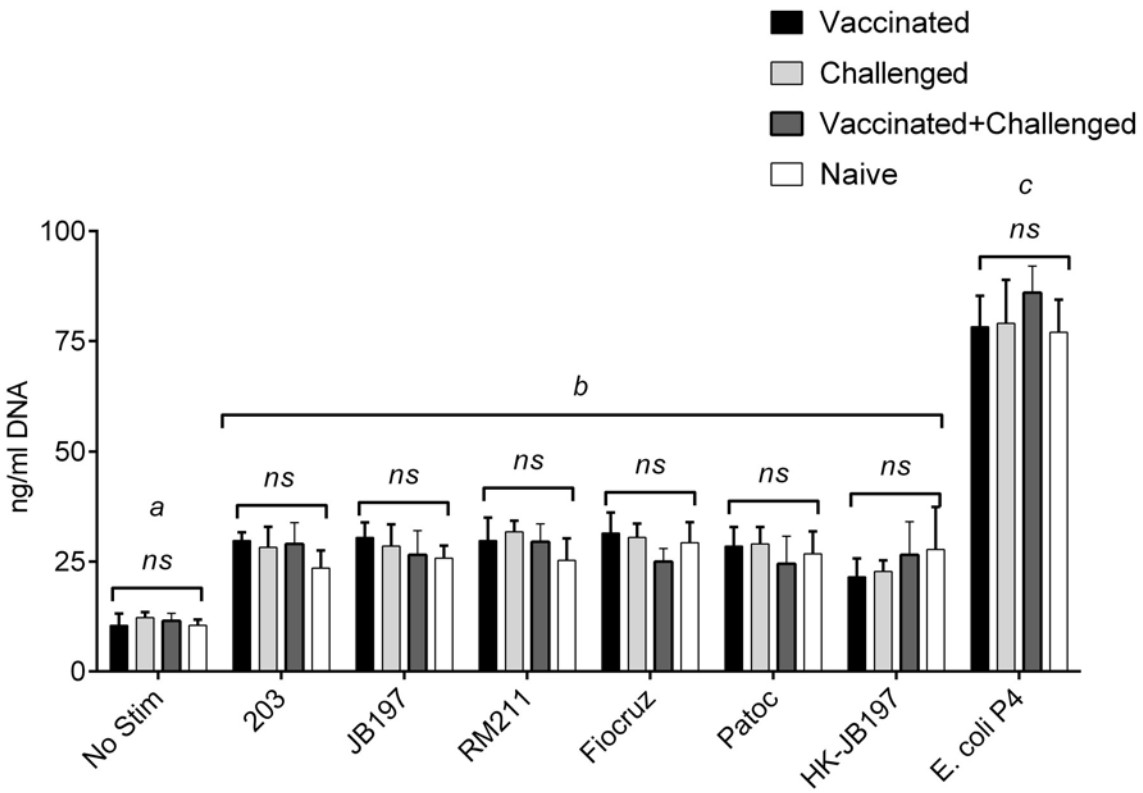

FIGURE 7 | Quantitation of extracellular DNA as an indication of NET formation by bovine PMNs in the presence of *Leptospira* and *Leptospira* reactive immune serum. Following incubation of isolated bovine PMNs in supplemented RPMI 1640 with 20% pooled serum from vaccinated, challenged, vaccinated + challenged, or naïve cattle, with media only (No Stim), *L. borgpetersenii* serovar Hardjo strain 203, *L. borgpetersenii* serovar Hardjo strain JB197, *L. interrogans* serovar Pomona strain RM211, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. biflexa* strain Patoc, heat-killed *L. borgpetersenii* serovar Hardjo strain JB197, or *E. coli* P4 in 96 well plates, extracellular DNA was released by digestion with Staphylococcal Endonuclease and supernatants assayed using fluormetric DNA quantitation assay (PicoGreen). Results depicted are mean and standard deviation (error bars) of cells from four individual animals assayed in two independent experiments. Leptospira strains (treatments) were compared across serum types (naïve, vaccinated, etc.) were compared using one-way ANOVA and effect of added immune serum was not significant ($p < 0.05$). Treatments were compared to each other by one-way ANOVA with Tukey's multiple comparisons post-test. Treatments with different letters (*a, b, c*) are statistically different from each other but not from treatments with the same letter ($p < 0.05$).

of *Leptospira* strains, again, with no difference to virulence or viability. NO_2, a product of NO and O_2^- can react with pathogen targets and effect innate immune pathogen clearance (Lowenstein and Padalko, 2004).

Neutrophil-derived cytokines can play a role in orienting immunity toward Th-1 type responses [production of IL-12, macrophage inflammatory protein (MIP)-1α] (Scapini et al., 2000). Interleukin-8 is produced in large amounts by neutrophils in response to external stimuli and can play a role in the inflammatory recruitment cascade (Hassfurther et al., 1994; Scapini et al., 2000). Neutrophils can also produce TNF-α which can elicit cytokine responses in an endocrine-like feedback loop for both neutrophil and surrounding cells (Walter and Morck, 2002). Production of MIP– 1α, 1β, and 1γ by neutrophils can be chemotactic and stimulatory for monocytes, macrophages, dendritic cells, NK cells, and Th1 lymphocytes (Scapini et al., 2000). After incubation with bacterial lipopolysaccharide, bovine neutrophils have been known to produce inflammatory cytokines including IL-8, IL-1β, and TNF-α (Worku and Morris, 2009). Hamster kidney following infection with virulent *Leptospira*, or hamster peripheral blood mononuclear cells (PBMCs) cultured with *Leptospira*, upregulate TNF-α, IFN-γ, IP-10, IL-10, and IL12p40 (Vernel-Pauillac and Merien, 2006; Lowanitchapat et al., 2010). Stimulation of human cell line THP-1, PBMCs or whole blood with *Leptospira* resulted in production of TNF-α and IL-6 (Goris et al., 2011). In the present study, we observed increases in IL-1β, IL-8, MIP-1α, and TNF-α cytokine gene expression. While increases in pro-inflammatory cytokine gene expression were observed from PMNs incubated with *Leptospira* these were far less that those observed with *E. coli* P4. Interestingly, cytokine expression was also dependent on live leptospires being present as heat-killed organisms did not induce the same magnitude of effect. While not reaching significance, there was a trend for the saprophyte Patoc to induce less cytokine gene transcript than the pathogenic (Hardjo 203 and JB197, Pomona RM211, and Fiocruz) strains used.

Although our data indicated modest evidence of NET activation, production of RNS, and slight increase in pro-inflammatory cytokine gene expression, we originally

hypothesized that bovine PMNs could still influence clearance of *Leptospira* by phagocytosis or some other method of bacterial killing. However, any PMN activation had no effect on *Leptospira* viability. We also did not observe killing of non-pathogenic strain of *Leptospira*, whereas others observed that non-pathogenic leptospires were able to be killed by human PMNs (Wang et al., 1984a,b). Using a most-probable-number procedure for quantitating viable numbers of *Leptospira*, they showed that while the pathogenic *Leptospira* became cell associated, they were not killed in the presence of neutrophils and immune serum (Wang et al., 1984a). More recently, Goris et al. showed that human whole blood was able to kill culture-adapted strains, but not host-adapted strains (Goris et al., 2011). Furthermore, THP-1 cells or PMBCs were not able to kill culture- or host- adapted strains (Goris et al., 2011). These differences in reported killing of *Leptospira* by whole blood or isolated cells may be attributed to differences in methods for *Leptospira* culture/viability or host cell type used in assays. We observed no reduction in *Leptospira* viability, including the non-pathogenic saprophyte strain, by bovine PMNs, as measured by dilution culture a more sensitive means of determining most probable numbers for difficult to culture organisms. Assays with *E. coli* P4 showed reductions in viability consistent with neutrophil killing assays, indicated that PMNs were capable of antimicrobial activities (Ermert et al., 2009). This further illustrates differences between acute and chronic/reservoir hosts in response to infection.

To test if opsonization was needed for *Leptospira*-PMN interaction or leptospiral killing, NET quantitation and leptospiral assays were conducted with the inclusion of immune serum. The serum titer or antibody reactivity was determined by MAT, which by its very description requires surface binding antibody. Inclusion of *Leptospira* specific antibodies did not enhance the results over naïve or non-immune serum nor did it change the results, all *Leptospira* strains resulted in similar levels of NETosis. Inclusion of *Leptospira* specific serum did not alter *Leptospira* viability either. While there is a trend for there to be less growth at the higher (−6 and −7 dilutions) for strain 203 in the presence of immune serum, the results are not consistent or striking enough to be of importance. Due to the free-thawing that the serum underwent, and the heat inactivation, it is unlikely that any active complement remained in the serum sample. Overall, current data indicates that clearance of *Leptospira* and *Leptospira* killing by innate cells is complex and may involve active complement, specific antibody and multiple phagocytic cell types (Wang et al., 1984b; Goris et al., 2011).

Differences between species in innate immunity function have been recently reported, illustrating that caution must be used when making assumptions regarding host-pathogen relationships. The differences in spirochete recognition by TLR receptors between mice and humans, and differences between bovine, mice and human TLR2 receptors at the level of amino acid homology, might explain differences in responses through activation of TLR receptors and subsequent inflammatory cascades. Further studies will be needed to confirm that bovine TLR2 does not recognize *Leptospira* antigens. This emphasizes the importance of studying pathogen-host interactions in native hosts.

Unlike other spirochetes such as *Treponema* or *Borrelia, Leptospira* species possess lipopolysaccharide (LPS) as part of their outer membrane (Nahori et al., 2005). Typically LPS is recognized by the pattern recognition receptor (PRR) toll-like receptor TLR4, leading to pro-inflammatory cytokine, and chemokine responses (Nahori et al., 2005). Recently it has been shown that *Leptospira* LPS is recognized by porcine, mouse and human TLR2, and neutrophils from these species, express surface TLR2 (Nahori et al., 2005; Thomas and Schroder, 2013; Guo et al., 2015). While bovine PMNs do express TLR2 and TLR4 (Swain et al., 2014), there are species differences in TLR structure that may impact functionality with certain bacterial moieties. Bovine TLR2 shares 77 and 65% homology at the amino acid level with human and murine TLR2, and 72 and 65% homology for TLR4 (Werling and Jungi, 2003). Ligation of bacteria to PRRs may promote neutrophil survival (increase in lifespan), ROS production (increase in myeloperoxidase activity), NET formation, and cytokine production, all of which may be detrimental to the bacterial pathogen (Yipp et al., 2012; Thomas and Schroder, 2013). In contrast to humans, the ability of mice to recognize *Leptospira* LPS and Lipid A through TLR4 and MyD88 independent mechanisms, may explain some of the observed differences between humans and mice regarding susceptibility to infection (Fraga et al., 2011).

CONCLUSION

Our data demonstrates *Leptospira* strains activate circulating PMNs of cattle greater than unstimulated cells, but not to the same levels of activation seen with another pathogen, a mastitis inducing strain of *E. coli*. Furthermore, this modest activation had no impact on the viability of the leptospires. Important to note, whether it was PMN activation or *Leptospira* survival, results were the same regardless of leptospiral strain used: cattle-host adapted, acute pathogenic or saprophytic. We speculate that nuances in bovine pattern recognition receptors, specifically TLR2, may explain our observation in cattle as compared to reports of activation of neutrophils by *Leptospira* in other species (human and mouse). This corresponds to phenotypic observations of wild rodents being chronic reservoirs of *Leptospira* carriage while humans are susceptible to acute but rarely chronic forms of the disease (Ganoza et al., 2010; De Silva et al., 2014; Loffler et al., 2014; Matsui et al., 2016). In comparison, cattle are susceptible to chronic infection with *Leptospira* and this correlates with the clinical presentation of a stealth disease residing in kidney and reproductive tissues. Furthermore, the results from this study, using bovine PMNs, contrast with those reported elsewhere using neutrophils or PMNs from humans and other acute hosts. These results show that differences in disease state (chronic or acute) may be host associated as well as *Leptospira* strain or specific. Continued study in the reservoir host is needed to fully understand the host-pathogen relationship.

AUTHOR CONTRIBUTIONS

DA and JW conceptualized original experiments. JW came up with overall experimental design. JW, AF, RH performed the experiments and were responsible for data analysis. JW, AF, RH, SO and DA all contributed to critical evaluation of the data, manuscript preparation and final editing of the manuscript.

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frontiers in Immunology

MINI REVIEW
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Hide and Seek: How Lyme Disease Spirochetes Overcome Complement Attack

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Overcoming the first line of the innate immune system is a general hallmark of pathogenic microbes to avoid recognition and to enter the human host. In particular, spirochetes belonging to the *Borrelia burgdorferi* sensu lato complex have developed various means to counter the immune response and to successfully survive in diverse host environments for a prolonged period of time. In regard to complement resistance, *Borrelia* utilize a plethora of immune evasion strategies involves capturing of host-derived complement regulators, terminating complement activation as well as shedding of cell-destroying complement complexes to manipulate and to expeditiously inhibit human complement. Owing to their mode of action, the interacting surface-exposed proteins identified among *B. burgdorferi* sensu stricto (s.s.), *Borrelia afzelii*, *Borrelia spielmanii*, and *Borrelia bavariensis* can be classified into at least two major categories, namely, molecules that directly interfere with distinct complement components including BBK32, CspA, BGA66, BGA71, and a CD59-like protein or molecules, which indirectly counteract complement activation by binding various complement regulators such as Factor H, Factor H-like protein 1 (FHL-1), Factor H-related proteins FHR-1, FHR-2, or C4Bp. The latter group of genetically and structurally unrelated proteins has been collectively referred to as "complement regulator-acquiring surface proteins" and consists of CspA, CspZ, ErpA, ErpC, ErpP, and the as yet unidentified protein p43. This review focuses on the current knowledge of immune evasion mechanisms exhibited by Lyme disease spirochetes and highlights the role of complement-interfering, infection-associated molecules playing an important part in these processes. Deciphering the immune evasion strategies may provide novel avenues for improved diagnostic approaches and therapeutic interventions.

Keywords: spirochetes, *Borrelia*, Lyme disease, complement, immune evasion, complement regulator, innate immunity

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INTRODUCTION

The genus *Borrelia* (*B.*) comprises the causative agents of Lyme disease (LD) and relapsing fever (1–3). Concerning LD spirochetes, there are distinct species belonging to the *Borrelia burgdorferi* sensu lato complex of which six species including *B. burgdorferi* sensu stricto (s.s.), *Borrelia afzelii*, *Borrelia garinii*, *Borrelia spielmanii*, *Borrelia bavariensis* (formerly referred to as *B. garinii* OspA serotype 4), as well as candidatus *Borrelia mayonii*, are associated with human LD (4, 5). While *Borrelia valaisiana*, *Borrelia lusitaniae*, and *Borrelia bissettii* have been detected in human biopsies,

the pathogenicity of these and the remaining borrelial species remains largely unclear (6–8).

To survive and establish a persistent infection in the human host, pathogens must evade the first line of host defense by counteracting complement as an essential part of innate immunity. This powerful surveillance system comprises a network of precursors, regulatory and inhibitory proteins that can be immediately activated upon recognition of invading microorganisms (9, 10). Despite the effectiveness and abundance of complement, LD spirochetes are able to overcome its destructive defense mechanisms (11–13). While attempting to decipher the molecular mechanisms of complement evasion, distinct complement-interfering and -inhibiting molecules of *B. burgdorferi* s.s., *B. afzelii*, *B. spielmanii*, and *B. bavariensis* have been identified (14, 15). This review focuses on the current knowledge of the molecular principles utilized by LD spirochetes to counteract complement at certain activation levels and on the borrelial proteins known to take part in complement inactivation.

ACTIVATION AND REGULATION OF THE COMPLEMENT SYSTEM

Complement operates as a cooperative network of inactive precursor molecules, fluid-phase and membrane-bound regulators, and inhibitors (16–20). The initiation of complement takes place in a cascade-like manner through three activation routes: the classical (CP), the lectin (LP), and the alternative pathway (AP), all of which converge in the generation of the highly reactive molecule, C3b (17, 19, 21, 22). The CP can be activated after binding of C1q to immune complexes (IgM and IgG) or charged molecules on the bacterial surface (23). In complex with C1q and C1r, C1s mediates cleavage of C4 and C2 leading to the formation of the C3 convertase, C4b2b. Activation of the LP is initiated by binding of mannan-binding lectin (MBL), ficolins (H-ficolin, L-ficolin, and M-ficolin), or collectins associated with MBL-associated serine proteases (MASP), to carbohydrates of microbial origin. After activation of MASP-2 by MASP-1, both proteases cleave C2 while MASP-2 is able to also cleave C4 to generate the identical C3 convertase. Finally, the AP is initiated by spontaneous hydrolysis of C3 followed by binding of C3b (opsonization) to different targets on the bacterial surface. Recruitment of Factor B (FB) followed by Factor D (FD)-mediated cleavage results in the formation of the membrane-bound C3 convertase C3bBb. To extend the half-life and to trigger the amplification of C3b (feedback loop), the C3 convertases of the AP are stabilized by properdin. Of note, deposition of large quantities of C3b on microbial surfaces is a prerequisite for opsonization and phagocytosis of invading pathogens. Upon binding of newly generated C3b molecules, the C4b2b and C3Bb complexes serve as precursors for the C5 convertases C4b2b3b and C3bBb3b. Cleavage of C5 into C5a and C5b by the C5 convertases initiates the unidirectional, sequential binding of the late components C6, C7, and C8 to C5b. Once the C5b–8 complex is formed, polymerization of multiple C9 molecules ensues, finally generating the pore-forming terminal complement complex [C5b–9, TCC, also referred to as membrane attack complex (MAC)], which promotes lysis of susceptible cells (19, 20). To avoid the detrimental effects of excessive complement activation, this surveillance system is tightly controlled by soluble and membrane-anchored regulators (21). The soluble regulators of the CP and LP include C1 esterase inhibitor (C1-INH) and C4b-binding protein (C4Bp), while the AP is primarily regulated by Factor H (FH) and Factor H-like protein 1 (FHL-1). Vitronectin, clusterin, and, in part, FH-related protein 1 (FHR-1) comprise the regulatory proteins of the terminal activation sequence (21, 24).

DIVERSITY IN COMPLEMENT SUSCEPTIBILITY

Initial investigations showed that LD spirochetes differ substantially in their susceptibility to human serum and finally led to the classification of spirochetes into three main categories, serum-resistant, intermediately serum-resistant/sensitive or partially resistant, and serum-sensitive (25–27). It is worth mentioning that categorizing of spirochetes in these particular groups largely depends on technical parameters, e.g., serum collection and storage, serum and cell concentrations, incubation period, and the method of choice used to determine borrelial survival, making it difficult to compare the data published. Changing the experimental conditions can lead to differences in the phenotypic classification, in particular of intermediately serum-resistant/sensitive strains. Among LD spirochetes, *B. burgdorferi* s.s., *B. afzelii*, *B. spielmanii*, *B. bavariensis*, and *Borrelia japonica* are resistant to complement-mediated killing, *B. bissettii* was classified as intermediately serum-resistant and *B. garinii*, *B. valaisiana*, and *B. lusitaniae* comprise the group of highly susceptible spirochetes (13, 25–35). Furthermore, differences in serum susceptibility have been reported among certain *B. valaisiana* and *B. garinii* isolates (29, 36). Strikingly, the serum susceptibility pattern of LD spirochetes almost matches pathogenicity in humans with the exception of *B. garinii* known to frequently cause LD. The underlying molecular principles of how *B. garinii* circumvent complement-mediated killing are largely unknown and are still a matter of controversy. It is likely that pathogen-associated factors produced solely in the infected host or that interaction with host or tick-derived proteins upon the transmission process, e.g., plasminogen (37), Tick Salivary Lectin Pathway Inhibitor (TSLPI) (38), or Salp20 (39), may protect *B. garinii* from complement attack.

BORRELIAL PROTEINS INTERACTING WITH HUMAN COMPLEMENT REGULATORS

Inactivation of the Alternative Pathway by Binding Complement Regulators FH and FHL-1

In 1997, two independent studies demonstrated that serum-resistant strains exhibit significantly lower amounts of deposited activation products (C3, C6, and TCC) compared to serum-susceptible strains, leading to the assumption that the complement cascade is inhibited at the level of C3 and/or C5 activation;

however, no underlying mechanism(s) were elucidated (26, 27). Several years later in 2001, OspE of *B. burgdorferi* s.s. (40) and the so-called Complement Regulator-Acquiring Surface Proteins (CRASP) of *B. burgdorferi* s.s. and *B. afzelii* (41, 42) were identified as ligands for FH and, in part, for FHL-1. Binding of these complement regulators by serum-resistant spirochetes inhibits activation at the central step of the complement cascade, C3 activation and the formation of C3 convertase, and thereby terminates the assembly and finally the integration of the TCC into the bacterial membrane (28, 42) (**Figure 1A**). Thereafter, several FH-binding proteins were detected in serum-resistant *B. spielmanii*, *B. japonica*, and *B. bissettii* isolates (31, 43, 44), while serum-sensitive *B. garinii*, *B. lusitaniae*, and *B. valaisiana* isolates did not bind functionally active FH (28, 30, 36, 42). The importance of disrupting complement activation at the level of C3 by surface-bound FH and FHL-1 was confirmed both in initial and follow-up studies investigating different borrelial species (29, 31–33). With the exception of serum-resistant *B. bavariensis* (45), almost all serum-resistant borrelial species are able to co-opt human FH and FHL-1 to protect themselves from complement-mediated killing, allowing LD spirochetes to survive in humans and in diverse, immune competent animal hosts (13).

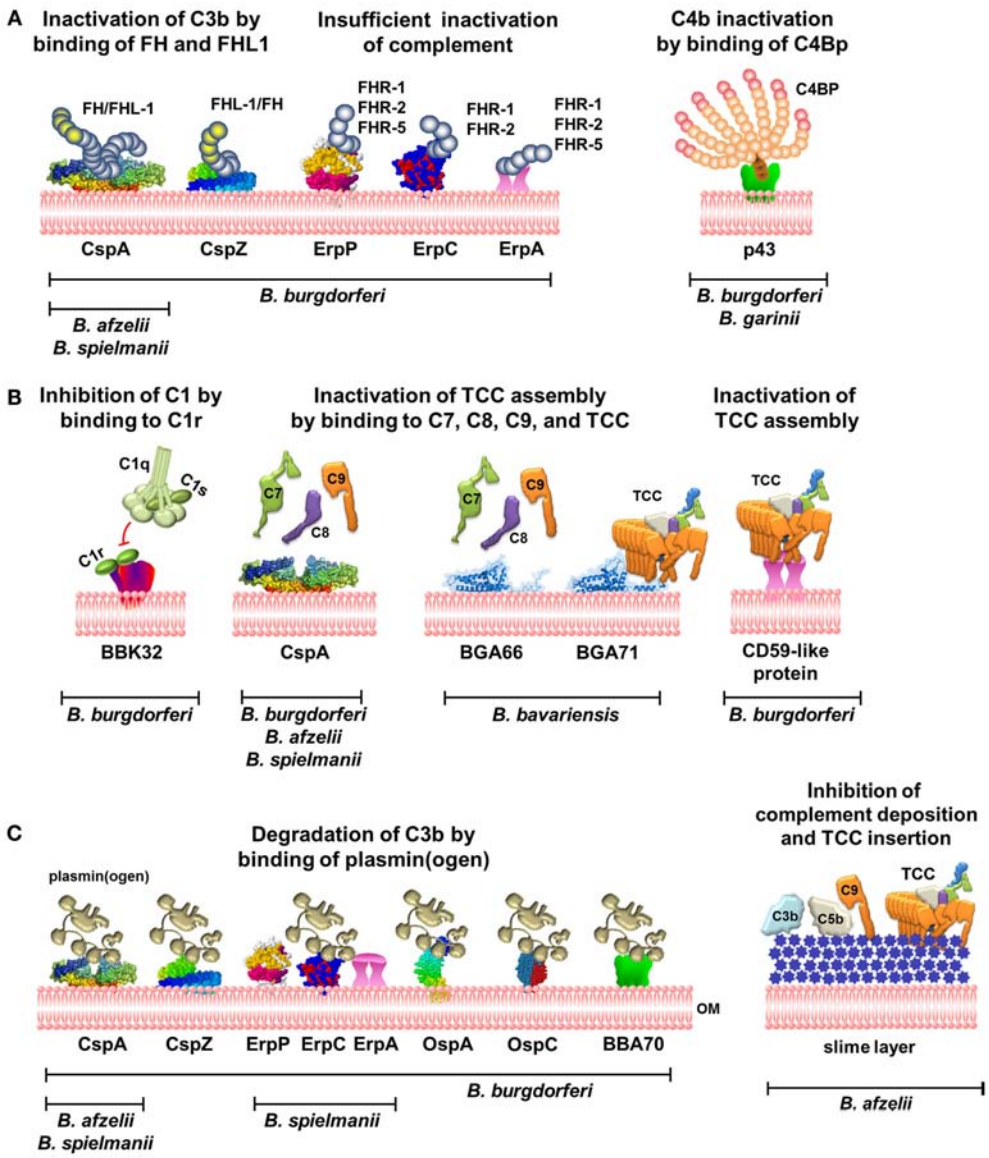

FIGURE 1 | **Complement evasion strategies of LD spirochetes**. **(A)** Inhibition of the AP and CP by binding of complement regulators FH and FHL-1 to CspA and CspZ or C4Bp to p43. Binding of FHRs to ErpP, ErpC, and ErpA does not terminate complement activation. **(B)** Inhibition of the CP and TP by direct interaction of diverse borrelial proteins produced by distinct genospecies with C1r or late complement components. **(C)** Inactivation of C3b by binding of plasmin(ogen) by diverse borrelial proteins and prevention of complement deposition by the production of a mucoid layer. OM, outer membrane; TCC, terminal complement complex; FH, Factor H; C4Bp, C4b-binding protein.

Concerning the FH/FHL-1/FHR interacting molecules, up to five distinct outer surface lipoproteins, collectively termed CRASP, have been identified, comprising three genetically unrelated groups with partially overlapping biological functions (**Table 1**) (14, 15, 41). Owing to the genetic composition, different combinations of these proteins can be exposed at the surface of a particular isolate. For historical reasons, a variety of names have been introduced at the time of description and are still found in the literature, leading to considerable confusion about their identities and biological functions. As a simplification, the synonyms and additional designations of the protein and gene names of CRASPs along with their specific functional properties are summarized in **Table 1**. With regard to the reference type strain *B. burgdorferi* s.s. B31, the identified FH/FHL-1/FHR-binding proteins consists of CspA (CRASP-1, BBA68), CspZ (CRASP-2, BBH06), ErpP (CRASP-3, BBN38), ErpC (CRASP-4), and ErpA (CRASP-5, BBP38) (14). Due to their different functions, structures, gene organization, etc., OspE homologous proteins are collectively referred to as OspE-related proteins (Erp) proteins (46).

CspA is the predominant FH and FHL-1 binding protein of *B. burgdorferi* s.s. and belongs to the paralogous protein family PFam54, of which 11 paralogs are produced in strain B31. Except for CspA, none of the other PFam54 members interact with FH and FHL-1, despite the high sequence homology, suggesting that these proteins possess other, as yet unknown functions (47). Moreover, irrespective of geographical origin, CspA paralogs among *B. burgdorferi* s.s. isolates are highly conserved (48). More importantly, structure refinements have disclosed a homodimer as the biologically relevant architecture of CspA (**Figure 1A**) (49). Although sequence differences within the C-terminal region may account for the inability of CspA paralogs to bind FH and FHL-1, further investigations are necessary to satisfactorily clarify this issue. Initial studies revealed a strong binding affinity of both complement regulators to CspA, accompanied by a powerful capacity to inactivate C3b in the presence of Factor I (50, 51) (**Figure 1A**). The importance of CspA in facilitating complement resistance of *B. burgdorferi* s.s. has been clearly demonstrated by generating a *cspA*-deficient mutant and strains complemented with the *cspA* gene (52–54). More recently, CspA has been demonstrated to possess additional functions: this protein directly interacts with components of the terminal pathway (C7, C8, and C9) as well as plasmin(ogen), thereby terminating TCC assembly and upon activation to plasmin also promoting degradation of C3b (54, 55) (**Figures 1B,C**). CspA orthologs, sharing identical biological functions, were also identified in *B. afzelii* and *B. spielmanii*. All of these orthologs belong to the PFam54 protein family, but the loci of the encoding genes differ from *cspA* of *B. burgdorferi* s.s. The orthologs display the same inactivating properties as CspA, impart resistance to complement-mediated killing, and bind complement components FH, FHL-1, C7, C8, and C9 as well as plasmin(ogen) (43, 55–57). These findings suggest that CspA is an important serum resistance factor of *B. burgdorferi* s.s., *B. afzelii*, and *B. spielmanii*. CspA is produced during tick feeding, shortly after transmission to the mammalian host and during transmission to feeding, naïve ticks but not in the midgut of unfed ticks, suggesting that CspA protect spirochetes from complement attack during established infection (58, 59).

Borrelia burgdorferi s.s. produces an additional FH and FHL-1-binding protein, *CspZ*, which independently provides borrelial cells with resistance to human complement (60, 61) (**Figure 1A**; **Table 1**). Once FH or FHL-1 binds to the CspZ-producing spirochetes, termination of the complement cascade takes place at the

TABLE 1 | Characteristics of complement-interacting proteins of LD spirochetes.

	CspA	CspZ	ErpP[a]	ErpC[a]	ErpA[a]	p43	BBK32	BGA66	BGA71	CD59-like protein
Synonyms and other designations	CRASP-1 BbCRASP-1 BBA68 ZS7.A68 FHBP	CRASP-2 BbCRASP-2 BBH06	CRASP-3 BbCRASP-3 BBN38	CRASP-4 BbCRASP-4	CRASP-5 BbCRASP-5 ErpI ErpN BBP38 BBL39 OspE	–	–	–	–	–
Gene name	*cspA*	*cspZ*	*erpP*	*erpC*	*erpA*	ND	*bbk32*	*bga66*	*bga71*	ND
Origin	Bb, Ba, Bs	Bb	Bb	Bb	Bb	Bb	Bb	Bba	Bba	Bb
Confers serum resistance	Yes	Yes	No	No	No	ND	Yes	Yes	Yes	ND
Interaction with complement regulators/components	FH FHL-1 C7, C8, C9, TCC	FH FHL-1	FHR-1 FHR-2 FHR-5	FHR-1 FHR-2	FHR-1 FHR-2 FHR-5	C4Bp	C1r	C7, C8, C9, TCC	C7, C8, C9, TCC	TCC
Interaction with plasmin(ogen)	Yes	Yes	Yes	Yes	Yes	ND	ND	ND	ND	ND
Affected complement pathways	AP, TP	AP	–	–	–	CP/LP(?)	CP	TP	TP	TP

[a]*Binding of FH has only been confirmed for recombinant proteins.*

ND, not determined; CRASP, complement regulator-acquiring surface protein; Erp, OspE/F-like protein; FH, Factor H; FHL, Factor H-like protein, FHR, FH-related protein; TCC, terminal complement complex; Bb, B. burgdorferi; Bba, B. bavariensis; Ba, B. afzelii; Bs, B. spielmanii; AP, alternative pathway; CP, classical pathway; LP, lectin pathway; TP, terminal pathway.

activation level of C3 as demonstrated by the decay of the C3 convertase, an increase of C3b degradation products, and the lack of deposited TCC (60, 61). Although *cspZ* sequences were identified in numerous genospecies associated with LD, including *B. afzelii*, *B. garinii*, *B. spielmanii*, *B. bavariensis*, and *B. bissettii*, none of the other CspZ proteins interact with FH and FHL-1 (62–65). Although CspZ is produced during mammalian infection and elicits a robust antibody response (66), additional studies revealed that this protein does not protect mice from infections and, if at all, is only partially required for infection (63, 67, 68). In addition, CspZ like CspA has been identified as a plasmin(ogen)-binding molecule, enabling *B. burgdorferi* s.s. to degrade surface-bound C3 and C3b (57) (**Figure 1C**). Of note, the efficacy in degrading C3/C3b by plasmin is much less pronounced, compared to the C3b inactivation capacity of FH and FHL-1 (55). In the case of strong complement activation initiated by the AP, generation of high amounts of C3b cannot be sufficiently inactivated by surface-bound plasmin; thus, more adequate inhibitors such as FH or FHL-1 are required to overcome the feedback loop.

Lyme disease spirochetes produce a number of polymorphic proteins belonging to the OspE/F paralogous protein family PFam162, of which *ErpA (BBP38)*, *ErpC*, and *ErpP (BBN38)* have been reported to bind FH as well as FHR-1, FHR-2, and in part FHR-5 (40, 50, 69–74) (**Figure 1A**; **Table 1**). Despite binding of FH to purified Erp proteins, there are several lines of evidence indicating that the same molecules, when exposed to the bacterial surface, do not confer protection of LD spirochetes from deposition of C3 and TCC (14, 70, 74). Spirochetes producing Erp proteins, but lacking CspA and CspZ, display a susceptible phenotype and are readily killed by complement (52, 70, 74). Owing to the Erp proteins strong affinity for FHRs (50), FH might be displaced from the bacterial surface, with a concurrent loss of its complement regulatory functions and, as such, is unable to protect the cells from the deleterious effects of complement.

Besides binding of complement components, ErpA, ErpC, and ErpP, as well as other Erp orthologs, are known to serve as potential ligands for plasmin(ogen) (**Figure 1C**) (75, 76). As mentioned previously, the role of activated plasmin in complement evasion of LD spirochetes requires further investigations. Although additional FH-binding Erp orthologs were identified in *in vitro* cultivated *B. garinii*, *Borrelia andersonii*, *B. japonica*, *Borrelia turdi*, and *Borrelia tanukii* isolates (44, 72, 77), the impact of these molecules on complement resistance has never been confirmed, in particular Erp proteins of *B. garinii*. Concerning additional FH/FHL-1-binding proteins, no data are currently available on other LD *Borrelia* species.

Inactivation of the Classical Pathway by Binding Complement Regulator C4Bp

The role of C4Bp, the key regulator of the CP, in immune evasion of LD spirochetes is still a matter of controversy. Pietikainen et al. have observed binding of C4Bp in serum-resistant *B. burgdorferi* s.s. and *B. afzelii* as well as in serum-sensitive *B. garinii* isolates (78) (**Figure 1A**). C4Bp bound to the borrelial surface in concert with Factor I maintained its complement regulatory activity and inactivated C4b (78). Along with the determination of C4Bp binding, a 43-kDa protein, tentatively designated *p43* was identified in *B. burgdorferi* strains B31 and N40, and *B. garinii* strains g46 and g50 (78). However, other studies failed to show C4Bp binding by *B. burgdorferi* s.s. LW2, *B. garinii* G1, *B. valaisiana* ($n = 3$), and *B. bavariensis* strains ($n = 8$) (36, 45), possibly due to different techniques or antibodies used for the detection of C4Bp. In addition, taking into consideration that *B. garinii* cells are killed in serum concentrations >20%, the physiological relevance of C4Bp in promoting complement resistance remains to be determined.

BORRELIAL PROTEINS DISPLAYING COMPLEMENT-INHIBITORY ACTIVITY

More recently, a novel immune evasion mechanism has been described by which *B. burgdorferi* s.s. specifically blocks CP activation (79). This study depicts *BBK32* as the first protein that binds to C1r in a non-covalent manner and thereby preventing autocatalysis of this proenzyme and subsequently the cleavage of C1s, leaving the C1q complex in an inactive enzymatic state (**Figure 1B**; **Table 1**). By interfering with C1r, BBK32 acts as a potent inhibitor of the CP without affecting the LP and AP.

Unlike other serum-resistant LD spirochetes, *B. bavariensis* binds neither FH/FHL-1 nor other complement regulators such as C4Bp or C1-Inhibitor (45). Detailed analysis revealed two proteins, *BGA66* and *BGA71* as novel complement inhibitors. They belong to the PFam54 protein family and share 51 and 41% sequence identities, respectively, to CspA. Both proteins interact with components of the terminal pathway, in particular C7, C8, and C9, and also with the assembled TCC (45) (**Figure 1B**; **Table 1**). Binding of the borrelial proteins to various components of the terminal pathway affects TCC formation by (i) inhibiting C9 auto-polymerization, (ii) terminating TCC assembly, and (iii) preventing integration of the functional pore-forming complex. Moreover, BGA66 and BGA71 are simultaneously produced in all *B. bavariensis* strains investigated, but each protein by itself displays anti-complement activity and renders transformed spirochetes resistant to complement-mediated killing. Despite the structural similarities to CspA, BGA66 and BGA71 do not bind the potent complement regulators FH and FHL-1 (45). One might speculate that termination of the final activation steps may result in a somewhat weaker complement inactivation capacity. However, this does not appear to be the case as CspA and CspZ-producing and FH/FHL-1-binding spirochetes did not show a different resistance phenotype compared to BGA66- or BGA71-producing cells, indicating that inhibition of the terminal pathway is as efficient as blocking complement activation at the level of C3.

Borrelia burgdorferi s.s. also produces a CD59-like protein, which preferentially bind to C9 and to some extent to the β-subunit of C8 (80) (**Figure 1B**; **Table 1**). By using anti-CD59 antibodies, the functional activity of this protein could be blocked, rendering the spirochetes susceptible to complement-mediated killing. Although this surface-exposed protein has never been identified, the binding properties of the CD59-like protein suggest a role in inactivating the terminal pathway of complement. Despite the overlapping complement-inhibitory activities, the CD59-like protein is not identical with BGA66 and BGA71.

FURTHER PROTEINS AND STRUCTURES INVOLVED IN COMPLEMENT RESISTANCE OF LD SPIROCHETES

Besides the already mentioned molecules, which interacting with complement in multiple ways, additional proteins have been described that also bind human plasmin(ogen), e.g., OspA, OspC, and BBA70 (81–83) (**Figure 1C**). For the latter, degradation of C3 and, in part, C5 has been demonstrated. Whether the interactions of OspC and BBA70, known to be expressed in the mammalian host, might support complement inactivation *in vivo* is not known.

Initial studies of the molecular principles of complement resistance, focusing on the amounts of deposited complement components, revealed an amorphous structure of high density that surrounds the entire cell envelope and, apparently, acts as a physical barrier, preventing the insertion of the formed TCC into the bacterial membrane of serum-resistant cells (**Figure 1C**) (32). Currently, no data are available on the composition and content of this so-called "slime layer" and whether this structure is present in LD spirochetes other than *B. afzelii* (32). Thus, further studies are required to verify the precise nature of this morphological substance.

FUTURE DIRECTIONS

Over the last decades, numerous molecules have been identified that interact with the innate immune system in multiple ways to influence or terminate complement at distinct activation levels, e.g., initiation of the CP, C3 activation by the AP, and formation of the TCC. Knowledge of the proteins involved in the interaction with complement has allowed for a better understanding of molecular principles of complement evasion developed by LD spirochetes. Future investigations will undoubtedly identify additional complement-interacting molecules required for evading the innate immune response of different animals, including reservoir hosts, and provide insight into whether these proteins might function in a host-specific manner during the infection process.

AUTHOR CONTRIBUTIONS

PK prepared the figure and table and wrote the manuscript.

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REVIEW
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Multifunctional and Redundant Roles of *Borrelia burgdorferi* Outer Surface Proteins in Tissue Adhesion, Colonization, and Complement Evasion

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Borrelia burgdorferi is the causative agent of Lyme disease in the U.S., with at least 25,000 cases reported to the CDC each year. *B. burgdorferi* is thought to enter and exit the bloodstream to achieve rapid dissemination to distal tissue sites during infection. Travel through the bloodstream requires evasion of immune surveillance and pathogen clearance in the host, a process at which *B. burgdorferi* is adept. *B. burgdorferi* encodes greater than 19 adhesive outer surface proteins many of which have been found to bind to host cells or components of the extracellular matrix. Several others bind to host complement regulatory factors, *in vitro*. Production of many of these adhesive proteins is tightly regulated by environmental cues, and some have been shown to aid in vascular interactions and tissue colonization, as well as survival in the blood, *in vivo*. Recent work has described multifaceted and redundant roles of *B. burgdorferi* outer surface proteins in complement component interactions and tissue targeted adhesion and colonization, distinct from their previously identified *in vitro* binding capabilities. Recent insights into the multifunctional roles of previously well-characterized outer surface proteins such as BBK32, DbpA, CspA, and OspC have changed the way we think about the surface proteome of these organisms during the tick–mammal life cycle. With the combination of new and old *in vivo* models and *in vitro* techniques, the field has identified distinct ligand binding domains on BBK32 and DbpA that afford tissue colonization or blood survival to *B. burgdorferi*. In this review, we describe the multifunctional and redundant roles of many adhesive outer surface proteins of *B. burgdorferi* in tissue adhesion, colonization, and bloodstream survival that, together, promote the survival of *Borrelia* spp. throughout maintenance in their multi-host lifestyle.

Keywords: Lyme disease, adhesion, *Borrelia*, colonization, complement, pathogenicity, virulence

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INTRODUCTION

Borrelia burgdorferi, a diderm motile spirochete bacterium, is the causative agent of Lyme disease in the U.S. Each year greater than 25,000 confirmed cases of Lyme disease are reported to the United States Centers for Disease Control and Prevention with about 96% of those cases reported from only 14 states in the Midwest and East (1). Lyme disease is also a significant health problem in parts of

Europe and Asia where it is more commonly caused by *Borrelia afzelii* and *Borrelia garinii* than *B. burgdorferi*.

Borrelia spp. are maintained in nature in a tick–mammal life cycle. *B. burgdorferi* is carried by several species of the *Ixodes* genus of tick and is transmitted to mammals through tick saliva (2). The spirochetes are maintained in the tick midgut as the tick progresses through its life stages, but the bacteria are not passed transovarially to its offspring (2). The primary mammalian reservoir for *B. burgdorferi* is the white-footed mouse, *Peromyscus leucopus* (2). This reservoir is not known to be physically affected by the infection (3). Additional small animals and birds can also serve as reservoirs, whereas large animals and humans can be accidental hosts for tick feeding and subsequent infection.

Human infection with *Borrelia* spp. often results in a number of generic symptoms including headache, fatigue, and general malaise, and for this reason, the infection is often misdiagnosed or goes untreated. A large percentage of individuals infected with *B. burgdorferi* will display a rash, termed erythema migrans, at the site of a tick bite (4). An untreated infection with *B. burgdorferi* can result in late stage symptoms including arthritis, carditis, and neurologic issues (5–7). The CDC reported from 2001 to 2010 that 31% of confirmed Lyme disease cases presented with Lyme arthritis, 14% with neurologic symptoms, and 1% with cardiac involvement (1). The results of a late stage *Borrelia* infection vary depending on the infecting species, with *B. garinii* most often associated with neurologic symptoms and *B. afzelii* infection commonly associated with a skin rash called acrodermatitis chronica atrophicans (8–10).

OUTER SURFACE PROTEINS OF *Borrelia burgdorferi*

Borrelia spp. are able to exist in the tick–mammal life cycle due to their ability to adapt to the environment in which they reside. In *in vitro* studies, *Borrelia* spp. are able to respond to changes in pH and temperature of the environment, as well as cell density of spirochetes, to differentially regulate the production of many of their outer surface proteins (**Figure 1**) (11–14).

One way in which *B. burgdorferi* is able to respond to changes in these environmental conditions is through the RpoN–RpoS signaling system (14, 16). RpoS, RpoN, Rrp2, and BosR are considered the master regulators of virulence gene expression in *B. burgdorferi* (17–23). RpoS and Rrp2 have been shown to be required for mouse infectivity (18, 24). One example of such control is the reciprocal expression of outer surface protein A (*ospA*) and outer surface protein C (*ospC*) tightly regulated by RpoN, RpoS, and Rrp2 (19–21, 25, 26).

Borrelia burgdorferi produces OspA on its surface while in the unfed tick (11). Upon the uptake of blood into the midgut of the tick, *ospA* expression is maintained until transmission into the mammal when expression is decreased and *ospC* expression is increased in conjunction with many other genes that encode outer surface proteins, to aid in survival within the mammal (15, 27, 28). Interestingly, OspC production is not necessary and is, in fact, detrimental to survival of the bacteria, likely due to the high immunogenicity of the OspC protein (29). In addition to regulation of surface protein production by RpoN, RpoS, Rrp2, or BosR, *B. burgdorferi* also utilizes other mechanisms to rapidly change the epitopes available on the surface inside the mammalian host, but not within the tick (30). For example, *B. burgdorferi* encodes a variable membrane protein-like sequence (Vls) antigenic variation system that enables evasion of recognition by the host-adaptive immune system by continual recombination of silent *vls* gene segments encoding different *vlsE* sequences into the expression site (31–34).

Outer Surface Proteins and Virulence

For years, a focus of the *Borrelia* field, as with any pathogen field, has been to identify bacterial proteins that could contribute to virulence. Due to the cumbersome nature of *Borrelia* genetics, the roles of very few proteins have been described in mammalian infection. Using traditional cloning methods, glycosaminoglycan (GAG) and fibronectin (Fn)-binding protein, BBK32, and GAG and decorin-binding proteins, DbpA and DbpB, were all identified as being important for the establishment or persistence of

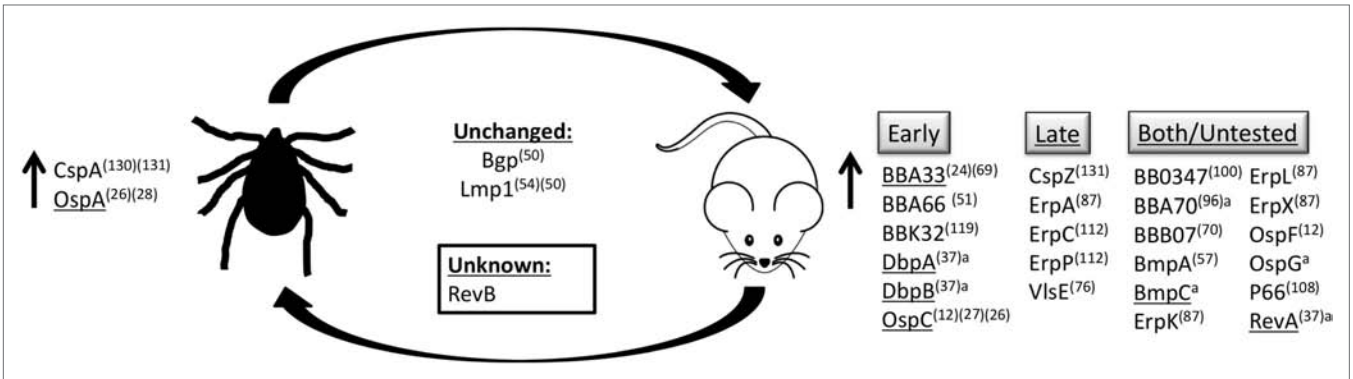

FIGURE 1 | Outer surface protein regulation. *B. burgdorferi* senses changes in temperature, pH, and cell density, as well as unknown stimuli to modulate production of proteins on the bacterial surface. Proteins listed are upregulated in their respective environments, in the tick vector or the mammalian host, or are produced at similar levels in both environments. Proteins produced during mammalian infection are grouped based on temporal expression pattern, expressed early during infection (early), during persistent infection (late), or those that have been detected both early and late during infection or have not been experimentally determined (both/untested). [a]Based on *in vivo* qRT-PCR and microarray data (15). [(#)]Reference. Underline indicates genes regulated by the RpoS regulon.

mammalian infection (35–44). Additionally, the Fn-binding protein, RevA, was also found to have an effect on bacterial virulence (45, 46), though the affinity of the interaction between RevA and Fn was found to be less than that of BBK32 and Fn (41). Likewise, deletion of the *ospC* gene from *B. burgdorferi* strain B31 also has a negative impact on the establishment of infection in mice (29, 38, 47). This may be due to the antiphagocytic properties of OspC on *Borrelia*, though the importance of this activity has not yet been elucidated *in vivo* (48). Recent headway has been made in identifying *Borrelia* genes involved in mammalian infection by the generation and utilization of a transposon library in *B. burgdorferi* (49). By inoculating mice with the signature-tagged transposon mutagenesis (STM) library, the list of virulence determinants of *B. burgdorferi* was expanded to include additional outer surface proteins, many with undescribed function including BBB07, Bgp, BmpC, ErpA, RevB, and VlsE (**Table 1**) (49, 50). Through the use of traditional cloning, it was shown that the lipoprotein, BBA66, is also required for mammalian infection (51, 52).

Ligand Binding Mediated by *Borrelia burgdorferi* Outer Surface Proteins

Borrelia burgdorferi is known to produce at least 19 adhesive proteins on its surface (**Table 1**) (35). Previous work has focused on describing the binding capability of three *B. burgdorferi* outer surface proteins to the ECM component, Fn, which is expressed by a variety of cells types and has been shown to be important in neural and vascular development (99): RevA, BBK32, and BB0347. RevA is a 17 kDa outer surface lipoprotein of *B. burgdorferi* produced within the mammalian environment, which has been shown to bind to Fn *in vitro*, though reports on the affinity of this interaction are conflicting (41, 45). Recently, it was found that production of RevA is required for the colonization of heart tissue

TABLE 1 | Adhesive outer surface proteins of *B. burgdorferi*.

Adhesin	Genetic locus[a]	*In vitro* binding	Reference	*In vivo* function	Reference
Adhesins with a role in mammalian infection					
Lmp1	*bb0210*	Chondroitin-6-sulfate	(53)	Not determined	(53–55)
BmpA	*bb0383*	Laminin	(56)	Joint persistence	(57)
BmpC	*bb0384*	Not determined		Not determined	(49)
Bgp	*bb0588*	Heparin, dermatan sulfate, GAGs, and aggrecan	(58–60)	Not determined	(49, 58)
P66	*bb0603*	Integrins αIIbβ3 and αvβ3	(61)	Heart and skin adhesion, dissemination, and vascular transmigration (integrin-binding domain)	(62–64)
DbpA	*bba24*	Decorin, GAGs	(65–68)	Joint colonization	(29, 35–44, 47, 49)
DbpB	*bba25*	Decorin, GAGs	(65–68)	Joint colonization	(36, 43, 44, 49)
BBA33	*bba33*	Collagen	(69)	Not determined	(69)
BBB07	*bbb07*	Integrin α3β1	(70)	Not determined	(49)
OspC	*bbb19*	Plasminogen, Salp15 (in tick saliva)	(71, 72)	Bloodstream survival	(29, 38, 47, 62)
RevB	*bbc10*	Fibronectin	(41, 45)	Not determined	(49)
VlsE	*bbf32*	Not determined		Not determined	(49, 73–76)
BBK32	*bbk32*	Fibronectin, GAGs, and complement component C1r	(77–80)	Vascular adhesion and joint colonization (GAG-binding domain)	(35, 39–42, 49, 62)
RevA	*bbm27*, *bbp27*	Fibronectin, laminin	(41, 45)	Heart colonization	(46, 49, 62)
ErpA (CRASP-5)	*bbp38*	Factor H, Factor H-related proteins 1, 2, and 5, and plasminogen	(81–86)	Not determined	(49, 87)
Adhesins with no role in mammalian infection					
OspA	*bba15*	TROSPA (in tick)	(88)	N/A	(89)
CspZ (CRASP-2)	*bbh06*	Factor H, Factor H-like 1	(82, 90)	N/A	(91)
Adhesins with an undetermined role in mammalian infection					
CspA (CRASP-1)	*bba68*	Factor H, Factor H-like 1, and complement components C7 and C9	(86, 92–95)	Not determined	
BBA70	*bba70*	Plasminogen	(96)	Not determined	
ErpC (CRASP-4)	*bbl39*	Factor H, Factor H-related protein 1, and plasminogen	(82, 83, 85)	Not determined	
ErpK	*bbm38*	Heparan sulfate	(97)	Not determined	
ErpP (CRASP-3)	*bbn38*	Factor H, Factor H-related proteins 1, 2, and 5, and plasminogen	(81–85, 98)	Not determined	
ErpL	*bbo39*	Heparan sulfate	(97)	Not determined	
ErpX	*bbq47*	Laminin	(85)	Not determined	
OspF	*bbr42*	Heparan sulfate	(97)	Not determined	
ErpG (OspG)	*bbs41*	Heparan sulfate, heparin	(97)	Not determined	

N/A, not applicable.

[a]*All gene designations are based on B. burgdorferi strain B31.*

1 month p.i. (46). In addition, *B. burgdorferi* produces BB0347 during mammalian infection (100), which has also been shown to bind to Fn with low affinity by surface plasmon resonance (41). RevA and BB0347 were both found to have a minimal role in vascular binding in mouse flank skin *in vivo* 1-h post infection (h.p.i.) (41). Much work has been done to decipher the role of the surface protein BBK32, produced during mammalian infection, and its high affinity interactions with Fn and GAGs, which are evenly distributed throughout the ECM of all mammalian tissues (41, 77, 101, 102). Tissue distribution and function of GAGs was expertly reviewed by Jinno and Park (86). Early work with BBK32 identified distinct regions of the protein required for binding to GAGs (residues 45–68) and Fn (residues 158–182) (41, 103). In contrast to other Fn-binding adhesins on *B. burgdorferi*, the region of Fn that interacts with BBK32 has been precisely defined (41). Interactions of BBK32 on the surface of *B. burgdorferi* with GAGs and Fn are important for tethering and dragging interactions with endothelial cells of the vasculature *in vivo*, respectively (33, 39, 98). This was demonstrated by a restoration of vascular adhesion to non-adhesive mutant *B. burgdorferi* upon BBK32 production as determined by intravital microscopy in mouse flank skin (35, 41, 104). Another component of the ECM, collagen, which is a structural component of bone, tendon, and ligaments [as reviewed in Ref. (99)] also acts as a ligand for *B. burgdorferi*. BBA33, was shown to bind to collagen type VI *in vitro*, and is required for mammalian infection (69). Additionally, the basement membrane glycoprotein, laminin, found in the epithelium [as reviewed in Ref. (99)], has been shown to be a ligand for *B. burgdorferi* adhesins BmpA, ErpX, and RevA (41, 56, 105). Studies using BmpA-deficient *B. burgdorferi* showed a role for this protein in bacterial persistence in the joints of mice (57).

Adhesive surface proteins of *B. burgdorferi* have also been identified that bind to integrins, integral membrane proteins found on the surface of all nucleated mammalian cells that function to bind to several ECM components [as reviewed in Ref. (106)]. One such protein is the 66 kDa putative porin, P66, which has been shown to be important for the establishment of mammalian infection (63, 107, 108). P66 was shown to bind to β3-chain integrins and is involved in bacterial dissemination from the site of inoculation in the skin (64, 109). *B. burgdorferi* encodes another integrin-binding protein, BBB07, which is produced during mammalian infection as evidenced by the presence of a specific antibody response in the serum of infected individuals (70). This protein has been shown to have a role in signaling through integrin α3β1 to induce the production of pro-inflammatory cytokines *in vitro*, though this activity has not yet been described *in vivo* (70).

Additional outer surface proteins (Osp) have been described on *B. burgdorferi* that are involved in binding to other host proteins, including the 22 kDa outer surface protein, OspC. OspC production is induced upon bacterial entry into mammalian tissue (**Figure 1**) (19) and has been shown to bind plasminogen *in vitro* (71). Plasminogen is a mammalian protein important for the degradation of the ECM to facilitate cellular migration. OspE protein family members, ErpA, ErpC, and ErpP, were all found to bind to plasminogen *in vitro*, as is seen with a number of other *Borrelia* proteins (85, 96, 110–112). However, the presence or role of plasminogen binding by these proteins *in vivo* has not yet been described. A function for plasminogen binding has been recently described for the adhesive *B. burgdorferi* protein, BBA70. Through *in vitro* experiments, it was shown that BBA70 binds to plasminogen, which cleaves and inactivates complement component C5, ultimately inhibiting membrane attack complex formation (96).

Along with producing proteins that bind to Fn, integrins, and plasminogen, *Borrelia* spp. also produce proteins, which have been shown to bind to a variety of GAGs. Just as was seen with BBK32, outer surface proteins DbpA and B, produced during mammalian infection (37), have been shown to bind to decorin, heparin, dermatan sulfate, and heparan sulfate *in vitro* (66–68, 113–115). OspF-related family members, ErpG, ErpK, and ErpL, were all found to bind to heparan sulfate, in addition to plasminogen, with varying affinities as determined by a series of *in vitro* assays (97). To add a layer of complexity, DbpA from different *Borrelia* species was found to bind to dermatan sulfate with differing affinities, which may contribute to the differences seen in clinical manifestations of disease caused by *B. garinii*, *B. burgdorferi*, and *B. afzelii* (114).

TISSUE COLONIZATION BY *Borrelia burgdorferi*

In order to colonize tissue sites distal to the site of tick bite in the mammal, it is thought that *Borrelia* travel within the vascular system of the host. Specific tissue colonization by the spirochetes is thought to occur by targeted exit from the vasculature dictated by the binding specificity of the outer surface proteins of *Borrelia* spp. This hypothesis is plausible, as characteristics of vascular beds differ depending on the tissue they are associated with as well as the size and type of vessel (116, 117). Tissue bed-specific endothelial cell surface receptors have been documented including VCAM1 in liver, CD36 in lung and heart, L-selectin (CD62L/SELL) in the spleen, and CD133 in the skin, brain, eye, and testicular microvasculature (116). Vascular beds can also vary in their production of proteoglycans, GAGs, and ECM components [as reviewed in Ref. (118)]. The ability of *B. burgdorferi* to bind to GAGs and ECM components has been specifically associated with effects on bacterial virulence and tissue colonization for *Borrelia* outer surface proteins such as DbpA and BBK32 (36, 39, 42–44, 51, 71, 77, 113, 119).

It is possible that *B. burgdorferi* are able to interact with differentially available endothelial cell surface proteins and utilize them to preferentially bind different tissues. Endothelial cell binding by *B. burgdorferi* has been observed to occur *in vitro* through interaction with cell surface proteoglycans such as dermatan sulfate, fibronectin, and heparin (120, 121). The binding of *B. burgdorferi* to the vasculature has been observed, *in vivo*, to occur in three stages (35, 104). Using intravital microscopy techniques on mouse flank skin, Norman et al. observed both tethering and dragging interactions to occur during the first stage of *B. burgdorferi* interactions with the vasculature (35). Using a flow chamber, tethering and dragging interactions with the endothelium were confirmed to occur both at the cell surface and at endothelial cell junctions (121). Stationary interactions, which occur after tethering and dragging, may be controlled by

B. burgdorferi integrin-binding proteins such as P66 and BBB07. After the spirochete is tightly associated with the endothelial cell junction, it is able to transmigrate into the tissue where it is presumed to colonize and replicate.

By inoculating mice with a library of filamentous phage-expressing *B. burgdorferi* N40 D10/E9 gene fragments, a number of outer surface proteins of *B. burgdorferi* that have a tropism for particular mouse tissues were identified (122). Several of the identified proteins in these experiments have been shown to bind to a small number of host proteins, as described in **Table 1**. This redundancy in protein function suggests a high importance for tissue tropic binding by the bacteria during a mammalian infection. The tissue targeting effects of the different outer surface proteins of *Borrelia* may be due to the presence of nutrients required for survival of the bacteria at those particular sites. The cumulative data suggest an association between *in vitro* ligand affinity and the ability to bind to particular tissues *in vivo*. For example, the GAG-binding proteins BBK32 and DbpA from *B. burgdorferi* have been shown to be tropic for joint tissue in mouse models of *Borrelia* infection (40, 114). Detailed work with BBK32 has shown the joint targeting effects of the protein to be mediated specifically by the GAG-binding domain (40). This association between ligand affinity and tissue targeting does not hold true with Fn-binding adhesins. Fn-binding proteins, BB0347 and BBK32, have been shown to preferentially bind joint tissue (40, 62), whereas the Fn-binding protein, RevA, was found to have a tropism for heart tissue *in vivo* (62). These data highlight the inherent difficulties in using *in vitro* binding data between two proteins to infer *in vivo* function of a protein. It is becoming increasingly clear that the outer surface proteome of *Borrelia* spp. during mammalian infection functions collaboratively to direct the bacteria to specific tissue sites. Further experiments using *in vivo* models will need to be performed to determine functions for each outer surface protein of Lyme disease *Borrelia*.

Interestingly, tissue tropism of *Borrelia* spp. appears not only to be determined by different bacterial proteins but also by allelic differences in a gene encoding a given protein taken from different *Borrelia* species. For example, *dbpA* gene sequences are variable between strains as well as species of *Borrelia*, and this variation not only affects ligand binding, as mentioned earlier, but also tissue tropism (114). It was shown that DbpA from *B. burgdorferi* strains B31-A3 and N40 D10/E9 is tropic for joint tissue adhesion and colonization, while DbpA from *B. afzelii* and *B. garinii* is not (114). DbpA from *B. afzelii* strain VS461 showed a tropism for skin, while DbpA from *B. garinii* strain PBr was uniquely tropic for heart tissue colonization among the *dbpA* alleles tested (75). These differences in tissue tropism of allelic gene variants from different strains and species of *Borrelia* could explain the differences observed in the symptoms of a late stage infection with *Borrelia* spp. DbpA may contribute to these symptoms as *B. burgdorferi* infection often results in arthritis symptoms, while late stage *B. afzelii* infection commonly presents with skin lesions.

COMPLEMENT COMPONENT BINDING

Rapid dissemination of *B. burgdorferi* during an infection is thought to be facilitated by the spirochetes traveling in the bloodstream of the mammalian host. During this dissemination process, the bacteria are exposed to the innate immune system of the host, designed to detect and clear invading pathogens. *B. burgdorferi* is also exposed to these blood products inside the midgut of a feeding tick during a blood meal. Resistance to killing by innate immune mechanisms in the blood and host tissues is, therefore, essential for maintenance of the enzoonotic lifestyle of *B. burgdorferi*.

One of the major innate immune components in the host blood and tissues is the complement cascade. The complement cascade is a series of proteolytic cleavage events in which inactive precursors are converted into active enzymes in the host serum and tissues. These proteolytic cleavage events are activated by three distinct pathogen-recognition mechanisms. The first is termed the "classical" complement cascade, mediated by antibodies that recognize the surface of the pathogen and recruit complement component C1q molecules, which then activate the cascade of enzymatic cleavage events. Mannose-binding lectin molecules present in the serum can bind to sugar moieties on the surface of an invading pathogen, activating the complement cascade by the "lectin" pathway. A third pathway known as the "alternative" pathway activates the complement cascade by random deposition of complement component C3 molecules onto the surface of the pathogen, activating the cascade. All three of the complement pathways converge on the activation of complement component C3, ultimately resulting in formation of the membrane attack complex pore in the pathogen membrane, and pathogen lysis.

The host has adapted many different regulatory mechanisms to control the activation of this pathway to minimize harm to host tissues. One such mechanism is the C1 inhibitor protein (C1-INH), which acts on the C1s and C1r molecules of the first step of the classical pathway (123). Additionally, the host produces C4-binding protein (C4BP), which inhibits the classical and lectin pathways by binding to C4b and inducing its proteolytic cleavage and subsequent inactivation [reviewed in Ref. (124)]. The host also has a number of different mechanisms that it employs for regulating the alternative complement cascade. The host produces Factor H, Factor H-like protein 1 (FHL-1), and complement Factor H-related (CFHR) proteins, which all inhibit the alternative complement cascade by acting on C3b. Factor H can act to sterically hinder the interactions of C3b with Factor B, compete with Factor Bb for binding to C3b, and induce its proteolytic degradation and inactivation into iC3b [reviewed in Ref. (125)]. Regulation of the complement cascade can occur at the end stages of the cascade by employing factors such as clusterin (ApoJ), vitronectin, and CD59. Vitronectin is known to inhibit the binding of complement factor complex C5–C7 to the pathogen membrane, as well as inhibit C9 oligomerization (126). CD59 functions in a similar fashion to vitronectin, intercalating into the membrane attack complex, and inhibiting its polymerization. Clusterin is a complement regulatory glycoprotein associated with apolipoprotein AI, a protein component of high-density lipoprotein (HDL) cholesterol molecules (125). Clusterin inhibits insertion of the membrane attack complex into the pathogen membrane by forming attack complex aggregates away from the pathogen surface (125).

Borrelia spp. produce several proteins on their surfaces, which are proposed to allow them to evade clearing by the complement cascade. Many of the proteins act by recruiting complement regulatory factors to the surface of *B. burgdorferi*, such as the surface proteins CspA (86, 92–95), CspZ (82, 90, 91), ErpP (94), ErpA (81, 98), and ErpC (127–129) as determined *in vitro*. Full length CspA and ErpP were found to be required for binding to purified human Factor H and FHL-1 *in vitro* using a series of C-terminal truncation proteins in a solid phase binding assay (92). The binding of Factor H and FHL-1 by CspA on the surface of *B. burgdorferi* was confirmed by far western blot and immunofluorescence assays (95) and contributes to cleavage and inactivation of complement component C3b (93). CspA has also been shown, *in vitro*, to interact with complement components C7 and C9, at a distinct location from the site of Factor H binding on CspA (92, 93, 95). Binding of CspA to C7 and C9 inhibits assembly of the membrane attack complex at the spirochete surface when incubated in active human serum as determined by immunofluorescence microscopy, contributing to bacterial resistance to lysis by human serum proteins *in vitro* (92, 93, 95). Interestingly, *cspA* was found to be expressed only in an unfed tick and not during mammalian infection, suggesting a necessary role for complement resistance of *Borrelia* within the tick (130, 131). Similar to CspA, another outer membrane protein of *B. burgdorferi*, CD59-like protein, also binds complement component C9 as well as C8β and inhibits the insertion of the membrane attack complex *in vitro* (132).

Borrelia burgdorferi also produces several OspE-related protein family members including ErpA, ErpP, and ErpC, which have been shown to recruit Factor H and CFHR proteins to the bacterial surface *in vitro* (81–83). By solid phase-binding assay and immunoblot, ErpA, ErpP, and ErpC were found to bind to CFHR-1, unlike CspA and CspZ, which showed no binding to CFHR-1 in active human serum *in vitro* (82). Additionally, recombinant ErpP and ErpA were found to bind full length recombinant Factor H with a low dissociation constant, suggesting a potential role for this interaction *in vivo*, though this has not yet been established (81, 83).

CspZ, a gene encoding another Factor H and FHL-1-binding protein on the surface of *B. burgdorferi*, has a reciprocal expression pattern to *cspA* (90). *CspZ* was found to be expressed primarily during mammalian infection but was not found to be required for establishment of mammalian infection (91). Consistent with this result, CspA and CspZ on the surface of *B. burgdorferi* were found to interact with human Factor H and FHL-1 *in vitro*, although production of CspZ was not found to be required for survival in active human serum *in vitro* (82, 91).

Recently, BBK32 was found to interact with a component of the classical complement cascade *in vitro* (80). BBK32 was shown to bind C1r, a member of the first protein complex in the classical complement cascade, whereby inhibiting its zymogen activity and effectively blocking attack complex formation *in vitro* (80). A C-terminal portion of BBK32 (residues 206–354) outside of the Fn- and GAG-binding domains, was found to be necessary for the interaction with C1 *in vitro* (80). We would predict based on the *in vitro* binding capabilities of BBK32 to C1, that BBK32 cooperates *in vivo* with additional *Borrelia* surface proteins to ensure successful inhibition of multiple branches of the complement cascade.

Experiments have been performed to discern the role of complement in the clearance of *Borrelia* during mammalian infection using C5-deficient mice (133). Complement component C5 is a point of convergence of all three pathways of the complement cascade. The activation of C5 ultimately results in the formation of the membrane attack complex pore in the pathogen membrane. *B. burgdorferi* recovery by culture from tissues of several infected strains of mice naturally deficient in C5, including A/J, AKR/J, B10.D2/oSnJ, DBA/2J, and SWR/J, was not found to be different than recovery from infected C5 sufficient C3H/HeJ mouse tissues at 2, 4, and 12 weeks p.i. (133). The authors concluded that complement activity is not required for clearance of *B. burgdorferi* during a mouse infection. Similar results were seen in experimentally infected C3-deficient mice where complement sensitive *B. garinii* was not recovered from complement-deficient mice (134). Additionally, it was shown that recruitment of Factor H to the surface of *B. burgdorferi in vivo* is not necessary for serum resistance of the bacteria as evidenced by similarities in bacterial burdens of WT and Factor H-deficient mice (135). Given what we now know about the ability of *B. burgdorferi* to interact with several host complement regulatory factors upstream of C3 and C5 *in vitro*, one would not predict to see a difference in WT bacteria survival in these experiments.

The evasion of the alternative complement cascade is not unique to *B. burgdorferi* and has been observed in *Borrelia hermsii*, a causative agent of relapsing fever (136, 137), *Borrelia bavariensis* (138), as well as other bacterial pathogens such as *Streptococcus pyogenes* (139), *Bordetella pertussis* (140, 141), *Neisseria gonorrhoeae* (142–145), *Escherichia coli* (146–148), *Leptospira interrogans* (149), *Yersinia pseudotuberculosis* (150, 151), *Salmonella enterica* serovar Typhimurium (152), and *Moraxella catarrhalis* (153). Many of these organisms obtain resistance to host complement by coating themselves in C4-binding protein (C4BP) and Factor H, similar to *B. burgdorferi* [reviewed in Ref. (124)].

SUMMARY

Borrelia spp. are highly adept at preventing clearance by the innate immune system of the host while adhering to and colonizing host tissues. The success of this mammalian pathogen can, so far, be attributed to the presences of a few bacterial proteins produced on the pathogen surface though there are many more adhesins whose *in vivo* functions have yet to be determined. For many years, researchers in the *Borrelia* field have identified and studied outer surface proteins of *Borrelia* spp. that may contribute to the efficiency of *Borrelia* as a pathogen. The host proteins that are specifically bound by different outer surface proteins are beginning to be revealed. *Borrelia* outer surface proteins BBK32, DbpA, RevA, CspA and Erps A, C, and P, have all been shown to bind to multiple macromolecules from the host cell surface and ECM. In addition to binding to multiple host cell surface proteins, *Borrelia* proteins BBK32, CspA, and Erps A, C, and P have all been found to bind to host complement components. The multifunctional and redundant capabilities of many of the proteins on the surface of *Borrelia* provide the bacteria with a

higher probability of successfully infecting the arthropod and mammalian hosts. As research of *Borrelia* outer surface proteins progresses, it becomes increasingly clear that redundancy in ECM and host protein-binding specificity acts to guarantee that the bacteria will successfully colonize host tissues while evading detection by the host immune system.

As suggested in this review, there is a strong correlation between ligand specificity of different bacterial outer surface proteins and the tissue tropism of those proteins. When examining the literature it becomes apparent that the binding affinity for host macromolecules such as GAGs by BBK32 and DbpA may be the necessary interactions required for joint tropism and colonization (40, 114). This is further evidenced by the documented species to species differences in GAG-binding capacity and joint colonization seen with the production of DbpA from *B. afzelli* and *B. garinii*, *Borrelia* species, which do not commonly cause arthritis in humans (114).

As discussed in this review, complement protein resistance is achieved by *Borrelia* spp. through the action of a few outer surface proteins with multiple levels of redundancy. One aspect of redundancy is the ability of multiple *Borrelia* proteins to bind to and recruit the same complement inhibitory protein. This functional redundancy ensures the successful inhibition of the complement cascade at different stages of the pathway by multiple *Borrelia* proteins. Redundancy in protein function is a common theme for both the tissue binding and complement protein recruitment aspects of *Borrelia* pathogenesis, and likely contribute largely to the pathogenic success of this bacterial genus.

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T Cells Exacerbate Lyme Borreliosis in TLR2-Deficient Mice

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Infection of humans with the spirochete, *Borrelia burgdorferi*, causes Lyme borreliosis and can lead to clinical manifestations such as arthritis, carditis, and neurological conditions. Experimental infection of mice recapitulates many of these symptoms and serves as a model system for the investigation of disease pathogenesis and immunity. Innate immunity is known to drive the development of Lyme arthritis and carditis, but the mechanisms driving this response remain unclear. Innate immune cells recognize *B. burgdorferi* surface lipoproteins primarily *via* toll-like receptor (TLR)2; however, previous work has demonstrated TLR2$^{-/-}$ mice had exacerbated disease and increased bacterial burden. We demonstrate increased CD4 and CD8 T cell infiltrates in *B. burgdorferi*-infected joints and hearts of C3H TLR2$^{-/-}$ mice. *In vivo* depletion of either CD4 or CD8 T cells reduced *Borrelia*-induced joint swelling and lowered tissue spirochete burden, whereas depletion of CD8 T cells alone reduced disease severity scores. Exacerbation of Lyme arthritis correlated with increased production of CXCL9 by synoviocytes, and this was reduced with CD8 T cell depletion. These results demonstrate T cells can exacerbate Lyme disease pathogenesis and prolong disease resolution possibly through dysregulation of inflammatory responses and inhibition of bacterial clearance.

Keywords: *Borrelia burgdorferi*, Lyme disease, mouse, toll-like receptor 2, T cells, arthritis

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INTRODUCTION

Lyme disease is the most commonly reported vector-borne illness in the United States, with an estimated 300,000 new cases each year (1). It is caused by infection with the spirochete, *Borrelia burgdorferi* (*Bb*), and is transmitted by *Ixodes* ticks (2). Humans infected with *B. burgdorferi* typically develop an expanding circular rash called erythema migrans as an early sign of infection (3). If not treated with antibiotics early during the infection, the spirochetes disseminate and colonize other tissues, potentially resulting in arthritis, carditis, or neurological disease that may be difficult to treat clinically (4). Despite much effort, the mechanisms *B. burgdorferi* uses to evade host immune-mediated clearance and persist in tissues resulting in disease pathogenesis are unclear and the focus of many infectious disease researchers.

In mice, innate immunity is considered to be the primary driver of Lyme arthritis and carditis pathogenesis (5, 6). Genetic control of disease resistance or susceptibility was shown to be independent of adaptive immunity (7). Innate phagocytes recognize *B. burgdorferi* primarily through TLR2-mediated recognition of spirochete surface lipoproteins (8). It was hypothesized that TLR2$^{-/-}$ mice would display an attenuated disease phenotype upon infection with *B. burgdorferi*. Surprisingly, TLR2$^{-/-}$ mice had increased Lyme arthritis severity in both disease-resistant C57BL/6 mice and -susceptible C3H/HeJ mice (9, 10). This response was attributed to an increased spirochete load in the tissues of TLR2$^{-/-}$ mice. However, increasing the infectious dose in WT

mice does not increase arthritis severity, except in BALB/c mice (11). In addition, arthritis susceptibility has been demonstrated to be independent of spirochete loads in joint tissues (11, 12). Following infection of TLR2$^{-/-}$/*scid* double mutant mice, Lyme arthritis severity was returned to WT control levels, suggesting a role for adaptive immune cells in driving the exacerbated disease severity in TLR2$^{-/-}$ mice (13). Subsequent work identified an increased presence of T cells in the joints of *B. burgdorferi*-infected C3H TLR2$^{-/-}$ mice, suggesting that these cells may drive the increased pathology in TLR2$^{-/-}$ mice (14). In addition, it was demonstrated that increased T cell infiltration into mouse joints was correlated with increased production of IFN-induced chemokines (CXCL9 and CXCL10) by synoviocytes. However, the exact role of increased T cell presence in the joints of TLR2$^{-/-}$ mice was not defined.

In the current study, we advanced this line of inquiry by depleting CD4$^+$ or CD8$^+$ T cells in C3H TLR2$^{-/-}$ mice infected with *B. burgdorferi*. We found that CD8$^+$ T cells were increased in the joints of TLR2$^{-/-}$ mice, whereas both CD8$^+$ and CD4$^+$ T cells were increased in infected hearts. Depletion of both T cell subsets decreased ankle swelling and joint spirochete loads, but only CD8$^+$ T cell depletion lowered arthritis or carditis severity scores. These results highlight the complex regulatory mechanisms that drive disease development and suggest CD8$^+$ T cells may have an underappreciated role in driving Lyme disease pathology.

MATERIALS AND METHODS

Animals

Female C3H/HeJ mice 4–6 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). C3H TLR2$^{-/-}$ mice at the N6 generation backcross were generously provided by Dr. Linda Bockenstedt (Yale University) and these were fully backcrossed onto the C3H/HeJ background (N10) in our colony. Animals were given sterile food and water *ad libitum* and housed in a specific pathogen-free facility. All works were done in accordance with the Animal Care and Use Committee of the University of Missouri.

Bacteria and Infections

Frozen stocks of a virulent, passage 8, clonal isolate of *B. burgdorferi* N40 strain were used for all infections. Stocks were added to 7-mL C-BSK-H medium (Sigma–Aldrich, St. Louis, MO, USA) and grown to log phase at 32°C. Spirochetes were enumerated using dark field microscopy and a Petroff–Hausser counting chamber (Hausser Scientific, Horsham, PA, USA). Spirochete dilutions were made in sterile BSK-H medium such that each mouse was inoculated in each hind foot pad with 50-μL medium containing 5×10^4 spirochetes.

Antibodies and Reagents

Antibodies for use in flow cytometry were obtained from eBioscience (CD45.2-PerCPCy5.5, CD16/CD32, CD3e-PEeFl.610, CD4-APCeFl.780, CD335-APCeFl.780, CD122-PECy7, IFN-γ-PerCPCy5.5, and F4/80-APCeFl.780) and Leinco (CD8-FITC and Ly6g-APC). Depleting antibodies were obtained from Leinco (GK1.5 for anti-CD4-depleting antibody and YTS-169 for anti-CD8-depleting antibody) and graciously donated by Dr. Helen Mullen (University of Missouri) (YTS-156 anti-CD8-depleting antibody). Collagenase/dispase Version 16 was purchased from Roche and resuspended according to manufacturer's directions. DNaseI DN-25 was purchased from Sigma and stored at −20°C in 2 mg/mL 50% glycerol and 75 mM NaCl.

In Vivo Depletion of T Cell Subsets

C3H TLR2$^{-/-}$ and WT C3H mice were treated with 400-μg CD8-depleting antibody (15) or 300-μg CD4-depleting antibody (16) i.p. 1 day prior to infection. As a negative control, mice were treated with sterile saline i.p. Every 7 days, CD8 or CD4 depletion was maintained by giving an additional i.p. injection of 250 or 100 μg antibody, respectively.

Cell Isolation for Flow Cytometry

C3H TLR2$^{-/-}$ and C3H WT mice were infected with *B. burgdorferi* and sacrificed at days 14, 21, 28, 42, and 49 postinfection. Hearts were perfused with 1× PBS, removed, and cut into fine pieces. Ankles were harvested from each mouse by removing the toes and carefully cutting through the knee joint, particularly to avoid bone marrow contamination. Excess muscle tissue was trimmed to reduce blood contamination. Ankles and hearts from each mouse were placed in appropriately labeled 15-mL conical tubes containing 5 mL 1× PBS + 4% FBS, 75 μL diluted DNaseI (0.03 mg), and 50 μL stock collagenase/dispase. These were placed on a rocker at room temperature for 1 h before being placed into sterile Petri dishes with 5 mL of additional RPMI supplemented with 10% FBS. Ankle tissue was carefully flayed apart using sterile rat tooth forceps. Cells from joints and hearts were strained through a 70-μm filter (BD Falcon) into a 50-mL conical tube. Cells were spun at 300 *g*, 4°C, 8 min. Supernatant was removed, and cells were washed with 5-mL 1× PBS + 4% FBS three times. Live cells were counted using 3% acetic acid with methylene blue (Stemcell Technologies).

Flow Cytometric Analysis

For flow cytometry, cells were stained for cell-specific markers. A total of 1×10^6 cells from each sample were placed in a 96-well U-bottom plate and Fc receptors blocked with anti-CD16/CD32 for 15 min at 4°C. Cells were then incubated with cell-specific marker antibodies listed above for 30 min on ice and then washed and fixed in 1% paraformaldehyde for 15 min. Cell analysis was run on a Dako Cyan flow cytometer using Summit V5.0 software. Samples were first gated on live cells, and then doublets were removed. Hematopoietic cells were selected by gating on CD45.2$^+$ cells and then specific cell types within that gate were analyzed. For T cells, CD3e$^+$ cells were selected and then CD4$^+$ or CD8$^+$ cell percentages were determined from the total CD3$^+$ cell population. Total cell numbers were determined by multiplying the total cells counted in the homogenized tissue × percent CD45.2$^+$ cells × percent cell-lineage-specific marker. For T cells, the total cells in the homogenized tissue × percent CD45.2$^+$ cells × percent CD3e$^+$ cells × percent CD4$^+$ or CD8$^+$ cells.

Assessment of Arthritis and Carditis Pathology

Ankle swelling was measured throughout the infection at the thickest craniocaudal portion of the joint using a metric caliper. Arthritis and carditis severity scores were determined, as described previously (17). Zinc–formalin-fixed, paraffin-embedded sections of ankle joints and hearts were stained with hematoxylin and eosin (H&E) and evaluated in a blinded manner on a scale of 0–4 with 0 representing no inflammation and 4 representing severe inflammation in more than half of the section evaluated.

Determination of *B. burgdorferi* Loads

DNA was extracted from bladders of untreated, CD4-depleted, and CD8-depleted TLR2$^{-/-}$ and WT mice by homogenization in TRIzol as per manufacturer's instructions. Real-time PCR reactions for *B. burgdorferi flagellin* normalized to copies of mouse *nidogen* within the same sample were performed using TaqMan Universal PCR Master Mix (Applied Biosystems). *Borrelia* loads are expressed as copies of *flagellin* per 1000 copies of *nidogen* as described (18).

Determination of Antibody Levels

Sera of infected animals were collected at sacrifice by cardiac puncture and *B. burgdorferi*-specific IgM and IgG levels were detected using enzyme-linked immunosorbent assays (ELISAs) on Immulon 2B ELISA plates as described (10).

Determination of Cytokine Levels in Tissues

Protein was extracted from joint or heart tissue, as described previously (18). Briefly, joint and heart samples were excised and immediately flash frozen in liquid nitrogen. The samples were wrapped in foil and pulverized with a hammer. The resultant powder was resuspended in HBSS-containing 0.2% protease inhibitor mixture (Sigma) and 0.4% Triton X-100. Samples were homogenized using a tissue homogenizer, and particles removed by centrifugation and filtration through a 0.45-μm filter. Samples were brought to 1.5 mL in homogenization buffer. Protein levels were determined using a BCA assay (Pierce), and cytokine levels were determined using a Cytokine Mouse 20-Plex Panel (Life Technologies).

Statistical Analysis

Statistical analyses were performed using Graphpad Prism software. For single comparisons, an unpaired Student's *t*-test was performed, and for non-parametric data, we used Mann–Whitney. Multiple comparisons were performed using ANOVA and Tukey *post hoc* test or Dunnett's test for comparison to a single control ($\alpha = 0.05$ for all tests).

RESULTS

Increased T Cell Infiltration in TLR2$^{-/-}$ Mice

Intradermal inoculation of *B. burgdorferi* into C3H TLR2$^{-/-}$ mice results in exacerbated arthritis compared with WT C3H and has been correlated with increased numbers of T cells within the joint tissue (9, 14). To investigate this phenomenon further, we infected C3H WT and C3H TLR2$^{-/-}$ mice with *B. burgdorferi* in both rear footpads and followed the development of arthritis. By the second week of infection, the TLR2$^{-/-}$ mice displayed significantly greater ankle swelling than the WT control mice, and this exacerbated response continued past day 35 postinfection (**Figure 1A**). Mice were sacrificed at various time points, and ankle and heart tissues were processed into single cell suspensions for analysis by flow cytometry. In agreement with a previous report, we found that T cells (CD3$^+$ cells) were significantly increased in joint tissue from TLR2$^{-/-}$ mice (**Figure 1B**). In addition, we also found that T cells were also increased in the inflammatory infiltrates in the hearts of *B. burgdorferi*-infected mice (**Figure 1C**). Thus, T cells are increased in the inflammatory infiltrates in Lyme arthritis and carditis in TLR2$^{-/-}$ mice.

The previous report by Wang et al. (14) did not report the phenotype of the T cells in the increased inflammatory infiltrate in TLR2$^{-/-}$ mice. Therefore, we next investigated the levels of CD4$^+$ and CD8$^+$ T cell phenotypes in the joints and heart tissue using flow cytometry. CD4$^+$ and CD8$^+$ T cells were identified

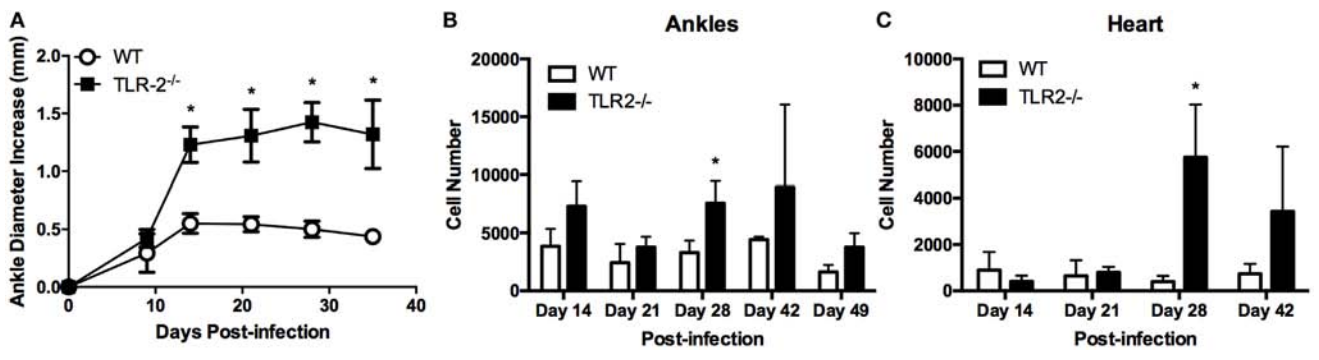

FIGURE 1 | Arthritis development and T cell infiltrates in TLR2$^{-/-}$ mice. (A) Ankle diameters of WT and TLR2$^{-/-}$ mice were measured weekly following footpad inoculation with 5×10^4 *B. burgdorferi*. At each indicated time point, three mice per strain were sacrificed and numbers of CD3e$^+$ T cells were determined from **(B)** ankles or **(C)** hearts by flow cytometry. Data are representative of two separate experiments. Symbols represent means ± SD. *$p > 0.01$ compared to WT control from same time point.

from CD45.2$^+$ CD3e$^+$ T cells. Foxp3$^+$ T regulatory cells could not be identified in either joint or heart cellular infiltrates (data not shown). Within both the ankle joint and heart tissue, CD4$^+$ T cells made up the majority of the T cells in both WT and TLR2$^{-/-}$ mice (**Figure 2**). However, whereas CD4$^+$ T cells in the joints of TLR2$^{-/-}$ mice were reduced compared to their WT counterparts, in the heart, CD4$^+$ cells were increased dramatically near the peak of inflammation (**Figures 2A,B**). CD8$^+$ T cell numbers trended higher at all time points evaluated within the joints and were significantly elevated in the TLR2$^{-/-}$ mice at day 28 postinfection (**Figure 2C**). In the hearts, CD8$^+$ T cells mirrored the increase in CD4$^+$ cells and significantly increased near the peak of inflammation (**Figure 2D**). These results demonstrate that lymphocytes can be differentially recruited into *B. burgdorferi*-infected tissues as has been demonstrated for macrophages and neutrophils (19, 20). In addition, CD8$^+$ T cell numbers are increased in the joints of *B. burgdorferi*-infected TLR2$^{-/-}$ mice, while both CD4$^+$ and CD8$^+$ cells were increased in the infected hearts.

We also investigated the presence of natural killer T (NKT) cells within the joint or heart tissue of *B. burgdorferi*-infected TLR2$^{-/-}$ mice. NKT cells have been reported to play a role in host defense in both Lyme arthritis and carditis (21, 22). In addition, we also examined the numbers of natural killer (NK) cells in the joints and hearts of TLR2$^{-/-}$ mice, as these cells have been implicated in the exacerbated development of Lyme arthritis in C57BL/6 IL-10$^{-/-}$ mice (23). We therefore looked for both NK (CD45.2$^+$NKp46$^+$CD122$^+$CD3e$^-$) and NKT (CD45.2$^+$NKp46$^+$CD122$^+$CD3e$^+$) cells in the infected tissues at day 21 postinfection using flow cytometry. Within the infected ankle joints, both NK and NKT cells were significantly decreased in TLR2$^{-/-}$ mice compared to WT mice (**Figures 3A,B**). Within the infected heart tissue, the numbers of NK cells and NKT cells were quite low and did not differ between WT and TLR2$^{-/-}$ mice (**Figures 3C,D**). These results suggest that neither NKT nor NK cells are likely responsible for the increased disease severity seen in the TLR2$^{-/-}$ mice.

In Vivo T Cell Subset Depletion in TLR2$^{-/-}$ Mice Reduces Lyme Arthritis to WT Levels

C3H WT and C3H TLR2$^{-/-}$ mice were depleted of either CD4$^+$ or CD8$^+$ T cells using intraperitoneal injections of either CD4$^+$ or CD8$^+$ T cell-depleting antibodies. Flow cytometry demonstrated the efficacy of the treatment (**Figure 4A**). One

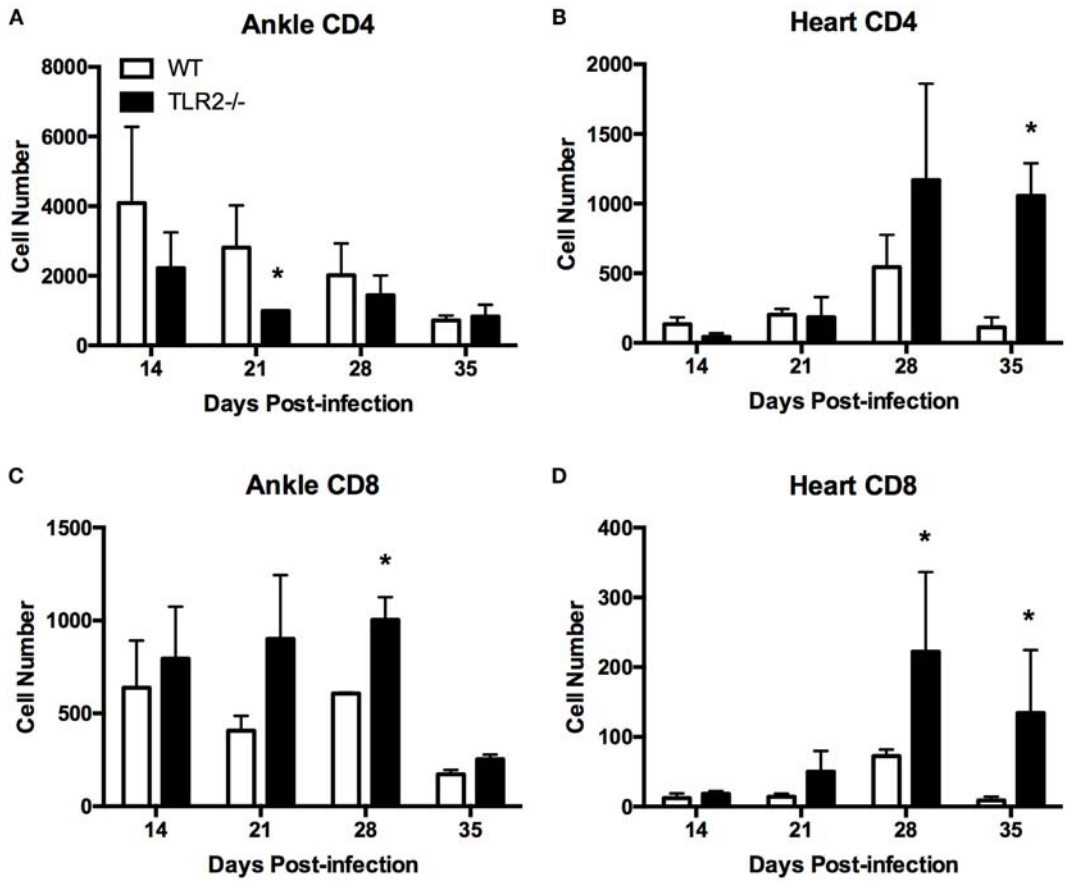

FIGURE 2 | T cell subsets in infected ankle and heart tissue. WT and TLR2$^{-/-}$ mice were infected with *B. burgdorferi* and three mice from each strain sacrificed at the indicated time points. T cell subsets in ankle **(A,C)** and heart **(B,D)** tissue were characterized for CD4 **(A,B)**, CD8 **(C,D)** by flow cytometry. Data are representative of two separate experiments. Bars represent means + SD. *$p > 0.05$ compared to WT control from same time point.

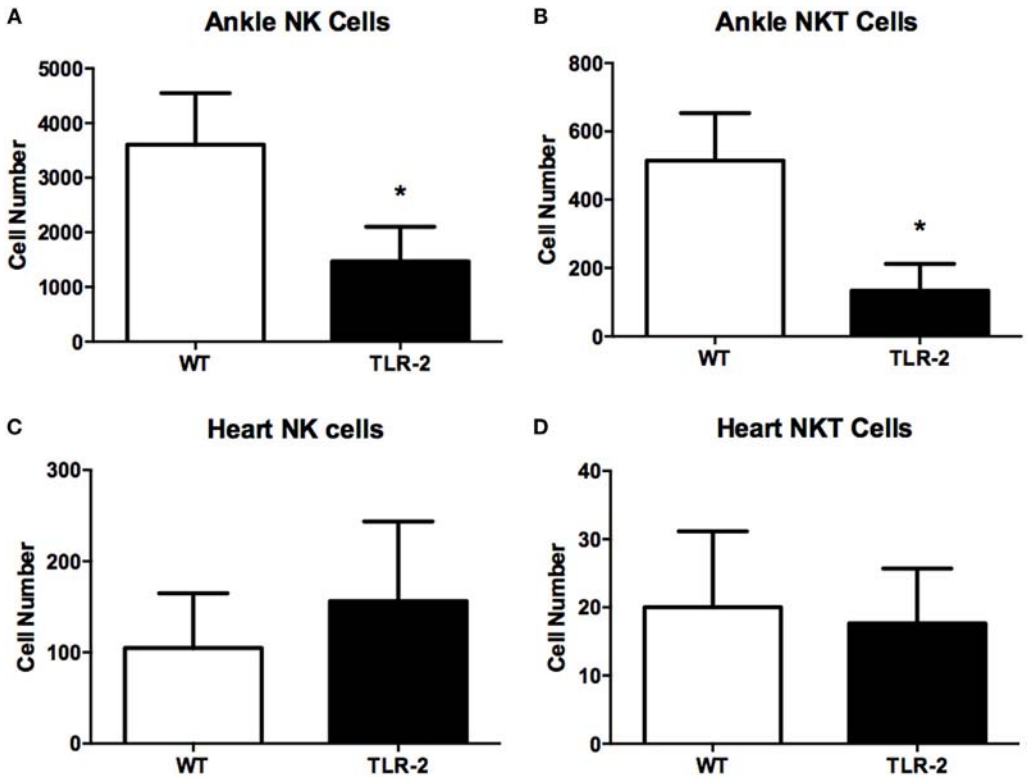

FIGURE 3 | NK and NKT cells in infected ankle and heart tissue. WT and TLR2$^{-/-}$ mice were infected with *B. burgdorferi* and three mice from each strain sacrificed at the indicated time points. NK (CD45.2$^+$NKp46$^+$CD122$^+$CD3e$^-$) and NKT (CD45.2$^+$NKp46$^+$CD122$^+$CD3e$^+$) cells were enumerated in ankle **(A,B)** and heart **(C,D)** tissue at day 21 postinfection. Data are representative of two separate experiments. Bars represent means + SD. *$p > 0.05$ compared to WT control from same time point.

day after administration of depleting antibodies, mice were infected with 1×10^5 *B. burgdorferi via* footpad inoculation. T cell depletion was maintained by weekly administration of depleting antibody throughout the infection time course. Ankle swelling was monitored every 7 days. WT mice depleted of either CD4$^+$ or CD8$^+$ T cells had slightly reduced swelling at all time points assessed compared to saline-treated controls, but these differences did not reach statistical significance (**Figure 4B**). TLR2$^{-/-}$ mice depleted of CD4$^+$ or CD8$^+$ T cells had significantly reduced ankle swelling compared to untreated controls, and the ankle diameter increase was similar to that typical of WT mice (compare **Figures 4B,C**). In the experiment shown, the anti-CD4 treated all died between days 21 and 28 postinfection. In a subsequent experiment, the anti-CD4-treated mice had a similar reduction in ankle swelling, but the mice did not die. The reason for this discrepancy is unknown, but in both instances the T cell depletion resulted in a significant decrease in ankle swelling. **Figure 4D** shows a representative ankle from a TLR2$^{-/-}$ CD8-depleted mouse and a saline-treated TLR2$^{-/-}$ control mouse at day 14 postinfection. The joint swelling in the CD8$^+$ T cell-depleted TLR2$^{-/-}$ mouse is dramatically reduced compared to the saline-treated TLR2$^{-/-}$ mouse. A similar reduction was seen in the CD4$^+$ T cell-depleted mice (data not shown).

Sections of both ankle joint and heart tissue from control-treated and T cell-depleted TLR2$^{-/-}$ mice were processed for histology by H&E staining and scored for arthritis and carditis severity (17). As previously reported, arthritis severity scores in TLR2$^{-/-}$ mice were significantly increased over WT control mice at day 21 postinfection (**Figure 5A**). Depletion of CD4$^+$ or CD8$^+$ T cell subsets had little effect on arthritis severity scores, with values similar to WT control-treated mice. In contrast, depletion of CD8$^+$ T cells, but not CD4$^+$ T cells, decreased arthritis severity scores to similar levels as WT mice. CD4$^+$ T cell severity scores remained high, similar to the TLR2$^{-/-}$ mice. In the hearts, carditis severity was not increased in the TLR2$^{-/-}$ mice compared to the WT control-treated mice (**Figure 5B**). Again, as in the joints, depletion of CD4$^+$ T cells had no effect on carditis severity scores in either WT or TLR2$^{-/-}$ mice. Depletion of CD8$^+$ T cells, however, lowered carditis severity in both the WT and TLR2$^{-/-}$ mice, indicating a role for CD8$^+$ T cells in carditis severity. Increased arthritis severity in TLR2$^{-/-}$ mice has been linked to an increase in spirochete burden in joint tissue (10). *B. burgdorferi* loads in tissue were significantly higher in the TLR2$^{-/-}$ control mice than in the WT control mice (**Figure 5C**). Depletion of T cell subsets had little effect on spirochete levels in WT mice, but significantly lowered *Borrelia* loads in the TLR2$^{-/-}$ mice compared with TLR2$^{-/-}$ control mice.

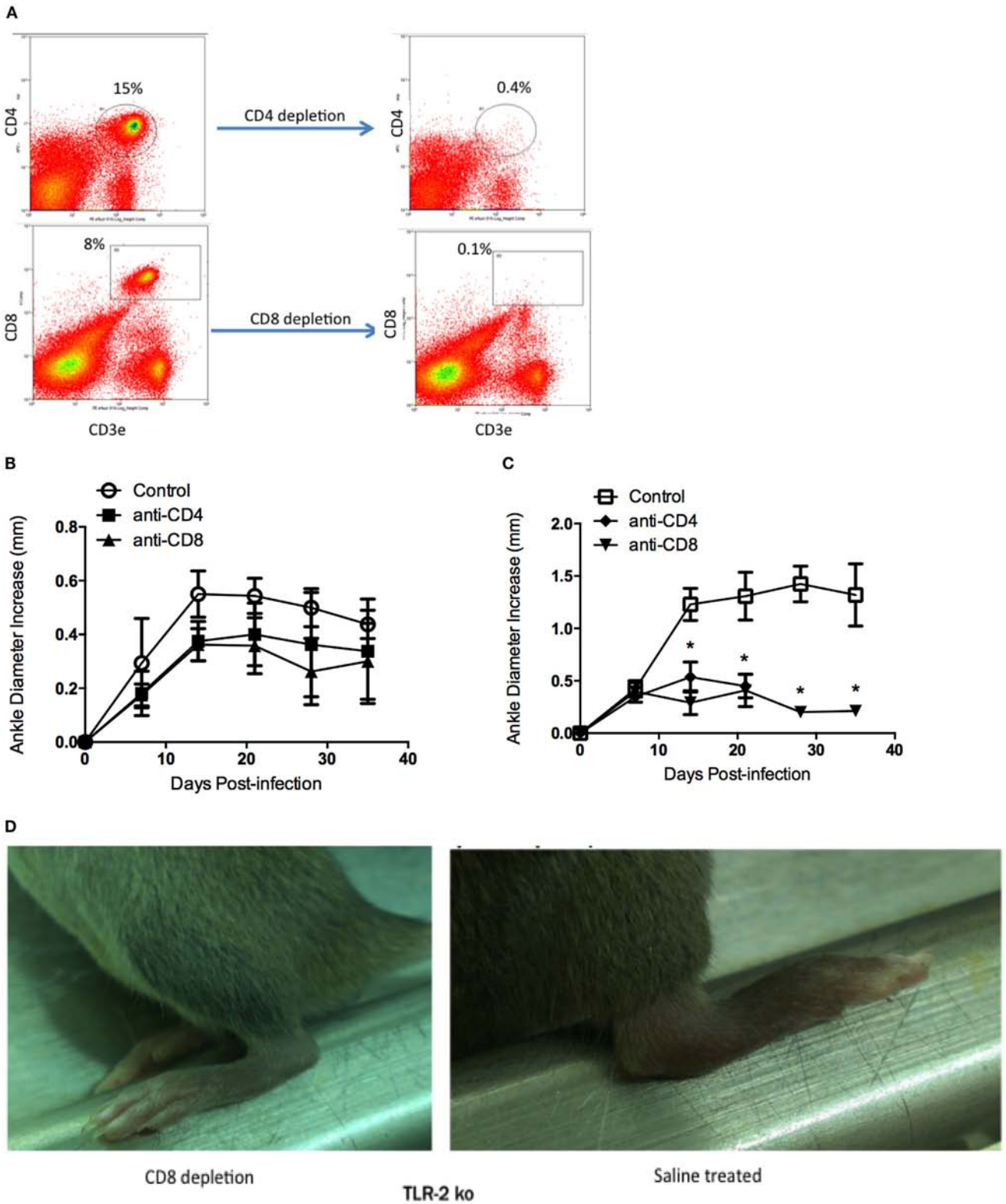

FIGURE 4 | **Lyme arthritis in mice depleted of CD4 or CD8 T cells**. Mice were treated with saline (control), CD4-depleting antibody, or CD8-depleting antibody and infected with *B. burgdorferi*. Flow cytometry plots demonstrate depletion of $CD4^+$ and $CD8^+$ T cells 6 days posttreatment **(A)**. Arthritis development was monitored over time for WT **(B)** and $TLR2^{-/-}$ **(C)** mice. Representative pictures of $TLR2^{-/-}$ mice depleted of CD8 T cells or saline treated were taken at day 14 postinfection **(D)**. Data are representative of two separate experiments. $n = 3$, symbols represent means ± SD. $^*p > 0.01$ compared to WT control from same time point.

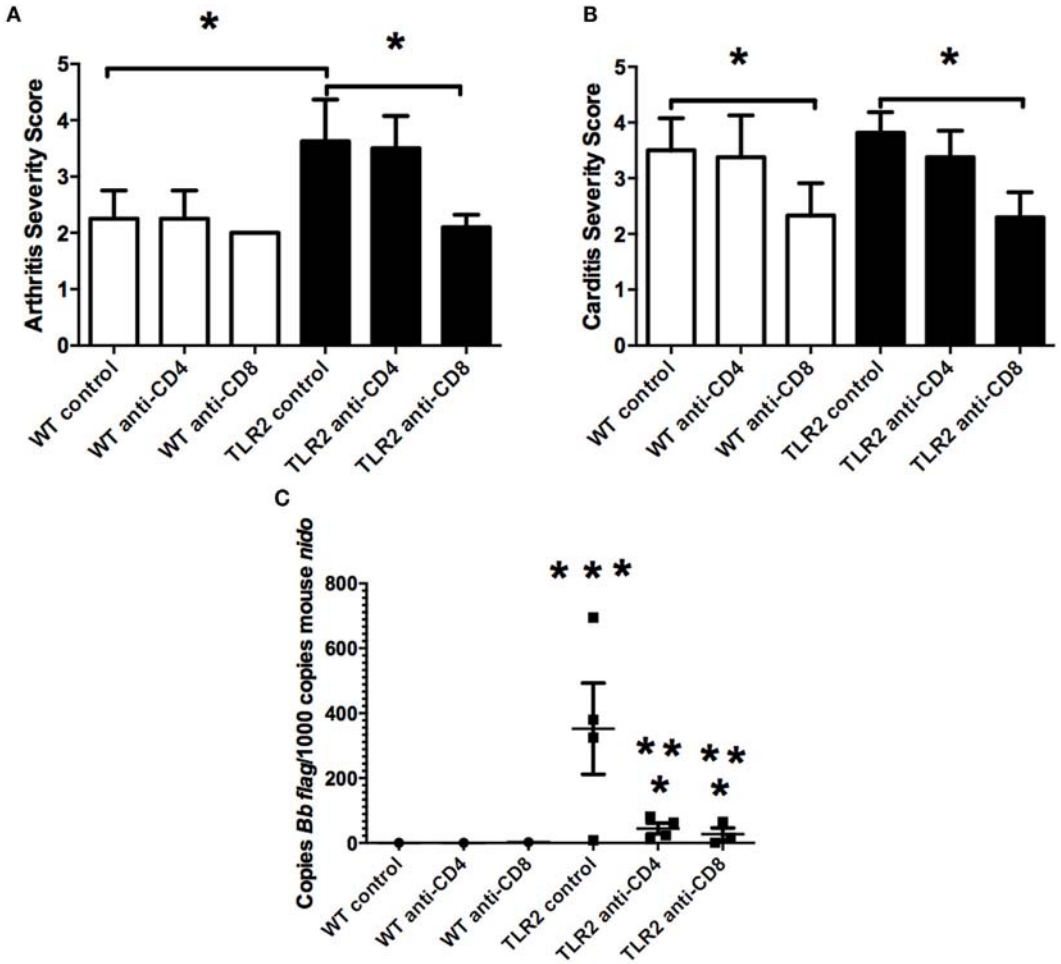

FIGURE 5 | **Arthritis and carditis severity scores**. Mice were infected and treated as in **Figure 4**. On day 21 postinfection, the mice were sacrificed and ankles **(A)** and hearts **(B)** were processed for histology and scored for lesion severity. Levels of *B. burgdorferi* in tissue were determined by qRT-PCR **(C)**. Data are representative of two separate experiments. $n = 8$, bars represent means ± SD. * vs. TLR2$^{-/-}$ control, ** vs. WT CD4 or CD8-depleted mice, *** vs. WT control, $p > 0.05$.

B. burgdorferi loads in the CD4$^+$ and CD8$^+$ T cell-depleted mice were still above levels in T cell-depleted WT mice. Thus, the defect in spirochete clearance in TLR2$^{-/-}$ mice can be mitigated by the removal of either CD4$^+$ or CD8$^+$ T cells, but does not return them to WT control levels.

Production of *Borrelia*-specific antibodies has been reported to be normal in TLR2$^{-/-}$ mice (10). We investigated the effect T cell subset depletion might have on their production. As previously reported, levels of *Borrelia*-specific IgM were not altered in TLR2$^{-/-}$ mice, and there was no effect of T cell depletion on their production (**Figure 6A**). Similarly, levels of *Borrelia*-specific IgG were no different between WT control and TLR2$^{-/-}$ mice (**Figure 6B**). However, CD4$^+$ T cell depletion significantly decreased *Borrelia*-specific IgG levels in both strains of mice and, thus, was not specific to the TLR2 deficiency, but rather more likely to the lack of T cell help for B cell class switching. CD8$^+$ T cell depletion had no effect on IgG levels in either the WT or TLR2$^{-/-}$ mice.

Neutrophils are the predominant inflammatory cell type in the joint in mice during Lyme arthritis, and both neutrophil and macrophage levels increase in the *B. burgdorferi*-infected joints of TLR2$^{-/-}$ mice (14). We therefore investigated the effect of T cell subset depletion on neutrophil and macrophage numbers in the joints of WT and TLR2$^{-/-}$ mice at day 21 postinfection using flow cytometry. Ly6g^{hi} cells, representative of mature neutrophils, were significantly increased in control TLR2$^{-/-}$ mice compared to control WT mice (**Figure 7A**). Interestingly, following either CD4$^+$ or CD8$^+$ T cell depletion, neutrophil numbers in joints of WT control mice were significantly increased, although this did not result in an increase in arthritis severity (**Figure 5A**). In contrast, depletion of either CD4$^+$ or CD8$^+$ T cells in TLR2$^{-/-}$ mice resulted in a reduction of neutrophil numbers to levels similar to the WT controls. Similar results were seen for macrophage numbers in the *B. burgdorferi*-infected joints, except that CD4$^+$ T cell depletion in WT mice did not increase macrophage levels (**Figure 7B**). These results demonstrate that depletion of T cell subsets in WT mice increases the recruitment of neutrophils and macrophages into the *B. burgdorferi*-infected joint, but does not increase arthritis severity. In contrast, TLR2$^{-/-}$ mice have exacerbated levels of neutrophils and macrophages in the infected

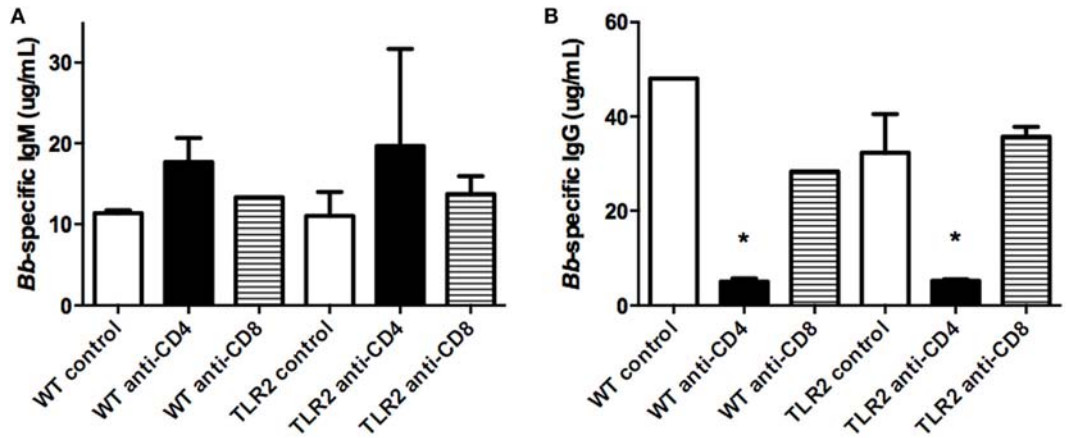

FIGURE 6 | ***Borrelia*-specific antibody production**. Mice were infected and treated as in **Figure 4**. On day 21 postinfection, the mice were sacrificed and assessed for *B. burgdorferi*-specific IgM **(A)** and IgG **(B)** levels in serum. Data are representative of two separate experiments. $n = 3$, bars represent means ± SD. *$p > 0.05$ compared to mouse strain control.

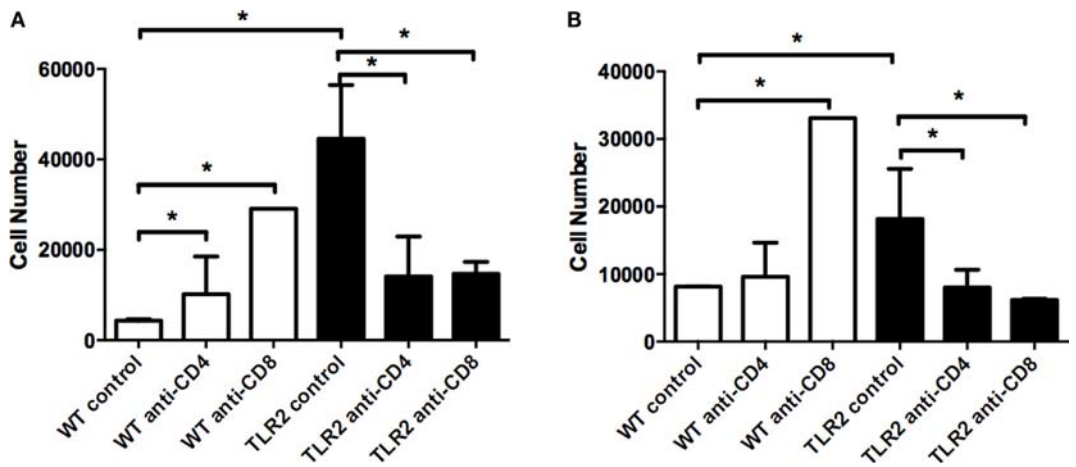

FIGURE 7 | **Neutrophil and macrophage numbers in infected ankle joints**. Mice were infected and treated as in **Figure 4**. On day 21 postinfection, the mice were sacrificed and neutrophil **(A)** and macrophage **(B)** numbers were assessed from joint tissue. Data are representative of two separate experiments. $n = 3$, bars represent means ± SD. *$p > 0.05$.

joints, and these levels are decreased with T cell depletion, but only $CD8^+$ T cell depletion appears to lower arthritis severity.

Bone marrow-derived macrophages from $TLR2^{-/-}$ mice were defective in their production of IL-6, IL-10, and TNFα when stimulated *in vitro* with *B. burgdorferi* recombinant outer surface proteins or sonicated spirochetes (10). In contrast, transcripts from several interferon-inducible chemokine genes were increased in joints of *B. burgdorferi*-infected mice (14). We therefore measured the levels of 20 chemokines and cytokines from the infected joints of control and $CD4^+$ or $CD8^+$ T cell-depleted WT and $TLR2^{-/-}$ mice at day 21 postinfection using a bead assay system. Most of the cytokines measured were present at low levels, and there were no statistically significant differences between the WT control and $TLR2^{-/-}$ mice (data not shown). Interferon-gamma levels are shown as a representative cytokine for this analysis (**Figure 8A**). No significant differences were seen between untreated or T cell-depleted WT and $TLR2^{-/-}$ mice. We also looked at an earlier time point, as we have previously shown that many pro-inflammatory mediators peak earlier during Lyme arthritis (18). Analysis of IFN-γ levels in ankle joints at day 7 postinfection yielded no difference between control WT and $TLR2^{-/-}$ mice (data not shown). The only inflammatory mediator that we found to be significantly altered in the joints of the $TLR2^{-/-}$ mice was CXCL9 (**Figure 8B**). Depletion of $CD4^+$ T cells had no effect on the levels of CXCL9, but $CD8^+$ T cell depletion dramatically reduced the level of CXCL9 within the joints, although this reduction was not statistically significant. Transcripts of CXCL10 were also reported to increase in joints of $TLR2^{-/-}$ mice infected with *B. burgdorferi* (14), although we found no difference in protein

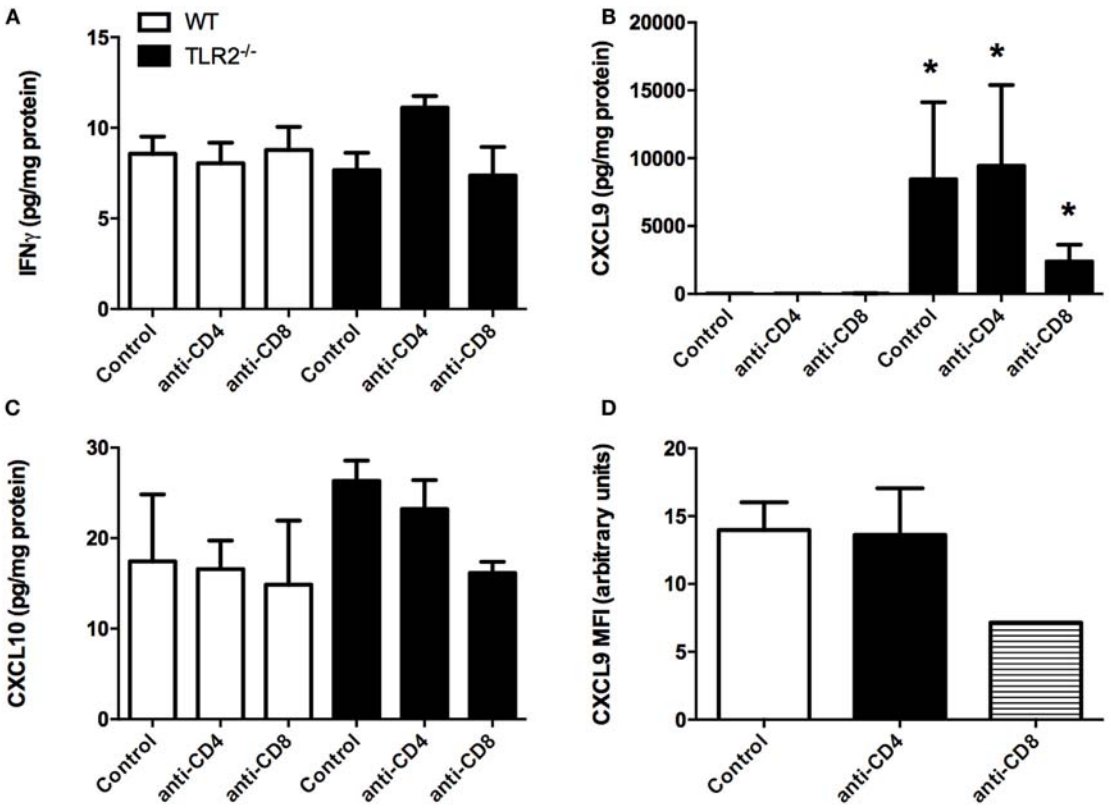

FIGURE 8 | **Cytokine production in joints of infected mice**. Mice were infected and treated as in **Figure 4**. On day 21 postinfection, the mice were sacrificed and levels of IFNγ **(A)**, CXCL9 **(B)**, and CXCL10 **(C)** were determined from homogenized joint tissue using a Lumina assay. CXCL9 levels within ICAM-1$^+$VCAM-1$^+$CD14$^-$ synoviocytes were assessed from TLR2$^{-/-}$ mice at 14 days postinfection by flow cytometry **(D)**. $n = 3$, data representative of one trial. Bars represent means ± SD. $^*p > 0.01$ compared to WT control.

levels in our study (**Figure 8C**). Synovial cell production of CXCL9- and CXCL10-mediated T cell recruitment and T cell production of mediators that stimulate synovial cell chemokine production set up a synergistic positive feedback loop that has been suggested to drive the enhanced inflammatory response in TLR2-deficient mice (14). We therefore examined the production of CXCL9 from synoviocytes *in vivo* using flow cytometry. Ankle joints from *B. burgdorferi*-infected TLR2$^{-/-}$ mice at day 14 postinfection were processed for flow cytometry. Synoviocytes (ICAM-1$^+$V-CAM-1$^+$CD14$^-$) were found to produce CXCL9 (**Figure 8D**). Depletion of CD4$^+$ T cells had no effect on the production of CXCL9 by these cells; however, CD8$^+$ T cell depletion caused about a 50% reduction in CXCL9 production. Due to the low numbers of animals in this experiment, this result was not statistically significant, but along with our previous data, suggests that CD8$^+$ T cells are the cells responsible for the increased inflammation and arthritis severity seen in TLR2$^{-/-}$ mice infected with *B. burgdorferi*.

DISCUSSION

Innate immune cells are the first line of defense against bacterial infection and their responses guide the developing immune response. These cells use an arsenal of pattern recognition receptors to recognize bacterial invaders and stimulate an appropriate response (24). Innate phagocytes recognize extracellular *B. burgdorferi* outer surface lipoproteins primarily *via* TLR1/2 heterodimers (8, 25) and possibly flagellin through TLR5 (26). Other intracellular receptors, such as NOD2, and endosomal TLRs (TLR7, TLR8, and TLR9) may also contribute to the response (27). Therefore, it was surprising when TLR2$^{-/-}$ mice were found to develop exacerbated Lyme arthritis (9, 10). This response was found to require adaptive immune cells (13) and correlated with increased recruitment of T cells into the infected joints (14). Here, we demonstrate that numbers of CD8$^+$ T cells, but not CD4$^+$ T cells, were increased in the joints of *B. burgdorferi*-infected TLR2$^{-/-}$ mice, but both T cell subsets were increased in the hearts. While depletion of either subset could lower joint swelling and spirochete loads, only CD8$^+$ T cell depletion lowered arthritis and carditis severity scores.

Similar to previous publications, we found that TLR2-deficient mice infected with *B. burgdorferi* displayed both increased ankle swelling and elevated numbers of CD3e$^+$ T cells within joint tissue at all time points assessed. In infected WT mice, T cells typically make up about 2% of the hematopoietic cell population within the joints. In infected TLR2-deficient mice,

this percentage jumped to 10. In the hearts, we found a similar phenomenon; T cell numbers were elevated during carditis. The role of T cells in mediating Lyme disease pathogenesis has been debated for some time. In the mouse model, the balance of $CD4^+$ T cell subsets (Th1/Th2) was suggested to mediate Lyme arthritis resistance or susceptibility through the production of IL-4 or IFNγ, respectively (28, 29). Treatment of C3H mice with anti-CD4-depleting antibody increased arthritis severity and treatment with anti-CD8-depleting antibody decreased arthritis severity, suggesting T cell responses could mediate Lyme disease pathogenesis (30). However, a subsequent study in arthritis resistant C57BL/6 mice deficient in $CD4^+$ cells showed no difference in the development of Lyme arthritis, but showed a delay in carditis resolution (31). Innate immunity was then shown to be capable of mediating both resistance and susceptibility to Lyme arthritis development (7). The small number of T cells in joints and hearts of *B. burgdorferi*-infected mice, however, suggests they might also have a role in shaping the developing immune response. This led to contrasting reports for the role of $CD4^+$ T cells in Lyme arthritis and carditis. Adoptive transfer of $CD4^+$ T cells into infected B6 $RAG^{-/-}$ mice led to exacerbated arthritis and myocarditis (32), while similar adoptive transfer into B6 $TCR\alpha^{-/-}$ mice resulted in no exacerbated disease and carditis resolution (33). These differences were suggested to be due to the differences in the lymphocyte compartment in the recipient mice and perhaps the induction of autoimmune disease (33). In the current study, we used serodepletion to remove $CD4^+$ or $CD8^+$ T cell subsets from naive WT or $TLR2^{-/-}$ mice. We found that depletion of $CD4^+$ or $CD8^+$ T cells had no effect on arthritis development in C3H mice, although depletion of $CD8^+$ cells reduced carditis severity at 3 weeks postinfection. These results are similar to those of Fikrig et al. (31) and contrasting with those of Keane-Myers and Nickell (30). The reasons for these differences are not clear, but most likely are due to differences in antibody treatments and efficiency of cell subset depletions. A role for $CD8^+$ T cells in driving exacerbated disease in the $TLR2^{-/-}$ mice, however, has not been previously reported and may represent a new target for therapy as $CD8^+$ T cells specific for *Borrelia* antigens have been reported in blood of patients with Lyme arthritis (34). We also looked the presence of T regulatory cells in the joints and hearts of WT and $TLR2^{-/-}$ mice, since these have been suggested to play a role in Lyme disease pathogenesis (35). T regulatory cells could not be found in either joint or heart tissue, and in the current, we did not look for γδT cells.

Natural killer cells are induced in mice following infection with *B. burgdorferi*, but their depletion had little effect on arthritis pathogenesis (36). They have been suggested to contribute to the increased inflammation seen in B6 $IL\text{-}10^{-/-}$ mice *via* their production of IFNγ (23). Prolonged NK cell activity has also been correlated with continued inflammation following antibiotic therapy in Lyme patients (37). We found decreased NK cell numbers in the joints of $TLR2^{-/-}$ mice and no change in heart tissue. Since there was no change in joint IFNγ levels in the $TLR2^{-/-}$ mice, it is unlikely the NK cells play a major role in the $TLR2^{-/-}$ mice. We found similar results for NKT cells. Low numbers of NKT cells were identified within infected joint tissue and almost none were found within heart tissue. NKT cells recognize bacterial glycolipids presented by CD1d (38), and infection of mice deficient in CD1 with *B. burgdorferi* led to impaired host defense, increased arthritis severity, and impaired spirochete clearance (39). NKT cells specifically recognize diacylglycerol antigens from *B. burgdorferi* (40). They limit spirochete dissemination by killing blood-borne spirochetes in the liver (41) and act as a cytotoxic barrier to prevent spirochete entry into the joint space (42). Deletion of NKT cells resulted in more severe and prolonged arthritis and reduced ability to clear spirochetes from tissues (22). It is tempting to speculate that the reduced numbers of NKT cells in the infected joints of $TLR2^{-/-}$ mice might be responsible for the increased arthritis severity and spirochete loads in this tissue. However, how depletion of $CD4^+$ or $CD8^+$ cells, both of which are expressed on some NKT cell subsets, could reduce *B. burgdorferi* tissue loads and increase arthritis severity in the $TLR2^{-/-}$ mice is unclear. More work is needed in this area.

Both C57BL/6- and C3H TLR2-deficient mice infected with *B. burgdorferi* display significantly worse arthritis and carditis (14, 43), and we have shown this is mediated primarily by increased recruitment of $CD8^+$ T cells into the infected joint. An increase in T cell numbers in the joints was associated with increased transcript levels of CXCL9 and CXCL10, giving a potential mechanism for the increased T cell recruitment (14). It was suggested that the enhanced arthritis of $TLR2^{-/-}$ mice was due to unregulated local chemokine production by synoviocytes. We measured chemokine production directly from infected joints and found CXCL9 was significantly unregulated in the joints of *B. burgdorferi*-infected TLR2-deficient mice. Protein levels of CXCL10 were only slightly elevated and at much lower levels than CXCL9. In the absence of $CD8^+$ T cells, CXCL9 levels were dramatically reduced, suggesting that $CD8^+$ T cells activate synoviocytes to produce CXCL9, thereby recruiting additional T cells into the joint and driving increased inflammation in the $TLR2^{-/-}$ mice. CXCL9 production can be induced by either type I or type II IFN (44). Production of type I IFN has been linked to arthritis development in *B. burgdorferi*-infected mice, and its production has been shown to be independent of TLR2 signaling (45). We measured levels of type II IFN (IFNγ) from the joints of *B. burgdorferi*-infected mice and found they were similar between WT and $TLR2^{-/-}$ mice. Thus, the mechanism responsible for increased CXCL9 expression in the joints of $TLR2^{-/-}$ mice is still unclear and is currently being investigated.

AUTHOR CONTRIBUTIONS

CL and CB designed the experiments, analyzed the data, and wrote the manuscript. CL, CP, KH, and JJ performed the experiments.

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Complement Evasion by Pathogenic *Leptospira*

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Leptospirosis is a neglected infectious disease caused by spirochetes from the genus *Leptospira*. Pathogenic microorganisms, notably those which reach the blood circulation such as *Leptospira*, have evolved multiple strategies to escape the host complement system, which is important for innate and acquired immunity. *Leptospira* avoid complement-mediated killing through: (i) recruitment of host complement regulators; (ii) acquisition of host proteases that cleave complement proteins on the bacterial surface; and, (iii) secretion of proteases that inactivate complement proteins in the *Leptospira* surroundings. The recruitment of host soluble complement regulatory proteins includes the acquisition of Factor H (FH) and FH-like-1 (alternative pathway), C4b-binding protein (C4BP) (classical and lectin pathways), and vitronectin (Vn) (terminal pathway). Once bound to the leptospiral surface, FH and C4BP retain cofactor activity of Factor I in the cleavage of C3b and C4b, respectively. Vn acquisition by leptospires may result in terminal pathway inhibition by blocking C9 polymerization. The second evasion mechanism lies in plasminogen (PLG) binding to the leptospiral surface. In the presence of host activators, PLG is converted to enzymatically active plasmin, which is able to degrade C3b, C4b, and C5 at the surface of the pathogen. A third strategy used by leptospires to escape from complement system is the active secretion of proteases. Pathogenic, but not saprophytic leptospires, are able to secrete metalloproteases that cleave C3 (central complement molecule), Factor B (alternative pathway), and C4 and C2 (classical and lectin pathways). The purpose of this review is to fully explore these complement evasion mechanisms, which act together to favor *Leptospira* survival and multiplication in the host.

Keywords: *Leptospira*, leptospirosis, complement system, immune evasion, serum resistance

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INTRODUCTION

Spirochetes of the genus *Leptospira* are highly motile Gram-negative bacteria that cause a worldwide zoonosis (1). This bacterium colonizes a wide range of hosts including humans, domestic and wild animal species. Patients with leptospirosis may present either very mild symptoms or subclinical disease (80–90% of infections) or a more severe illness characterized by jaundice, acute renal failure and bleeding (Weil's disease), or pulmonary hemorrhage syndrome [reviewed in Ref. (2)].

The genus *Leptospira* comprises bacteria having distinct ecological adaptations: exclusively non-pathogenic free-living species, exclusively host-dependent organisms and pathogenic species capable of surviving both inside and outside the host for long periods (3). Molecular phylogenetic analysis of

16S rRNA gene sequences allowed clustering of *Leptospira* species into three groups, comprising pathogens, non-pathogens, and an intermediate group (4).

Upon infection, pathogenic leptospires spread and propagate in susceptible hosts because they have evolved diverse immune evasion strategies. Conversely, saprophytic *Leptospira* strains are highly susceptible to serum bactericidal activity, an observation already made by Johnson and Muschel in the mid-1960s (5). Since our insights into complement evasion mechanisms of *Leptospira* have substantially increased during the last 10 years, we aim here to provide a comprehensive overview of the interactions of this relevant human pathogen with the complement system.

THE ROLE OF THE COMPLEMENT SYSTEM IN THE HOST'S DEFENSE AGAINST PATHOGENS

The complement system is composed of more than 50 plasma proteins and receptors. Traditionally considered as one of the first lines of defense against invading microorganisms due to its opsonic, inflammatory, and lytic activities, complement roles extend far beyond pathogen killing [reviewed in Ref. (6)]. Complement effector functions result from activation of three different pathways: classical, alternative, and/or lectin pathways (CP, AP, and LP, respectively). While the AP and LP participate in the innate immunity, the CP is generally activated by the presence of IgG or IgM specifically bound to antigens. The AP is initiated by the spontaneous hydrolysis of an intrachain thioester bond located in the C3 molecule, while the LP is activated when lectins, such as mannose-binding lectin or ficolins, bind to carbohydrates commonly found on microorganisms' surfaces. During activation, fragments C3b and C4b are generated and they bind covalently to acceptor surfaces such as immune complexes, foreign, and host cells located on the vicinity of the activation site. On these surfaces, C3 and C5 convertases are formed which further lead to the formation of the membrane attack complex culminating with microorganism lysis. As a consequence of activation, particles opsonized with iC3b, C3b, and C4b are more efficiently internalized by neutrophils, monocytes, and macrophages once bound to complement receptors present on these cells' membranes. CR2 promotes activation and proliferation of B lymphocytes in the presence of C3d/C3dg fragments covalently bound to antigens inducing the production of antibodies. In addition, C3a and C5a fragments are important anaphylatoxins. They are also chemoattractant factors for inflammatory cells [reviewed in Ref. (7)]. In order to protect the host against self-damage, complement activation is tightly controlled at all stages of the cascade by several soluble and cell surface regulators. C1 inhibitor, Factor I (FI), Factor H (FH), and C4b-binding protein (C4BP) are soluble complement regulators whereas complement receptor type 1 (CR1 or CD35), membrane cofactor protein (MCP or CD46), decay accelerator factor (DAF or CD55), and CD59 are cell-anchored regulatory receptors [reviewed in Ref. (7, 8)].

COMPLEMENT EVASION STRATEGIES BY *LEPTOSPIRA*

Pathogens use a range of strategies to avoid complement attack, and *Leptospira* is no exception to this phenomenon. While pathogenic *Leptospira* strains resist complement-mediated killing, saprophyte *Leptospira* strains are highly susceptible to serum killing (9, 10). Concerning the group of leptospires of intermediate pathogenicity, such as *Leptospira licerasiae*, nothing is known about their response to complement. Pathogenic *Leptospira* escape from complement-mediated killing through: (i) recruitment of host complement regulators; (ii) acquisition of host proteases that cleave complement proteins on the bacterial surface; and (iii) secretion of proteases that inactivate complement in the *Leptospira* surroundings (**Figure 1**). These mechanisms are universal strategies employed by diverse pathogens including bacteria, viruses, and fungi to circumvent complement attack [reviewed in Ref. (11)].

Recruitment of Mammalian Host Complement Soluble Regulatory Proteins

Pathogenic *Leptospira* are potentially able to control all pathways of the complement system by acquiring soluble negative host regulators. Control of the AP is achieved by interaction of *Leptospira* with FH, a 155 kDa plasma glycoprotein [443 ± 106 μg/mL in human serum (12)] composed of 20 globular domains (termed complement control protein domains, CCPs). FH inhibits AP activation by preventing binding of Factor B (FB) to C3b, by accelerating the decay of the C3-convertase C3bBb and by acting as a cofactor for the cleavage of C3b by FI (13–15). Serum-resistant *Leptospira* strains bind three members of the FH family: FH itself, Factor H-like protein 1 (FHL-1), and Factor H-related protein 1 (FHR-1) (9, 16). Once bound to the *Leptospira* surface, FH remains functional and promotes FI-mediated cleavage of C3b, thus generating the iC3b fragment (9, 16). Moreover, *Leptospira* survival in FH-depleted serum was shown to be impaired by 60%, and reconstitution of this serum with purified FH up to physiological concentrations restored bacterial survival in a dose-dependent manner, further supporting a role for FH in *Leptospira* serum resistance (16). Control of the CP by pathogenic *Leptospira* is mediated by surface-bound C4BP, a 570 kDa plasma glycoprotein [335 ± 83 μg/ml (12)] that is found in three isoforms with different subunit composition. The major isoform, α7/β1, is a complex of seven α-chains and one β-chain. The other C4BP isoforms in plasma are α7/β0 and α6/β1. Each α-chain is comprised of eight CCPs, and the β-chain is comprised of three CCPs (17). C4BP inhibits CP and LP activation by interfering with the assembly and decay of the C3 convertase (C4bC2a) and by acting as a cofactor for FI in the proteolytic inactivation of C4b (18, 19). Both virulent and culture-attenuated *Leptospira* strains express ligands for C4BP, in contrast to non-pathogenic strains, which have been shown to bind insignificant amounts of this complement regulator (10). As expected, C4b is more efficiently cleaved by FI in the presence of C4BP bound to *Leptospira interrogans* virulent strains, which may probably explain their higher survival rate in normal human serum as compared to culture-attenuated

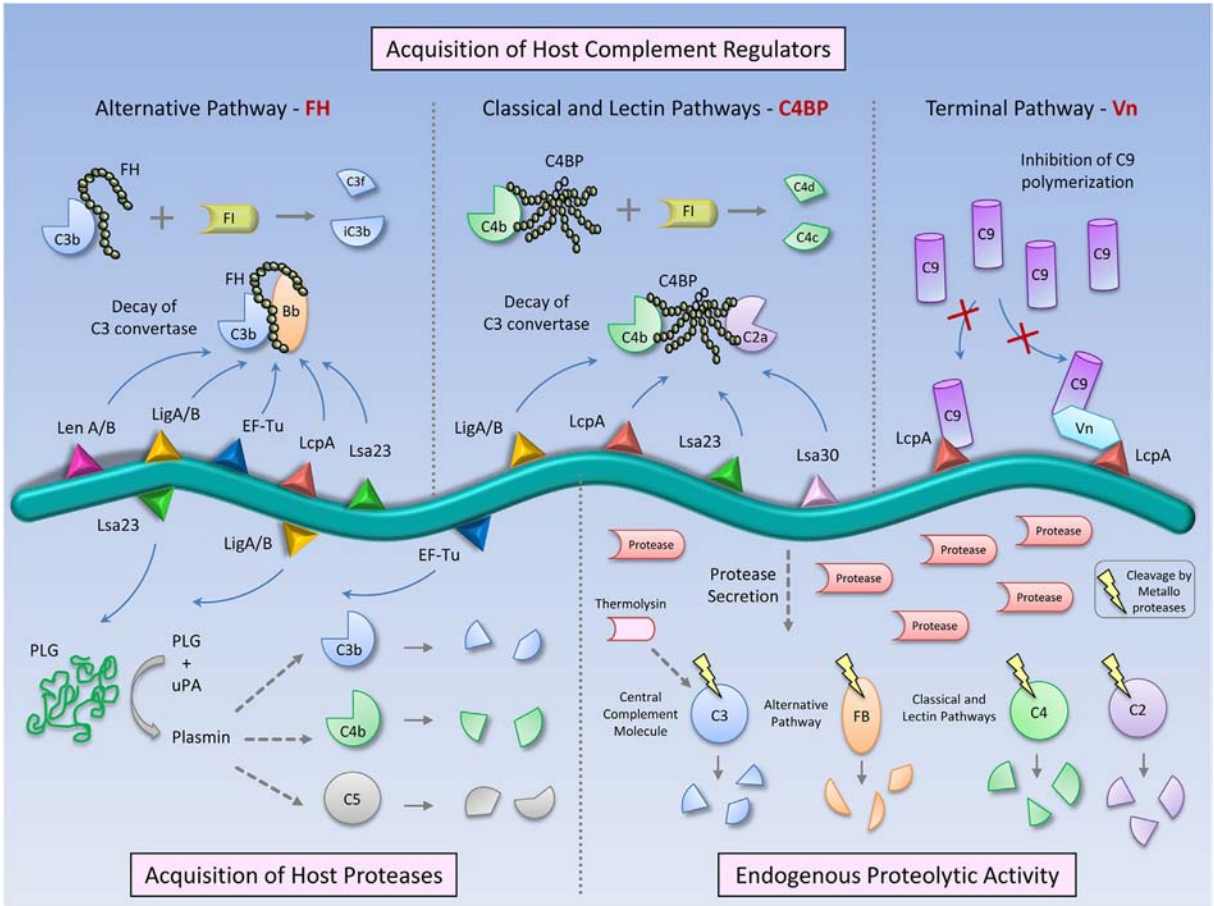

FIGURE 1 | **Complement evasion strategies of pathogenic *Leptospira***. To circumvent the complement system, *Leptospira* has evolved different immune evasion strategies: (i) acquisition of host soluble complement regulators: Factor H (FH)—AP regulator, C4b-binding protein (C4BP) —CP and LP regulator, and vitronectin (Vn) —terminal pathway regulator. FH and C4BP accelerate the decay of the C3 convertases (C3bBb and C4b2a, respectively) and act as cofactors for Factor I in C3b and C4b cleavages. Vn and the leptospiral protein LcpA bind C9 and inhibit its polymerization, thus potentially blocking MAC formation; (ii) acquisition of host proteases: pathogenic *Leptospira* binds plasminogen, which in the presence of activators, such as Urokinase-type plasminogen activator (uPA), is converted in the enzymatically active plasmin. This serine protease cleaves C3b, C4b, and C5, promoting a downregulation of complement activation on the *Leptospira* surface, and (iii) Direct inactivation of complement proteins by *Leptospira* endogenous proteases. Metalloproteases secreted by pathogenic *Leptospira* strains are able to cleave and inactivate the complement proteins: C3 (central complement molecule), Factor B (from AP), and C2 and C4 (CP and LP). Thermolysin is one of the proteases responsible for these cleavages, degrading C3. The combination of host-derived and endogenous factors from pathogenic *Leptospira* enables the bacteria to successfully establish infection and colonize target organs of the host.

strains (10). Leptospires also acquire vitronectin (Vn) on their surfaces (20). Vn is a glycoprotein that circulates in the bloodstream as a monomer [65–75 kDa, 104 ± 25 μg/mL (12)] or is deposited in the extracellular matrix (ECM) as a multimer that interacts with several macromolecular components, including glycosaminoglycans and collagens (21, 22). Vn plays multiple roles in many biological processes including the regulation of the terminal pathway of complement by inhibiting C5b7 complex formation and C9 polymerization. Once bound to the bacterial surface, it may protect the microorganism against lysis by impairing MAC formation. A number of strains belonging to different *Leptospira* species have been shown to interact with human Vn (20). Acquisition of this terminal pathway regulatory protein may assist *Leptospira* to evade complement attack.

Leptospira Ligands for Host Complement Regulators

Pathogenic *Leptospira* bind soluble host complement regulators *via* surface proteins and multiple ligands for those regulators have been described. The most extensively characterized complement evasion molecules from *Leptospira* are (i) leptospiral endostatin-like proteins A and B [LenA and LenB (23, 24)], (ii) *Leptospira* immunoglobulin-like (Lig) proteins A and B [LigA and LigB (16, 25)], and (iii) Leptospiral complement regulator-acquiring protein A [LcpA (26)]. All of these proteins have been shown to bind more than one complement regulator and seem to be involved not only in immune evasion but also in adhesion and invasion by interacting with ECM and plasma proteins such as plasminogen (PLG) (27).

Leptospira interrogans strains encode up to six distinct paralogous proteins called LenA–F, harboring domains that presumably share structural and functional similarities with mammalian endostatins (24). Two proteins of this family, LenA (formerly called LfhA and Lsa24) and LenB, have affinities for complement regulators. While LenA binds both FH and FHR-1, LenB has been shown to interact only with FH (23, 24).

LigA and LigB are multifunctional proteins capable of interacting with the ECM, cell lines, and complement regulators *in vitro*. The family of Lig proteins is composed of LigA, LigB, and LigC, which respectively consist of 13, 12, and 13 Ig-like domains. In certain *Leptospira* species *ligC* is a pseudogene (28). The *lig* genes are present only in pathogenic *Leptospira* species, and they are expressed during mammalian infection (28). Recombinant LigA and LigB bind FH, FHL-1, FHR-1, and C4BP, thus potentially allowing control of all complement activation pathways (16). FH CCP5 and CCP20 domains interact with both LigA and LigB (16). C4BP CCP4, CCP7, and CCP8 domains are involved in the interaction with both LigA and LigB (29). Fine mapping of the LigA and LigB domains involved in binding to C4BP has demonstrated that interactions occur through the bacterial immunoglobulin-like (Big) domains 7 and 8 (LigA7–8 and LigB7–8) of both LigA and LigB and also through LigB9–10 (29). As FH and C4BP do not compete for binding to Lig proteins, they probably have distinct binding sites on these molecules and may then interact with their targets simultaneously (16). It has been shown that ectopic LigB expression promotes survival of the saprophyte *Leptospira biflexa* in normal human serum (30). LigB binds C3b and C4b directly through repeats 9–11 (LigB9–11) and inhibits both the alternative and classical pathways in hemolytic assays with erythrocytes (30). Given the susceptibility of non-pathogenic *Leptospira* to the alternative pathway (9, 10), the increased resistance of *ligB*-transformed *L. biflexa* to complement killing may be attributed to the acquisition of C3b and FH by these bacteria (30). Further studies extended this observation by demonstrating that expression of both *ligA* and *ligB* genes enhances *L. biflexa*'s resistance to serum killing, as demonstrated by a reduced MAC deposition on *lig*-transformed *L. biflexa* compared to the wild type strain (31).

Pathogenic *Leptospira* species also bind host's negative complement regulators through a 20-kDa surface-exposed lipoprotein named LcpA. First described as a C4BP-interacting protein (26), LcpA was later shown to bind FH and Vn as well as the terminal pathway component C9 (20). Usually, microorganisms bind FH *via* a common site located inside CCP20 (32). LcpA is no exception to this rule, since a monoclonal antibody directed against CCP20 inhibited binding of FH to LcpA (20). CCP7 and CCP8 domains mediate the interaction of C4BP with LcpA (29). Both FH and C4BP have been shown to remain functional once bound to LcpA, thus being able to act as cofactors for FI (20, 26). LcpA also interferes with the terminal pathway of complement by binding to C9, a molecule that has a key role in MAC formation on bacterial cells. In the presence of LcpA, Zn^{2+}-induced C9 polymerization is inhibited *in vitro* and MAC formation on sheep erythrocytes is partially impaired, preventing cell lysis (20). Competitive binding assays indicate that LcpA interacts with C4BP, FH, and Vn through distinct sites (20).

Based on binding affinities, other *Leptospira* proteins have been shown to acquire complement regulators (**Table 1**). Lsa30 binds C4BP whereas Lsa23 binds both C4BP and FH (33, 34). Interestingly, the moonlighting protein EF-Tu, shown to be surface-exposed in *Leptospira*, also acquires the complement regulator FH (35).

Acquisition of Host Proteases That Cleave Complement Proteins on the *Leptospira* Surface

Proteolytic activity is a fundamental tool employed by diverse pathogens to both overcome tissue barriers and evade the immune system (37). Degradation of ECM components favors pathogen spreading and dissemination, while cleavage and inactivation of immune effector molecules dampen the host defense system, allowing an effective establishment of the infection (38, 39).

Pathogenic leptospires circumvent complement attack by the cleavage and inactivation of key complement molecules from the three activation pathways. The degradation of complement proteins may occur indirectly, using host-acquired proteases such as PLG, or directly, by the activity of endogenous proteases produced by pathogenic *Leptospira* strains, as discussed in the next section.

It is well-known that leptospires are able to bind human PLG (40, 41). PLG is a single-chain glycoprotein (92 kDa) that is a key component of the host fibrinolytic system. This proenzyme is found in plasma and extracellular fluids at concentrations of 180–200 μg/mL (42).

Although both saprophytic and pathogenic leptospires bind purified PLG, only pathogenic strains are able to acquire PLG from human plasma (40, 41). The interaction of PLG with *Leptospira* is mediated by bacterial membrane proteins (**Table 1**), and involves lysine residues, which are probably positioned at the PLG kringle domains. Another interesting finding is that

TABLE 1 | Host molecules that interact with *Leptospira* ligands to evade the complement system.

Host molecule	Ligands (*Leptospira* proteins)	Reference
Factor H (FH)	LenA and LenB (leptospiral endostatin-like proteins A and B)	(24)
	LigA and LigB (*Leptospira* immunoglobulin-like proteins A and B)	(16)
	EF-Tu (elongation factor Tu)	(35)
	LcpA (leptospiral complement regulator-acquiring protein A)	(20)
	Lsa23 (23 kDa adhesin)	(34)
C4b-binding protein	LcpA	(26)
	LigA and LigB	(16)
	Lsa30 (30 kDa adhesin)	(33)
	Lsa23 (23 kDa adhesin)	(34)
Vitronectin	LcpA	(20)
Plasminogen	EF-Tu	(35)
	Lsa23	(34)
	LigA and LigB	(36)

Leptospira cell integrity is preserved, since cellular growth is not impaired by PLG binding (40). Once bound to the *Leptospira* surface and in the presence of urokinase-type plasminogen activator (uPA), PLG is converted to enzymatically active plasmin (40, 41). Plasmin is a serine protease that cleaves diverse important biological substrates, including ECM proteins, like fibrinogen, and complement molecules, such as C3b and C5 (43). In this way, pathogenic leptospires coated with plasmin showed reduced deposition of C3b and IgG on their surface, which was probably related to proteolytic degradation of these molecules, potentially reducing opsonization (44). Furthermore, *L. interrogans* serovar Pomona also displayed enhanced survival in human serum when bound to plasmin (44), which reinforces its role in complement resistance.

Several *Leptospira* membrane proteins have been described as PLG ligands (33, 41, 45–56). However, only a few of them were indeed shown to directly interfere with complement activation: the elongation factor Tu [EF-Tu (35)]; LigA and LigB (36) and *Leptospira* 23 kDa surface adhesion [Lsa23 (34)], whose interactions with PLG resulted in the cleavage of C3b, C4b, and/or C5 (**Table 1**).

Secretion of Leptospiral Proteases That Directly Inactivate Complement

The *Leptospira* evasion strategies described until know focused on host molecules hijacked by the pathogen to inactivate complement on its surface. Recently, we demonstrated that pathogenic *Leptospira* can produce molecules that are able to directly interfere with the complement system, in a manner independent of the host machinery. It was observed that the culture supernatants of pathogenic, but not of saprophytic *Leptospira* strains, were able to specifically inhibit all the three activation pathways of complement. The inhibitory effect observed could be directly correlated to the proteolytic activity present in these culture supernatants. Indeed, the leptospiral proteases were able to target a wide range of substrates including C3, a key factor in the amplification of the complement cascade, FB from AP, C2, and C4, from CP and LP. These cleavages were observed both with purified complement proteins or normal human serum, which indicates that the leptospiral proteases exert their function in a physiological context and may contribute to bacterial virulence (57).

The proteolytic activity found exclusively in pathogenic *Leptospira* supernatants was almost completely abolished by 1.10-phenanthroline, indicating a major role of metalloproteases in the degradation of complement proteins. A recombinant metalloprotease from the thermolysin family, present only in *Leptospira* pathogenic species, seems to contribute to these cleavages, since it was able to degrade the central complement protein C3 (57).

The degradation and functional inactivation of complement is a key strategy for attenuating diverse immune responses that are dependent on the proper activation of this system (58). The secretion of proteases that directly cleave complement proteins may contribute to *Leptospira* immune evasion, as demonstrated for a wide range of other pathogens (59).

CONCLUDING REMARKS

Complement is a precisely regulated system composed of numerous specific factors that are activated in a cascade-like manner. This multifactorial cascade nature provides diverse targets for possible interferences by pathogen-derived evasion molecules (58). Most successful human pathogens have developed multiple parallel mechanisms of evading the complement system (60). *Leptospira*, which is a highly invasive spirochete, is a good example of a pathogen that employs diverse strategies to circumvent complement activation (**Figure 1**). The combination of host-derived and endogenous factors enables these spirochetes to successfully establish the infection and colonize target organs of the host. Therefore, *Leptospira* ligands of host regulators and secreted proteases constitute potential sites for immune interference, either as vaccine candidates or as targets for therapeutic agents in the development of new treatments and prophylactic approaches in leptospirosis.

AUTHOR CONTRIBUTIONS

TF participated in the drafting of the article and prepared the tables and figures. LI performed a critical revision and approved the final version to be published. AB participated in the drafting of the article and performed a critical revision of the final version.

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REVIEW
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Host Immune Evasion by Lyme and Relapsing Fever Borreliae: Findings to Lead Future Studies for *Borrelia miyamotoi*

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The emerging pathogen, *Borrelia miyamotoi*, is a relapsing fever spirochete vectored by the same species of *Ixodes* ticks that carry the causative agents of Lyme disease in the US, Europe, and Asia. Symptoms caused by infection with *B. miyamotoi* are similar to a relapsing fever infection. However, *B. miyamotoi* has adapted to different vectors and reservoirs, which could result in unique physiology, including immune evasion mechanisms. Lyme *Borrelia* utilize a combination of *Ixodes*-produced inhibitors and native proteins [i.e., factor H-binding proteins (FHBPs)/complement regulator-acquiring surface proteins, p43, BBK32, BGA66, BGA71, CD59-like protein] to inhibit complement, while some relapsing fever spirochetes use C4b-binding protein and likely *Ornithodoros*-produced inhibitors. To evade the humoral response, *Borrelia* utilize antigenic variation of either outer surface proteins (Osps) and the Vmp-like sequences (Vls) system (Lyme borreliae) or variable membrane proteins (Vmps, relapsing fever borreliae). *B. miyamotoi* possesses putative FHBPs and antigenic variation of Vmps has been demonstrated. This review summarizes and compares the common mechanisms utilized by Lyme and relapsing fever spirochetes, as well as the current state of understanding immune evasion by *B. miyamotoi*.

Keywords: *Borrelia miyamotoi*, spirochetes, relapsing fever, Lyme disease, complement, factor H, antigenic variation, immune response

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INTRODUCTION

Tick-borne diseases are among the top reported diseases to the US Centers for Disease Control and Prevention. This group of diseases include an array of viral, bacterial, and parasitic pathogens (e.g., Lyme disease, tick-borne relapsing fever, anaplasmosis, rickettsiosis, Powassan virus, tick-borne encephalitis virus, Colorado tick fever, Heartland virus, babesisosis) transmitted by the bite of certain species of hard and soft shell ticks from four genera (*Ixodes*, *Dermacentor*, *Amblyomma*, *Ornithodoros*) (1–3).

Intense research efforts are occurring worldwide in an attempt to understand, detect, control, treat, and eradicate these pathogens and their diseases. One step toward preventing and treating infectious diseases is to understand how pathogens evade host defenses to establish infection. Pathogenic Lyme and relapsing fever borreliae establish infection through one or more of the following mechanisms: physical barriers (e.g., slime layer of glycoproteins), migration to

immunoprivileged sites, and hijacking host processes [e.g., inactivation of complement with factor H-binding proteins (FHBPs)]. The mechanisms utilized by the emerging relapsing fever pathogen, *Borrelia miyamotoi*, are currently uncharacterized. Herein, we review some mechanisms Lyme and relapsing fever *Borrelia* utilize to inhibit and evade host complement and humoral immune responses and relate these to mechanisms that might be used by *B. miyamotoi*.

Lyme Disease and Tick-Borne Relapsing Fever: Spirochetes, Vectors, and Diseases

Approximately 20 closely related pathogenic and non-pathogenic species of *Borrelia* form the *B. burgdorferi sensu lato* complex. Of these 20 species, at least five are classified as causative agents of Lyme disease (US: *B. burgdorferi sensu stricto*; Europe and Asia: *B. afzelii*, *B. garinii*, *B. spielmanii*, *B. bavariensis*) (4–10). Lyme borreliae are carried and transmitted by several species of *Ixodes* ticks (Ixodidae, hard shell) though the most common species are *I. scapularis* and *I. pacificus* in the US and *I. ricinus* and *I. persulcatus* in Europe and Asia.

Species of *Ornithodoros* ticks (Argasidae, soft shell) carry and transmit relapsing fever spirochetes. Several *Borrelia* spp. cause relapsing fever but *B. hermsii*, *B. turicatae*, *B. crocidurae*, *B. hispanica*, *B. duttonii* are more commonly encountered.

While the general rule is *Ixodes* transmit spirochetes of the *B. burgdorferi s.l.* complex and *Ornithodoros* transmit relapsing fever borreliae, there are exceptions. *B. recurrentis* is a louse-borne relapsing fever spirochete endemic mainly to sub-Saharan Africa. *B. theileri* causes bovine borreliosis and is transmitted by *Rhipicephalus microplus*, a hard shell tick that parasitizes livestock (11). *B. lonestari* and *B. turcica*, spirochetes genetically similar to relapsing fever borreliae, are found in the hard shell ticks, *Amblyomma americanum* and *Hyalomma aegyptium*, respectively (12, 13). Finally, *B. miyamotoi* is a relapsing fever spirochete vectored by the same *Ixodes* spp. that transmit species of the *B. burgdorferi s.l.* complex.

In terms of disease, several tick-borne diseases are associated with non-specific symptoms (i.e., a possibly self-limiting "influenza-like" illness characterized by malaise, fatigue, aches, fever, and chills) (**Table 1**). While infection with *Borrelia* spp. generally results in similar symptoms, some species-specific symptoms can arise (14, 15). Erythema migrans and arthritis are commonly associated with a *B. burgdorferi s.s.* infection but rarely with *B. afzelii* infection, which more commonly manifests in the dermatological condition, acrodermatitis chronica atrophicans. *B. garinii* is more commonly associated with neurological symptoms. Relapsing fever is characterized by recurring spirochetemia corresponding to recurrent episodes of high fever not seen with *B. burgdorferi s.l.* infections.

Borrelia miyamotoi

Borrelia miyamotoi s.s. strains were first isolated and cultured in Japan in 1995 from *I. persulcatus* and the blood of *Apodemus argenteus* (small Japanese field mouse) (16). Since this initial isolation, *B. miyamotoi* DNA has been identified in *I. scapularis*, *I. pacificus*, *I. ricinus*, and *I. persulcatus* across the Northern hemisphere (17–84). *B. miyamotoi* DNA has also been identified in humans with a suspected tick-borne disease; while *B. miyamotoi* is associated with disease, teasing out the details of an infection with this spirochete has proven difficult for several reasons (85–92).

First, diagnoses based on serology can be problematic and lead to false-negative diagnoses. Several antigens, including 4 of the 10 assayed in a Lyme Western blot, are shared among Lyme, relapsing fever, and *B. miyamotoi* spirochetes (93, 94). Although Lyme and relapsing fever *Borrelia* cause different diseases and occupy different niches, species in this genus share a high degree of genetic homology (95–98). Therefore, some degree of cross-reactivity occurs between *B. miyamotoi* antibodies and *B. burgdorferi s.l.* antigens (91).

Second, an adequate and appropriate immunocompetent animal model to study *B. miyamotoi* infection is only now beginning to take shape. Without an optimal animal model to identify characteristic symptoms and pathologies, we are left to interpret and extrapolate symptoms from complex human cases where disease pathology can be complicated by underlying or unreported medical conditions or coinfections. Previous attempts to infect immunocompetent *Peromyscus leucopus* mice (a common reservoir for *B. burgdorferi* in the US) with *B. miyamotoi s.l.* LB-2001 (US strain) had been unsuccessful leaving severe combined immune deficient (SCID) mice as the only available animal model (17). SCID mice infected with *B. miyamotoi* exhibit sustained spirochetemia, similar to infection with relapsing fever spirochetes (99). Recently, however, Wagemakers et al. (100) were able to successfully infect immunocompetent C3H/HeN mice with LB-2001 and demonstrate spirochetemia 2 days post infection (dpi). Three of the eight mice infected exhibited relapsing spirochetemia at 5 and 6 dpi. More studies are required to determine the optimal animal model for *B. miyamotoi* infection (101–103).

Finally, *B. miyamotoi*'s status as a pathogen has only recently been established. The first confirmed human infections were reported in Russia in 2011 (85) with more cases subsequently described in the US, Europe, and Japan (86–91, 104–107).

B. miyamotoi Infection and Disease

Much of the data available on *B. miyamotoi* infections come from retrospective serological analyses of banked patient samples, which provide valuable epidemiological information but can lack the detailed patient history or clinical aspects required to sufficiently define a disease. The available data depict an illness, currently termed *B. miyamotoi* disease or hard tick-borne relapsing fever that is similar to relapsing fever.

The patients described by Platonov et al. (85) reported tick bites, developed moderate or severe disease, and were hospitalized as a precautionary measure against more severe tick-borne diseases, particularly viral tick-borne encephalitis. In total, 46 patients were classified as having a confirmed *B. miyamotoi* infection with no detected current *B. burgdorferi s.l.* coinfection by PCR. Sera from all 46 patients reacted with whole cell lysates of *B. burgdorferi*, *B. afzelii*, and *B. garinii*. The most common

TABLE 1 | Overview of *Borrelia* diseases.

Disease	Vector[a]	Causative agent(s)[a]	Clinical symptom(s)
Lyme disease	*I. scapularis* (USA) *I. pacificus* (USA) *I. ricinus* (Europe, Asia) *I. persulcatus* (Europe, Asia)	*B. burgdorferi sensu stricto* (USA) *B. afzelii* (Europe, Asia) *B. bavariensis* (Europe, Asia; formerly *B. garinii* OspA serotype 4) *B. garinii* (Europe, Asia) *B. spielmanii* (Europe, Asia)	Symptom onset after exposure: early stage generally 3–30 days Influenza-like (e.g., mild fever, malaise, myalgia/arthralgia; *B. burgdorferi s.s.*) Erythema migrans (*B. burgdorferi s.s.*, *B. afzelii*) Symptom onset after exposure: late stage generally >30 days Arthritis Acrodermatitis chronica atrophicans (*B. afzelii*) Neurological (Lyme neuroborreliosis, e.g., numbness, Bell's palsy, stiffness of neck, declining memory, sleep disorders; *B. burgdorferi s.s.*, *B. bavariensis*)
Tick-borne relapsing fever	*O. hermsi* *O. turicata* *O. parkeri* *O. moubata*	*B. hermsii* *B. turicatae* *B. parkerii* *B. duttonii*	Symptom onset: ca. 7 days Influenza-like Recurring high fever Headache Myalgia Arthritis Approximately 3–10 febrile episodes (relapses) occur; mortality rates are variable but generally less than 5%
Hard tick-borne relapsing fever/*Borrelia miyamotoi* disease	*I. scapularis* *I pacificus* *I ricinus* *I. persulcatus*	*B. miyamotoi*	Symptom onset after exposure: ca. 15 days (85) Influenza-like Most common: Fever Malaise Headache Chills Arthritis/arthralgia Meningoencephalitis (immunocompromised patients) Rare (less than 10% of patients): Rash/erythema migrans Gastrointestinal (e.g., vomiting, nausea, diarrhea) Cardiac/respiratory (shortness of breath) Neurological (e.g., dizziness, confusion) Stiffness of neck
Louse-borne relapsing fever	*P. humanus humanus*	*B. recurrentis*	Symptom onset after exposure: ca. 4–8 days Recurring high fever Malaise Headache Chills Meningism Myalgia Nausea Vomiting Approximately 3–5 relapses occur; mortality rate varies greatly (30–70% without treatment during outbreaks)

[a]*Commonly encountered and studied vectors and causative agents are listed.*

symptoms were fever, headache, and malaise or fatigue (**Table 2**). Five patients reported recurrent fever with an average duration of 3.4 days, and 9 days between relapses, similar to infections with relapsing fever spirochetes. All patients were successfully treated with ceftriaxone or doxycycline.

A similar series of cases were reported in the US in 2015 (91). Ninety-seven of 11,515 patient samples submitted by clinical laboratories for tick-borne disease analysis were PCR-positive for *B. miyamotoi*. Patients with known or suspected *B. burgdorferi* coinfection or a history of Lyme disease were omitted from further analysis. Fever, headache, and malaise were commonly reported among US patients with two patients reporting recurrent fever (**Table 2**). The duration of febrile episodes and the time between relapses were not reported. Spirochetemia was noted in US patients but was either not reported or documented in Russian patients. Strikingly, a rash or single erythema migrans of unknown origin was reported in 8 and 9% of US and Russian patients, respectively.

Some symptoms were different between the US and Russia patients, which suggest clinical manifestations vary by *B. miyamotoi* strain, similar to that seen with *B. burgdorferi s.l.* strains (**Table 2**) (108). Arthralgia was more common in US (76%) compared to Russian patients (28%), and leukopenia, thrombocytopenia, and elevated liver enzymes were found in some US patients but in none of the Russian patients. These differences may be explained by genetic differences between American

TABLE 2 | Comparison of symptoms reported from US (91) and Russian (85) patients.

Symptom	US ($n = 51$)	Russia ($n = 46$)
Fever, chills	96%	98%, 35%[a]
Headache	96%[b]	89%
Myalgia	84%	59%
Arthralgia	76%	28%
Malaise/fatigue	82%	98%
Rash/EM[c]	8%	9%
Gastrointestinal symptoms[d]	6%	30% (nausea) 7% (vomiting)
Respiratory symptoms[e]	6%	na[f]
Neurological symptoms (dizziness, confusion, vertigo)	8%	na
Stiff neck	na	2%

[a]*Fever and chills were reported in separate categories.*
[b]*Authors noted in most patients the headaches were severe.*
[c]*US patients were described as having a rash. Russian patients were noted for having a single erythema migrans.*
[d]*For US patients, GI symptoms included nausea, abdominal pain, diarrhea, anorexia. For Russian patients, GI symptoms included nausea and vomiting.*
[e]*Labored breathing or short of breath.*
[f]*Not reported.*

and Asian type *B. miyamotoi*. Genetic analyses of *B. miyamotoi* isolates have revealed heterogeneity between, and a high degree of homology among, strains from the US (American types; *I. scapularis, I. pacificus*), Europe (European type; *I. ricinus*), and Asia (Asian type; *I. persulcatus*) (59, 109).

Detailed case reports are currently available for nine patients in the US, Europe, and Japan. For immunocompetent patients, symptoms were similar to those observed in the aforementioned studies (e.g., fever, headache, malaise) (86, 89, 90, 106, 107). One US patient did not seek treatment, providing additional evidence that *B. miyamotoi* can result in recurrent fever and be self-resolving, similar to other relapsing fever infections (92, 110). This patient experienced two episodes of fever separated by 3 weeks, significantly longer than in other *B. miyamotoi* or relapsing fever patients, with each episode lasting 4–5 days, on par with *B. miyamotoi* or relapsing fever patients.

The pathology of *B. miyamotoi* infection is dramatically different in immunocompromised patients, specifically those treated for non-Hodgkin's lymphoma (NHL) with rituximab. Two patients treated with rituximab for NHL, one from the US (88) and one from the Netherlands (87), with reported recent tick bites developed meningoencephalitis. Motile spirochetes were detected in cerebral spinal fluid in both cases. Interestingly, *glpQ* was amplified and sequenced from both patient's samples yet no anti-GlpQ antibodies were detected in the blood or cerebral spinal fluid of the European patient. IgM against *B. burgdorferi* was negative for both patients. Neither patient reported any of the commonly associated symptoms of a *B. miyamotoi* infection (e.g., fever, headache, myalgia, malaise). Instead, both patients exhibited neurological symptoms (cognitive processing defects, disturbed gait). A third patient from Germany, also treated with rituximab for NHL, developed Lyme neuroborreliosis-like symptoms (dizziness, vomiting, and headache) (111).

THE COMPLEMENT SYSTEM

The complement system, composed of the classical, lectin, and alternative branches, is a crucial component of the immune system (**Figure 1**). Components of complement continuously circulate in blood making complement one of the first lines of defense against pathogens. Complement initiates an immune response by: (1) triggering phagocytosis through opsonization, (2) mediating inflammation through the release of chemotactic peptides, and (3) lysing cells *via* the membrane attack complex (MAC, also called the terminal complement complex or TCC) (**Figure 1**) (112).

The classical pathway is generally mediated by non-specific antibodies, immunoglobulin G (IgG) or IgM, binding a bacterial antigen. Importantly, recent studies have shown *Borrelia*-specific IgM is produced by a subset of B cells during infection and plays a crucial role in clearing *Borrelia* (113–121). The C1 complex, composed of C1q, C1r, and C1s, forms upon recognition of bound IgG or IgM. C1 cleaves C2 (C2a, C2b) and C4 (C4a, C4b). C4b covalently binds the target's cell surface and complexes with C2a to form C3 convertase, which cleaves C3 into C3a and C3b. C3b covalently binds the target cell surface (opsonization, facilitates phagocytosis of foreign cells and cellular debris), while C3a remains soluble to act as a mediator of inflammation. C5 convertase forms when C3b binds C3 convertase. Not surprisingly, C5 convertase cleaves C5 into C5a, a soluble inflammatory mediator, and C5b. C5b binds the target cell surface and C6 forming C5b6, which binds C7 (C5b–7) then C8. The C5b–8 complex binds C9 (C5b–9) and facilitates polymerization of several additional C9 proteins. These polymerized C9 proteins form the transmembrane pore of the MAC allowing an influx of extracellular fluid and subsequent lysis of the target cell.

The lectin pathway is very similar to the classical pathway, differing only in the initiation steps. The lectin pathway is typically initiated through mannose-binding lectins, a group of pattern recognition receptors (PRRs) on host cells, binding specific sets of carbohydrates on foreign cells (pathogen-associated molecular patterns, PAMPs). The lectin and classical pathways converge at the cleavage of C2 and C4 by different mechanisms. In the lectin pathway, C4 and C2 cleavage occurs through mannose-binding lectin-associated serine proteases (112).

Like the classical and lectin pathways, the alternative pathway forms a C3 convertase, C5 convertase, and results in the formation of the MAC. Unlike the classical and lectin pathways, the alternative pathway may not require antibody–antigen or PAMP–PRR interactions for activation. Rather, this pathway is initiated through hydrolysis of C3 to C3(H_2O), which is thought to occur continuously at low levels. The pathway is propagated through interactions with bacterial antigens or a lack of host surface markers (e.g., sialic acid, glycosaminoglycans, sulfated polysaccharides) (112).

Factor B, after binding C3(H_2O), is cleaved by factor D into Ba and Bb resulting in C3(H_2O)Bb, the fluid-phase C3 convertase (cleaves C3 to C3a and C3b). C3b binds the bacterial cell surface where it complexes with additional factor B. Factor D again cleaves factor B, which results in the second, predominant and

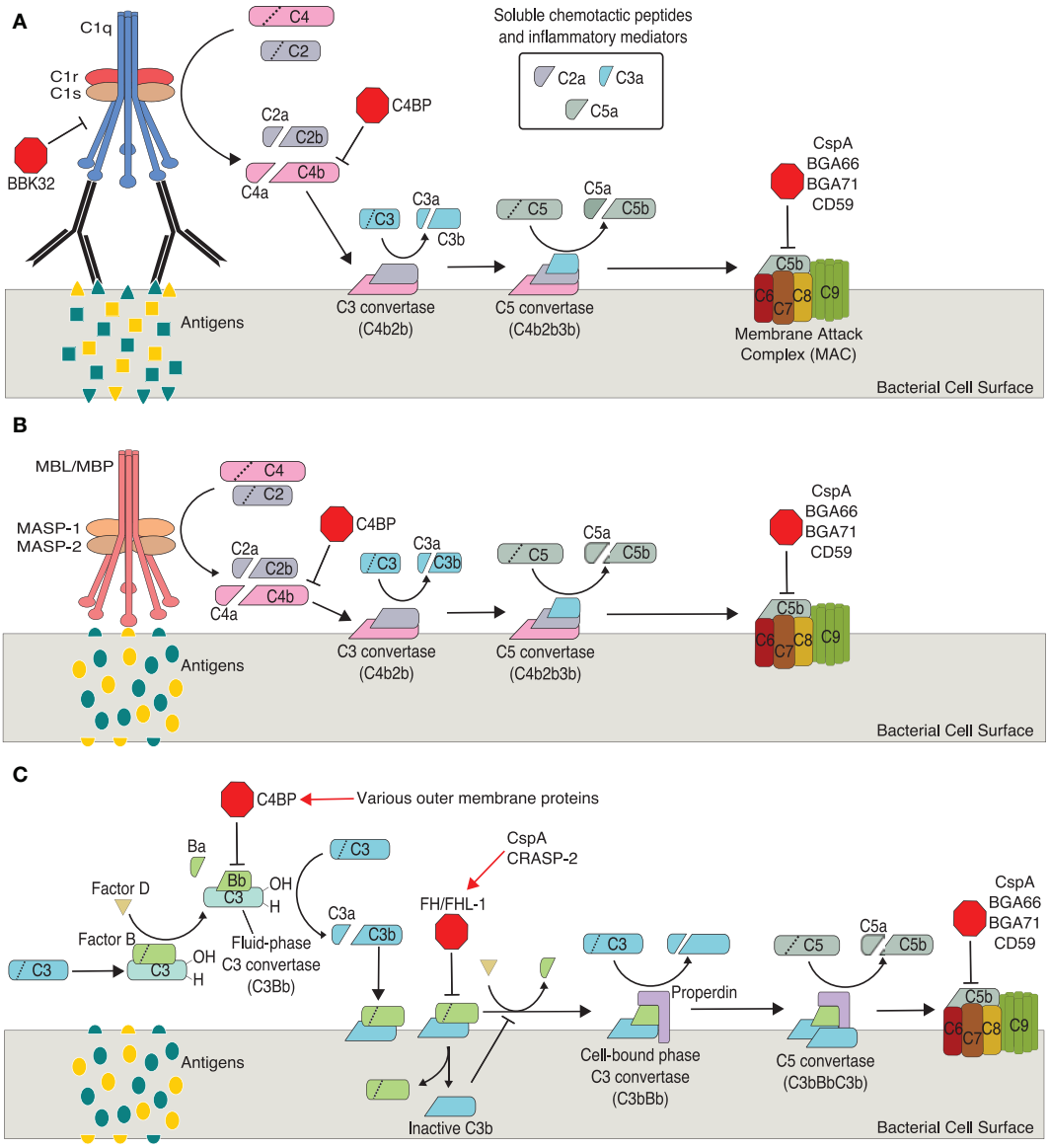

FIGURE 1 | Activation and regulation of complement pathways relevant to *Borrelia* spp. infection. **(A)** Classical pathway. **(B)** Mannose–lectin pathway. **(C)** Alternative pathway. Points of complement inhibition utilized by *Borrelia* spp. are indicated by red octagons. Red arrows indicate borrelial proteins that interact with host regulatory proteins.

cell-bound C3 convertase (C3bBb). This cell-bound C3 convertase is stabilized by properdin (C3bBbP). Binding of additional C3b to C3 convertase results in the formation of C5 convertase (C3bBbC3b), which cleaves C5 and initiates the formation of the MAC as described above.

INHIBITION OF THE MAMMALIAN COMPLEMENT SYSTEM BY *Borrelia* AND *Ixodes*

Regulation of complement is critical for survival of host cells (122, 123). Numerous mechanisms have evolved in hosts to prevent aberrant activation of complement on host cells including the use of complement regulatory factors and host cell surface components (e.g., sialic acid). Pathogens that inhibit host complement use mechanisms that are inextricably tied to host regulatory processes. *Borrelia* use several native proteins to inhibit complement [i.e., FHBPs or complement regulator-acquiring surface proteins (CRASPs), p43, BBK32, BGA66, BGA71, CD59-like protein] (124). The following sections focus on the complement regulators factor H (FH), factor H-like protein-1 (FHL-1), factor I (FI), C4b-binding protein (C4BP), and CD59.

At least for Lyme borreliae, resistance to complement varies by strain and species (125–130). Roughly, 10% of *B. burgdorferi s.s.* are serum resistant, and 90% are intermediately resistant

to serum; 75% of *B. afzelii* isolates are resistant, and 25% are intermediate; 100% of *B. garinii* isolates are sensitive (specifically, OspA serotypes 3, 5, 6, 7); *B. bavariensis* (formerly *B. garinii* OspA serotype 4) is intermediately resistant. To the best of our knowledge, similar comparisons of multiple strains and species have not been published for relapsing fever *Borrelia*, though complement resistance is not universal among relapsing fever species among the strains observed. Resistance to complement is important for the transmission, survival, and dissemination of some *Borrelia* spp. in mammalian and rodent hosts and reservoirs (131). Many *Borrelia* spp., particularly Lyme borreliae, are masters of complement evasion due to the native anticomplement proteins some possess and the ability all infectious strains possess to co-opt tick and host complement regulatory proteins.

FH, FHL-1, and FI

Factor H is an ubiquitous 150-kDa soluble protein produced by diverse cell types throughout the human body (e.g., hepatic cells, fibroblasts, monocytes, endothelial cells) (132). FH consists of 20 short consensus repeats, while FHL-1 is a truncated variant of FH consisting of the FH N-terminal short consensus repeats 1 through 7. Both FH and FHL-1 are major direct regulators of the alternative complement pathway. In addition, FH and FHL-1 can directly regulate the classical and lectin pathways, though the regulatory roles in these pathways are minor compared to other classical and lectin regulatory mechanisms. Regulation is achieved through the recognition of self and non-self molecules *via* domains located on the C- and N-terminals, respectively (133–135). The C-terminal discriminates self from non-self through interactions with sialic acids, glycosaminoglycans, and sulfated polysaccharides, which are typically found only on host cells (136–140). FH binds self molecules with high affinity to prevent activation of complement. FH regulates the classical and lectin pathways by acting as a co-factor for FI. In this capacity, FH facilitates the serine protease activity of FI in cleaving and inactivating C3b. The alternative pathway is regulated through FH targeting factor Bb, which prevents the formation of fluid-phase C3 convertase and promotes decay ("decay acceleration activity") of C3 and C5 convertases (141). For comprehensive reviews of FH and FHL-1, see Ref. (132, 141, 142).

FHBPs and CRASPs

Interactions with FH are the best-studied mechanism for *Borrelia* complement inactivation, and complement resistance is correlated with binding FH (143). *Borrelia* spp. bind FH and/or FHL-1 through various native proteins collectively termed FHBPs or CRASPs (125, 144, 145). CRASPs can be grouped by their ability to bind only FH or both FH and FHL-1 as well as the species specificity of binding (that is, whether a FHBP can bind FH from only one or several host species) (125, 145): CRASP-1 (CspA) and CRASP-2 (CspZ) bind both FH and FHL-1, while CRASP-3 (ErpP), CRASP-4 (ErpC), and CRASP-5 (ErpA) bind only FH. CRASPs bind soluble FH and maintain it in an active conformation thereby allowing FH to inhibit completion of the complement response (i.e., MAC formation).

Several relapsing fever spirochetes bind FH *in vitro* (125, 146–151). Two FHBPs, FhbA and BhCRASP-1, have been identified in *B. hermsii* strains YOR and HS1, respectively (152, 153). However, binding FH is not as important for relapsing fever spirochetes to establish infection as it is for Lyme disease *Borrelia* (154, 155). Further supporting the non-essential nature of binding FH, Woodman et al. (154) found that despite FhbA being surface exposed and strongly binding FH *in vitro*, only 16% of *B. hermsii* recovered from the blood of infected mice had detectable levels of bound FH.

C4b-Binding Protein

C4b-binding protein (C4BP) has regulatory roles in all three pathways, though is the major regulator of the classical and lectin pathways. C4BP facilitates inactivation of C4b (classical, lectin) and fluid-phase C3b (alternative) by binding C4b, displacing C2a, and facilitating FI-mediated inactivation of C3 and C5 convertases (156).

Some Lyme and relapsing fever *Borrelia* spp. bind human and various animal C4BP (143, 148, 149, 157, 158). A comprehensive analysis identified outer surface proteins (Osps) associated with C4BP including OspA, Vlps, variable membrane proteins (Vmps), and several unidentified Osps (159). However, other studies have observed no binding of C4BP by *Borrelia* spp (143, 150, 160). These contradictory data may be due to differences in experimental design including the use of different strains, growth medium, temperatures, growth phases, and the use of recombinant versus native human C4BP. A putative C4BP receptor, p43, has been identified in *B. burgdorferi s.l.* (157). The relapsing fever spirochetes *B. recurrentis* and *B. duttonii* produce CihC, a surface lipoprotein homologous in sequence and function to fibronectin-binding proteins of other relapsing fever spirochetes, which also binds C4BP (148).

FHBP, C4BP, and *Borrelia* Niche

Resistance to complement is positively correlated to the infectivity of some *Borrelia* strains (130). With a higher resistance to complement, the more likely a bacterium can survive, disseminate, and proliferate. Co-opting tick proteins will protect spirochetes during the initial stages of transmission and dissemination but sustained dissemination requires *Borrelia* to resist complement *via* its own native mechanisms.

This leads to the question of how complement sensitive strains can cause infection. An interesting hypothesis was developed regarding complement resistance and spirochete niche when a relationship was noted between binding of the complement inhibitors, C4BP and FH (157, 158, 161). Neurotropic strains (e.g., *B. bavariensis*, *B. garinii*, *B. turicatae*, *B. duttonii*, and to a lesser extent *B. hermsii*) do not have to be highly resistant to complement in immunoprivileged sites, such as the central nervous system. Finding neurotropic species strongly bind C4BP and very weakly bind FH and FHL-1, while species that are not neurotropic bind C4BP but preferentially bind FH and/or FHL-1 supports this hypothesis (157). Alitalo et al. (162) did find *B. garinii* strains isolated from neuroborreliosis patients not only express FHBPs not expressed by strains cultured *in vitro*

for an extended time but the FHBPs also bind FH. This implies complement resistance, though this was not reported and one of the isolates (LU59) was later reported to be highly but not completely sensitive to complement (163). It is possible that strong binding of FH is an artifact seen *in vitro*, similar to that observed with relapsing fever spirochetes (see FHBPs and CRASPs). Thus, binding FH is not required for neurotropic strains. Perhaps C4BP is sufficient to prevent complement activation during migration of neurotropic species from the site of inoculation to immunoprivileged sites. On the other hand, binding FH may be important for neurotropic strains to resist complement during migration and the incomplete sensitivity observed by Sandholm et al. (158) may be due to *in vitro* culturing resulting in the population losing its ability to bind FH. It could also be that neither C4BP nor FHBPs play a role in complement-sensitive borreliae disseminating and a novel mechanism is utilized by complement-sensitive strains.

CD59-Like Protein

Little information is available regarding the CD59-like protein of *B. burgdorferi*. Pausa et al. (164) demonstrated an increase in serum sensitivity and MAC formation in a serum-resistant *B. burgdorferi* isolate treated with anti-CD59 antibodies compared to the control treated *B. burgdorferi* and the serum-sensitive *B. garinii* isolate. In eukaryotic cells, CD59 is a surface-exposed membrane protein that prevents C9 polymerization and thus the formation of the MAC (19, 20). Still, it is not clear *Borrelia* possesses a protein homologous to mammalian or rodent CD59. While human anti-CD59 antibodies bound a surface-exposed integral membrane protein (29 kDa), this protein has never been identified though several known borrelial proteins can and have been ruled out based on molecular weight (e.g., BGA66, BGA71, OspA, OspB, OspC) (124). Given the demonstrated complement resistance conferred by this unknown borrelial protein, more attention should be given to identifying and clarifying the role this protein plays in complement resistance.

Complement Inhibition by *Ixodes* and *Ornithodoros* Salivary Proteins

A large number of proteins with a vast array of functions have been identified in the saliva of feeding *Ixodes* spp. with more being identified and characterized (165–169). While the details and mechanisms for some of these proteins remain to be elucidated, the beneficial nature of *Ixodes* salivary proteins to spirochete transmission and survival has been established (170–177). *Ixodes* saliva contains adaptive and innate immunomodulatory and anticomplement proteins (165, 178–183). A recent study demonstrated changes in the salivary protein profile over the course of a feeding, which has implications for the efficacy of the host immune response at the feeding pit and for transmitting spirochetes (168). Currently, several members of the anticomplement family of proteins have been characterized from *I. scapularis*, *I. ricinus*, and *I. persulcatus* including Salp15, Salp20, Isac, Irac I, Irac II, and Ixac-B1, -2, -3, -4, -5.

Salp15 is able to inhibit both adaptive and innate immune responses (184, 185). Salp15 binds OspC, which both serum-resistant and serum-sensitive *B. burgdorferi s.l.* produce, to inhibit deposition of the MAC and block the recognition and binding of antibodies to OspC (172, 186–188). In addition, Salp15 expression increases when ticks are infected with *B. burgdorferi* (172). Interestingly, mice passively immunized with anti-Salp15 antibodies were protected from infection with *B. burgdorferi* (189).

Salp20 inhibits the alternative complement pathway through binding properdin, which prevents stabilization of C3 convertase and propagation of the alternative pathway (183, 190, 191). In addition, Salp20 enhances the activity of FH to inhibit the alternative pathway (183). Incubating a serum-sensitive *B. garinii* strain with Salp20 protected the strain from complement activation and lysis (190). The mechanism(s) by which Salp20 confer(s) protection to *B. garinii* is unknown.

The Isac-like family of proteins include *Ixodes scapularis* anticomplement (Isac), *I. ricinus* anticomplement (Irac I), Irac II, and Ixac-B1 through -5 (*I. ricinus* anticomplement). Proteins in this family are similar in function to Salp20 (180, 182, 192). Inhibition of the alternative complement pathway is achieved through targeting C3 convertase *via* interactions with properdin, as Salp20 does, and by preventing factor B from binding C3b or displacing factor B from C3 convertase.

Ornithodoros salivary gland extracts also possess proteins that inhibit the host immune response (193). To date, however, one complement inhibitor has been identified and characterized from one *Ornithodoros* spp. *O. moubata*, found in Africa, is the vector of the relapsing fever spirochete *B. duttonii* (194). *O. moubata* complement inhibitor (OmCI) is a lipocalin that binds to and prevents cleavage of C5 (195, 196). OmCI was found to be effective at inhibiting C5 cleavage in different mammalian and rodent hosts (196). It is unknown if OmCI protects *B. duttonii* or if homologous proteins are found in other *Ornithodoros* spp.

EVASION BY *Borrelia* OF THE MAMMALIAN HUMORAL IMMUNE RESPONSE BY SURFACE PROTEIN VARIATION

Evasion of complement is undoubtedly a vital mechanism to ensure spirochetes survive and establish infection. However, *Borrelia* will elicit an humoral immune response, and there are clear roles for this immune response in controlling and preventing *Borrelia* infection (113–118, 197, 198). These responses form the basis of an intense research effort for effective Lyme vaccines. Fortunately for *Borrelia*, they are quite adept at evading the host humoral response primarily through variation of surface-exposed proteins. Lyme disease *Borrelia* possess Osps and variable membrane protein-like [Vmp-like sequences (Vls)] proteins, while relapsing fever *Borrelia* possess Vmps (includes variable large and variable small proteins) (199–203). Some species hide antigens by inducing erythrocyte rosetting (204).

Osps and VlsE of Lyme *Borrelia*

The Osps, particularly OspC, are one of the most studied group of *Borrelia* proteins. For comprehensive reviews of *Borrelia* Osps, see Ref. (145, 205, 206). OspE and OspF are discussed above with FHBPs. Less is known about OspA, a protein predominantly involved in uptake and survival in ticks. OspA is immunogenic and able to block antibody binding to another surface-exposed protein, P66 (207, 208).

OspC has diverse roles, many of which are essential for transmission from *Ixodes* and establishing infection in mammals (209–216). These studies were key in demonstrating that *ospC* is upregulated during the early stages of infection, downregulated after infection has been established, and deleting or overexpressing *ospC* results in spirochetes that are quickly cleared from a host.

A handful of immune evasion functions have been identified for OspC. As discussed above, OspC protects *Borrelia* by binding Salp15. OspC also prevents phagocytosis by macrophages (216). In addition, several OspC types have been identified and correlated with a strains ability to establish infection in hosts and reservoirs (217–222). However, as each Osp is present as a single-copy locus, genetic variation is seen at the population level. That is, outside of random mutation or horizontal gene transfer events, a single spirochete cannot produce different OspC types *in situ*.

In contrast, the Vls system can change the expressed surface antigen *in situ* (**Figure 2**). Antigenic recombination of VlsE is

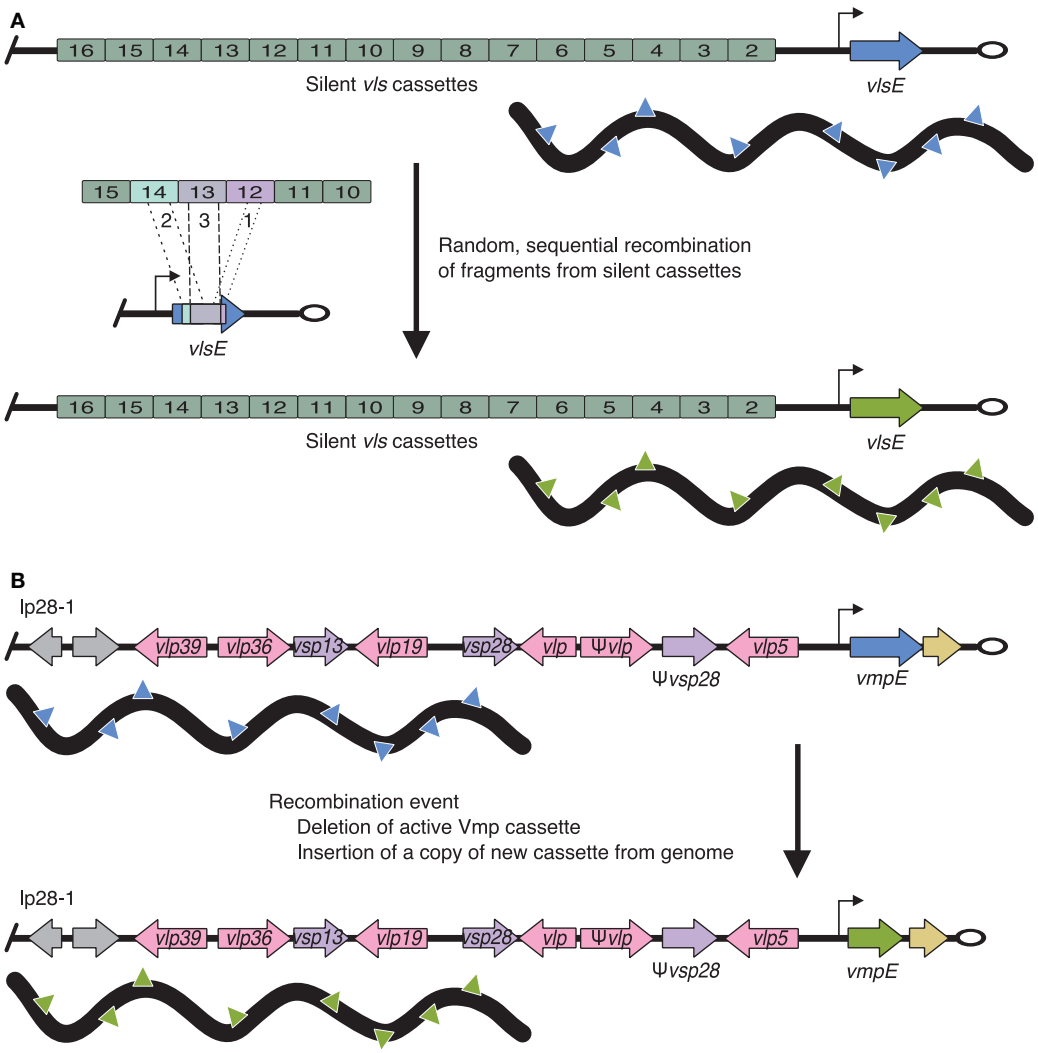

FIGURE 2 | Antigenic variation of Lyme borreliae VlsE and relapsing fever borreliae Vmp systems. (A) VlsE: the expression locus (*vlsE*) is located near the telomere (open oval) of linear plasmid (lp) 28-1 (blue or green arrow, promoter is indicated by a black arrow). Silent *vls* cassettes are located upstream and in the opposite orientation of *vlsE*. Antigenic variation occurs through the random and sequential insertion of silent cassette fragments (labeled 1, 2, and 3). **(B)** *vlp* (pink arrows) and *vsp* (purple arrows) cassettes are located throughout the genome on lp28-1, 28-2, 28-3, 28-4, and 32-1. The expression locus (blue or green arrow, promoter is indicated by a black arrow) is found on lp28-1 near the telomere (open oval). Changing the expressed Vmp cassette is achieved through deletion of the current cassette (blue arrow) followed by insertion of a copy of a new cassette (green arrow *via* recombination events) resulting in a change in the expressed Vmp on the surface of the bacterium (denoted by blue or green triangles, respectively). Gray arrows indicate non-Vmp ORFs; tan arrows indicate downstream homology sequences (DHS, sequences found throughout the genome and required for mapping recombination events at the Vmp expression locus).

important in maintaining infection in mammals and helps Lyme *Borrelia* evade the humoral immune response (223–236). The Vls system is composed of approximately 16 *vls* cassettes (the exact number varies by strain) and one expression locus, *vlsE*. All of the identified *vls* cassettes are located on the same plasmid (lp28-1) in close proximity to but in the opposite direction of *vlsE*. Expression at *vlsE* occurs through the random recombination of segments of multiple *vls* cassettes rather than recombination of an entire, single *vls* cassette. Thus, recombination events result in thousands of unique VlsE variants, all approximately 36 kDa.

Vmps of Relapsing Fever *Borrelia*

Variable membrane proteins, a system similar to Vls, are one of the best characterized immune evasion mechanisms (199, 237–240). *B. hermsii* has approximately 60 unique and promoterless *vmp* cassettes (i.e., silent cassettes) scattered throughout its genome and one promoter-driven *vmp* expression locus (**Figure 2**). A single *vmp* cassette is expressed when the entire cassette is moved to the expression locus.

The majority of spirochetes are cleared from the host through specific anti-Vmp IgM antibodies raised against the predominantly expressed Vmps, which results in a significant decrease in spirochete load (from approximately 10^5–$10^7$to $<10^4$ spirochetes/mL). The remaining spirochetes consist of a small population expressing different cassettes. Since the host has not raised a strong antibody response to these non-dominantly expressed Vmps, this minority population of spirochetes can proliferate to high concentrations until an antibody response is mounted and the majority of spirochetes are once again cleared. This cycle of *vmp* conversion, peaking spirochete loads, and antibody-mediated clearing repeats a minimum of two times resulting in the characteristic symptoms of a relapsing fever illness.

MECHANISMS OF IMMUNE EVASION BY *B. miyamotoi*: WHERE WE ARE

Given the genetic similarity of *B. miyamotoi* to relapsing fever spirochetes, it is likely *B. miyamotoi* utilizes some homologous mechanisms to evade host immune responses. While *B. miyamotoi* is resistant to complement *in vitro* (241, 242), complement inactivation is not required for relapsing fever spirochetes to establish infection. OspE homologs have been identified in *B. miyamotoi* FR64b (isolated from the blood of *A. argenteus*); however, McDowell et al. were unable to demonstrate FH-binding (125). This suggests, as is the case for relapsing fever spirochetes, inactivation of complement may not be required to resolve spirochetemia during infection with *B. miyamotoi* (115, 243).

Instead, it appears *B. miyamotoi* utilizes a Vmp system (244), and Wagemakers et al. (100) recently demonstrated antigenic variation of Vmps in *B. miyamotoi*. C3H/HeN mice infected with *B. miyamotoi* LB-2001 produced anti-Vsp1 IgM and IgG antibodies that were effective in clearing the initial spirochetemic peak of *B. miyamotoi* from SCID mice. Despite this clearing, a second spirochetemic relapse occurred. Analyses of the secondary *B. miyamotoi* population revealed expression of *vlpC2*, not *vsp1*, as would be expected in the case of antigenic variation. They also noted *vlpC2* was present in the initial *B. miyamotoi* population in a much lower prevalence compared to *vsp1*.

MECHANISMS OF IMMUNE EVASION BY *B. miyamotoi*: WHERE WE NEED TO BE

Even though *B. miyamotoi* is genetically similar to relapsing fever spirochetes, it has evolved and exists in different vectors (*Ixodes* not *Ornithodoros*) with different enzootic cycles and different co-pathogens compared to relapsing fever spirochetes. We should not assume *B. miyamotoi* utilizes the same set of mechanisms as other relapsing fever spirochetes. *B. miyamotoi* may use a combination of relapsing fever and Lyme *Borrelia* mechanisms as well as completely novel mechanisms.

The role of IgM in clearing *B. miyamotoi* has not been demonstrated. As discussed above, IgM is key in clearing relapsing fever infections. During *B. hermsii* infections, IgM targets FhbA and other surface proteins (113). IgM likely is important in clearing *B. miyamotoi*. All immunocompromised patients diagnosed with a *B. miyamotoi* infection developed meningoencephalitis. A shared factor with these patients has been treatment with rituximab, a monoclonal anti-CD20 antibody targeting IgM-producing CD20-positive B cells. Depletion of B cells may explain how *B. miyamotoi* is able to migrate to the CNS and causes meningoencephalitis in patients treated with rituximab. The presence of unknown complement inhibitors, however, could contribute to the complement resistance of *B. miyamotoi* and may be useful in establishing infection (241, 242).

The effects of tick saliva on *B. miyamotoi* survival have not yet been studied. However, being vectored by *Ixodes*, *B. miyamotoi* likely takes advantage of the protective proteins in tick saliva. In addition, understanding interactions between host, vector, and pathogen will aid in the development of Lyme and relapsing fever prevention strategies and thus requires more attention.

CONCLUDING REMARKS

Infection with *B. miyamotoi* in immunocompetent patients generally results in non-specific symptoms (e.g., headache, malaise), recurrent fever, and spirochetemia characteristic of relapsing fever. However, additional symptoms characteristic of relapsing fever have not been demonstrated, namely rapid symptom onset with a crisis event suggesting *B. miyamotoi* infection is not synonymous with relapsing fever and is rather a relapsing fever-like illness (90). This should not be surprising given the different lifestyle of *B. miyamotoi* compared to the vast majority of relapsing fever spirochetes.

The ability to evade the immune response is important for any successful pathogen but many of the mechanisms *B. miyamotoi*

utilizes remain undiscovered. Hindered by the lack of a robust animal model, the relatively long-standing inability to culture *in vitro*, and being unaware of its pathogenicity, our understanding of *B. miyamotoi* is still in its infancy. However, we are making large strides forward with recent advances in culture techniques, animals models, physicians actively considering *B. miyamotoi* infection, as well as a growing wealth of epidemiological data that will allow us to clarify the details of infection, genetics, and physiology of this emerging pathogen.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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Tick Humoral Responses: Marching to the Beat of a Different Drummer

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Ticks transmit a variety of human pathogens, including *Borrelia burgdorferi*, the etiological agent of Lyme disease. Multiple pathogens that are transmitted simultaneously, termed "coinfections," are of increasing importance and can affect disease outcome in a host. Arthropod immunity is central to pathogen acquisition and transmission by the tick. Pattern recognition receptors recognize pathogen-associated molecular patterns and induce humoral responses through the Toll and Immune Deficiency (IMD) pathways. Comparative analyses between insects and ticks reveal that while the Toll pathway is conserved, the IMD network exhibits a high degree of variability. This indicates that major differences in humoral immunity exist between insects and ticks. While many variables can affect immunity, one of the major forces that shape immune outcomes is the microbiota. In light of this, we discuss how the presence of commensal bacteria, symbionts and/or coinfections can lead to altered immune responses in the tick that impact pathogen persistence and subsequent transmission. By investigating non-insect arthropod immunity, we will not only better comprehend tick biology, but also unravel the intricate effects that pathogen coinfections have on vector competence and tick-borne disease transmission.

Keywords: tick-borne diseases, Lyme disease, vector, ticks, humoral immunity

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INTRODUCTION

Ticks are increasingly important disease vectors that transmit a variety of pathogens relevant to public and veterinary health (de la Fuente et al., 2008; Stromdahl and Hickling, 2012; Hartemink and Takken, 2016; Kernif et al., 2016). The most prevalent vector-borne illness in the Northern hemisphere, Lyme disease, is transmitted by *Ixodes* spp. ticks and is caused by the spirochete *Borrelia* spp. (Mather and Mather, 1990). Ticks are first colonized by pathogens when they take a bloodmeal from an infected host. The microbes will then lie dormant throughout digestion and molting. Subsequent transmission to a new vertebrate host occurs during the second bloodmeal, where pathogens migrate to the salivary glands and are injected along with saliva. Multiple obstacles within the vector can impact pathogen survival and persistence (Liu and Bonnet, 2014), including the arthropod's immune system. This is the foremost defense against invading microbes and largely impacts the ability of an arthropod to be a competent vector for pathogens (Hillyer et al., 2003; Garver et al., 2009; Blumberg et al., 2013).

Arthropod immunity lacks adaptive components and is limited to innate processes, which can be categorized as either cellular or humoral (Ganesan et al., 2011; Buchon et al., 2014; Myllymaki et al., 2014). Humoral immunity involves innate signaling cascades, such as the Toll and Immune Deficiency (IMD) pathways. Immune defenses are triggered by pathogen-associated

molecular patterns (PAMPs), which are sensed by pattern recognition receptors (PRRs) (Hillyer, 2016). Both pathogenic and commensal bacteria can elicit immune responses in arthropods, which makes the composition of the microbiota a significant force in determining vector competence as well (Cirimotich et al., 2011). For the purposes of this article, the microbiome/microbiota will be defined as all microorganisms present in the arthropod including symbionts, commensals and pathogens.

Although insect immunity has been heavily studied and is well understood, owing to the model organism *Drosophila melanogaster*, recent data demonstrates that non-insect arthropods, such as ticks, are significantly different (Palmer and Jiggins, 2015; Gulia-Nuss et al., 2016; Rosa et al., 2016; Shaw et al., 2017). Genome sequencing data shows that ticks lack several genes involved in innate immunity when compared to insects including some PRRs, pathway signaling molecules and antimicrobial peptides (AMPs) (Severo et al., 2013; Smith and Pal, 2014; Palmer and Jiggins, 2015; Bechsgaard et al., 2016; Gulia-Nuss et al., 2016; Rosa et al., 2016). Nevertheless, immune pathways within ticks remain functional, suggesting that there are undiscovered principles governing non-insect arthropod immunity (Kopacek et al., 1999; Sonenshine et al., 2002; Simser et al., 2004; Pelc et al., 2014). Herein, we will discuss the current understanding of tick humoral signaling pathways in the context of disease transmission both with and without confounding factors, such as coinfections and the microbiota.

HUMORAL IMMUNITY

Two of the best studied immune signaling cascades in arthropod immunity are the Toll and IMD pathways. Both are initiated by distinct PAMPs and orchestrate the production of microbiocidal AMPs (Hillyer, 2016). The Toll pathway responds primarily to Gram-positive bacteria and fungi whereas the IMD pathway recognizes Gram-negative bacteria (Hillyer, 2016). Herein, we will describe our current understanding of tick humoral immunity in comparison to insects.

The Toll Pathway

In *Drosophila*, Lysine-type peptidoglycan from the cell wall of Gram-positive bacteria is recognized by peptidoglycan recognition receptor proteins (PGRPs)-SA. β1-3-glucan from fungi is sensed by Gram-negative binding proteins (GNBPs) (Michel et al., 2001; Kanagawa et al., 2011) (**Figure 1**). Most of the components that comprise the Toll pathway in insects are conserved in the tick genome, although there are a few deviations (**Figure 1**; **Table 1**) (Palmer and Jiggins, 2015; Bechsgaard et al., 2016). For example, there are eight Toll receptors found in *Drosophila,* whereas only four have been identified in the *Ixodes scapularis* genome (Palmer and Jiggins, 2015). *I. scapularis* ticks also lack genes encoding *GNBPs* (Palmer and Jiggins, 2015; Gulia-Nuss et al., 2016). Despite the reduction in receptor repertoire, evidence for functional Toll signaling in ticks exists. *In vitro* challenge of *Rhipicephalus microplus* with *Enterobacter cloacae*, *Micrococcus luteus* and *Saccharomyces cerevisiae* lead to upregulation of *toll*, *myD88*, *tube*, *pelle*, and *cactus* suggesting pathway functionality (Rosa et al., 2016).

Drosophila transcriptional regulators controlled by the Toll pathway, Dif and Dorsal, regulate the expression of *defensin* and other AMPs (Meng et al., 1999). Interestingly, instances of cooperation between transcription factors have been described (Meng et al., 1999). Optimal induction of *defensin* was reported when the IMD pathway-regulated transcription factor, Relish, formed heterodimers with Dif or Dorsal (Han and Ip, 1999). These experiments were performed *in vitro* with stably transfected cell lines and thus the *in vivo* relevance is unclear, but suggests interesting potential for defenses orchestrated by multiple immune pathways. Ticks also produce several Defensin-like AMPs (Johns et al., 2001b; Sonenshine et al., 2002; Ceraul et al., 2003, 2007; Lai et al., 2004; Hynes et al., 2005; Zhou et al., 2007; Wang and Zhu, 2011; Chrudimska et al., 2014; Pelc et al., 2014). Although the mechanism of *defensin* regulation in ticks is not characterized, the highly conserved nature of the Toll pathway suggests that it may act similarly to insects. Moreover, tick Defensins are secreted in response to both Gram-positive and negative bacteria, suggesting that there may be a similar mechanism of cross-talk in non-insect arthropods (Sonenshine et al., 2002).

The IMD Pathway

Diaminopimelic acid (DAP)-type peptidoglycan from Gram-negative bacteria stimulates the IMD pathway in *Drosophila*, which is recognized by both transmembrane and soluble PGRPs (Boutros et al., 2002; Hillyer, 2016). Ticks lack several key components of the IMD pathway such as transmembrane PGRPs, *imd, dFADD,* and IMD pathway-specific AMPs (**Table 1**; **Figure 1**) (Severo et al., 2013; Palmer and Jiggins, 2015; Gulia-Nuss et al., 2016; Rosa et al., 2016). Despite lacking key components, the IMD pathway is functional in ticks (Shaw et al., 2017). The *I. scapularis* Relish is activated in response to *Anaplasma phagocytophilum* infection and knocking down regulatory components from the IMD pathway (*relish*, *capsar*, *uev1a,* and *bendless*) lead to altered pathogen burden levels with both *A. phagocytophilum* and *Borrelia burgdorferi* (Shaw et al., 2017). A separate study also showed that bacterial infection of *R. microplus* lead to transcriptional upregulation of IMD signaling components (*tak1*, *tab2*, *ikk*β, *ikk*γ, and *relish*) (Rosa et al., 2016). Taken together, these studies provide evidence for a functional IMD pathway in ticks.

Drosophila PGRP-LC and PGRP-LE are IMD pathway receptors (Kaneko et al., 2006) and PGRP-SD is an IMD co-receptor (Iatsenko et al., 2016). Transmembrane PGRP-LC and soluble PGRP-LE multimerize after binding to DAP-type peptidoglycan and initiate signaling by recruiting IMD to the RIP Homotypic Interaction Motif (RHIM) (**Figure 1**) (Kaneko et al., 2006). PGRP-SD, initially thought to activate Toll signaling (Bischoff et al., 2004), elicits the IMD pathway by interacting with PGRP-LC (Iatsenko et al., 2016) and DAP-type peptidoglycan (Leone et al., 2008). Although there are four encoded *PGRPs* in the *I. scapularis* genome, none are predicted to be transmembrane proteins or to have the IMD-interacting RHIM domain (Palmer and Jiggins, 2015). This is consistent with the lack of *imd* in the

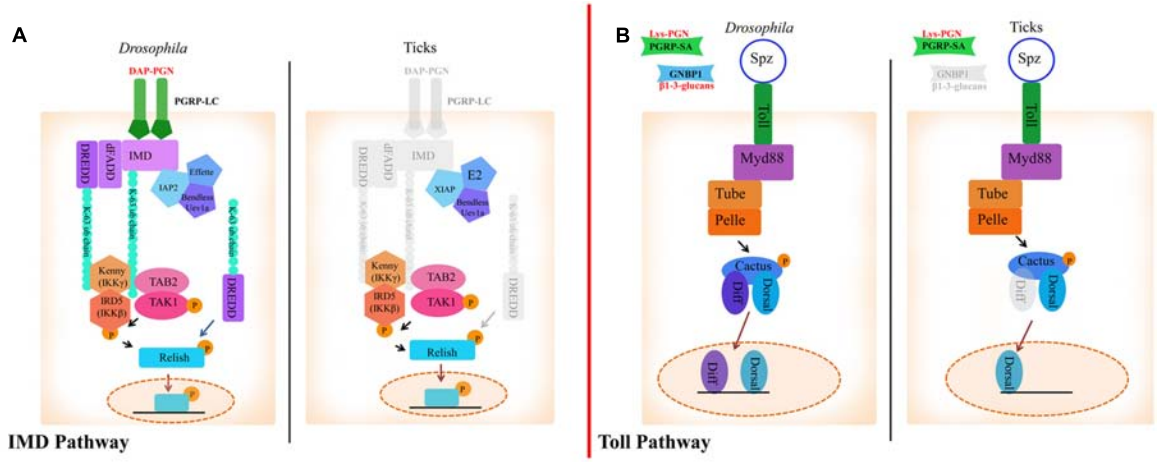

FIGURE 1 | The Immune Deficiency (IMD) and Toll pathways in *Drosophila* and ticks. (A) Activation of the IMD pathway in *Drosophila* is initiated by PGRP-LC binding to diaminopimelic acid (DAP)-type peptidoglycan. This leads to IMD, dFADD, and DREDD recruitment. IMD is cleaved by DREDD, exposing an ubiquitylation site and is polyubiquitylated by IAP2, Effete, Uev1a and Bendless in a K63-dependent manner. K-63 polyubiquitin chains are believed to serve as the recruiters for the proteins TAB2, TAK1, and IKK (IKKγ and IKKβ), which transfer a phosphate group to Relish. Relish is then cleaved by DREDD, removing the C-terminal ankyrin repeats. The N-terminal portion of Relish is translocated to the nucleus where it induces the transcription of AMPs (Vandenabeele and Bertrand, 2012). In ticks, transmembrane PGRPs, IMD, dFADD and possibly DREDD are missing (shaded gray). XIAP is suggested to regulate the IMD pathway in ticks through direct interaction with Bendless (Shaw et al., 2017). **(B)** In *Drosophila*, the Toll pathway is activated by PGRPs and GNBPs binding to Lysine-type peptidoglycan or β1-3-glucan, respectively. PAMP binding to PRRs leads to activation of ModSP (Modular Serine Protease) and Grass in the extracellular milieu. Spz is then cleaved and binds to Toll receptors. Following Spz binding, MyD88 dimers interface with the Toll receptor and recruit Tube, an adaptor molecule that interacts with the protein kinase Pelle. Cactus is then phosphorylated and degraded, which leads to translocation of Dif (Dorsal-related immunity factor) and/or Dorsal to the nucleus and AMP upregulation (Lindsay and Wasserman, 2014). The tick genome encodes all components of the Toll pathway, with the exception of *GNBPs* and *dif*.

genome, suggesting an alternative mode of pathway activation (**Figure 1**) (Palmer and Jiggins, 2015; Bechsgaard et al., 2016). The role of secreted PGRPs in ticks is unknown, although a recent study showed that silencing the soluble *I. scapularis PGRPs* did not have a significant effect on *A. phagocytophilum* colonization (Shaw et al., 2017).

K63-dependent polyubiquitylation of IMD and dDREDD (Death related ced-3/Nedd2-like caspase) by the E3 ubiquitin ligase, inhibitor of apoptosis protein 2 (IAP2), is necessary for signal transduction in *Drosophila* (Paquette et al., 2010; Meinander et al., 2012). A different E3 ubiquitin ligase in ticks, X-linked inhibitor of apoptosis (XIAP), has been shown to influence *A. phagocytophilum* burden (Severo et al., 2013) by interfacing with the IMD pathway (Severo et al., 2013; Shaw et al., 2017). XIAP physically interacts with the IMD pathway E2 ubiquitin conjugating enzyme, Bendless, and carries out K63-dependent polyubiquitylation together with Uev1a (Shaw et al., 2017). Moreover, double knockdown of *bendless-uev1a* heterodimers and *xiap* lead to increased colonization by both *A. phagocytophilum* and *B. burgdorferi*, suggesting a defect in pathogen control (Shaw et al., 2017).

In addition to alternative signaling modes, there is evidence that PAMPs other than DAP-type peptidoglycan can trigger the IMD signaling cascade. Reports of virus and parasite-induced IMD pathway activation in insects lend support to this hypothesis (Baton et al., 2009; Costa et al., 2009). In ticks, the IMD circuitry senses infection-derived lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-34 phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyl diacylglycerol (PODAG), and leads to Relish activation (Shaw et al., 2017). Moreover, priming ticks with these lipids induced protection against *A. phagocytophilum* and *A. marginale* infection both *in vitro* and *in vivo*, respectively (Shaw et al., 2017). These findings coupled with the lack of transmembrane PGRPs and key signaling molecules suggest that a non-canonical IMD pathway exists in ticks.

OTHER IMMUNE SIGNALING PATHWAYS

The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway is not part of the humoral innate response in insects, but does have a role in immunity through crosstalk with IMD and Toll signaling (Myllymaki and Ramet, 2014). The JAK/STAT pathway is activated by the receptor Dome through recognition of the cytokine signaling molecule, Unpaired (Upd) (Harrison et al., 1998; Brown et al., 2001). This interaction results in phosphorylation of Hop proteins and translocation of Stat92E to the nucleus, which stimulates expression of cytokines and members of the *tot* family (Harrison et al., 1995; Agaisse et al., 2003; Bach et al., 2003; Lamiable et al., 2016). The *I. scapularis* JAK/STAT pathway is important for the control of *A. phagocytophilum* and regulates the expression of a gene family that encodes 5.3 kDa antimicrobial peptides (Liu et al., 2012). Comparative analysis demonstrates that the JAK/STAT pathway is well conserved between ticks and *Drosophila* (Palmer and Jiggins, 2015; Bechsgaard et al., 2016), with the exception of *upd* (Liu et al., 2012; Rosa et al., 2016).

TABLE 1 | Arthropod humoral networks.

Pathway	Component	Insects			Crustacea		Arachnids		
		Drosophila[α]	*Anopheles*[β,ε]	Pea aphid[θ]	*Daphnia* spp.[α,ε]	Shrimp[ζ]	Mites[α]	*Ixodes* spp.[α,δ]	*Rhipicephalus* spp.[δ]
Toll Pathway	PGRP	+	+	−	−	−	+	+	+
	GNBP	+	+	+	+	−	−	−	−
	Spz	+	+	+	+	+	+	+	+
	Toll	+	+	+	+	+	+	+	+
	MyD88	+	+	+	+	+	+	+	+
	Tube	+	+	+	−	−	−	+	+
	Pelle	+	+	+	+	+	+	+	+
	Dif/Dorsal	+	+	+	+	+	+	+	+
	Cactus	+	+	+	+	+	+	+	+
IMD pathway	Transmembrane PGRPs	+	+	−	−	−	−	−	−
	Soluble PGRPs	+	+	−	−	−	+	+	+
	IMD	+	+	−	+	+	−	−	−
	dFADD	+	+	−	+	−	−	−	−
	DREDD	+	+	−	+	−	−	−	−
	IAP2	+	+	+	+	+	+	+	+
	Bendless	+	+	+	+	+	+	+	+
	Uev1a	+	+	+	+	+	+	+	+
	Effette	+	+	+	+	+	+	+	+
	XIAP	+	+	+	+	+	+	+	+
	TAB2	+	−	+	−	−	−	+	+
	TAK1	+	+	+	+	−	+	+	+
	IKKγ	+	+	−	+	−	−	+	+
	IKKα/β	+	+	+	+	+	+	+	+
	Relish	+	+	−	+	+	+	+	+
	Caspar	+	+	+	+	+	+	+	+
	Caudal	+	+	+	+	+	+	+	+

α = *(Palmer and Jiggins, 2015)*; β = *(Waterhouse et al., 2007)*; θ = *(Gerardo et al., 2010)*; δ = *(Rosa et al., 2016)*; ε = *(McTaggart et al., 2009)*; ζ = *(Liu et al., 2009; Li and Xiang, 2013; Udompetcharaporn et al., 2014)*.

Beyond pathogen control, the JAK/STAT pathway has an important role in physiological maintenance. *Drosophila* midgut homeostasis is influenced by the microbiota, which is regulated by phagocytic cells and the IMD pathway. This in turn impacts JAK/STAT signaling (Guillou et al., 2016). Mutation of either the CD36 phagocytic receptor, Croquemort, or Relish causes overexpression of Upd3 and dysregulated gut integrity, leading to increased mortality (Guillou et al., 2016). The mechanistic details involved in JAK/STAT activation in ticks are currently unknown, although the absence of *upd* is intriguing. A recent study showed that mammalian-derived interferon (IFN)-γ, present in the bloodmeal, stimulated the tick JAK/STAT pathway (Smith et al., 2016). This cytokine cross-talk upregulated the tick Rho-like GTPase (IGTPase) and induced expression of domesticated amidase effector (DAE2), an AMP homologous to eukaryotic effectors that hydrolyzes bacterial peptidoglycans (Chou et al., 2015; Smith et al., 2016). This is an interesting example of cross-species cytokine signaling and could indicate that midgut homeostasis in ticks and the microbiota are influenced by mammalian-derived signaling molecules.

THE MICROBIOME

Non-symbiotic and Symbiotic Commensals

The microbiome is comprised of commensal bacteria in the gut and other endosymbionts (Narasimhan and Fikrig, 2015). Ticks harbor less complex microbial communities, likely due to their blood-only diet, than other vectors that are not exclusively hematophagous, such as mosquitoes (Hawlena et al., 2013; Clayton et al., 2015; Rynkiewicz et al., 2015). *Proteobacteria, Actinobacteria, Enterobacter, Sphingobacterium, Firmicutes, Pseudomonas,* and *Bacteroidetes* have all been associated with ticks, although bacterial composition varies depending on geographic region and sex (Van Treuren et al., 2015). Interestingly, there is evidence that the microbiota impacts the arthropod through involvement with the immune system (Hawlena et al., 2013). Commensal bacteria stimulate gut epithelium renewal through JAK/STAT signaling in *Drosophila* (Buchon et al., 2009). Similarly, the tick microbiota also impacts midgut epithelium and peritrophic membrane integrity (Narasimhan et al., 2014; Narasimhan and Fikrig, 2015).

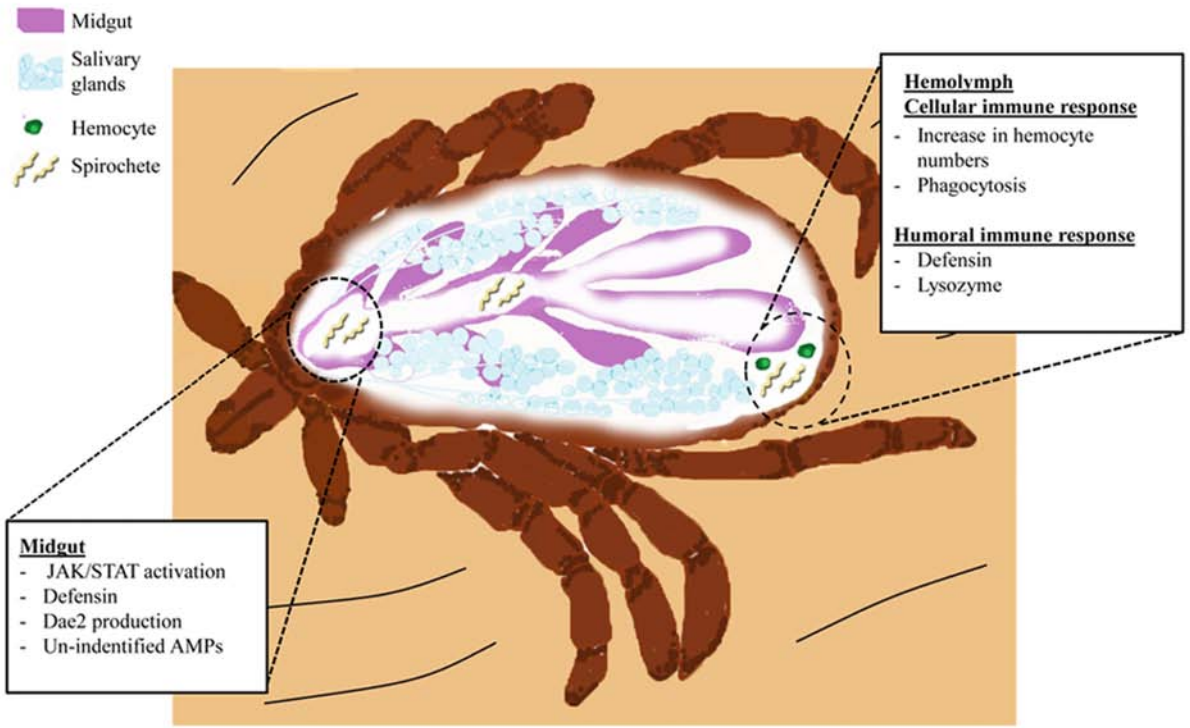

FIGURE 2 | The *I. scapularis* response to *B. burgdorferi* infection. Spirochetes (light yellow) enter the tick midgut (purple) during blood feeding. Spirochetes interact with gut tissues and trigger activation of the JAK/STAT pathway. Induction of JAK/STAT signaling and possibly other pathways leads to AMP production (Defensins and DAE2). Spirochete migration into the hemolymph elicits cellular and humoral immunity. Cellular responses include increased prevalence of hemocytes (green) and initiation of phagocytosis. Humoral immunity results in the secretion of Defensins (originating from hemocytes and the fat body) and Lysozyme (hemocytes) into the hemolymph (Johns et al., 2001b; Ceraul et al., 2003, 2007). Niche-specific immune responses, such as those originating from the salivary glands (light blue structures), remain elusive.

Although blood is a nutrient-rich source, it lacks some metabolites that are essential for survival. Endosymbiotic relationships can provide these nutrients and have been observed in many hematophagous arthropods including tsetse flies, bed bugs, lice, reduviid bugs and ticks (Rio et al., 2016). For example, a *Coxiella*-like endosymbiont provides vitamins and co-factors to *Amblyomma americanum* ticks and is required for adequate fecundity (Smith et al., 2015). A combination of mechanisms is likely used to ensure balance between the arthropod and endosymbiont. The arthropod host must control endosymbiont numbers to avoid over stimulation of immune responses and/or nutrient deprivation. In contrast, endosymbiotic bacteria must evade or suppress immune recognition to avoid clearance (Herren and Lemaitre, 2011; Masson et al., 2015; Shokal et al., 2016). Limited information is known about these relationships, owing to the difficult nature of *in vitro* symbiont cultivation, although a few studies have been reported (Kurtti et al., 2015, 2016). For example, the intracellular *Dermacentor andersoni* endosymbiont, *R. peacockii,* is 150-fold more resistant to AMPs than extracellular bacteria, illustrating a mechanism of immune tolerance (Baldridge et al., 2005). Avoidance mechanisms remain largely understudied, but likely vary depending on the endosymbiont and tick host species.

Pathogen Coinfection

Simultaneous colonization by multiple pathogens is termed "coinfection" and is becoming a major health concern worldwide (Steiner et al., 2008; Schulze et al., 2013). In Europe, over half of all surveyed *I. ricinus* ticks are coinfected (Moutailler et al., 2016), with the most prevalent instances occurring in areas that are forested and endemic for Lyme disease (Swanson et al., 2006). Coinfections can increase the severity of illness, as demonstrated with babesiosis and Lyme disease (Diuk-Wasser et al., 2016). Moreover, simultaneous infection of *Peromyscus leucopus* mice with the parasite, *Babesia microti*, and *B. burgdorferi* increased the number of parasites acquired by ticks during a bloodmeal. This was likely due to heightened parasitemia in the mouse during coinfection (Diuk-Wasser et al., 2016). Conflicting reports have been published about coinfections with *B. burgdorferi* and *A. phagocytophilum*. One study reported no observable differences in acquisition and transmission with *I. scapularis* ticks (Levin and Fish, 2000), whereas another demonstrated that *B. burgdorferi* burden in ticks increased when fed on mice coinfected with *A. phagocytophilum* (Thomas et al., 2001). *E. ruminantium* levels increased during *in vitro* coinfection with *B. burgdorferi* as well (Moniuszko et al., 2014). Importantly, coinfections are not a phenomenon limited to *Ixodes* ticks,

as both *Rhipicephalus* sp. and *Hyalomma rufipes* ticks can harbor between two to four pathogens (Berggoetz et al., 2014). Taken together, this information suggests that coinfection is a previously unappreciated phenomenon that likely impacts tick-borne disease transmission and outcome.

B. burgdorferi AND TICK IMMUNITY

Lyme disease is the most important vector-borne disease in the Northern hemisphere and approximately 30,000 cases are reported annually in the United States (Kugeler et al., 2015; Diuk-Wasser et al., 2016). *B. burgdorferi* colonizes ticks during a bloodmeal, where they will persist during digestion and molting (Radolf et al., 2012). Transmission subsequently occurs during a second bloodmeal when spirochetes are introduced into a new host with the saliva injected by a feeding tick (Radolf et al., 2012) (**Figure 2**).

Different species of ticks vary in their ability to transmit *Borrelia* spp. (Mather and Mather, 1990; Dolan et al., 1998). *Dermacentor* ticks, for instance, are not able to acquire or transmit *B. burgdorferi* (Mather and Mather, 1990). Spirochetes injected into *D. variabilis* are cleared from the hemocoel, whereas artificially infected *I. scapularis* retain the pathogen (Johns et al., 2001a). Inoculation of *B. burgdorferi* results in a rapid increase of hemocytes, lysozyme, and AMPs in *D. variabilis* (Johns et al., 2000; Sonenshine et al., 2002), which are likely major factors influencing this species' competence. How and why these responses are not also induced in *Ixodes* ticks remains unknown, but is an intriguing topic.

The microbiome also influences vector competence. Ticks with a modified microbiota, termed "dysbiosed", maintain lower *B. burgdorferi* numbers as compared to normal ticks (Narasimhan et al., 2014). Interestingly, this reduction in spirochetes appears to be related to midgut homeostasis and epithelial renewal controlled by JAK/STAT pathway-regulated expression of *peritrophin-1* (Narasimhan et al., 2014). A graphic representation of the humoral and cellular responses of ticks during *B. burgdorferi* infection can be found in **Figure 2**.

CONCLUSION

Although ticks are of increasingly importance, little is known about what dictates their competence as disease vectors. It is known that immune networks heavily influence insect vector competence. However, there are fundamental differences in tick immunity when compared to insects. For example, the repertoire of Toll receptors found in ticks is reduced when compared to *Drosophila* (Palmer and Jiggins, 2015) and the IMD pathway has a significant amount of gene loss, yet both remain active (Severo et al., 2013; Smith and Pal, 2014; Palmer and Jiggins, 2015; Bechsgaard et al., 2016; Gulia-Nuss et al., 2016; Rosa et al., 2016). Unknown immune networks are likely present in ticks that facilitate the recognition of invading pathogens. Exploiting the long co-evolutionary history between ticks and the pathogens they can transmit, such as *Borrelia*, *Anaplasma*, *Ehrlichia,* and/or *Rickettsia*, is one avenue for approaching this gap in knowledge. For example, a non-canonical IMD network in ticks has recently been identified using both *A. phagocytophilum* and *B. burgdorferi* (Shaw et al., 2017).

Other confounding factors influencing pathogen transmission are coinfections and/or interactions with the microbiota. For instance, simultaneous infection of ticks with *A. phagocytophilum* and *B. burgdorferi* leads to higher spirochete burdens (Thomas et al., 2001). It is tempting to speculate that *A. phagocytophilum* virulence proteins exert an immunosuppressive effect on the tick that inadvertently confers a survival advantage for *B. burgdorferi*. Another point of interest is the recent evidence that mammalian-derived cytokines can cross-react with the tick immune system (Smith et al., 2016). This discovery sheds new light on what we know about vector competence because coinfection in the mammal will inevitably skew the cytokine profile of the host and thus the bloodmeal taken by a tick. Investigating alternative immune circuitry and agonists will not only lead to better understanding of tick biology and pathogen transmission, but will also illuminate how coinfections are maintained.

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REVIEW
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Borrelia burgdorferi Manipulates Innate and Adaptive Immunity to Establish Persistence in Rodent Reservoir Hosts

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Borrelia burgdorferi sensu lato species complex is capable of establishing persistent infections in a wide variety of species, particularly rodents. Infection is asymptomatic or mild in most reservoir host species, indicating successful co-evolution of the pathogen with its natural hosts. However, infected humans and other incidental hosts can develop Lyme disease, a serious inflammatory syndrome characterized by tissue inflammation of joints, heart, muscles, skin, and CNS. Although *B. burgdorferi* infection induces both innate and adaptive immune responses, they are ultimately ineffective in clearing the infection from reservoir hosts, leading to bacterial persistence. Here, we review some mechanisms by which *B. burgdorferi* evades the immune system of the rodent host, focusing in particular on the effects of innate immune mechanisms and recent findings suggesting that T-dependent B cell responses are subverted during infection. A better understanding of the mechanisms causing persistence in rodents may help to increase our understanding of the pathogenesis of Lyme disease and ultimately aid in the development of therapies that support effective clearance of the bacterial infection by the host's immune system.

Keywords: immune evasion, immune exhaustion, germinal center, complement inhibition, persistent infection, lyme disease

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INTRODUCTION

Borrelia burgdorferi sensu lato is a species complex of spirochetal bacteria that infects a wide variety of mammals, birds, and reptiles. It includes, most notably, *Borrelia burgdorferi* sensu stricto (*Borrelia burgdorferi*), *Borrelia afzelii*, and *Borrelia garinii* (1), the three most prevalent causative agents of Lyme disease in humans (2). According to CDC reports, Lyme disease caused by *B. burgdorferi* is currently the most common vector-borne disease in the United States (3). In Europe, infections with *B. afzelii* and *B. garinii* are more prevalent than those with *B. burgdorferi*. In Asia, of the three primary disease-causing species, only *B. afzelii* and *B. garinii* are present (4). Other *Borrelia* species, including *Borrelia bavariensis* and *Borrelia spielmanii*, can also cause infection and disease in humans (5). Thus, *B. burgdorferi* sensu lato infections are an important global public health concern.

The bacteria are transmitted between hosts by ticks of the genus *Ixodes*. *B. burgdorferi* infection is therefore only common in areas where these vector species thrive. Deer mice (*Peromyscus*

leucopus) are often considered the major reservoir host in the United States (6). In mice, spirochetes form persistent, non-resolving infections (7). However, these infections do not cause noticeable manifestations of disease in *P. leucopus* or certain common laboratory strains of mice [*Mus musculus*] (8–10). This suggests that *B. burgdorferi* has developed immune evasion strategies that allow it to persist in the face of a mammalian immune system. Such mechanisms may be the products of co-evolution with reservoir hosts, minimizing host disease manifestations while maximizing bacterial growth and transmission.

Laboratory mouse studies have been used to better understand *B. burgdorferi* pathogenesis in humans (11), just as they have for many other disease processes. They also provide an opportunity for better understanding the amplification and spread of *B. burgdorferi* in wild rodents, which in turn affects the infection risk of humans in endemic areas. Furthermore, *B. burgdorferi* in the mouse is an excellent model system for better understanding the mechanisms by which certain pathogens can achieve persistence in immunocompetent hosts (12, 13). Here, we summarize known mechanisms by which *B. burgdorferi* circumvents innate and adaptive immune responses to establish lifelong persistence in the mouse host. We examine interference with both the innate and adaptive immune systems. We emphasize bacterial persistence over disease and tissue pathology, because persistence of *B. burgdorferi* in wild rodents is a prerequisite for human infections.

EVIDENCE FOR CO-EVOLUTION OF *B. burgdorferi* WITH VERTEBRATE HOSTS AND VARIATION IN HOST RESPONSES

A reservoir host is one in which a particular pathogen can survive with minimal affect to that host. The pathogen can subsequently be transmitted to other species that may experience ill effects. Incidental, or "dead-end," hosts do not facilitate transmission of the pathogen to another host, but often experience manifestations of disease. This is the case for *B. burgdorferi*, where the reservoir hosts, including wild rodents [*Peromyscus* spp. (14, 15)] and passerine birds [canary finches (16)], are largely asymptomatically infected, whereas incidental hosts like humans can sometimes suffer a severe array of diseases, including arthritis, carditis, and skin and neurological disease manifestations (11, 17).

As outlined earlier, *Borrelia* species require both an invertebrate vector (ticks of the *Ixodes* genus) and a vertebrate host to complete their life cycle (11). Many ecological and evolutionary factors affect prevalence, persistence, and disease development by *Borrelia* infections in both vector and host. These factors include population dynamics, dispersal/migration, and evolution of all three players, as well as environmental landscape and climate (4). Further complicating the situation is the fact that both spirochetes (*B. burgdorferi* sensu lato) and the vectors (*Ixodes ricinus* species complex) are part of large species complexes, which have their own unique evolutionary patterns (4). Therefore, individual effects on *Borrelia* persistence can be difficult to untangle.

The existence of co-evolution with reservoir, but not incidental hosts like humans, remains to be rigorously tested with population genomics approaches (18). However, current evidence supports this hypothesis. For example, differential resistance to complement, an important and evolutionary conserved innate immune defense mechanism, has been suggested to drive host specializations of various *Borrelia* species to mammals, birds, and reptiles (19). In addition, there is evidence that selection acts on *B. burgdorferi*, within the reservoir host, to generate sequence diversity and polymorphisms relevant to virulence (20).

Co-evolution of host and pathogen must achieve a balance between the induction of immune mechanisms that reduce pathogen burden and pathogen-induced diseases, without clearing the infection. How this is achieved is incompletely understood. The continued presence of the pathogen in its host should provide ongoing triggers for both innate and adaptive immune response induction. Current research suggests that the immune system has important immune checkpoints that regulate immune responses, leading to a state of "immune exhaustion" during chronic infections. The process of immune exhaustion was first identified in chronic LCMV infection (21), leading to the discovery of distinct functions manifested in distinct transcriptional profiles of "exhausted T cells" in both mice and humans in response to a variety of chronic infections (22–25). The state of immune exhaustion goes beyond suppression of the T cell compartment alone, encompassing alterations in both innate and adaptive immune responses [reviewed in Ref. (26)]. While immune exhaustion has not been studied in the context of persistent infection with *B. burgdorferi*, it is conceivable that this process is also involved in the establishment of *Borrelia* persistence in its natural reservoir host. Indeed, mounting evidence suggest that the adaptive immune response is suppressed during *B. burgdorferi* infection.

EVIDENCE OF *B. burgdorferi* PERSISTENCE

Persistent infection of reservoir hosts increases the odds that *B. burgdorferi* will be passed on to new hosts. It is challenging to distinguish between high prevalence of reinfection and/or true persistence occurring in the natural habitat of a reservoir host, such as the white-footed mouse *P. leucopus* (14, 15, 27). Experimental infections of *P. leucopus* have confirmed, however, that persistence of *B. burgdorferi* can occur, at least in laboratory settings (20). Experimental evidence also shows that *B. burgdorferi* persists in various laboratory mouse strains, either with or without disease manifestations, depending on the strain used (7, 8). Persistence after experimental infection was also observed in chipmunks (28), dogs (29), canaries (16), and non-human primates (30, 31). **Figure 1** summarizes mechanisms that can support Borrelia persistence.

INTERFERENCE OF *B. burgdorferi* WITH THE INNATE IMMUNE SYSTEM

Ticks provide the first defenses for *B. burgdorferi* against the innate immune system of the mammalian host. *B. burgdorferi* is transmitted from the tick to the mammalian host within <16–72 h after onset of tick feeding (32), where it encounters

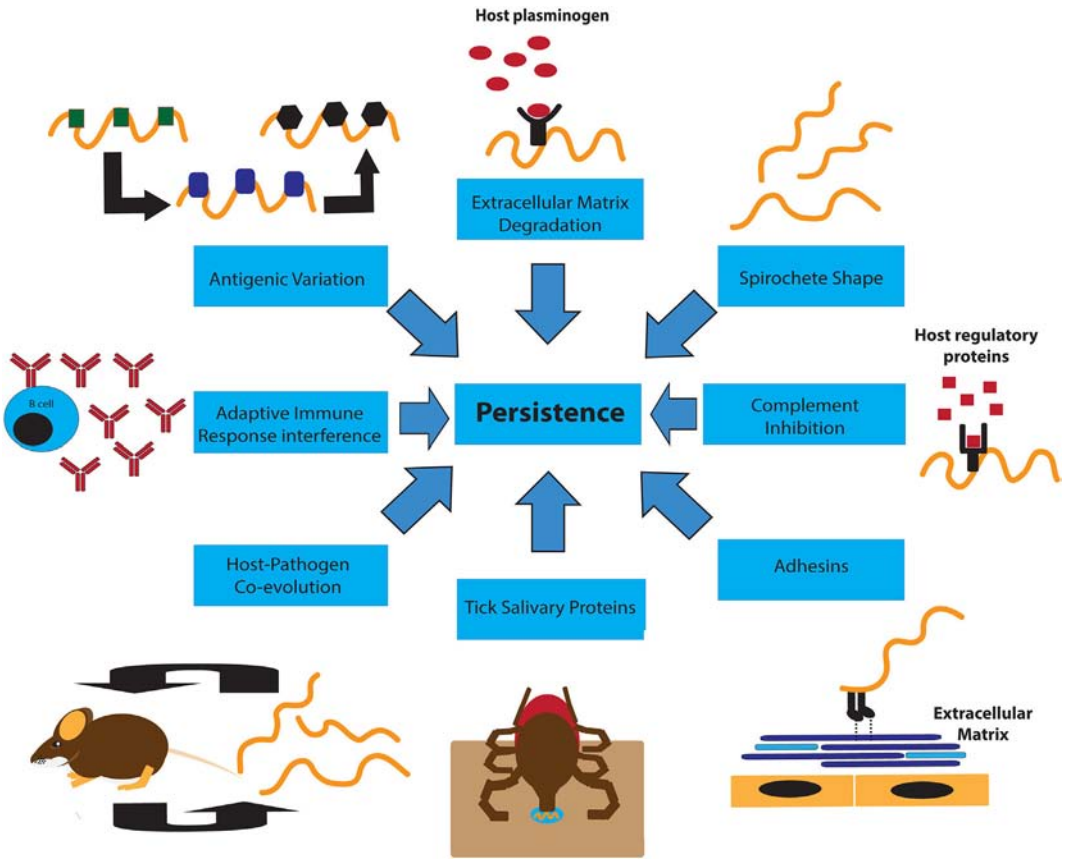

FIGURE 1 | An overview diagram of factors contributing to persistence of *Borrelia burgdorferi* in rodent hosts. Shown are eight bacterial characteristics and mechanisms that *B. burgdorferi* may use to establish persistence in the rodent host: Spirochete shape (38, 123), antigenic variation and changes in gene expression (77, 83, 89), plasminogen binding and destruction of the extracellular matrix (52), interference with the adaptive immune response (69, 74, 75), host–pathogen co-evolution (20, 124), tick salivary protein-mediated immunosuppression (34), adhesins allowing entrance into the vasculature and tissues (49, 50), and interference with complement *via* CRASPs, and BBK32 (45, 60, 62, 64).

host defenses present in the skin. Tick salivary proteins play a role in suppressing the host immune system as long as the tick vector is attached. This includes the inhibition of vigorous activation of skin-resident macrophages and dendritic cells, including the suppression of cytokine and chemokine production, and inhibition of granulocyte recruitment to the site of the tick bite [reviewed in Ref. (33, 34)]. Multiple tick salivary proteins are known to interfere with the activation of the alternative complement pathway, potentially further supporting pathogen transmission [reviewed in Ref. (33)]. Mast cells also appear to be direct targets of salivary protein-mediated immune suppression (35). Inhibition of mast cells was facilitated through secretion of the salivary protein sialostatin L, which was shown to inhibit the induction of IL-9 production in the skin. IL-9 is known as an important regulator of pathogen-induced immune responses (35–37).

The morphology of spirochetes facilitates versatile motility that is predicted to play a role in the dissemination and persistence of *Leptospira*, *Treponema*, and *Borrelia* genera (38, 39). *B. burgdorferi* itself also expresses known immunomodulatory surface proteins, which help to modulate immune responses of the host. These proteins, specifically lipoproteins, have been studied extensively, and their wide variety of functions is reviewed elsewhere (40). We focus here on known functions of some proteins that are likely to contribute to persistence of *B. burgdorferi* in the rodent host (**Table 1**).

One important group of such proteins is the adhesins, which mimic host integrins, molecules that facilitate attachment and migration. Thus, *B. burgdorferi* seems to subvert existing mechanisms regulating immune cell migration for its benefit. Adhesins are an important category of bacterial virulence factors that protect bacteria from clearance by physical forces such as mucociliary clearance, facilitate homing to and entrance into host tissues that act as important pathogen niches and trigger signaling events in host cells (41). BBK32 is one such adhesin. At the initial site of infection, BBK32 was shown to create "catch bonds" that slow bacterial movement enough for flagella-driven entrance of *B. burgdorferi* into the vasculature (42). This helped to explain why bacterial loads in the blood are higher when BBK32 is expressed (43). Once in the blood, BBK32 seems to help *B. burgdorferi* to target joint tissues for colonization *via* binding to glycosaminoglycan (44). However, recent studies suggested

TABLE 1 | *B. burgdorferi*'s interface with mammalian hosts and its effect on *Borrelia* survival.

Immune response modulators	Effects	Reference
Co-evolution of *B. burgdorferi* and its hosts	Host specialization and evolution of virulence- and infectivity-associated genes	(19, 20)
Tick salivary proteins	Suppression of pro-inflammatory responses in the host	(33, 34, 125)
Spirochete morphology and motility	Increase in *B. burgdorferi* dissemination and persistence	(38, 39)
B. burgdorferi adhesins	Interactions with host tissues, contributing to dissemination and persistence	(49, 50)
Host interactive proteins	Binding to host enzymes, such as plasmin/plasminogen; facilitates extracellular matrix degradation	(50, 52)
CRASPs	Decreased and inhibited complement activation	(60, 62, 63)
Modulation of protein expression	Adaptation to host, downregulation of immunogenic proteins, and antigenic variation	(79, 80)
Inappropriate macrophage activation	Extracellular matrix degradation	(54)
Antibodies with IgM-skewed isotype profile and of low affinity	Decreased antibody response quality which may contribute to persistence	(see text footnote) (73–75)
Loss of demarcated T and B cell zones in secondary lymphoid tissues and collapse of germinal centers	Reduced antibody class switch recombination and somatic affinity maturation. Failure to induce long-lived plasma cells and memory B cells in a timely manner	(69, 73, 74, 110)

that BBK32 has additional, complement-inhibitory, functions (45). Specifically, it was found to bind to the C1 complex and thereby to inhibit the classical pathway of complement activation, i.e., antibody-mediated bacterial clearance (45).

Another adhesion protein of *B. burgdorferi*, p66, was shown to interact with ligands on the host vasculature to facilitate *B. burgdorferi* extravasation from blood vessels into tissues (46). *B. burgdorferi* also expresses two decorin-binding proteins (Dbp), DbpA and DbpB. Surface expression of these proteins seems to increase the level of tissue colonization (47). They seem to support persistence of *B. burgdorferi* in tissues that express high levels of decorin (joint tissue and skin) (48). Further details about *B. burgdorferi*-expressed adhesins have been summarized in recent reviews (49, 50).

Once bacteria have gained access to a specific organ or tissue, they require proteases that degrade the extracellular matrix (ECM), enabling the bacteria to move between cells deeper into the tissues. This is particularly important for *B. burgdorferi*, which targets ECM-rich connective tissues (50, 51). The genome of *B. burgdorferi* does not seem to contain any known ECM-degrading proteases. Instead *B. burgdorferi* is able to bind host urokinase and plasminogen, a multifunctional serum protein that can initiate ECM and fibrinogen degradation (52). Plasminogen can also inhibit complement activation and promote complement degradation (53).

Furthermore, *B. burgdorferi* can induce host cells such as macrophages to secrete matrix metalloproteases (MMPs), particularly the gelatinase MMP-9, *via* TLR2-mediated immune activation (54). MMP-9 was shown to be selectively upregulated in erythema migrans skin lesions during acute *B. burgdorferi* infections of humans (55) and is thought to help *B. burgdorferi* tissue dissemination by enabling the degradation of the ECM. However, studies by Hu and colleagues recently demonstrated that MMP-9 expression is not required for *B. burgdorferi* dissemination. Instead, it regulated the influx of inflammatory cells, and thereby Lyme arthritis, indirectly by the degradation of collagen in joints (56). Plasminogen-binding proteins have not been shown to increase *B. burgdorferi* persistence *per se*, but they do facilitate the entrance of *B. burgdorferi* into the ECM of tissues, where bacteria maybe protected from innate immune response mechanisms such as complement-mediated degradation and/or neutralization by early-induced IgM antibodies.[1]

Interference of *B. burgdorferi* with the Complement System

As mentioned earlier, inhibition of the complement system is an important immune evasion strategy employed by many pathogens, including *B. burgdorferi* (57–59). *B. burgdorferi* proteins that interfere with complement activation allow for survival and dissemination of the pathogen from the initial site of infection (60).

The complement system consists of an evolutionarily highly conserved family of proteins that are found in all body fluids and serve three main functions during infection: trigger inflammation, opsonize pathogens, and form the "membrane attack complex" (formation of a pore in the cell membrane that causes cell lysis). Classical, alternative, and lectin are the three distinct pathways by which complement-mediated signaling and bacterial killing can be initiated [described in more detail in Ref. (61)]. Independent of the initial trigger, all pathways lead to the formation of a protease, the C3 convertase, which cleaves the complement component C3 into its activated components C3a (an inflammatory mediator) and C3b (an opsonin and immune stimulatory protein). The complement component C3b can also form the C5 convertase, another protease that cleaves complement component C5 into C5a and C5b, leading to the formation

[1]Hastey CJ, Elsner RA, Olsen KJ, Tunev SS, Escobar ED, Barthold SW, et al. *Borrelia burgdorferi* infection-induced IgM controls bacteremia but not bacterial dissemination or tissue burden. (submitted).

of the bactericidal "membrane attack complex" (61). C5a, like C3a, is a strong inducer of inflammation. During the process of complement activation, another complement component, C4, is cleaved into C4a and C4b. The latter also acts as an opsonin. Because of these multiple and highly pro-inflammatory effects, systemic activation of complement can cause septic shock if not appropriately regulated. Regulators of overshooting complement activation include components of the complement system itself: the Factor H family proteins and the C4b-binding protein. These proteins inhibit complement activation by a variety of mechanisms, including by accelerating the decay of C3 convertases, thus interrupting the complement activation cascade.

Borrelia burgdorferi has evolved complex mechanisms to evade complement-mediated killing by binding to the inhibitory host-regulatory factors [reviewed in Ref. (60, 62)]. *B. burgdorferi* expresses a diverse family of complement regulator-acquiring surface proteins, which recruit Factor H family proteins [reviewed in Ref. (63)]. Factor H and its relatives primarily inhibit activation of the alternative complement pathway. More recently, it was discovered that *B. burgdorferi* also binds to host C4-binding proteins, which primarily inhibit activation of the classical and lectin pathways (64). And, as outlined earlier, BBK32 seems to inhibit the classical pathway of complement activation *via* binding to the C1 complex (45). Thus, *B. burgdorferi* seems to target all three activation pathways of the complement cascade. The effects of complement inhibition on adaptive immune responses are outlined below.

INTERFERENCE OF *B. burgdorferi* WITH THE INDUCTION OF ADAPTIVE HUMORAL IMMUNE RESPONSES

High levels of *B. burgdorferi* antigen-specific antibodies are produced during infection, and they have the capacity to prevent reinfection with the same *B. burgdorferi* strains (65–67). The antibody response also results in reduction, but not elimination, of *B. burgdorferi* from tissues (68). Both T-independent and T-dependent antigens are targeted by the humoral immune response, representing a wide variety of surface proteins with different functions (20, 67, 69). How then does *B. burgdorferi* avoid antibody-mediated clearance?

B. burgdorferi-Induced Humoral Immunity

The strong production of *B. burgdorferi* antigen-specific antibodies, as well as the strong increases in *B. burgdorferi* tissue load in SCID mice and B cell-deficient mice compared to wild-type controls (70, 71), has long been considered evidence of a robust and effective B cell response against *B. burgdorferi* infection. However, while the data provide clear evidence that B cells play an important role in the control of *B. burgdorferi* infection, this does not mean that these responses are optimally induced. Considering that *B. burgdorferi* infection results in persistent infections of mice despite these robust antibody responses, it is important to also consider the *quality* of the *B. burgdorferi*-specific antibody response. Several factors are known to affect the efficacy of humoral immune responses, including the epitope specificity of the induced antibodies, their immunoglobulin isotype profile, binding avidity to their cognate antigens, and various posttranslational modifications that can affect their effector functions (6, 20).

IgM is the first antibody isotype secreted during an immune response. It is important in controlling bacteremia and in activating the classical complement pathway. Immunoglobulin class switch recombination (CSR) to IgG typically occurs soon after an infection, and the four subtypes of murine IgG work together effectively to clear most pathogens (72). However, during *B. burgdorferi* infection, serum IgM levels remain high throughout infection (see text footnote). Moreover, hybridomas generated from lymph nodes of mice on days 8 and 18 postinfection showed that nearly half of *B. burgdorferi* antigen-specific cells were producing IgM, and the ratio of IgM to IgG never significantly changed throughout the infection (see text footnote) (73). The strong and ongoing production of IgM cannot be explained entirely by a predominance of T-independent responses, because depletion of CD4 T cells decreased the number of IgM-antibody-secreting cells (ASCs) (74). Thus, infection with *B. burgdorferi* induces an antibody response that is characterized by the continued production of IgM and IgG. Further studies will need to determine whether the strong production of IgM is evidence of a strong beneficial immune response or whether *B. burgdorferi* might be subverting the B cell response to generate this unusual Ig-isotype profile. Our studies have failed to find any beneficial effect of IgM on control of bacterial dissemination or *B. burgdorferi* tissue loads (see text footnote).

Given the important role of T cells in the regulation of CSR by B cells, the data may indicate a deficiency in T helper cell activation and/or functionality. *In vitro* data indeed provided some evidence that the CD4 T helper cell response induced by *B. burgdorferi* infection is distinct in function from that of CD4 T cells induced by immunizing mice with inactivated *B. burgdorferi* (75). T-dependent B cell responses usually also result in significant affinity maturation, i.e., an increase in the binding avidity of antibodies to their cognate antigens over time. Measuring antibody avidities of serum antibodies to a representative T-dependent antigen on *B. burgdorferi* N40, arthritis-related protein (Arp), however, failed to provide evidence for affinity maturation in the serum response to *B. burgdorferi* (75). Instead, we found that the binding avidity of the serum antibodies to Arp initially increased for the first 6 weeks of infection, but then peaked and steadily decreased thereafter to levels seen at the beginning of the infection. The rate of drop in antibody affinity was consistent with the normal half-life and decays kinetics of serum antibodies (75), suggesting that the ASC that generated the higher-affinity antibodies were short-lived.

Both CSR and hyperaffinity maturation of antibodies are T-dependent processes that usually occur in germinal center (GC) reactions in secondary lymphoid tissues. As we will outline below in more detail, we noted a collapse of the GC responses that coincided with the peak and then reversal of the antibody avidities. The data strongly suggest that the T-dependent GC responses are not fully functional during *B. burgdorferi* infection. The fact that immunization with inactivated *B. burgdorferi* infection resulted in robust GC responses suggests that exposure

to live *B. burgdorferi* results in a subversion of the B cell response (73, 74). On the basis of these data, we propose that although present in large quantities, the B cell responses to and functionality of the serum antibody response to *B. burgdorferi* are suboptimal, enabling *B. burgdorferi* persistence, while also controlling *B. burgdorferi* tissue loads and thus overwhelming infection. Alterations in the B cell response quality and/or antibody functionality could provide a powerful immune evasion strategy for bacteria that are clearly susceptible to antibody-mediated immune clearance mechanisms (65, 66, 70, 71, 76, 77).

Modification of *B. burgdorferi* Outer Surface Protein Expression

Although *B. burgdorferi* surface proteins have many functions in evading recognition by the host immune system, they are also antigens that trigger antibody responses. Additional immune evasion strategies therefore likely exist that inhibit recognition and antibody-mediated clearance of *B. burgdorferi*.

Borrelia burgdorferi is known to undergo major protein expression changes over the course of its life cycle, including expression of outer surface proteins. During transmission from the tick to a mammalian host, environmental signals trigger extensive global changes in gene expression (78, 79). Changes in surface protein expression also occur within a mammalian host during the course of infection. At least some proteins that trigger strong antibody responses are downregulated during the chronic phase of infection (80). One important example is OspC, a T-dependent antigen that is essential for initial colonization of mammalian hosts. Shortly after infection, this lipoprotein is no longer required, and if not downregulated triggers a strong and effective antibody response. However, expression is usually rapidly lost upon infection (81–84). This appears to be an effective immune evasion strategy, because constitutive expression of OspC prevents spirochete persistence, whereas bacteria that successfully downregulate OspC outcompete expressors *in vivo* (76, 85).

Antigenic variation is a process by which a pathogen varies the sequence of an expressed protein to avoid the deleterious effects of antibodies raised against it. Antibody-target switching has been demonstrated as an effective immune evasion strategy for numerous pathogens, including the closely related relapsing fever pathogen *Borrelia hermsii* (86–88). The *B. burgdorferi* genome contains the variable surface antigen E (*vlsE)* locus, which has received much attention as a major immunodominant surface protein *of B. burgdorferi* that undergoes extensive and rapid antigenic variation in mammalian hosts [reviewed in Ref. (89)]. In *B. burgdorferi* strain B31, VlsE recombination seems to be critical for *B. burgdorferi* persistence and the ability of *B. burgdorferi* to reinfect a host following antibiotic treatment (77, 90–92). It is interesting to note that vlsE recombination also occurs in antibody-deficient SCID mice, but not *in vitro*, suggesting that host triggers other than the antibody response direct this process (93). While it seems that a lack of variation of vlsE results in the rapid clearance of *B. burgdorferi*, it is less clear whether this process alone can explain the effective evasion of *B. burgdorferi* from antibody-mediated clearance. Recently, mathematical models have been put forward to suggest that a strongly immunodominant variable surface protein may prolong immune responses long enough to drive immune exhaustion (94), an intriguing idea that requires testing in the context of *B. burgdorferi* infection.

Finally, extensive genetic variation exists between the various *Borrelia* species and between individual bacteria (95–98). This allows adaptation to selective pressure from the host immune system, as well as to larger ecological niches, allowing strains to become fitter in a given geographic area (95). It also applies during the course of a chronic infection, as bacterial variants compete with each other (85, 99). Variation between strains is large enough in the context of the antibody response that different strains can infect the same mouse (100). Reinfection with the same strain has been tested in experimental settings after antibiotic treatment of mice. Studies by Piesman et al. and by Elsner et al. suggested that antibody-mediated protection wanes over time, even to the same strain of *B. burgdorferi* (69, 101). Given that long-term antibody production is usually induced following an infection, facilitated by the development of long-lived plasma cells that migrate to and then reside in the bone marrow (102, 103), the data further suggest that the antibody response to *B. burgdorferi* infection lacks some of these key components of successful humoral responses. It can be expected that each of the above outlined immune evasion mechanisms is unlikely to be solely responsible for persistence; instead they may act in concert. Whether these multiple apparent strategies represent redundant or synergistic effects remains to be established.

B. burgdorferi Modulates the Adaptive Immune Response

Modeling of population dynamics supports the establishment of equilibrium between *B. burgdorferi* and the host's adaptive immune response (104). This is consistent with recent findings suggesting that the humoral immune response and, more specifically, the T-dependent B cell responses are a particular target of *B. burgdorferi*-induced immune suppression.

After escaping the site of infection, *B. burgdorferi* disseminates to other tissues *via* blood and lymph (43, 105). Mechanisms of entrance into the blood are better studied, but live *B. burgdorferi* is also capable of entering, persisting in, and traveling through the lymphatics (73, 105). Dissemination *via* the lymphatics has been described in other infections as an effective technique for immune evasion due to the specialized cell populations and altered immune milieu in these vessels compared to the bloodstream (106–108). Shortly after infection with cultured or host-adapted spirochetes (HAS), live *B. burgdorferi* enter lymph nodes draining the site of infection, resulting in massive lymph node enlargement (73, 109).

In secondary lymphoid tissues such as the lymph nodes, naïve T cells encounter antigens for the first time, and activated antigen-specific B cells receive the signals they need to proliferate. During *B. burgdorferi* infection, the presence of live spirochetes seems to interfere with these processes. Between days 5 and 7 postinfection of C57BL/6 mice with HAS, the separation of T cell zones and B cell follicles completely was lost within the draining lymph nodes. This degradation was not seen in mice that were inoculated with heat-inactivated

spirochetes, suggesting that an active bacterial process causes the disruption of the normal immune response of the host (73, 74, 110). Interestingly, similar observations have been made also following infections with other pathogens, including infections with *Plasmodium* and *Salmonella* (111, 112). While during *Salmonella* infection lymph node architecture disruption was dependent on TLR4-mediated signaling (112), the observed changes during *B. burgdorferi* infection were independent of MyD88 and TRIF signaling (110). Given the importance of T and B cell trafficking within the lymph nodes for initiating and maintaining T–B interaction and immune response regulation, the data suggest that multiple pathogens, including *B. burgdorferi*, may have evolved strategies to interfere with the earliest processes of adaptive immune induction.

Furthermore, the presence of live (but not heat-killed) *B. burgdorferi* caused a disproportionate recruitment and proliferation of naïve follicular B cells, but not CD4 T cells, to the affected lymph nodes. This appeared to be a separately controlled process, but one also mediated specifically by live bacteria. B cell recruitment and/or retention required signaling through the type I interferon receptor by non-hematopoietic cells, likely lymph node stromal cell compartments (110). The resulting accumulation of large numbers of B cells in the lymph nodes explains the lymphadenopathy that is also observed in infected humans and dogs (113, 114). This pattern of lymph node architecture disruption followed by influx/retention of B cells is recapitulated later in more distant lymph nodes as they are infiltrated by the migrating spirochetes (73, 110).

The data support the concept that *B. burgdorferi* interferes with the induction and maintenance of adaptive immune responses by altering or hijacking innate immune signaling pathways. What effect does this have on persistence of *B. burgdorferi*? The organized lymph node structure and the regulated migration of lymphocytes within secondary lymphoid tissues is crucial for effective interaction of T and B cells and the induction of adaptive immune effector mechanisms. Disruption of this structure is liable to have long-term negative consequences for the quality of the immune response and thus increases the likelihood of pathogen persistence. We outline below evidence to this effect.

Interference of *B. burgdorferi* with GC Formation and Maintenance

Activated B cells in the draining lymph nodes proliferate rapidly and initially form structures called extrafollicular foci (73). In extrafollicular foci, B cells become plasmablasts with or without T cell help and produce large quantities of *B. burgdorferi*-specific antibodies rapidly. B cells in extrafollicular foci, however, do not undergo extensive affinity maturation, nor do they form memory B cells or long-lived plasma cells (115). For this, GCs are needed. GCs are a complex and highly organized structures that form within the B cell follicles of secondary lymphoid tissues that consist of GC B cells, CD4 T follicular helper cells (T_{FH}), and follicular dendritic cells (FDCs). B cell activation and proliferation in GCs is facilitated by interaction with CD4 T cells and presentation of antigens on FDCs. High-frequency insertion of random mutations into the antigen-binding variable domain of the antibody molecule during this process, and the competition of the newly generated B cells for binding to antigen and for T cell-help, is thought to drive B cells with the highest affinity for antigen to outcompete other clones with weaker antigen binding. Although mechanisms are still unclear, GC responses lead to the development of two distinct cell populations: memory B cells, i.e., non-ASCs that circulate and can respond vigorously to repeat infection, and long-lived plasma cells, which continuously produce antibodies from their bone marrow niches and contribute to immune protection from reinfections (116–119). The GC environment also promotes CSR to IgG.

Despite the early presence of *B. burgdorferi* in the draining lymph nodes, GC B cell and T_{FH} numbers remain low for the first 2 weeks in experimentally infected mice (74) and then become measurable. However, although live *B. burgdorferi* remained present in the lymph node for at least 1 year after infection, and thus, *B. burgdorferi* antigens were presumably continuously available, the GCs collapsed around 1 month after infection, and associated B and T cell numbers decreased steadily over the next month (69). As outlined earlier, the decline in the avidity of serum antibodies against Arp follows the collapse of GCs (69).

Consistent with the short-lived nature of the GC responses, their functional ineffectiveness was demonstrated by experiments showing a complete lack of memory recall responses to both *B. burgdorferi* antigens and a co-administered vaccine antigen for many months after infection with *B. burgdorferi*. Stable continued antibody production by long-lived ASCs in the bone marrow was also strongly delayed for at least 3 months after infection (69, 74). The delay in these important B cell response outcomes is especially dramatic considering a mouse's relatively short life span and likely frequent exposure in the wild. Although mice do not clear infection with *B. burgdorferi*, impairing the memory response could be advantageous to *B. burgdorferi* by leaving the host susceptible to secondary or superinfections. It might also prevent a timely and strong response to antigens that are dynamically upregulated and downregulated by *B. burgdorferi* as the infection progresses.

By decreasing the capacity of the host to produce effective antibodies against *B. burgdorferi*, the GC collapse may help *B. burgdorferi* evade clearance. The signals and mechanisms leading to the collapse, however, are unknown. One possible mechanism is the interference of *B. burgdorferi* with the complement system. Continued antigen presentation is crucial for hyperaffinity maturation, and components of the complement system are known to be involved in this process. Specifically, activated C3 and C4 fragments bound to antigen and adhere to complement receptors 1 and 2 (CR1 and CR2). These receptors are present on the major antigen-presenting cells in the GC, the FDC, and on GC B cells. It was shown previously that GCs will form normally in mice lacking CR1 and CR2, but collapse prematurely, before GCs can perform their important functions (120). This phenotype is strikingly similar to that seen in wild-type mice infected with *B. burgdorferi*. Interestingly, in *B. burgdorferi*-infected mice, although CR1 and CR2 are present on FDCs and GC B cells, C4 is not detectable (69). C4 is typically deposited on the surface of FDCs supporting antigen presentation. Interference with C4 deposition could inhibit antigen

presentation by FDCs to GC B cells and thereby lead to GC collapse. *B. burgdorferi* interference with activation of complement could also have various indirect effects on GCs: changing the cytokine milieu, reducing antigen presentation to naïve B cells *via* CR1 on APCs outside the GC, reducing naïve B cell activation *via* co-stimulation with CR2, and reducing opsonization (and thus uptake) of antigens. Exploring the role of complement and complement inhibition by *B. burgdorferi* during infection are important subjects for future studies.

CONCLUSION

The phenomena described earlier represent potential novel mechanism(s) for manipulation of the adaptive immune system by a pathogen that establishes persistent infections in its reservoir host. Elucidation of these mechanisms has important translational and clinical applications. A better understanding of how *B. burgdorferi* persists long term in rodents would be useful for understanding public health risks and devising appropriate preventative measures in endemic areas. Given the extensive similarities in the immune system of rodents and humans, it seems likely that the mechanisms of immune evasion and suppression outlined here may also be active in at least some infected humans. The induction of diseases such as carditis, arthritis, acrodermatitis chronica atrophicans, and neuroborreliosis seen in some patients with Lyme disease and infected companion animals, but rarely in mice, suggest maladaptation of *B. burgdorferi* to these hosts. Humans developing these inflammatory diseases to *B. burgdorferi* infection may have an immune system that is ineffectively suppressed by *B. burgdorferi*. There is good experimental evidence that a block of pro-inflammatory T cell responses, such as facilitated through blockade of IL-12, will cause reductions in arthritis development in C3H mice, but it also causes increases in *Borrelia* tissue loads (121, 122). Development of therapeutics that can shift the balance toward immune activation and bacterial clearance without causing inflammation-induced diseases might provide superior tools to the current antibiotic therapies.

Much remains to be elucidated about the mechanisms by which *Borrelia* evades the host response. This area of research provides a particularly rich ground for collaboration among evolutionary biologists, ecologists, microbiologists, and immunologists.

AUTHOR CONTRIBUTIONS

KT and NB conceived of and wrote the article.

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REVIEW
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Animal Models of Leptospirosis: Of Mice and Hamsters

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Pathogenic *Leptospira* sp. are spirochetal bacteria responsible for leptospirosis, an emerging worldwide zoonosis. These spirochetes are very successful pathogens that infect a wide range of hosts such as fish, reptiles, birds, marsupials, and mammals. Transmission occurs when chronically infected animals excrete live bacteria in their urine, contaminating the environment. *Leptospira* sp. enter their hosts through damaged skin and mucosa. Chronically infected rats and mice are asymptomatic and are considered as important reservoirs of the disease. Infected humans may develop either a flu-like, usually mild illness with or without chronic asymptotic renal colonization, or a severe acute disease with kidney, liver, and heart failure, potentially leading to death. Leptospirosis is an economic burden on society due to health-care costs related to elevated morbidity of humans and loss of animals of agricultural interest. There are no effective vaccines against leptospirosis. *Leptospira* sp. are difficult to genetically manipulate which delays the pace of research progress. In this review, we discuss in an historical perspective how animal models have contributed to further our knowledge of leptospirosis. Hamsters, guinea pigs, and gerbils have been instrumental to study the pathophysiology of acute lethal leptospirosis and the *Leptospira* sp. genes involved in virulence. Chronic renal colonization has been mostly studied using experimentally infected rats. A special emphasis will be placed on mouse models, long thought to be irrelevant since they survive lethal infection. However, mice have recently been shown to be good models of sublethal infection leading to chronic colonization. Furthermore, congenic and transgenic mice have proven essential to study how innate immune cells interact with the pathogen and to understand the role of the toll-like receptor 4, which is important to control *Leptospira* sp. load and disease. The use of inbred and transgenic mouse models opens up the field to the comprehensive study of immune responses to *Leptospira* sp. infection and subsequent pathophysiology of inflammation. It also allows for testing of drugs and vaccines in a biological system that can avail of a wealth of molecular tools that enable understanding of the mechanisms of action of protective vaccines.

Keywords: ***Leptospira interrogans*, leptospirosis, animal models, hamsters, mouse models, virulence factors, TLR4, TLR2**

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Abbreviations: sp., species; ko, knockout; TLR, toll-like receptor; DAF-1, decay-accelerating factor 1; LPS, lipopolysaccharide; NLR, nod-like receptor; PBMC, peripheral blood mononuclear cell; BMM, bone marrow derived macrophages; Ig, immunoglobulin; LRR, leucine rich repeat; Na/K-ATPase, potassium pump; GLP, glycolipoprotein; IL, interleukin; NO, nitric oxide.

INTRODUCTION

The most important factor in the development of animal models of leptospirosis is that experimental infection closely recapitulates natural disease in humans. Only then, these tools can be used in fundamental research of *Leptospira* sp. pathogenesis and disease, host–pathogen interactions leading to eradication or persistence of *Leptospira* sp., characterization of pathogen associated virulence factors, immune responses to infection and subsequent pathophysiology of inflammation. Under the realm of applied research, these models can be used to test vaccines to prevent infection or disease progression and to test therapeutics for cure or to mitigate signs and symptoms of the illness. Given the limited availability of properly validated biological samples from human leptospirosis patients, animal models also provide a source of material (especially urine) than can be used to develop proof-of-principle versions of new diagnostic assays.

We start the review by defining the enzootic cycle of pathogenic *Leptospira* sp. and the clinical presentation of the disease in human patients to frame how animal models that address distinct components of the cycle contribute to the understanding of how reservoir hosts contaminate the environment and enable transmission of pathogenic *Leptospira* sp. to humans and how we can use these animals to better understand disease pathogenesis. We describe the animal models used to study the forms of lethal, sublethal and chronic leptospirosis with an emphasis on mouse models.

The mouse is a versatile animal model to study *Leptospira* sp. infection because we can avail of a vast number of reagents and genetic backgrounds tailored to providing answers to specific questions.

The Enzootic Cycle of Pathogenic *Leptospira* sp.

Leptospirosis is an emerging zoonotic disease with a worldwide distribution caused by infection with any of the several pathogenic serovars of *Leptospira* sp. The disease affects virtually all vertebrates and has a broad range of clinical signs and symptoms, from mild, subclinical infection to multiple-organ failure and death. *Leptospira* sp. penetrate abraded skin or mucous membranes, enter the bloodstream, and disseminate throughout the body. The pathogens are easily maintained in sylvatic and domestic environments mostly by transmission through rodent species. In these reservoirs, infection produces chronic, asymptomatic carriage. Some pathogenic *Leptospira* sp. such as Canicola and Hardjobovis are maintained in non-rodent mammal reservoirs. *Leptospira* sp. can then infect livestock and domestic and wild animals and cause a range of disease manifestations and carrier states. Maintenance of *Leptospira* sp. in these populations is due to their continued exposure to animal reservoirs or to transmission within animal herds. Accidental hosts like humans can be infected by direct contact with reservoir animals or by exposure to environmental surface water or soil that is contaminated with their urine (1).

Clinical Presentation of the Disease in Human Populations

Human leptospirosis ranges in severity from a self-limited febrile illness to a fulminant life-threatening illness, also called Weil's disease. When illness occurs, a broad array of organ systems may be involved, reflecting the systemic nature of the infection. As a result, the signs and symptoms of leptospirosis are frequently mistaken for other causes of acute febrile syndrome (2). A recent systematic review of published cases estimated that leptospirosis causes ~1 million cases a year resulting in ~6% death rate (3).

Most leptospirosis cases are mild and resolve spontaneously (>90%). It typically presents as a biphasic disease, with an initial acute illness lasting about 1 week characterized by fever, myalgia, and headache that may be confounded with other entities such as influenza and dengue fever. In this phase, *Leptospira* sp. are found in blood or in the cerebrospinal fluid. The second phase is characterized by the presence of *Leptospira* sp. in urine and the immune response to *Leptospira* sp. is detectable by traditional serological methods. A low percentage of patients (<10%) progress to multisystem organ failure and have widespread hematogenous dissemination of pathogens resulting in non-oliguric (high-output) renal dysfunction or oliguric renal failure (2, 4). Hemorrhagic complications are common and are associated with coagulation abnormalities. Severe pulmonary hemorrhage syndrome due to extensive alveolar bleeding has a fatality rate of >50% (2).

If we consider the clinical outcomes in human populations described above, we can group leptospirosis as sublethal and lethal infections. Given the most recent numbers (3) on mortality rates in humans afflicted with leptospirosis, it is reasonable to expect that >90% of people go on to develop sublethal cases of the disease. In lethal forms of the disease, either the kidney or the lung are the affected organs. In addition, patients may also present altered mental status due to neuroleptospirosis (5, 6), and liver and other organs may also be involved. Although leptospirosis is primarily a zoonosis, with humans considered as accidental hosts, it is worth noting that transient *Leptospira* sp. shedding does occur during human infection and human-to-human infection, although extremely rare, has been reported (2, 7, 8). Moreover, chronic asymptomatic leptospirosis has recently been shown in a Peruvian population (9), suggesting that the renal colonization is not a peculiarity of some animal carrier but rather it may follow severe acute leptospirosis. Thus, there is a wealth of information to be learned from sublethal and/or chronic models of *Leptospira* sp. infection.

PHYSIOPATHOLOGY OF ANIMAL LEPTOSPIROSIS

In this part, we discuss in an historical perspective the use of animal models of leptospirosis to better understand the physiopathology of the disease, with an emphasis on the mouse model that has been largely overlooked.

Historic Perspective on the Use of Animal Models of Leptospirosis

The first animal model of acute *Leptospira* sp. infection and disease was reported by Inada and colleagues in 1916. They injected blood from a patient with Weil's disease into monkey, rabbit, rat, and guinea pig and observed that 7 days post-infection only the guinea pig developed signs and symptoms consistent with Weil's disease. They followed up with microscopic examination of the liver and detected a bacterium morphologically identical to the spirochete they observed in specimens of blood, intestinal walls, and adrenal glands obtained from patients who succumbed to the disease. They concluded that this spirochete was the pathogenic cause of Weil's disease and named it *Spirochaeta icterohaemorrhagiae* (10).

In the mid-twentieth century, it was reported that golden Syrian hamsters were particularly susceptible to *Leptospira* sp. infection (11). Guinea pigs, golden hamsters, and dogs were then used for laboratory studies of pathogenic *Leptospira* sp. infection (12–15). Over the same period, it was also shown that young white mice were extremely susceptible to infection with *Leptospira interrogans* serovar Icterohaemorrhagiae (16) and that different strains of mice varied greatly in susceptibility to this organism (17). Others reported that mice were susceptible to some of the *Leptospira* sp. serotypes isolated in Northern Australia and that survivors became permanent renal carriers (18). In 1963, a lack of availability of golden Syrian hamsters in Australia led Spradbrow to establish a model of *Leptospira* sp. infection in mice (15–20 g, ~6 weeks old), which produced acute disease with over 50% of mortality and chronic persistent renal infections in the surviving animals. To address the need for complete cure of carriers of chronic renal infections, Spradbrow used this model to study the effectiveness of antibiotic treatments in clearance of *Leptospira* sp. from urine and kidney and found that streptomycin was the only antibiotic of which a single administration regularly cured chronic renal infections (19). Interestingly, as recently as 2001, streptomycin was found to be the most effective anti-*Leptospira* sp. antibiotic in patients diagnosed with Weil's disease (20).

Although guinea pigs and golden hamsters were the animals most commonly used in laboratory studies of *Leptospira* sp. infections in the mid-twentieth century, it was also recognized that both species are less convenient to handle than mice, which are the ideal laboratory animal. Fast forward 50 years and the same questions are debated today worldwide. For example, in the USA, the Animal Welfare Act (AWA) (7 U.S.C. § 2131) is the federal law that regulates the care and use of animals in research. The AWA provides protections for certain species (such as hamsters, guinea pigs, dogs, and non-human primates) while excluding others such as mice (*Mus* sp.) and rats (*Rattus* sp.) bred for research. The fact that guinea pigs and hamsters are covered under AWA protected species leads to onerous regulation (housing, medical records, transportation) and to United States Department of Agriculture official yearly inspections, which in turn results in a considerably lower use of these animals in US laboratories (5–10% use of all protected species). Cumbersome regulations provide the global research community with an opportunity to develop additional mouse or rat models of research for leptospirosis.

Lethal Leptospirosis in Hamsters and Guinea Pigs

Throughout the last quarter of the twentieth century, hamsters have been used as the primary model for acute leptospirosis. The route of infection is via intraperitoneal injection, usually with *Leptospira* sp. resuspended in EMJH medium. Young hamsters (4 weeks old) infected with a broad range of pathogenic serovars of *Leptospira* sp. develop fulminant, disseminated infection, which reproduces the severe form of human leptospirosis with the presence of *Leptospira* sp. in the tissues, destruction of hepatocyte junctions that leads to jaundice, leukocytosis, hemorrhage, endothelial alteration, thrombotic glomerulopathy, and interstitial nephritis (21–23). Hamsters are desert animals traditionally not exposed to the humid conditions that mediate transmission of *Leptospira* sp. Possibly for that reason, hamsters are exquisitely sensitive to leptospirosis since one single organism is able to cause disease (23). The severe leptospirosis induced by pathogenic *Leptospira* sp. in hamsters is associated with enhanced expression of pro-inflammatory cytokines and mediators by peripheral blood cells, such as IL-10, IL-1α, TNF-α, and the cyclo-oxygenase 2 (24). Upon infection with *L. interrogans* serovar Icterohaemorrhagiae strain Verdun, mRNA levels of these immunomodulators have been found to be higher in hamsters that will not survive the infection compared to survivors (25). Also, the intensity of the pro-inflammatory response varies according to the strain used, as do the bacterial loads in organs. Hence, infection with the highly virulent (V) serovar Manilae generates higher levels of cytokine transcripts in lungs and liver compared to the infection with *L. interrogans* Hebdomadis (26). These data suggest that the uncontrolled cytokine production found in hamsters upon infection with pathogenic *Leptospira* sp. may mimic the adverse cytokine storm described in sepsis.

Common applications of the hamster model of leptospirosis include determination of strain infectivity, routes of infection (27), restoring virulence to culture-attenuated strains, assessing usefulness of potential vaccines or diagnostic antigens, and examining pathology of kidney (23).

Severe pulmonary leptospirosis has been studied using the guinea pig model (28) as it replicates the pulmonary hemorrhage and respiratory failure seen in humans. Studies of this model revealed thrombocytopenia, extensive hemorrhage of the lungs, absence of intravascular coagulation, and extensive deposition of immunoglobulin and complement along the alveolar basement membrane, which suggested that an immune process may be involved in the etiology of fatal pulmonary hemorrhage in leptospirosis (28, 29). The same pathology was replicated in dogs, which could also be used as a natural disease model for human leptospirosis (30).

Mouse Models of Leptospirosis

Rats and some strains of mice (31) are generally unsuitable hosts for acute lethal leptospirosis because they develop severe signs of disease only within a short window of time after birth, before 3–4 weeks, but afterward no longer succumb to infection (32). This suggests that a mature immune system achieved by 5 weeks of age in mice (33) is required to control leptospirosis to ensure survival. However, a number of inbred wild-type (WT),

immunosuppressed, or transgenic mice have been used as models of lethal, sublethal and chronic leptospirosis. A summary of mice as animal models to study leptospirosis is provided below and in **Table 1**.

Mouse Models of Lethal Leptospirosis

As the use of inbred strains of mice became ubiquitous in laboratory testing, adult BALB/c mice were infected in the mid-70s with several genospecies of pathogenic *Leptospira* sp. These mice presented with non-lethal dissemination of the bacteria in blood and in tissues, and thus, this mouse strain was associated with resistance to acute *Leptospira* sp. infection (38). However, there are a number of examples of lethal infection of several strains of mice such as infection of adult C57BL/6 mice with 10^8 *L. interrogans* serovar Manilae (38), infection of immunosuppressed BALB/c mice with *L. interrogans* serovar Pomona (43). Adult C57BL/6 mice deficient for toll-like receptor 4 (TLR4) or myeloid differentiation factor 88 (MyD88) (44), μMT mice devoid of B cells (44) as well as C57BL/6 mice deficient for the decay-accelerating factor (DAF)-1ko (37), were shown to die after infection with *L. interrogans* serovar Copenhageni. Furthermore, infection of 4-week old C3H–HeJ mice with *L. interrogans* serovar Icterohaemorrhagiae led to lethality with pulmonary hemorrhage (39). C3H/SCID mice devoid of B and T cells were also sensitive and died from the infection with *L. interrogans* serovar Copenhageni, without lung hemorrhage (40). Those murine models presenting targeted deficiencies in immune system receptors have been instrumental in the discovery of many key factors required to control *Leptospira* sp. in mammalian hosts and are discussed in part 2 of this review. Lethal infection of mice is observed using high doses of inoculum of *Leptospira* sp. (usually 10^6–10^8), whereas in the hamster model, lethal doses of inoculum of *Leptospira* sp. range between 10^2 and 10^3. However, infection of 5-week-old hamsters with doses ranging from 10^2 to 10^6 led to a progressive increase in animal survival with a 50% survival rate in hamsters infected with 10^6 *Leptospira kirschneri* (45), which constitutes an interesting paradox. Although *Leptospira* sp. numbers such as 10^2–5×10^3 were previously quantified in urban slum water gutters in the Peruvian Amazon region of Iquitos (9), the minimum or median *Leptospira* sp. infectious dose in humans is unknown.

Mouse Models of Sublethal Leptospirosis

Lethal outcome inconsistencies in the former studies raised questions regarding the potential usefulness of mice in understanding pathogenesis and clinical disease progression. In the last decade, there has been a surge in the number of studies investigating outcomes of experimental leptospirosis among different strains of mice such as A, CBA, BALB/c, C57BL/6, and C3H–HeJ mice, all showing sublethal infections after intraperitoneal inoculation (34–36, 41, 46) (**Table 1**). Infection of mice strains A, CBA, and C57BL/6 with *L. interrogans* serovar Copenhageni strain Cop led to dissemination of *Leptospira* sp. to the kidneys one month post-infection and to nephritis without apparent colonization. In BALB/c mice, no kidney lesions were observed confirming that this strain is more resistant to infection (34). Two models of sublethal leptospirosis using adult mice have been developed recently (38, 41). Sublethal infection of 7- to 10-week-old C57BL/6 mice with 10^6–10^7 of a bioluminescent version of *L. interrogans* serovar Manilae strain L495 allowed for live imaging of infected albino animals. The advantage of using live imaging is to allow quantification of live *L. interrogans*. Mice exhibited biphasic disease with a self-resolving hematogenous dissemination followed by renal colonization (38). In another study, 10-week-old C3H–HeJ mice were inoculated with 10^6–10^7 *L. interrogans* serovar Copenhageni Fiocruz L1-130. Infection led to bloodstream dissemination of *L. interrogans*, which was followed by urinary shedding, body weight loss, hypothermia, and colonization of the kidney by live spirochetes 2 weeks after infection. In addition, infection triggered inflammation of the kidney but not of the liver or the lung. Infection of mice with pathogenic *Leptospira* sp. seems to depend on infectious dose and on the mouse genetic background. The sensitivity of the techniques used to detect *Leptospira* sp. in the biological samples tested as well as the lack of well-established clinical scores such as weight loss and temperature to record disease course may account for the inconsistencies reported in the literature. As an example, long-term colonization of kidney was also observed in BALB/c mice infected with 10^7 of the bioluminescent version of *L. interrogans* serovar Manilae strain L495 (38).

Mouse and Rat Models of Chronic Leptospirosis

A few years after its discovery, more than 100 years ago (10), human leptospirosis was associated with the presence of rats and mice, identified as asymptomatic renal carriers of a live spirochetal bacterium, named "interrogans" because of its question mark's shape (47). Yet, experimental infection and characterization of the disease in these animals is quite recent (48, 49), most probably because they are relatively resistant to acute disease and therefore were not considered as *bona fide* models of severe human leptospirosis. Nevertheless, knowledge of the biology of pathogenic *Leptospira* sp., the histopathology, and their survival in the reservoir host, as well as their transmission to other hosts is of utmost importance to better understand and counteract the infection in susceptible hosts, like humans.

Two studies characterized renal lesions in brown Wistar rat (*Rattus norvegicus*) experimentally infected through intraperitoneal route with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. At a high dose of 10^8 bacteria, 1 month post-infection, all infected rats were asymptomatic, without any loss of weight compared to non-infected rats, and presented dense *Leptospira* colonization of renal tubules, without evidence of major inflammation. A total of 2–4 months post-infection around 70% of the rats presented renal interstitial nephritis, also observed in kidneys of 50% of captured wild rats found positive for *Leptospira* sp. culture (4, 50).

Interestingly, C57BL/6 mice intraperitoneally infected with 10^7 of a bioluminescent version of *L. interrogans* serovar Manilae strain L495 (38) showed the same features as previously observed in rats (4). Renal colonization remained stable for the lifetime of C57BL6/J mice (38). *Leptospira* sp. persistence may be different in rats, depending on the bacteria serovar used for the infection.

TABLE 1 | Mice as animal models to study disease caused by pathogenic *Leptospira* sp.

Mouse strain	Genotype	Model of disease	Pathogenic *Leptospira* sp. serovar and strain	Biological variables			Tissue dissemination (technique, dpi)	Major findings	Reference
				Sex	Age (w.o.)	Infection route/dose			
A/J	*ahl4* mutation	Asymptomatic	Icterohaemorrhagiae strain Cop	F	4–5	IP/10^3, 10^6	Kidn (IF, dpi 28)	High kidney colonization	Santos et al. (34)
CBA	*pde6b* mutation	Asymptomatic	Icterohaemorrhagiae strain Cop	F	6–7	IP/10^3, 10^6	Kidn (IF, dpi 28)	Inflammatory lesions and interstitial nephritis	Santos et al. (34)
BALB/c	Wild type (WT)	Asymptomatic	Copenhageni strain Cop	ND	ND	IP/10^6, 10^7	Kidn (HP, dpi 28)	No signs of disease	Bandeira et al. (35)
	CB17 SCID	Acute					Kidn, Liv, lun (HP, dpi 28)	Pulmonary hemorrhage	
C57BL/6	WT	Asymptomatic	Icterohaemorrhagiae strain Cop	F	4–5	IP/10^3, 10^6	Kidn (IF, dpi 28)	Inflammatory lesions and interstitial nephritis	Santos et al. (34)
	WT	Asymptomatic	Copenhageni strain Cop	ND	ND	IP/10^3, 10^6 IP/10^6, 10^7	Lun, Kidn (HP, dpi 28)	Interstitial nephritis	Bandeira et al. (35)
	iNOSko	Asymptomatic							
	Rag1-ko	Acute					Kidn, Liv, and Lun (HP, necropsy dpi 7–10)	Pulmonary hemorrhage	
	WT	Asymptomatic Renal colonization	Copenhageni strain Fiocruz L1–130 and Manilae strain L495	F	8–10	IP/2 × 10^8	Liv, Lun, Kidn (qPCR, HP, dpi 15, 30, 60, 90, 180)	Renal fibrosis model Chronic infection	Fanton d'Andon et al. (36)
	WT	Asymptomatic Renal colonization	Copenhageni strain Fiocruz L1–130	ND	3–4	IP/10^6	Bl and Kidn (qPCR, HP, dpi 14, 90)	Renal fibrosis model Chronic infection	Ferrer et al. (37)
	Albino	Asymptomatic Renal colonization	Manilae strain L495 (bioluminescent)	F	7–10	IP/10^7, 10^8	Live imaging (dpi 1–142)	Model of biphasic leptospirosis Chronic infection	Ratet et al. (38)
C3H	C3H/HeJ (*tlr4* point mutation)	Acute	Icterohaemorrhagiae	ND	3	ND/~10^7	Lun, Kidn (HP, dpi 14, 20, 180)	Pulmonary hemorrhage Defects in CD4+ and CD8+ T cells correlate with disease progression	Pereira et al. (39)
	C3H/HeJ and C3H/HeJ/SCID	Sublethal and acute	Copenhageni strain RJ16441	ND	3–6	IP/10^6	Liv, Kidn (HP, dpi 3–17)	No lung hemorrhage	Nally et al. (40)
	C3H/HeJ (*tlr4* point mutation)	Sublethal	Copenhageni strain Fiocruz L1-130	F	8–16	IP/10^6, 10^7	Uri, BL, Kidn (qPCR, HP, dpi 15)	Kidney inflammation Increased CD4+ effector T cells in spleen	Richer et al. (41)
OF1	WT	Asymptomatic	*L. borgpetersenii* Ballum strain B3-13S	ND	6–8	IP/10^8	Kidn, Lun, Liv (HP, qPCR, dpi 14, 21, 28)	Renal carriage No nephritis	Matsui et al. (42)

ND, not described; F, female; w.o., weeks old; IP, intraperitoneal; HP, histopathology; IF, immunofluorescence; BL, blood; Kidn, kidney; Liv, liver; Lun, lungs; Uri, urine; dpi, day post-infection.
Highlighted in gray are the mouse models leading to acute leptospirosis.

In Wistar rats, *L. interrogans* serovar Icterohaemorrhagiae persisted for 220 days but *L. interrogans* serovar Grippotyphosa persisted only 40 days (48) and *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 persisted for 4 months (4). Another study using a different strain of the same bacteria serovar showed that shedding of *L. interrogans* serovar Copenhageni strain RJ16441 in the urine of Sprague-Dawley rats ceased 2–3 months post-infection (51). Altogether, these data suggest that some rats clear *Leptospira* sp. from the kidney. Nevertheless, the fact that both mice and rats shed *Leptospira* sp. for several months after kidney colonization is clearly established. The mechanism leading to the sterilization of the kidney is extremely important and remains to be investigated.

Another common feature of the rat and mouse models is a threshold of infection required to get renal colonization. In rats, the dose of 10^4 bacteria injected intraperitoneally allows for the renal colonization of 50% of rats (4). In C57BL/6 mice, the lower limit to obtain 100% of renal colonization is 10^6 bacteria (36, 38), but this threshold depends on the *Leptospira* sp. serovar and strain, since intraperitoneal injection with 10^3 *L. interrogans* serovar Copenhageni strain Cop is enough to colonize the kidneys of mice (34). Given that the rat can shed up to 10^7 *Leptospira* sp. per ml of urine (52), we may speculate that high concentration of *Leptospira* sp. in water may associate with higher transmission rates. However, the reverse may not necessarily be true.

Renal fibrosis, usually associated with inflammation, is characterized by the pathological accumulation of extracellular matrix components, such as collagen, and may compromise the kidney function of patients with leptospirosis (53). Fibrosis has also been observed in some wild rats (50) and dogs naturally infected with *L. interrogans* (54), and more recently in surviving hamsters experimentally infected with *Leptospira borgpetersenii* serovar Ballum (42). Two recent studies found that mild fibrosis occurs in kidneys of C57BL/6 mice infected with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 and serovar Manilae strain L495 (36, 37). Both studies found interstitial nephritis in infected mice 2 weeks post-infection, decreasing therafter, and sustained fibrosis from 2 weeks until 3 or 6 months post-infection. The use of antibiotic showed that fibrosis is associated with the presence of live bacteria colonizing the kidneys and not antigens (36), as previously suggested *in vitro* (55). Hence, antibiotic therapy initiated in mice 1 day after infection allowed for sterilization of kidneys, and fibrosis was not observed 15 days post-infection. However, when antibiotic treatment started 3 days post-infection, it failed to eradicate *Leptospira* sp. and fibrosis was still observed, although the kidney presented only minimal inflammation (38). In mice, it was also shown that the lack of DAF-1, an early regulator of complement cascades, aggravates fibrosis induced by infection with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (37). Of note, the bacterial load in the kidneys of hamsters, OF1 mice (42), and C57BL/6 mice (36, 37) at the chronic phase of leptospirosis does not correlate with the extent of fibrosis, suggesting that the initial insult of penetration in the tubule, rather than colonization itself or the ensuing inflammation, is important for establishment of fibrosis.

Antibiotics administered at the chronic phase, when *Leptospira* sp. are established in the renal tubules, do not easily eradicate the pathogen in mice (19, 38) or in humans (2). In the chronic stage, Wharthin–Starry staining of rats' kidneys has shown very dense colonization of proximal tubules by *L. interrogans* (50). Whether *Leptospira* sp. form biofilm in the tubules, as *in vitro* (56), remains to be demonstrated but it would explain the *Leptospira* sp. resistance to antibiotics in this phase of infection, in contrast to the acute phase, when antibiotics are effective if administered early (38). Interestingly, in mice, several rounds of the same antibiotic treatment at the chronic phase of disease resulted in stepwise reductions of *Leptospira* sp. (38). Together these studies suggest that a small number of bacteria reach a low number of renal tubules, where they multiply until they completely fill their niche, between 15 days and 3 weeks post-infection. Once the niche is filled, the multiplication rate would compensate the shedding, potentially explaining the observed sustained level of colonization (38). Altogether, these data suggest that the establishment of *Leptospira* sp. in the renal proximal tubules is an early event, occurring in the very first days post-infection, only when the number of *Leptospira* sp. is high enough to overwhelm the natural blood defenses. These data also imply that after the initial entry in a tubule, *Leptospira* sp. do not colonize new tubules, in mice (38). This phenomenon could be due to the efficient immunoglobulin (Ig) response (44, 47) that would control any *Leptospira* sp. leaving a tubule to go back into circulation, before it may infect another nephron. Only two studies in the 1980s addressed the mechanism of entry of the *Leptospira* sp. in the tubules, using experimentally infected swine with *L. interrogans* serovar Pomona, or mice. They described a biphasic infection with first a hematogenous dissemination of *Leptospira* sp. in the kidney, followed in the first 4 days post-infection by the tubular phase, where *Leptospira* sp. cross the tubules till the lumen [reviewed in Ref. (52)]. Thereafter, the proximal tubule of the kidney constitutes a safe niche where *Leptospira* sp. are protected from the activity of the immune system and do not cause major lesions, aside from mild fibrosis, a reminder of the initial tubular insult. The reason why *Leptospira* sp. localizes in the proximal tubules is unknown.

Since chronic asymptomatic leptospirosis with prolonged shedding of *Leptospira* sp. in the urine has also been observed in humans (9, 57), these results should emphasize the fact that prophylactic and antibiotic treatments in humans, like serum therapy (58), are important to be administered early after infection to avoid renal colonization that in the long term may weaken the kidney (59).

Although the intraperitoneal route of infection has been widely used in leptospirosis animal models, it may not reflect the whole process of infection since *Leptospira* sp. penetrate the body through abraded skin or mucosa. A recent study compared infection with 10^7 *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 in 7-week-old Wistar rats through mucosal, subcutaneous and intraperitoneal routes. Rats infected through all routes remained largely asymptomatic. One month post-infection, kidneys of all rats infected intraperitoneally were colonized, without any sign of interstitial nephritis, although only five out of eight rats infected through the mucosal route and only one out of eight rats infected subcutaneously were colonized (60), suggesting that

natural infection does not systematically lead to renal colonization in 7-week-old Wistar rats.

Mouse models of sublethal and chronic infection may allow us to better understand leptospirosis and host factors that lead to immune evasion, which can result in acute or chronic disease and susceptibility or resistance to infection (41).

PROFILING IMMUNE RESPONSES TO PATHOGENIC *Leptospira* USING MOUSE MODELS

The wide availability of genetically defined congenic strains of mice is currently leading to a new trove of knowledge on the engagement of immune system components with pathogenic *Leptospira* sp., which informs our understanding of the pathways to, and the markers for, protective immunity (61).

Protective Role of Antibodies against Lipopolysaccharide (LPS) and Roles of TLR4 and TLR2

The humoral response against pathogenic *Leptospira* sp., described as a "bactericidal substance for the spirochaetae in the blood of patients" and its ability to destroy *Leptospira* sp. has been demonstrated since 1916. In those studies, serum from immunized horses and goats or from convalescent patients with leptospirosis was administered to guinea pigs experimentally infected with *Leptospira* sp. or to leptospirosis patients, respectively (10, 58). The authors drew very robust conclusions about their data. Indeed, to test the efficiency of sera to clear *Leptospira* sp. from the blood of infected animals or patients suffering from leptospirosis, they injected the blood, with or without *Leptospira* sp., into naïve guinea pigs that subsequently did or did not develop leptospirosis (58). More recently, mostly mouse models of leptospirosis produced important clues about the immune cells and receptors involved in susceptibility to leptospirosis (40, 43, 44, 62, 63) (highlighted in **Table 2**). Not surprisingly, B cells appeared to be key players and are important to control leptospirosis. Indeed, BALB/c mice chemically depleted of B cells were shown to be susceptible to lethal leptospirosis induced by *L. interrogans* serovar Pomona in contrast to untreated WT mice (43). This observation was confirmed using C3H/SCID mice and μMT mice on a C57BL6/J background, both genetically deficient for B cells. These mice also died from acute leptospirosis when infected with *L. interrogans* serovar Copenhageni (40, 44). Interestingly, the antibody response to *Leptospira* sp. has been shown to appear quickly after infection (58), as early as 3 days in the case of IgM after intraperitoneal infection with *L. interrogans* serovar Copenhageni (43, 44).

It is known that most of the protective antibody response to *Leptospira* sp. infection are directed against the LPS. However, *Leptospira* sp. LPS is different from one serovar to another, which undermines serovar cross-protection (68). Definite evidence was provided in 1986 with a monoclonal antibody elicited against LPS that protected guinea pigs against leptospirosis (69). A more recent study confirmed passive immunization of guinea pigs with agglutinating monoclonal antibodies against LPS (70). The fact that Nude mice, unable to produce T cells, were still able to mount a protective immune response suggested that protection was T cell independent (64), which was consistent with the fact that LPS is the target of the antibodies since this molecule is known to mediate T independent responses. This observation was confirmed using CD3ko mice, lacking T cells (44). The crucial humoral response is mediated by TLR4, a member of the toll-like receptor family of innate receptors. Those receptors are involved in microbial recognition, through conserved molecular patterns, such as LPS, and are involved in the expression of antimicrobial peptides, chemokines, cytokines, and molecules of costimulation, leading to recruitment of immune cells, which culminates in the elimination of the pathogen, and therefore protection. Indeed, C3H/HeJ mice, known for decades to be resistant to LPS endotoxin shock, were often used as model of infection since they are more susceptible to many different pathogens (i.e., *Borrelia burgdorferi*). They were subsequently shown to have a mutation in TLR4 (71). Four-week-old C3H/HeJ are sensitive to acute leptospirosis upon infection with *L. interrogans* serovar Icterohaemorraghiae (39). Later on, C3H/HeN mice that do not have the point mutation in TLR4 were infected in parallel with C3H/HeJ mice, and proven resistant to death, providing further evidence that TLR4 is important to control leptospirosis (63). One of the mechanisms of susceptibility linked to TLR4 has been shown to rely on the early production of IgM directed against the LPS, and on the production of IgG relying on both TLR4 and TLR2 (44). The leptospiral LPS recognition by TLR4 is most probably the major factor influencing the susceptibility or resistance to infection and disease progression, since the lack of this receptor is enough to confer susceptibility to *Leptospira* sp. infection (44). LPS from *L. interrogans* is atypical and is not recognized by TLR4 in human cells, whereas mice that are able to recognize the *Leptospira* sp. LPS are resistant to lethal leptospirosis (72, 73), providing an explanation why humans are sensitive to acute leptospirosis. Mice are therefore excellent reservoir hosts in the enzootic cycle that maintains this pathogen in the environment. It is tempting to speculate that TLR4 from hamsters, gerbils, and guinea pig would also not be able to recognize the *Leptospira* sp. LPS. Recently, a study using mice showed that TRIF, the adaptor of TLR4 and also TLR3, the receptor of viral RNA, has a protective role during leptospirosis (74). However, the role of TRIF has not been further studied (74), but the results showing decreased humoral defense or higher *L. interrogans* burden in the organs of TRIFko mice suggest that the role of TRIF is indeed linked to TLR4. Also, LPS from *Leptospira* sp. is atypically recognized by TLR2, the receptor of lipoproteins, and this is not conferred by the lipid A moiety, but most probably by a lipopeptide linked to the O antigen (72, 73), which is still unknown. Hence, intraperitoneal injection of purified LPS from *L. interrogans* serovar Icterohaemorrhagiae strain Verdun is able to kill C57BL6/J WT mice but not TLR2ko mice, in a model of LPS toxicity leading to liver injury and mortality, after sensitization of mice with D-galactosamine and IFNγ (72). Whether this atypical TLR2 reactivity of the LPS is linked to virulence is unknown.

LipL32, the major outer membrane (OM) lipoprotein of *Leptospira* sp. is recognized by TLR2, and therefore is able to

TABLE 2 | Mice as animal models to study innate and adaptive immune responses to *Leptospira* sp. infection.

Mouse strain	Genotype	Model of disease	Pathogenic *Leptospira* sp. serovar and strain	Biological variables			Tissue dissemination (technique, dpi)	Major findings	Reference
				Sex	Age (w.o.)	Infection route/dose			
BALB/c	Wild type (WT)	Asymptomatic	Pomona and Hardjo	M, F	~6	IP/~10^8 10^9	BL (dpi 1–30)	B cells are important to control *Leptospirosis*	Adler and Faine (43)
	Immunosuppressed	Acute							
	Athymic nude	Asymptomatic	Pomona L10 and Copenhageni L45	ND	5–6	IP/~3 × 10^8	BL (dpi 2–10)	Humoral response crucial to control *Leptospirosis*	Adler and Faine (64)
	Immunosuppressed	Acute							
	WT	Asymptomatic	Copenhageni strain Fiocruz L1-130	ND	ND	IP/2 × 10^8	Kidn (HP dpi 28)	No interstitial nephritis	Athanazio et al. (46)
	IL4ko	Asymptomatic							
	WT	Asymptomatic	Icterohaemorrhagiae strain Cop	F	4–5	IP/10^6	BL, Kidn (IF dpi 28)	High IgG response No kidney lesions	Santos et al. (34)
C57BL/6	WT, TNFRko, IFNγko	Asymptomatic	Copenhageni strain Fiocruz L1-130	ND	ND	IP/2 × 10^8	Kidn (HP dpi 28)	TNFR involved in interstitial nephritis	Athanazio et al. (46)
	WT, CD3ko, TLR2ko	Asymptomatic	Copenhageni strain Fiocruz L1–130	F	8–10	IP/2 × 10^8	BL, Kidn (qPCR, qRT-PCR dpi 3)	Toll-like receptor 4 (TLR4) and TLR2-dependent IgM, IgG, iNOS and IFN-γ responses	Chassin et al. (44)
	TLR4ko; TLR2/4dko; MyD88ko	Lethal							
	μMT; Rag2ko	Lethal							
	CD3ko	Asymptomatic	Copenhageni strain Fiocruz L1-130	F	8–10	IP/10^6	Kidn (qPCR, ELISA, qRT-PCR dpi 3)	Downregulation of transporters in kidneys NLRP3/TLR2/4 dependent IL1β secretion	Lacroix-Lamande et al. (65)
	WT	Asymptomatic							
	TLR2/4dko	Sublethal infection							
	WT, TLR2ko; TLR3ko; TLR5ko; TLR9ko	Asymptomatic chronic renal colonization	Copenhageni strain Fiocruz L1–130 and Manilae strain L495	F	8–10	IP/10^6	Liv, Lun, and Kidn (qPCR and HP dpi 15)	TLRs, NLRs, T and B-cells are not involved in fibrosis iNOS participates in fibrosis	Fanton d'Andon et al. (36)
	TLR4ko; TLR2/4dko; MyD88ko	Sublethal infection							
	Nod1ko; Nod2ko; Nod1/2dko, Casp1ko	Asymptomatic							
	μMT	Sublethal							
	iNOSko	Asymptomatic							
	CD3ko	Asymptomatic							
	Daf1ko	Lethal chronic renal colonization	Copenhageni strain Fiocruz L1–130	ND	3–4	IP/10^6	BL and Kidn (qPCR and HP dpi 14 and 90)	Lack of Daf1 enhances nephritis and fibrosis	Ferrer et al. (37)
	WT	Asymptomatic	Copenhageni strain Fiocruz L1–130 and Manilae strain L495	M	4	IP/5 × 10^6	BL, Kidn, and Liv (qPCR and HP dpi 3)	Pathogenic *Leptospira* sp. infection trigger formation of neutrophil extracellular traps	Scharrig et al. (66)
C3H	C3H/HeJ (*tlr4* point mutation)	Lethal and sublethal	Icterohaemorrhagiae strain HAI188	F	3–6	IP/6 × 10^8 IP/10^8	Heart, Spl, Kidn, Lun, and Liv (qPCR and HP dpi 21)	TLR4 response important to control *Leptospira* sp. infection	Viriyakosol et al. (63)
	C3H/SCID								
	C3H/OuJ (functional TLR4)	Asymptomatic							
	C3H/HeJ	Lethal and sublethal	Copenhageni strain Fiocruz L1-130	ND	3	IP/10^7	Kidn (qPCR and HP dpi 14)	Protective role of NO against *Leptospira* sp.	Pretre et al. (67)

dko, double knockout; ND, not described; F, female; M, male; w.o., weeks old; IP, intraperitoneal; BL, blood; Kidn, kidney; Liv, liver; Lun, lungs; Spl, spleen; HP, histopathology; IF, immunofluorescence; dpi, day post-infection. Highlighted in blue are the mouse models potentially leading to lethal infection.

induce inflammation *in vitro* in proximal kidney cells (72, 75, 76). However, the relevance for kidney infection is not clear, since the lack of TLR2 did not change fibrosis and did not reduce inflammation in kidneys of mice 15 days post-infection with *L. interrogans* (36). Fibrosis was also induced only in the presence of live bacteria, not in the presence of LipL32 antigens, present after an antibiotic treatment (36). Nevertheless, *Leptospira* sp. antigens from the OM, including the lipoprotein LipL32 may be responsible for nephritis observed in other animals, as was very elegantly demonstrated in zebrafish larvae (77). Posttranslational modifications of LipL32 have recently been studied using *Leptospira* sp. retrieved from urine of rats chronically infected with *L. interrogans* serovar Copenhageni strain RJ16441 (78). The authors showed acetylation or trimethylation of lysines of LipL32, only in the case of *Leptospira* sp. retrieved from urine but not from *Leptospira* sp. grown in culture. They further showed that the lysine modifications lowered the reactivity toward sera from infected patients, suggesting a role in escape from the immune response and helping the maintenance of *Leptospira* sp. in the proximal tubules. Of note, the peculiar *Leptospira* sp. lipid A was also shown to be methylated, the methyl group inactivating a phosphate group (79), known to be important for human TLR4 recognition of lipid A. Whether posttranslational methylation modification is a general strategy of *Leptospira* sp. to escape the immune response remains to be investigated.

Protective Host Mediators in Leptospirosis

IL-1β is a pleiotropic cytokine, central in inflammation. Its expression is tightly regulated by two signals, the first deriving from a NF-κB pathway such as TLR or TNF stimulation. The second signal activates the inflammasome, a platform of proteins leading to activation of Caspase 1, able to cleave the pro-IL-1β to mature IL-1β, which can be secreted. Using mouse bone marrow-derived macrophages *in vitro*, it has been shown that *Leptospira* sp. trigger these cells to produce IL-1β, through the Nod-like receptor protein 3 (NLRP3) inflammasome (65). In mice, LPS and lipoproteins are recognized by TLR4 and TLR2, which participate in IL-1β secretion by priming mRNA expression of the pro-IL-1β and NLRP3. *Leptospira* sp. activate the NLRP3 inflammasome through a dysregulation of the potassium flux (65), due to glycolipoprotein action, known to downregulate the potassium pump (80). This has been confirmed *in vivo* after infection with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 of TLR2ko or TLR4ko mice. It was shown that all the transporters were downregulated and the IL1β decreased in both TLR2ko and TLR4ko mice, 3 days post-infection (65).

Another inflammatory cytokine, IFN-γ is generally recognized as a protective cytokine, able to prime the phagocytic activity of macrophages. IFN-γ mRNA expression peaks in hamsters between 8 and 18 h post intraperitoneal infection with *L. interrogans* serovar Icterohaemorraghiae strain Verdun in blood (24). Upon infection with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, IFN-γ mRNA is expressed in the organs of mice 3 days post-infection through a TLR2- and TLR4-dependent pathway (44). IFN-γ has been shown to be produced by T cells in kidney and also by parenchymal cells, and in liver, its production is linked to the presence of B cells (44). The beneficial effect of T cells, potentially through IFN-γ production, has been shown in the lungs and kidneys of C3H/HeJ mice infected with *Leptospira* sp. and depleted in CD4 and CD8 T cells (65) and in CD3ko/C57BL/6 mice (44).

Nitric oxide (NO) is an antimicrobial compound, produced by the inducible nitric oxide synthase (iNOS) in macrophages and endothelial cells. Upon infection with *Leptospira* sp., iNOS is expressed 3 days post-infection in kidneys and lungs of mice in a TLR2- and TLR4-dependent manner (44). NO is secreted by parenchymal cells in kidneys of infected mice (44), and it plays a damaging role in nephritis (35) and in renal fibrotic lesions induced by *Leptospira* sp. (36). Experiments conducted with both Syrian hamsters and C3H/HeJ mice treated with a specific inhibitor of iNOS showed increased mortality and aggravated renal lesions upon infection with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, suggesting that overall NO plays an important antimicrobial role against *Leptospira* sp. (67). Of note, in renal lesions and in pulmonary hemorrhage induced by *L. interrogans* serovar Copenhageni, the potential contribution of autoimmune response has been ruled out since in both cases the lesions were still present in mice without B and T cells (35, 36).

Other than protective responses resulting from the stimulation of both TLR4 and TLR2, it was shown that leptospirosis induced a TLR2- and TLR4-independent inflammation, also independent from other TLRs. Indeed, in mice deficient for MyD88, the adaptor of almost all TLRs except TLR3, the inflammation upon infection with *L. interrogans* was equivalent to the inflammation found in double TLR2/TLR4ko mice (44). The receptor responsible for this detrimental inflammation remains to be determined.

Role of Neutrophils and Macrophages in Leptospirosis

Although neutrophilia is a common feature of human leptospirosis, only few neutrophils were identified in kidneys of mice 3 days post-infection with *L. interrogans* (44, 66) and depletion of neutrophils did not change the overall course of the disease (44, 66, 81), suggesting that neutrophils are not major players in murine leptospirosis. However, a recent study in C57BL/6 mice showed that *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 triggered, in the dissemination phase, the generation of neutrophil extracellular DNA traps (66). NETs from human neutrophils killed *L. interrogans in vitro*. The depletion of neutrophils in mice resulted in increased bacteria 3 days post-infection in blood and also in kidneys 14 days post-infection, although nephritis was not changed, compared to untreated infected mice. These results suggest that NETs are an important mechanism of host defense occurring early, impairing the dissemination of bacteria in the tissues. However, *Leptospira* sp. that manage to settle in the kidneys are obviously able to escape this defense (66), suggesting that these bacteria reached the kidneys before the onset of NET generation 1–2 days post-infection. Alternatively, NET escape may be due to a nuclease activity found in pathogenic but not in saprophytic *Leptospira*

sp. (66). Unchanged nephritis despite higher bacterial colonization is difficult to interpret but reminds us that fibrosis is not proportional to the bacterial load (36).

Several *in vitro* and one *in vivo* study depleting macrophages with silica particles suggest that macrophages can phagocyte *Leptospira* sp., at least when they are opsonized with specific Ig (43, 64, 82). This has been recently confirmed *in vivo* using zebrafish (83). Indeed, zebrafish embryos are a powerful model to study host–pathogen interactions. They have a functional innate immune system close to the mammalian immune system. The embryos are transparent and allow tracking of fluorescent bacteria. Using Syto-83 dye labeled and unstained *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, the authors showed rapid encounter and internalization of *Leptospira* sp. in phagocytes 2 h post intravenous injection and survival of *Leptospira* sp. in these cells until 48 h post-infection (83). Strikingly, the infected phagocytes changed morphology and specifically migrated in a dorsal part of the embryo, which has never been observed with other pathogens, suggesting that phagocytes could help *Leptospira* sp. traffic inside the host (83). Whether this observation could be relevant in mammals is unknown.

Human Genetic Polymorphisms Associated with Susceptibility to Leptospirosis

Human genetic polymorphism studies are very useful to understand the mechanisms involved in immune response to diseases. Only a few studies have been performed on polymorphisms in innate immune genes known to be associated with infectious diseases that could be relevant for leptospirosis (84–86). A study on an Argentinian population suggested that polymorphisms on both *tlr2* and in *tlr1* genes may confer enhanced susceptibility to severe leptospirosis (86). It has also been suggested that, among other factors, polymorphisms in *IL-4* and *IL-4R* (84) and *IL-1*β, *IL-12R*, and *CISH* (multiple cytokines inducible SH2-containing protein gene) (85) correlated with susceptibility to leptospirosis. However, these studies produced discordant results, despite the fact that two were performed a few years apart in two distinct cohorts of patients from Terceira (84) and São Miguel (85) neighboring islands in the Azores archipelago, Portugal. The role of IL-4 has been studied in mice, but the resistance of BALB/c/IL-4ko mice to the infection with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was comparable to WT BALB/c, suggesting that IL4 does not play a major role in leptospirosis in these mice (46). The association of *tlr2* polymorphism with leptospirosis (86) was not found in the Terceira Island study (84), which also did not find the *IL-1*β polymorphism found in the São Miguel Island (85). Interestingly, the São Miguel study did not find polymorphisms in TLR4 associated with susceptibility to leptospirosis (85), which is consistent with the finding that the atypical LPS from *Leptospira* sp. does not signal through TLR4 in humans (69, 70). The small cohorts (around 100) of leptospirosis patients may be one of the factors decreasing the significance of these results. Thus, larger studies with multifactorial analysis are needed to confirm the potential protective roles of cytokines and TLRs in human leptospirosis.

A summary of the use of mice as animal models to study innate and adaptive immune responses to *Leptospira* sp. infection is provided in **Table 2**, and a diagram of the immune response to *Leptospira* sp. infection is provided in **Figure 1**.

VIRULENCE FACTORS OF *Leptospira* sp. ASSESSED WITH ANIMAL MODELS OF LEPTOSPIROSIS

To establish whether a gene encoded by a pathogen can contribute to its virulence, the classical approach involves genetic inactivation and study of the outcome of infection with the mutant strain. Targeted genetic manipulation of *Leptospira* sp. is still not achievable on a routine basis; however, random mutagenesis by transposon insertion made it possible to generate mutant strains that can be tested in animal models to evaluate the role of the mutated gene in virulence. In a limited number of studies, complementation of the mutant was achieved (87–90) and allowed to fulfill the molecular Koch's postulates (91), since the complementation of the mutated non-virulent (NV) strains restored virulence.

This section describes the *Leptospira* sp. virulence factors characterized in studies fulfilling the following criteria: (1) no difference in the *in vitro* growth rate at 30°C between the WT and mutant strains; (2) the WT *Leptospira* sp. strain caused death of all the infected individuals (lethal challenge), whereas the mutant strain, at equivalent dose, results in the survival of all animals. Moreover, we discriminate the bacterial phenotype as V, causing death of the host, or NV, which does not kill the host and is not found in organs and kidneys (**Figure 2**). A mutant strain is considered NV attenuated when it still colonizes target organs, including kidneys. Hamsters, guinea pigs, and gerbils used for studying acute leptospirosis will succumb to the infection in a period ranging from 5 to 10 days for V strains, whereas they will survive with no clinical signs of the infection when NV or attenuated strains are administrated. Some of the studies also included a test in rats or mice to check kidney colonization.

As shown in **Figure 2** and **Tables 3** and **4**, around 20 genes have been tested for their potential role in virulence in leptospirosis. Half of them have been identified as essential for virulence (highlighted in **Tables 3** and **4**). Two components of the cell wall, the endoflagella and LPS, appear to be true virulence factors, since mutations in several genes involved in motility or LPS synthesis, result in the loss of virulence (**Figure 2**; **Table 3**).

Endoflagella As a Virulence Factor

A mutant of the flagellar subunit FlaA2 (92) resulted in a flagellum more flexible than the WT. This bacterium lost translational motility and was not virulent in hamsters. Moreover, 5 days post-infection, the FlaA2 mutant was not found in organs, suggesting that motility is also essential for early dissemination in tissues (92). Similarly, an abundant protein exposed at the surface of the flagellum filament, called Flagellar coiling protein (Fcp)A was recently characterized (88). Mutants in the *fcp*A gene, either from clinical isolates or constructed by allelic exchange, lost the hook shape, presented uncoiled flagella, and lost motility (88). This mutant also lost its ability to translocate *in vitro* across polarized kidney cells (88), suggesting that the step of active penetration in

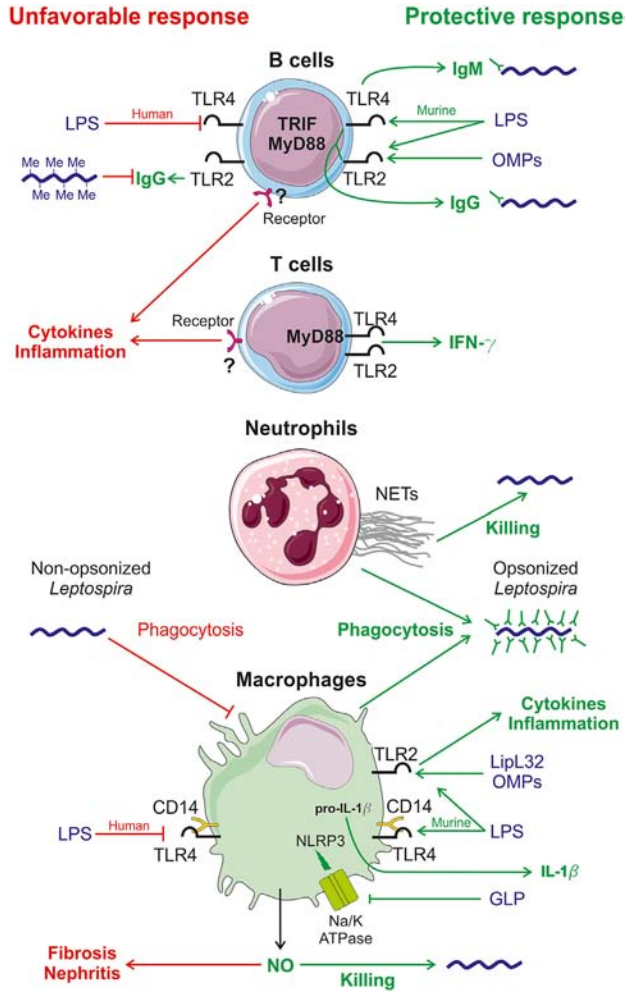

FIGURE 1 | **Continued**

Diagram of immune responses induced by *Leptospira* sp. infection in mice. Known innate responses to *Leptospira* sp. involve neutrophils, macrophages but also B and T cells. Recognition of *Leptospira* sp. mostly occurs through the TLR2 pathway, sensing outer membrane proteins (OMPs) such as the lipoprotein LipL32, the major leptospiral OMP and the atypical LPS (72), which is also recognized by toll-like receptor 4 (TLR4) in mice (73). Protective host responses are depicted in green on the right side, whereas potential unfavorable responses are in red, on the left side. In mouse B cells, TLR4 stimulation by leptospiral LPS leads to the early production of partially protective IgM (44), via the TRIF adaptor (74). TLR2 and TLR4 responses, through the Myd88 adaptor, also control the production of protective IgG (44). *In vivo* methylation of LipL32 in rat has been shown to reduce its recognition by human antiserum (78). In humans, leptospiral LPS is not recognized by TLR4 (73), potentially leading to disease, as observed with TLR4 mutant mice (39, 44, 63). In mouse T cells, *Leptospira interrogans* signal through the MyD88-dependent activation of TLR4 and TLR2 receptors and trigger the production of the protective pro-inflammatory cytokine IFN-γ that activates macrophages (44). Both T and B cells, by sensing leptospiral components through an unknown receptor, are involved in the production of an unfavorable pro-inflammatory cytokine response (44). In humans, neutrophils play a slight protective role for the host against *Leptospira* sp. infection due to the production of bactericidal neutrophil extracellular traps (NETs) (66). Neutrophils and macrophages barely phagocytize non-opsonized *Leptospira* sp. but opsonized *Leptospira* sp. with specific IgG are readily killed by phagocytose (43, 82). Macrophages produce pro-inflammatory cytokines leading to a protective inflammatory state by sensing OMPs, including LipL32, via TLR2 (72). The atypical leptospiral LPS is also detected by TLR2 and CD14, the co-receptor of TLR4 (72). Acting in concert with TLR2 and TLR4 activation, leptospiral glycoprotein blocks the Na/K-ATPase pump that triggers the activation of the Nod-like receptor protein 3 (NLRP3) inflammasome and enables the secretion of the pro-inflammatory cytokine IL-1β (65). Upon *L. interrogans* infection, macrophages and other cells also produce nitric oxide (NO), which has a positive and a negative effect. NO has a protective effect through the action of its antimicrobial role (67) and the negative effect is that NO activity favors kidney fibrosis (36) and nephritis of infected hosts (35, 67).

the tubule might require bacterial motility. Moreover, the intraperitoneal or conjunctival infection of FcpA mutant strains in hamsters did not result in disease or colonization of the kidneys, although complementation restored the flagella coiling, translational motility, and virulence. Mutations on different components of the flagellar motor switch, such as FliY (93) and FliM (89), resulted in mutant strains that are deficient in motility in soft agar plates and were not able to cause disease in guinea pigs or hamsters, respectively. Of note, the trans-complementation with a WT copy of the *fli*M gene on the pMaori replicative plasmid (105) restored motility and virulence. These results indicate that when the flagellar motor is not able to propel the flagella or when the bacteria do not have a fully functional flagellum, pathogenic *Leptospira* sp. are impaired in their ability to move, which as a consequence impairs the dissemination in host tissues and prevents disease progression.

LPS As a Virulence Factor

Several mutant strains in genes annotated as LPS biosynthesis genes showed a NV phenotype (**Table 2**; **Figure 2**), demonstrating that this cell wall component is crucial for virulence. The LPS is a complex molecule encoded by a huge locus larger than 100 kb, rather conserved among pathogenic *Leptospira* sp. strains (106). LPS is constituted of three parts, the lipid A moiety that anchors the LPS in the OM, a conserved core oligosaccharide, and a polysaccharide component, the immunogenic O antigen, which is highly variable in composition and length. Antigenic diversity constitutes the basis of the serological classification of pathogenic *Leptospira* sp. Two mutants in the LPS biosynthesis locus of the *L. interrogans* Manilae strain L495, M895 altered in *Lman-1456* (LA1641) and M1352 altered in *Lman-1408* (94) have been obtained by random mutagenesis (94). Both of the genes were of unknown function and *Lman-1408* was specific to the Manilae strain. According to electrophoretic profiles after silver staining, M895 was truncated in the O antigen but not M1352. Both presented altered recognition by a polyclonal serum against the WT Manilae L495 strain and were NV in the hamster model, even at the very high dose of 10^7 bacteria. Interestingly, it was later shown that both mutants M895 and M1352 were also impaired in their ability to colonize the kidney of BALB/c mice (107) (**Table 3**).

Two other mutant strains of *L. interrogans* serovar Manilae L495 have been obtained by random mutagenesis in two different genes, both annotated *lpx*D, coding for LpxD, the

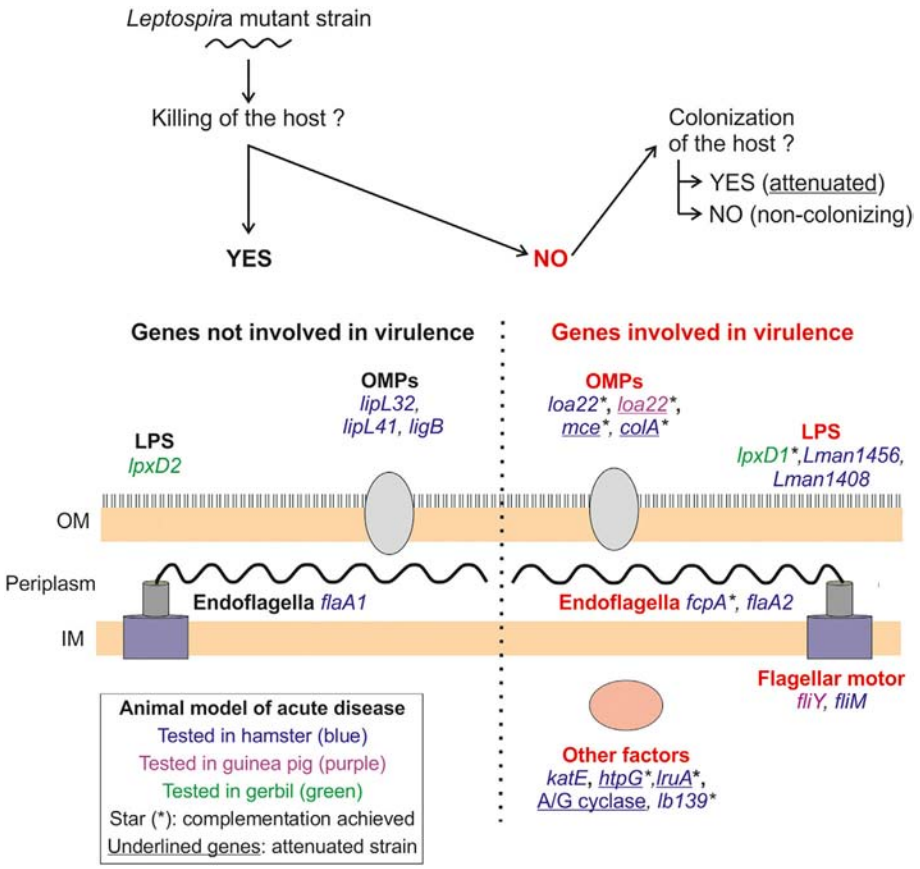

FIGURE 2 | *Leptospira* sp. virulence factors identified in animal models of lethal disease. The panel depicts the different factors (in capitals) and genes tested for virulence in different animal models (summarized in **Tables 3** and **4**) and their localization in *Leptospira* sp. The mutant strains were inoculated via the intraperitoneal route, using both lethal and sublethal bacterial doses, in different animal models for acute disease: hamster (blue), guinea pig (purple), and gerbil (green). In all cases, the mutant strains were compared with their wild-type counterpart to assess the effect on virulence of the mutated genes. As schematized on top of the panel, we discriminate the genes in two groups; the genes not involved in virulence (on the left side of the panel), since the mutant strain killed the host, and the virulence genes (right side of the panel) determined since the mutant strain did not cause the death of animal. In this category, we further distinguish the non-virulent (NV) mutants that did not colonize target organs and the NV attenuated ones that did. Attenuated mutants are indicated by underlined names. Complementation of mutated genes was not always achieved, and the complemented mutants are indicated with a star (*). OM, outer membrane; IM, inner membrane; OMP, outer membrane protein.

N-acyltransferase of lipid A biosynthesis (**Table 3**). In different bacteria, change in temperature upon entry in the host regulates the function of LpxD, which modifies the number or length of acylated chains in the lipid A. Since LPS is an abundant component of the OM, the modified hydrophobicity of the lipid A can directly influence the OM fluidity. This mechanism appears to be important for the host adaptation to temperature changes, and therefore to virulence (108). The mutant strain in the gene la0512 (90) annotated *lpx*D1, was NV in gerbils. Compared to the parental strain or to the second mutant lpxD2, still V, the LpxD1 mutant had altered growth at 37°C, although growth at 30°C was not altered. The LpxD1 mutant also had reduced resistance to the antimicrobial peptide polymyxin B at 37°C, reflecting altered OM integrity. Complementation of the LpxD1 mutant restored all altered phenotypes, as well as virulence (90). However, the structure of the lipid A from the WT and mutant strains grown *in vitro* at different temperatures were analyzed by Maldi-MS but surprisingly it did not show major modifications of the structure (90), found identical to the lipid A structures from *L. interrogans* serovar Icterohaemorraghiae strain Verdun and serovar Pomona strain L170 (79). These results raised questions about the biochemical function of the *Leptospira* sp. enzymes annotated as LpxD that potentially could acylate components other than lipid A. Nevertheless, the LPS of the LpxD1 mutant was not recognized by a polyclonal serum against L495, suggesting that the enzyme is indeed modifying the LPS structure. Moreover, in *L. interrogans* serovar Copenhageni strain L1-130, able to cause acute disease in the guinea pig and asymptomatic chronic renal colonization in rats, it has been shown that the O antigen content of the LPS is decreased in bacteria retrieved from the liver of moribund guinea pigs, compared to the LPS of bacteria retrieved from the kidneys of rats. The latter also showed the same electrophoretic profiles than the LPS prepared from bacteria grown in EMJH (51). These data suggest that the modulated expression of the O antigen

TABLE 3 | Animal models to study *Leptospira interrogans* mutants in motility and LPS biosynthesis genes.

Function	*L. interrogans* serovar and strain	Gene/name	Biological variables				Bacterial phenotype	Tissue dissemination (technique, dpi)	Reference
			Animal	Sex	Age (w.o.)	Infection route/dose			
Motility	Australis *702*	*fliM* (LIC11836) Flagellar motor switch protein	Hamster	F	6	IP/10^6, 10^8	NV (**c**$^+$)	ND (dpi 21)	Fontana et al. (89)
	Manilae strain L495	*flaA1* (LIC10787) Flagellar subunit	Hamster	M	4	IP/10^3, 10^6	V	Kidn+; Liv+ (qPCR, dpi 5–7)	Lambert et al. (92)
	Manilae strain L495	*flaA2* (LIC10788) Flagellar subunit	Hamster	M	4	IP/10^3, 10^6	NV	Kidn−; Liv− (qPCR, dpi 21)	Lambert et al. (92)
	Lai strain Lai	*fliY* (LA2613) Flagellar motor switch protein	Guinea pig	ND	3	IP/6×10^8, 1.2×10^9, 3×10^9, 6×10^9	NV	ND	Liao et al. (93)
	Copenhageni strain Fiocruz LV2756	*fcpA* Flagellar coiling protein	Hamster	M	3–6	IP/10^8	NV (**c**$^+$)	Kidn−; Liv−; Lun−; Spl−; Eye−; BL− (qPCR, dpi 21)	Wunder et al. (88)
						CJ/10^8	NV	Kidn−; Liv−; Lun−; Spl−; Eye−; BL− (qPCR, dpi 21)	
LPS synthesis	Manilae strain L495	*lpxD1* (LA051) LPS modification enzyme	Gerbil	ND	ND	IP/10^4	NV (**c**$^+$)	Kidn− (qPCR, culture, dpi 20)	Eshghi et al. (90)
	Manilae strain L495	*lpxD2* (LA4326) LPS modification enzyme	Gerbil	ND	ND	IP/10^4	V	Kidn+ (qPCR, culture, dpi 20)	Eshghi et al. (90)
	Manilae strain L495	*Lman-1456* (LA1641) LPS synthesis	Hamster	ND	ND	IP/10^3	NV	Kidn− (culture, dpi 21)	Murray et al. (94)
						CJ/10^6	NV	ND	
	Manilae strain L495	*Lman-1408* (LA1641) LPS synthesis	Hamster	ND	ND	IP/10^3	NV	Kidn− (culture, dpi 21)	Murray et al. (94)

*M, male; F, female; ND, not described; w.o., weeks old; IP, intraperitoneal; CJ, via conjunctiva; NV, non-virulent; V, virulent; **c**$^+$, complementation restored virulence; BL, blood; Kidn, kidney; Liv, liver; Lun, lungs; Spl, spleen; dpi, day post-infection.*

The numbers in brackets refer to the corresponding annotated genes in L. interrogans Copenhageni strain Fiocruz L1-130 (LIC), or in L. interrogans Icterohaemorraghiae strain Lai (LA). Highlighted in light orange are the genes involved in virulence.

part of the LPS is important for virulence in the acute model of infection. These studies showed that *Leptospira* sp. LPS is crucial for virulence although the underlying mechanism remains to be understood. Potentially, the decreased O antigen could help the bacteria escape early antibody response, shown in mice to occur as soon as 3 days post-infection (44). At the chronic phase in the rat, the O antigen is fully expressed, possibly protecting *Leptospira* sp. from the IgG response. We could speculate that this complete form of LPS could provide bacteria shed in urine with some advantage in survival in the environment or in the early phase of infection.

LoA22, Mammalian Cell Entry (Mce), and ColA OMPs Are Virulence Factors

The OMP LoA22, whose function is still unknown, is one of the few OM candidate protein (**Table 3**; **Figure 2**) shown to be a virulence factor (87). LoA22 was found to be the most upregulated OMP in the liver of moribund guinea pigs infected with *L. interrogans* serovar Copenhageni (109). In hamster, the *loA22* mutant of the *L. interrogans* serovar Lai was NV, and the complementation restored the virulence. Interestingly, the same mutant was found only attenuated in guinea pigs, since it did not kill the animals and was not found in the liver but still colonized the kidneys (87). These results suggest that LoA22 displays an important role at the acute phase of the infection during multiplication in blood or dissemination in tissues, but may not be a key player in permeation of the tubules.

Other OMPs that might be considered as involved in invasion of *Leptospira* sp. are the Mce protein (98), which has homologs in other pathogenic bacteria, and the collagenase A protein ColA (97), encoding for a protein involved also in host–pathogen interactions during invasion and transmission. Both mutants for Mce and ColA proteins are attenuated.

LipL32, LipL41, and LigB OMPs Are Not Virulence Factors

All the other OM components tested were not involved in virulence (**Table 4**; **Figure 2**). Particularly striking is the case of LipL32, which is the most abundant lipoprotein of the OM and is highly immunogenic, and also expressed during acute infection of guinea pig (109), but whose expression is downregulated in the blood of OF1 mice and hamsters (110). Knockdown of LipL32 did not abrogate virulence of the strains tested in the acute and the chronic model of the disease, leading to death of the hamsters and kidney colonization of rats in the same extent of the WT strain (95). Moreover, this mutant was

TABLE 4 | Animal models to study *Leptospira interrogans* mutants in outer membrane proteins (OMPs) genes and other factors.

Function	*L. interrogans* serovar and strain	Gene/name	Biological variables				Bacterial phenotype	Tissue dissemination (technique, dpi)	Reference
			Animal	Sex	Age (w.o.)	Infection route/dose			
OMPs	Manilae strain L495	*lipL32* (LIC11352) OMP	Hamster	ND	ND	IP/10^3	V	Kidn+; Lun+; Liv+ (HP, dpi 7–14)	Murray et al. (95)
				ND	ND	CJ/10^6	V	ND	
			Wistar rat	ND	6	IP/10^8	V	Kidn+ (HP, dpi 15)	
	Manilae strain L495 Pomona strain LT993	*lipL41* (LA0615) OMP	Hamster	M/F	4–6	IP/10^3, 10^4	V	Kidn+ (culture, dpi 21)	King et al. (96)
	Lai strain Lai 56601	*loa22* (LA0222) OmpA-like protein	Hamster	M	6–8	IP/5 × 10^7, 10^8	NV (**c**$^+$)	ND	Ristow et al. (87)
			Guinea pig	M	2–3	IP/2 × 10^8, 4 × 10^8	NV (**c**$^+$) A	Kidn+; Liv– (culture, dpi 21)	
	Lai strain Lai 56601	*colA* (LA0872) Collagenase	Hamster	M	ND	IP/10^6	NV (**c**$^+$) A	Kidn+; Lun+; Liv+; Uri+; BL+ (CFU, dpi 14)	Kassegne et al. (97)
	strain Lai	*mce* Mammalian cell entry protein	Hamster	M	4	IP/10^5, 10^6, 10^7, 10^8, 10^9	NV (**c**$^+$) A	Uri+ (CFU, dpi 14)	Zhang et al. (98)
	Copenhageni strain Fiocruz L1-130	*ligB*	Hamster	M	5–8	IP/10, 10^2,10^4 10^6	V	Kidn+	Croda et al. (99)
			Wistar rat	ND	4–5	IP/10^8	V	Kidn+ (culture, dpi 9)	
Other factors	Manilae strain L495	LIC12327 Adenylate/guanylate cyclase	Hamster	F	4	IP/10^6	NV A	BL+; Kidn+; Liv+ (qPCR, dpi 4)	Lourdault et al. (100)
	Manilae strain L495	LB139	Hamster	ND	ND	IP/10^6	NV (**c**$^+$)	BL–; Kidn–; Liv– (qPCR and IF dpi 5 and 25)	Eshghi et al. (101)
						CJ/10^7	NV (**c**$^+$)	ND	
	Manilae strain L495	*htpG* (LB058) High-temperature protein G	Hamster	M/F	4–6	IP/10^3, 10^5, 10^7	NV (**c**$^+$) A	Kidn+; Lun+; Liv+ (qPCR, dpi 5)	King et al. (102)
	Manilae strain L495	*lruA* (LIC11003)	Hamster	M	4	IP/10^3	NV A	Kidn+ (qPCR, dpi 21)	Zhang et al. (103)
	Manilae strain L495 Pomona strain LT993	*katE* (LA1859; LIC12032) Catalase	Hamster	M	4	IP/10^6	NV (L495)	ND (dpi 21)	Eshghi et al. (104)
							NV (LT993)	ND (dpi21)	

M, male; F, female; ND, not described; w.o., weeks old; IP, intraperitoneal; CJ, via conjunctiva; NV, non-virulent; V, virulent; NV A, attenuated or partially virulent; **c**$^+$*, complementation restored virulence; BL, blood; Kidn, kidney; Liv, liver; Lun, lungs; Uri, urine; CFU: colony forming units; HP, histopathology; IF, immunofluorescence; dpi, day post-infection.*
The numbers in brackets refer to the corresponding annotated genes in L. interrogans Copenhageni strain Fiocruz L1-130 (LIC), or in L. interrogans Icterohaemorraghiae strain Lai (LA). Highlighted in light orange are the genes involved in virulence and in light yellow the attenuated mutant strains, non virulent but still colonizing the kidneys.

still V when administered through the conjunctiva, showing that Lip32 is not necessary in the first steps of the infectious process of penetration through the mucosa (95). It is important to note that absence of LipL32 has a deep impact on the cell, with 46 genes modulated, as shown by microarray analysis (95), although LipL32 does not affect the virulence process. Likewise, the third most abundant OM lipoprotein LipL41 is not necessary for virulence of *Leptospira* sp. in the acute model of the disease, since all the animals infected with the mutant strain succumbed to the infection (96). These results, both from LipL41 and LipL32, suggest either that these lipoproteins, of unknown function, may be important for the survival of *Leptospira* sp. in the environment or that the numerous lipoproteins encoded in the *Leptospira* sp. genome could have redundant functions, which could allow bacteria mutated in a single lipoprotein gene to retain virulence. Notably, a targeted mutant of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 in the protein LigB, considered to be involved in *Leptospira* sp. adhesion to the host and upregulated 24 h post-infection *in vivo* in blood of both OF1 mice and hamsters (110), retained its virulence in both models of acute and chronic disease (99) (**Table 4**).

Other Virulence Factors

The catalase KatE (104) is another factor linked with loss of virulence, which could be involved in escape of bactericidal activity

from neutrophils and macrophages due to detoxification of ROS produced by phagocytes. This hypothesis is supported by the fact that KatE is located in the periplasmic space of *Leptospira* sp. cell wall where it can participate in ROS resistance. Mutants of the chaperone HtpG (102), which is related to virulence in other bacterial species and of the protein LruA, a 28 kDa surface-exposed lipoprotein that might interact with the serum apolipoprotein A1 (103) lead to an attenuated phenotype in the hamster model of acute infection.

Also, a putative regulatory locus of *L. interrogans* Manilae L495 (*lb139*), important for multiple gene regulation, including motility genes has been recently shown to be a virulence factor. Indeed, the lb139 insertion mutant did not kill hamsters, nor did it colonize the organs (101).

A high-throughput method has been developed to screen for new virulence factors in *L. interrogans* serovar Manilae strain L495. The technique consists in injecting in the hamster or in the BALB/c model of renal colonization, a pool of mutants obtained by random mutagenesis. Then, presence of each mutant is tested by specific PCR, in the pool of *Leptospira* sp. retrieved in culture from the kidneys of moribund animals, compared to the pool of mutants grown in culture. The mutants not found in organs are assumed to be NV (107). Recently, an improved version of this technique, allowing for direct quantification of mutants in organs and blood, and therefore able to assess their relative fitness, has been performed in hamsters. A new virulence factor, an adenylate/guanylate cyclase gene, has been identified. This mutant was attenuated when tested individually in hamsters (100). Interestingly, a soluble adenylate cyclase was previously identified as a putative virulence factor using comparative sequencing of a V strain of *L. interrogans* serovar Lai strain 56601 versus an isogenic culture derivative strain. This protein was shown *in vivo* to be highly upregulated in the hamster model compared to the EMJH culture, and *in vitro* to elevate the intracellular cyclic AMP in macrophages (111), therefore potentially reducing the host innate TNF response, as previously shown in *Mycobacteria*-infected macrophages (112).

Another recent study using RNA-Seq compared the transcriptome of V *L. interrogans* serovar Copenhageni grown *in vitro* in EMJH, to *Leptospira* sp. cultivated *in vivo* within a dialysis chamber implanted in the peritoneal cavity (DMC) of Sprague-Dawley rats (113), mimicking the host adapted state. 10 days post inoculation, motile *Leptospira* sp. harvested in exponential growth were used to prepare RNAs. Other than a core of genes identically regulated between the two conditions, the authors found 166 genes differentially expressed, most of them being specific of the pathogenic but not the saprophytic strain (113). The analysis of the upregulated genes, including a comparison with other serovars, provides a comprehensive picture of the genes important in the host–pathogen interactions. Some genes already known as virulence factors, such as collagenase A, were expressed 50 times more in DMC than *in vitro*. Also found in these studies were lipoproteins, hemolysins, and flagellar components, but most of them are unknown genes that remain to be studied (113).

A summary of the virulence factors of leptospirosis is presented in **Figure 2**.

CONCLUSION AND FUTURE STUDIES

Animal models, such as guinea pigs, gerbils, and in particular hamsters, have been instrumental to understand the pathophysiology of lethal leptospirosis, as well as to get insight into the *Leptospira* sp. genes involved in virulence. Using these animal models, high-throughput methods, relatively easy to perform, spare animal use and can be applied each time a new random mutagenesis (99, 114, 115) is performed and might facilitate the discovery of new virulence factors of pathogenic *Leptospira* sp. These models have also been very important to test vaccines, which was not the focus of the present review. Vaccines for leptospirosis have been recently reviewed by Adler (116).

The overlooked mouse model has recently proved its usefulness to provide insight both in sublethal and chronic leptospirosis, in particular the host immune mechanisms that control pathogenic *Leptospira* sp., thus unlocking an avenue of research into the immunological mechanisms of susceptibility or resistance to *Leptospira* sp. infection. Moreover, these versatile murine models are appropriate to test new vaccine candidates and drugs to treat leptospirosis. In the near future, the availability of CrispR/Cas 9 technology should provide new tools to design both new deficient mice and targeted mutants of *Leptospira* sp. to study the contribution of unique genes in host–pathogen interaction studies. The new genetic tools available should also help shift research from the current descriptive forms to a more complex mechanistic evaluation of the pathophysiology induced by pathogenic *Leptospira* sp. with the goal of finding the function of specific genes. Indeed, transgenic mice models, devoid of certain genes or cell subsets that can further be studied in different compartments using the Cre/Lox system, are unique means to understand the crucial question of how *Leptospira* sp. overcome their hosts' immune responses.

Alternative, non-traditional models could also contribute to the understanding of chronic infection. Natural outbred strains of mice (*Mus musculus,* Swiss Webster) can be used to study how pathogenic *Leptospira* sp. establish effective infection in reservoir hosts without causing disease. Natural hosts of human pathogens such as *Leptospira* sp. develop tolerance to the pathogen and are usually asymptomatic when infected, which is the opposite to an immune response developed by the "accidental" host (human) that succumbs to the pathogenic effect of the agent. These non-traditional animal models could be used in comparative studies with strains of mice engineered to be susceptible to *Leptospira* sp. dissemination. In the "One Health" context, research on resistance and tolerance in reservoir hosts is required for development of Public Health measures targeting natural reservoirs and for understanding the mechanisms of human leptospirosis and other infections.

Human leptospirosis studies are mostly epidemiologic, using blood or urine as biological samples, and can only provide a limited amount of information about mechanisms of host–pathogen interaction. Long-awaited human polymorphisms studies have not provided clear answers about innate immune genes involved in protection against leptospirosis. Therefore, notwithstanding ethical concerns about experimentation in humans

and animals, increased regulations, and justified restrictions about animal use in research, it is extremely important to keep developing and using animal models of leptospirosis to further our understanding of the disease as well as to identify vaccine candidates, therapeutics, and diagnostic assays. New vaccines and immuno- or other therapies to prevent rapid progression of *Leptospira* sp. through tissues and colonization of the kidney could help prevent severe cases of the disease and save lives. We may think that the clinical picture of leptospirosis is rather well known, but some unexpected findings may challenge our current knowledge. For example, *Leptospira* sp. were found in lungs of asymptomatic wild rats carrying the spirochete in their kidneys, adhering to the ciliated surface of the bronchi (117). In addition to the intraperitoneal route of infection, necessary to precisely determine infectious dose, more physiological routes of infection, such as the conjunctival, transdermal, and other mucosal routes that have recently been studied in rats (60) and hamsters (27), should be investigated in different hosts, including mice, gerbils, and guinea pigs. Using a natural route of infection may be how we could develop a long-awaited model for neuroleptospirosis (118). In addition, disease progression might be different if *Leptospira* sp. is deposited in the peritoneum or if it is deposited in skin or mucosal surfaces and expected to waddle through the natural barriers including skin, extracellular matrix, and the immune system before it reaches target organs.

As an alternative to mammal use, the development of the *Zebrafish* model of leptospirosis (77, 83) should in the future also help with innate immunity studies. Indeed, the NF-κB and Interferon signaling pathways are conserved through evolution, and the generation of genetic mutants in Zebrafish is amenable (119). Likewise, the development of a *Caenorhabditis elegans* model, that is well established for characterization of molecular mechanisms involved in pathogen activation of innate immune pathways, as well as pathological mechanisms, would be welcomed to study leptospirosis since the genome of this worm is reduced and the genetic tools are available (120). However, it is not known yet whether *Leptospira* sp. are pathogenic for this nematode, like are other human or zoonotic pathogens such as *Coxiella*, *Salmonella*, or *Pseudomonas* sp.

Leptospirosis is a zoonosis, and humans are just one among many other vertebrates to be affected by the disease (3). Epidemiologic data show that less than 7% of people infected with pathogenic *Leptospira* sp. die from severe forms of leptospirosis (3, 121). The current view that humans mostly suffer from acute severe disease is outdated and has recently been questioned given that clinical manifestations of human disease recapitulate all the different clinical profiles and stages found in animals. It ranges from asymptomatic to lethal, encompassing sublethal as well as chronic forms. Indeed, chronic renal infection that occurs in endemic areas of leptospirosis and asymptomatic disease, revealed by seropositive serum against *Leptospira* sp., has recently been shown to favor chronic kidney disease (3, 9, 59). Interestingly, all these clinical forms can be studied using the animal models presented in this review. These data suggest that the dichotomy between acute and chronic diseases may be a man-made artifact and that the classification of leptospirosis according to the symptoms may not represent the continuum of a biphasic disease, with blood dissemination and seeding of target organs with *Leptospira* sp., which may be severe enough to impair organ function and cause death, or depending on the capability of the host immune response to control *Leptospira* sp. dissemination, the disease can be mild allowing for natural recovery and kidney colonization.

Leptospirosis is a neglected disease with a limited number of groups working on it. Compared to *B. burgdorferi*, another spirochete that causes Lyme disease, we still lack a lot of useful tools, such as GFP or RFP fluorescent *Leptospira*, sp. allowing for efficient imaging in cells and tracking of bacteria in the host. Hopefully, recombinant labeled strains will be constructed, potentially using the replicative plasmid pMaori, adapted to pathogenic *Leptospira* sp. (105), or CrispR/Cas 9 technology. Although not allowing sensitive imaging in organs, bioluminescent strains have been developed and may also give some clues about the kinetics and localization of *Leptospira* sp. in different hosts, as well as horizontal or vertical routes of transmission of *Leptospira* sp. within hosts, and in the environment (38).

An important topic that until now has been overlooked is the species-specificity adaptation of *Leptospira* sp. to their hosts. We know that some *Leptospira* sp. serovars are more commonly associated with particular hosts, such as Ballum with mice, Canicola with dogs, Hardjo with cattle, or Icterohaemorrhagiae with rats (122, 123). Lately, sequencing of hundreds of leptospiral genomes highlighted specific features of pathogenic strains (123, 124), but the basis of the host preferences of *Leptospira* sp. is unknown. Immune factors, such as TLR4 and TLR2, contribute to the resistance of the host, and most probably shape the nature of the pathogen interaction with a particular species. On the other hand, recent work showed that the LPS of pathogenic and intermediate *Leptospira* sp. were different (125). Therefore, future structure–function and expression studies of TLRs and NLRs from different animals toward molecules from different serovars should give interesting clues about the sensitivity of a given species. Hopefully, it would explain the leptospirosis symptoms, and target organs that may vary from one host to another, such as the ocular manifestation in horses, placenta infection in cattle, or renal insufficiency in dogs, for example.

Summary

In addition to other animal models, congenic and transgenic mouse models of leptospirosis offer a myriad of possibilities to advance knowledge of host–pathogen interactions especially as it concerns the study of early innate and adaptive immune responses to pathogenic *Leptospira* sp. Mice can be used for profiling immune responses to dose-dependent sublethal, lethal, and chronic leptospirosis, which could inform understanding of the natural disease progression in longitudinal studies. From an applied research standpoint, mice are well-accepted experimental models that can be readily adapted to higher throughput testing of either vaccine candidates or therapeutic options.

AUTHOR CONTRIBUTIONS

MGS wrote the introduction and summary, contributed to the writing of the physiology part, and edited the manuscript and tables. IS contributed to the writing of the virulence part and did the figures and tables. CW wrote and edited the manuscript and tables.

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REVIEW
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Borrelia burgdorferi Keeps Moving and Carries on: A Review of Borrelial Dissemination and Invasion

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Borrelia burgdorferi is the etiological agent of Lyme disease, a multisystemic, multistage, inflammatory infection resulting in patients experiencing cardiac, neurological, and arthritic complications when not treated with antibiotics shortly after exposure. The spirochetal bacterium transmits through the *Ixodes* vector colonizing the dermis of a mammalian host prior to hematogenous dissemination and invasion of distal tissues all the while combating the immune response as it traverses through its pathogenic lifecycle. The innate immune response controls the borrelial burden in the dermis, but is unable to clear the infection and thereby prevent progression of disease. Dissemination in the mammalian host requires temporal regulation of virulence determinants to allow for vascular interactions, invasion, and colonization of distal tissues. Virulence determinants and/or adhesins are highly heterogenetic among environmental *B. burgdorferi* strains with particular genotypes being associated with the ability to disseminate to specific tissues and the severity of disease, but fail to generate cross-protective immunity between borrelial strains. The unique motility of *B. burgdorferi* rendered by the endoflagella serves a vital function for dissemination and protection from immune recognition. Progress has been made toward understanding the chemotactic regulation coordinating the activity of the two polar localized flagellar motors and their role in borrelial virulence, but this regulation is not yet fully understood. Distinct states of motility allow for dynamic interactions between several *B. burgdorferi* adhesins and host targets that play roles in transendothelial migration. Transmigration across endothelial and blood–brain barriers allows for the invasion of tissues and elicits localized immune responses. The invasive nature of *B. burgdorferi* is lacking in proactive mechanisms to modulate disease, such as secretion systems and toxins, but recent work has shown degradation of host extracellular matrices by *B. burgdorferi* contributes to the invasive capabilities of the pathogen. Additionally, *B. burgdorferi* may use invasion of eukaryotic cells for immune evasion and protection against environmental stresses. This review provides an overview of *B. burgdorferi* mechanisms for dissemination and invasion in the mammalian host, which are essential for pathogenesis and the development of persistent infection.

Keywords: *Borrelia burgdorferi*, Lyme disease, dissemination, invasion, vascular interaction, protease, motility, chemotaxis

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INTRODUCTION

Lyme disease, the leading tick-borne infection in the United States, occurs in multiple stages and is a multisystemic disease due to the etiologic agent *Borrelia burgdorferi* (1–4). This disease was first recognized during the 1970s when a cluster of rheumatoid-like arthritis cases occurred in patients who were predominantly children in Lyme, Connecticut (5). Lyme disease has become a significant emerging infectious disease with the Centers for Disease Control (CDC) estimating approximately 300,000 new cases in 2013 in the United States (6). The spirochetal bacterium is transmitted when an *Ixodes* tick vector colonized with *B. burgdorferi* takes a blood meal on reservoir mammals, such as small rodents and birds, or accidental human hosts, resulting in the colonization of dermal tissue and develops into a localized infection (7). This earliest stage of Lyme disease is characterized by a painless bulls-eye rash, called an erythema migrans, experienced by approximately 70–80% of patients at the site of the tick bite (1–3). In the absence of the distinctive erythema migrans, Lyme disease can be difficult to diagnose due to non-specific flu-like symptoms including headache, neck stiffness, malaise, fatigue, myalgia, and fever. During localized infection, the number of *B. burgdorferi* cells increases in the dermal tissue in preparation for dissemination to sites of secondary colonization. Days to weeks after infection, *B. burgdorferi* progresses onto a disseminated stage when the pathogen travels away from the site of the tick bite through the bloodstream and/or lymphatic system to invade and colonize various tissues, such as the heart, synovial fluid of joints, and the nervous system. Patients can develop secondary erythema migrans at distal locations on the skin from the original site of infection. Late infection, the third stage of disease, develops months to years after exposure to *B. burgdorferi* and patients can experience different manifestations including neuroborreliosis, Lyme carditis, and/or arthritis. Following antibiotic treatment, a subset of patients continues to present with arthritic symptoms that has been designated postinfectious, antibiotic-refractory Lyme arthritis (8).

Borrelia burgdorferi is a gram-negative "like" bacterium that is a member of the Spirochaetaceae family known by its distinctive spiral morphology and motility propelled by the endoflagellum (9, 10). Motility of *B. burgdorferi* is essential for pathogenesis throughout the enzootic cycle, though swimming traits differ greatly in the *Ixodes* tick than that observed in the mammalian host (11, 12). The segmented genome of *B. burgdorferi* is unlike most bacterial pathogens consisting of a 910,725 bp linear chromosome along with up to 23 circular and linear plasmids ranging in size from 5 to 56 kb (13, 14). The borrelial genome is minimal in size relative to other bacterial pathogens but is also limited in genes annotated to encode metabolic capabilities, virulence, and defense mechanisms. The glycolytic pathway is the only complete metabolic pathway identified in the annotated genome while pathways for generating energy, amino acids, and lipid synthesis, to name a few, are noticeably incomplete or absent (15, 16). *B. burgdorferi* scavenges from its environment to fulfill basic needs for survival and the pathogen goes as far as incorporating host lipids into the outer membrane (OM) that could also serve as an evasion mechanism (17). The simple bilayer membrane structure and limited number of detoxification genes provides little protection to *B. burgdorferi* from the onslaught of the host immune response, specifically oxidative and nitrosative stress (17–22). Borrelial oxidative stress regulator (BosR) has been associated with regulating genes involved in combating oxidative stress and is important for establishing mammalian infection (23–31). A single superoxide dismutase (sodA) aids in the resolution of reactive oxygen species (ROS), but to date a functional catalase has not been identified (32, 33). The reduced form of coenzyme A (CoASH) has been shown to serve in the place of catalase in *B. burgdorferi* by reducing hydrogen peroxide (22). Dynamic temporal and spatial regulation of borrelial genes is crucial for successful colonization, dissemination, and invasion of *B. burgdorferi* in the tick vector and mammalian host (7, 34, 35). Borrelial two-component pathways HK1/Rrp1 and Rrp2-RpoN-RpoS in the tick vector and mammalian host, respectively, regulate genes with a variety of functions including metabolism, chemotaxis, antigenic variation, and adhesion (7, 34). *B. burgdorferi* gene regulation also contributes to immune evasion as it progresses through the different stages of murine infection by inducing the recombination events of an antigenic variation gene (vlsE) and the activation or repression of lipoprotein genes in a tissue specific and temporal manner (36). The lack of secretion systems or toxins limits the ability of *B. burgdorferi* to modulate the resulting inflammatory disease.

Escape of *B. burgdorferi* from the tick midgut to the hemolymph during a blood meal is an important step for transmission through the salivary glands to a mammalian host providing the necessary environmental cues, such as temperature and pH, for adaptation (7, 34). *B. burgdorferi* gene regulation in response to environmental cues is the best characterized for the early localized stage of infection, but much remains to be understood about the mechanisms for dissemination and invasion necessary for the later stages of Lyme disease (7, 35, 37). Dissemination is a necessary step in pathogenesis, and this process inhibits clearance by the immune system, but the specific mechanisms and host–pathogen events that initiate and complete this stage of infection are not well understood. Invasion of *B. burgdorferi* allows the pathogen to reach immunoprotected niches in the mammalian host where the pathogen is not cleared, but induces inflammation. The intimate interaction between *B. burgdorferi* lipoproteins and vascular tissue, in particular with extracellular matrices (ECM), has been shown to be integral to borrelial pathogenesis (38–41). *B. burgdorferi* adhesin interactions with the ECM and/or requirements for mammalian infection have been thoroughly reviewed by several contributors in the field (38–40), but the specific roles for many of these adhesins in dissemination and/or invasion has not been evaluated, therefore, were not addressed in this review. The purpose of this review is to highlight the mechanisms utilized by *B. burgdorferi* for dissemination and invasion of tissues of human and reservoir host.

MORPHOLOGY, MOTILITY, AND CHEMOTAXIS REGULATION

A discussion of dissemination and invasion would be incomplete without briefly describing the unique morphology, motility, and

chemotaxis of *B. burgdorferi* that has been previously thoroughly reviewed (10, 12, 42). *B. burgdorferi* is often referred to as a gram-negative "like" bacterium because it is void of lipopolysaccharide (LPS) in its OM, but has a similar membrane organization. The unique helical shape of spirochetes is due to its composition of the lipid bilayer OM, a periplasmic space containing peptidoglycan and endoflagella, and a second lipid bilayer inner membrane (IM) forming the inner most compartment of the cell structure (also called the protoplasmic cell cylinder). *B. burgdorferi* cells are long in length and thin in diameter with dimensions of 10–20 μm and approximately 0.3 μm, respectively. We have limited knowledge about the role of peptidoglycan in the morphology and motility of *B. burgdorferi*, but recent work has demonstrated that the temporal and spatial regulation of peptidoglycan synthesis occurs separately from cell elongation and septum formation take place separately, thus potentially contributes to the unique cell shape (43). In the periplasmic space, a flagellum is attached to each pole then travels toward the midline of the cell and potentially overlaps with the flagellum anchored at the opposite end of the cell (10, 12). Multiple flagellum, ranging from 7 to 11 individual flagellum, cluster at the poles of the cell and arrange themselves into a ribbon to form the borrelial endoflagella that wraps around the periplasmic cylinder generating the characteristic helical or planar flat-wave morphology. The concealment of the flagella within the periplasmic space preventing recognition by the host innate immune response is a passive means of immune evasion by *B. burgdorferi*.

The intricacy of the borrelial cell structure and complexity of its motility program makes the accompanying regulation astoundingly complex. Motility of each flagella in the cluster requires the coordinated activities of the basal body, hook, and filament (10, 12) (**Figure 1**). The basal body includes the export apparatus (FliH and FliI), C-ring switch complex (FliG and FliM), MS ring (FliF), collar structure, FliL, rod (FliE), P-ring, and the motor stator (MotA and MotB). The export apparatus and C-ring are located on the cytoplasmic face of the IM and associates with the MS ring that is flanked by FliL and stator embedded in the IM. FliL is important for the orientation of the motor and localizes between the stator and rotor. MotB, a motor protein, is required for *in vivo* helical morphology and motility (44). The rod extends from the MS ring into the P ring

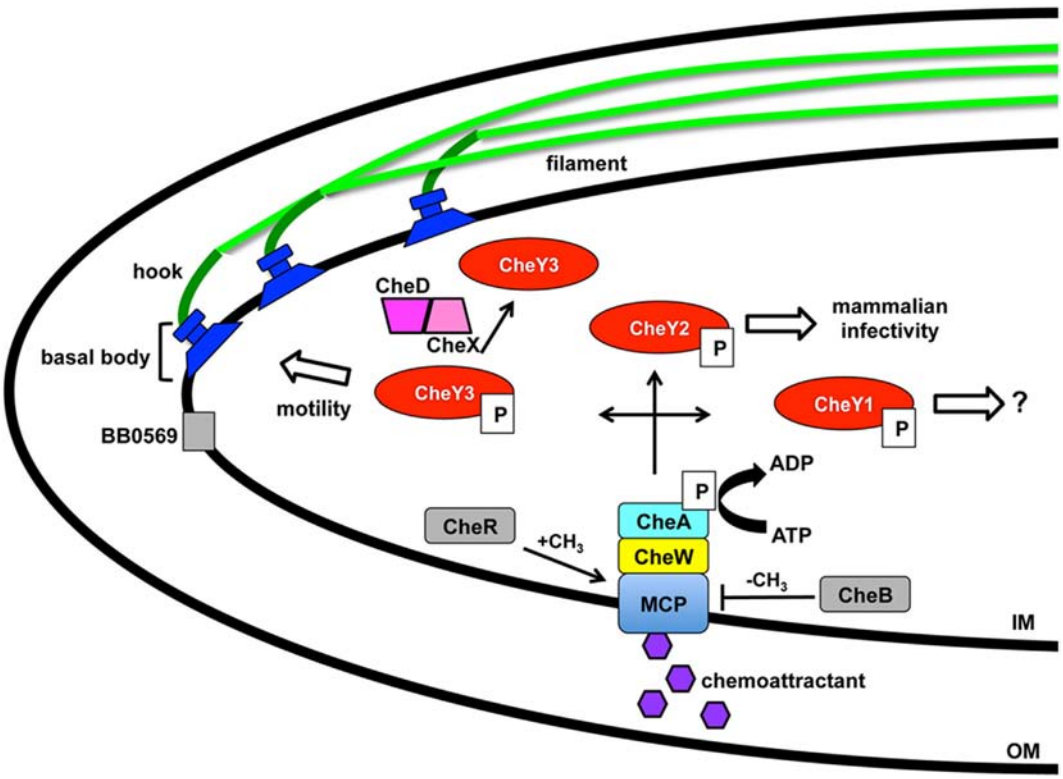

FIGURE 1 | *Borrelia burgdorferi* motility and associated regulation. Motility of *B. burgdorferi* is driven by multiple flagellum in the periplasmic space that are comprised of an inner membrane anchored basal body, hook, and filament. Coordinated rotation of the flagellar motors drive the direction of swimming or causes flexing of the spirochete. A two component regulatory system controls the motility in response to chemoattractant signals through the membrane-bound chemoreceptor and methyl-accepting chemotaxis protein (MCP). A complex is formed between MCP, linker protein CheW, and histidine kinase CheA with the latter able to autophosphorylate and transfer the phosphate to response regulators CheY1, CheY2, and CheY3 resulting in distinct outcomes. Levels of CheY-P can be reduced by the phosphatase CheX that forms a complex with CheD and increases its activity. The borrelial proteins designated in gray are chemotaxis homologs that have not been characterized to date. BB0569 localizes to the poles of the cell near flagellar motors and influences motility, but its specific function is unknown. CheR and CheB are potential methyltransferase and methylesterase proteins, respectively, which may modify MCP. Modification of Xu et al. (54).

prior to transitioning into the flagellar hook. The rod and P-ring flank the collar and genes involved in collar formation have not been identified in *B. burgdorferi*. Recently identified, FlbB is a novel flagellar motor protein that associates with the basal body to achieve the proper orientation of the flagella and collar assembly (45). The primary contributor to the flagellar hook is FlgE that connects to the filament composed of FlaA and FlaB that are exported by a SecA-mediated pathway and Type III flagellin-specific pathway, respectively (10, 12). Coordinated motility of polarized endoflagella is a complex process for directional swimming or non-directional flex modality of borrelial cells. Endoflagella anchored on opposite ends rotation is coordinated to drive swimming in a single direction by one flagella moving clockwise and the second counterclockwise. Reversal of swimming occurs when both endoflagella change direction of rotation. The non-directional mode of motility occurs when the both endoflagella move in a clockwise or counterclockwise rotation resulting in a flex state that is similar to the tumble pattern of movement observed for other bacteria (46). *B. burgdorferi* motility in conventional liquid cultivation is widely homogeneous with the cells in a constant swim state. However, the *in vivo* environments in which the pathogen traverses throughout the enzootic cycle are rarely consistent. *In vitro* cultivated *B. burgdorferi* in a media complex with 3% gelatin displayed heterogeneous motility patterns not unlike that observed in mammalian dermis (47). Another morphology taken on by *B. burgdorferi* is a round body, also referred to as a cystic formation, which is a proposed reversible survival mode when under conditions of stress (48–53). The mechanism for round body formation and the role of borrelial endoflagella in this process is unknown.

Borrelial motility is required for virulence, but more specifically is essential for the invasion of tissues in response to environmental signals (10, 12). A two-component signal transduction pathway regulates *B. burgdorferi* chemotaxis and utilizes a membrane-bound chemoreceptor and methyl-accepting chemotaxis protein (MCP) that forms a complex with linker protein CheW and histidine kinase CheA to sense environmental cues (**Figure 1**). CheA autophosphorylates prior to transferring the phosphate to three response regulators, CheY1, CheY2, and CheY3, encoded by *B. burgdorferi*. A low concentration of CheY-P initiates a run motility and high CheY-P concentrations causes *B. burgdorferi* to flex. CheX phosphorylase rapidly reduces the amount of CheY-P and is enhanced by forming a complex with CheD (55, 56). CheY3 is the only CheY response regulator that has a role in motility and CheY1 or CheY2 are not able to complement the function of CheY3 (57). Phosphorylated CheY3 interacts with the flagellar switch proteins to regulate the rotation of the motors. Xu et al. recently demonstrated a requirement of CheY2 for infectivity. However, this protein does not play a role in chemotaxis or motility (54). *B. burgdorferi* encodes homologs for chemotaxis adaptation proteins CheB and CheR, but to date, these proteins have not been characterized. In *E. coli*, CheR and CheB alter the methylation of MCP by methyltransferase and methylesterase activity, respectively, in response to positive and negative stimuli (46). The number of chemotaxis homologs encoded in the *B. burgdorferi* genome is greater and far more complex than other motile pathogens, such as *E. coli* or *S. enterica* (13, 14). Additional genes, *csrA* and *bb0569,* influence the motility regulation of *B. burgdorferi* (58, 59). Carbon storage regulator, CsrA, is involved in posttranscriptional regulation of flagellar genes and contributes to cell morphology (59, 60). BB0569, annotated as a hypothetical protein, has sequence similarity to MCPs and was found to localize to the poles of the borrelial cell and important for motility and chemotaxis (58). *B. burgdorferi* motility is regulated throughout the enzootic cycle with the distinct behavior of intermittent swimming in the tick vector that includes periods of a non-motile state relative to the persistent swimming observed in the murine host (44, 61).

DISSEMINATION

Borrelia burgdorferi disseminates from the site of the tick bite within the mammalian host to secondary colonization sites requiring dramatic adaptation of the pathogen in response to environmental changes and obstacles presented by the host innate immune response (7, 62–65). A bottleneck occurs during the *B. burgdorferi* initial colonization of dermal tissue when establishing localized infection. This process is in part mediated through the MyD88 pathway, which potentially alters the spirochetal population progressing into the dissemination stage when it infects distal tissues (66–70). *B. burgdorferi* dissemination in mammals occurs by hematogenous and non-hematogenous routes, such as through the lymphatic system or direct spread through the tissues, with the former being the most well understood (71, 72). *B. burgdorferi* hematogenous dissemination has been the focus of much research identifying distinct infectivity classifications of environmental or human isolates by the ribosomal spacer type (RST) or ospC sequence heterogeneity (73). Three RST genotypes have been defined for *B. burgdorferi* as RST1, RST2, and RST3 in descending order of dissemination capabilities (71, 74). At least 16 different *ospC* genotypes that vary in the ability to disseminate and cause disease have been recognized (73, 75–80). More human infections have been associated with *ospC* types A, B, I, H, and K, but there is no correlation with the frequency distribution of *ospC* genotypes in infected *Ixodes scapularis* (73, 81). Genotypes A and B are part of RST1, type I is in RST2 and H and K in RST3 (73). Genotypic differences can predict the ability of a *B. burgdorferi* strain to disseminate and cause infection. The effectiveness or lack of physical barriers, host factors, and immune response can also influence the outcome of borrelial infection.

B. burgdorferi Hematogenous Vascular Interactions

After the establishment of a localized dermal infection, *B. burgdorferi* begins to disseminate throughout the mammalian host *via* the hematogenous route (3, 71, 82). *Borrelia hermsii*, etiologic agent of relapsing fever, reaches high bacterial loads in the blood. However, *B. burgdorferi*, which also disseminates by hematogenous route, is difficult to isolate from blood due to the low number of bacterial cells present. The monitoring of bioluminescent *B. burgdorferi* in real time during murine experimental infection

with an *in vivo* imaging system (IVIS) demonstrated the pathogen established a strong localized infection signified by elevated emission of light centered around the site of inoculation followed by dissemination throughout the skin (83). The inoculum dose altered the spatial and temporal dissemination of *B. burgdorferi* constitutively expressing codon-optimized firefly luciferase, but the general pattern of dissemination was the same independent of dose (83, 84). Upon reaching specific tissues or target sites, *B. burgdorferi* traverses dense extracellular matrix (ECM) and crosses tissue barriers through yet to be elucidated mechanisms.

Hematogenous dissemination of pathogens within mammals is poorly understood due to the difficulty of evaluating bacteria under the shear stress conditions in the blood stream. Shear stress is a type of tangential stress that acts along a parallel surface due to friction caused by fluid viscosity that is overcome by bacteria for dissemination and invasion (85). The utilization of intravital microscopy (IVM) and spinning disk confocal intravital IVM allowed the evaluation of biofluorescent *B. burgdorferi* in real time under *in vivo* shear stress conditions that characterized the multiple stages of vascular interaction prior to transmigration (86–88). GFP-expressing *B. burgdorferi* visualized by IVM in murine ears approximately 3–4 weeks after infection demonstrated swimming and flexing motility commonly observed under *in vitro* cultivation (86). Microvascular interactions visualized in the murine skin flank by epifluorescence and spinning disk confocal IVM following intravenous injection of biofluorescent *B. burgdorferi* found that bacterial cells did not localize to arterioles, but localized to capillaries, postcapillary venules, and large veins. The study defined *B. burgdorferi* dissemination interactions as transient tethering-type associations, dragging interactions, and stationary adhesion (**Figure 2**). It is important to note that not all of the *B. burgdorferi* cells associated with the vasculature and thus were not directly assessed (86). Rapid extravasation of *B. burgdorferi* that did not form prior interactions was observed from postcapillary venules. Transient tethering-type interactions occurred briefly when *B. burgdorferi* slowed down relative to the velocity of the blood stream to associate with the endothelium at one region or tip of the cell in a manner that appeared to tether the cell to the surface followed by a detachment. Tethering stabilizes interactions by reducing the dissociation rates of *B. burgdorferi* from the endothelium (89). Dragging, another type of short-term interaction, was distinguished from tethering by *B. burgdorferi* coming into contact with the endothelium over the length of the cell and being dragged along the vascular surface in the direction of blood flow (86). The final stage of vascular interaction prior to transmigration is stationary adhesion where the *B. burgdorferi* cell is at a complete stop and no longer progressing in the direction of the blood stream. Borrelial cells maintained stationary adhesion for an average of 10 min. 3D visualization was performed to determine the localization of interactions in relation to endothelial junctions labeled with PECAM-1 that found stationary adhesion primarily occurred at the junctions while tethering and dragging took place along the endothelial cell surface (86). Non-infectious borrelial cells lacking genes for OM lipoproteins did not display the same interactions with the endothelium as infectious *B. burgdorferi* indicating pathogen ligands that can bind to specific host receptors (87). An *in vitro* flow chamber model was developed to evaluate *B. burgdorferi* interactions with human endothelia under a controlled environment for shear force (89). *B. burgdorferi* endothelial interactions in postcapillary venules by IVM and those in the flow chamber were consistent, thus providing an opportunity to understand

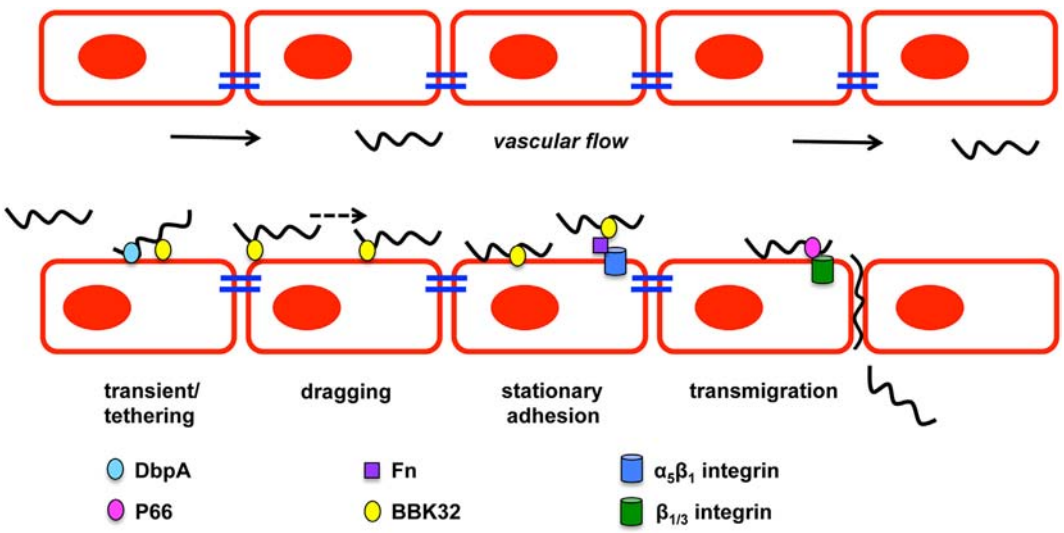

FIGURE 2 | *Borrelia burgdorferi* vascular interactions and transmigration. *B. burgdorferi* forms three types of vascular interactions: transient/tethering, dragging, and stationary adhesion. Decorin-binding protein A (DbpA) promotes transient/tethering with endothelial cells. Fibronectin-binding protein (BBK32) is involved in all three types of interactions. Stationary interaction through BBK32 may be through direct interaction with the endothelial surface or through a fibronectin bridge (Fn) to a host integrin. P66 also interacts with integrins and is important for transmigration of *B. burgdorferi*. Figure is a modification of Moriarty et al. (86).

the specific mechanics behind tethering, dragging, and stationary adhesion in controlled conditions.

Numerous borrelial lipoproteins have previously been shown to bind components of the ECM (39, 41, 90–94). Norman et al. examined potential mechanisms mediating the interactions with the vasculature through fibronectin (Fn) and glycosaminoglycans (GAGs) (87). Biofluorescent *B. burgdorferi* was incubated with dextran sulfate to block potential GAG interactions and evaluated *in vivo* by epifluorescence IVM resulting in a 30 and 80% reduction in transient and dragging interactions, respectively. Stationary adhesion was reduced to a similar level as dragging when cells were treated with dextran sulfate (87). This indicates that GAGs are involved in mediating the host–pathogen interaction of *B. burgdorferi* in the vasculature. The role of Fn in *B. burgdorferi* binding to the vasculature was assessed by treating *B. burgdorferi* that expresses fibronectin-binding protein, BBK32, with anti-Fn IgG prior to IV injection. Short-lived reduction in transient and dragging interactions were observed, suggesting that Fn plays an important role in the ability of *B. burgdorferi* to interact with the host vascular endothelium (87). Fibronectin C-terminal heparin-binding domain and integrin-interacting cell-binding domain that were responsible for *B. burgdorferi* vascular interaction was evaluated by competition with peptides representing the binding domains under *in vivo* conditions. *B. burgdorferi* was able to readily interact with the endothelium at all stages when the RGD sequence of the integrin-interacting cell-binding domain was in circulation to compete with plasma Fn for binding. Heparin-binding domain peptide decreased *B. burgdorferi* transient interactions by 52% and further reduced dragging and stationary adhesion by 84%, suggesting that host GAGs are responsible the observed vascular interactions. The heparin-binding domain of Fn and GAGs are directly or indirectly important for *B. burgdorferi* interactions with the host endothelial surface.

BBK32 is one of several *B. burgdorferi* proteins shown to bind Fn and its role in mammalian infectivity has been assessed as a ligand under *in vivo* conditions (83, 87, 88, 95–97). Fibronectin binding by *B. burgdorferi* may build a bridge for binding integrins allowing further host–pathogen interactions to occur (92, 98, 99). *B. burgdorferi* lacking *bbk32* attenuates the infectivity phenotype and increases the ID_{50} about 15-fold as determined by cultivation of murine tissues 3 weeks after inoculation (95). High dose infection, specifically 10^5, with the *bbk32* mutant strain results in colonization of disseminated tissues at a slightly lower level than wild-type when bacterial loads are quantitatively determined (97). *In vivo* imaging of bioluminescent *bbk32* deletion strain demonstrated a similar dose dependent phenotype, but also that the role for *bbk32* may be more important for earlier stages of infection (83). Light emission from mice infected with this strain was significantly less 1 week after inoculation when compared to wild-type infected controls. Gain-of-function experiments utilized non-infectious *B. burgdorferi* lacking several plasmids and an adhesin protein necessary for dissemination or invasion (86–88). Non-infectious *B. burgdorferi* that was complemented with *bbk32* driven by the *ospC* promoter caused increased vascular interactions relative to the non-infectious parent strain lacking the transgene (87). Mutagenesis of *bbk32* in infectious *B. burgdorferi* allowed the specific role of this fibronectin-binding protein to be characterized for Fn binding and vascular interactions (88) (**Figure 2**). Biofluorescent *bbk32* deletion strain displayed a 20 and 50% reduction in the murine skin flank and joint, respectively, in tethering and dragging. Epifluorescence IVM requires a high dose inoculation by IV injection and may contribute to the different outcomes of *bbk32* infectivity studies using lower dose intradermal inoculum that resulted in more pronounced virulence attenuation. The disruption of two regions of *bbk32* (Fn-binding, Δ158–182, or GAG-binding sequences, Δ45–68) caused a reduction of tethering and dragging, respectively, indicating these binding domains serve distinct mechanistic functions for *B. burgdorferi* endothelial interactions. BBK32 has a fast binding association and disassociation with Fn, but GAG binding is longer lasting and more stable (88). These differences in binding kinetics may explain the distinct binding interactions observed by *in vivo* IVM. Flow chamber studies evaluated the specific mechanism by which BBK32 associates with the endothelium and determined it contributes to stabilization of interactions and increases the range of force and shear stress through catch bond mechanism (89). A catch bond occurs when the higher the tensile force the longer the life of a non-covalent bond between a receptor and ligand (100). It is not clear if BBK32 is directly binding the endothelium utilizing lectin-binding sites or if the interactions are occurring through host ligands. Other possible fibronectin-binding proteins, RevA, RevB, and BB0347, were not able to restore vascular interactions in non-infectious *B. burgdorferi* (88). It is likely that a yet to be identified borrelial proteins that are able to bind fibronectin contribute to vascular interactions in addition to BBK32.

It is likely that many more players are involved in vascular interactions and possibly do so in a tissue specific or spatial manner. A prime candidate is borrelial lipoprotein decorin-binding protein A (DbpA) that is able to bind decorin, heparin, and dermatan sulfate GAG (101–103). *dbpA* is co-transcribed with highly conserved *dbpB* that has a decreased decorin-binding ability and does not impact infection to the degree observed for *dbpA* (13, 14, 101, 104–106). Environmental signals, such as temperature, pH, and CO_2, regulate *dbpBA* expression in a RpoS-dependent manner (107–116). Incubation of *B. burgdorferi* with neuroglial or human umbilical vein endothelial cell (HUVEC) cells induced the expression of *dbpBA* among other virulence associated lipoproteins (117). *B. burgdorferi* lacking *dbpBA* has a severely attenuated infectivity phenotype with reduced colonization of the skin, heart, and joint when needle inoculated, but tick transmission of the *dbpBA* mutant resulted in an infectivity phenotype similar to wild-type (83, 118–122). Over time high dose inoculum of the *dbpBA* mutant can overcome the limitations to dissemination and reach wild-type bacterial burden in tissues despite reduced pathology in the heart and joint (118). Expression of *dbpA* is detectable up to 8 weeks after inoculation in several tissues, which is dissimilar to another RpoS-regulated gene *ospC* that is downregulated about a week after infection (123, 124). The importance of decorin binding by *B. burgdorferi* is further demonstrated in the resistance of decorin-deficient mice to infection (125). Transcripts of *dbpA* are readily detectable from blood during infection, but there is also some indication that DbpA

contributes to lymphatic dissemination of *B. burgdorferi* (118). The *dbpBA* mutant has reduced colonization of lymph nodes of immunocompetent mice relative to wild-type *B. burgdorferi*, but there was no difference observed in immunodeficient mice (118). The ability of DbpBA to aid in vascular interactions was assessed using an *in vitro* flow chamber seeded with HUVECs to mimic shear stress of the blood stream (126). Non-infectious *B. burgdorferi* B313 missing numerous plasmids was complemented with *dbpBA* on a shuttle vector and formed the short-lived tethering interaction (126) (**Figure 2**). DbpA has not been evaluated under *in vivo* conditions with IVM. In addition, its specific contribution to invasion as assessed by its ability to cross cell culture monolayers or to move in *in vivo* transmigration assays has not been determined. Vascular interaction is merely one step in the borrelial pathogenic process with stage each building upon the other for the end result of persistent infection in several mammalian tissues.

INVASION

Borrelial dissemination and subsequent invasion of mammalian tissues in the latter stages of disease is an essential step for pathogenesis and immune evasion. Invasion of *B. burgdorferi* by entering into a tissue through transmigration or extravasation results in the colonization of secondary infection sites that become the foci of disease. *B. burgdorferi* has the ability to preferentially escape into heart or joint tissue and cross the blood–brain barrier (BBB) stimulating an inflammatory immune response to cause carditis, arthritis, and neuroborreliosis, respectively (1). The endothelial surface of the BBB has an unique organization and increased level of protection in comparison to other endothelial tissues targeted for invasion by *B. burgdorferi*. Invasion of the BBB by *B. burgdorferi* results in activation of the host plasminogen activation system (PAS), matrix metalloproteases (MMP), and host calcium signaling (127–129). It is likely that *B. burgdorferi* utilizes unique invasion strategies in a tissue-specific manner in addition to mechanisms common to other pathogens for entry into immunoprotected niches.

Early host–pathogen studies evaluated the ability of *B. burgdorferi* to cross cell culture monolayers of various types (130–135). Infectious *B. burgdorferi* is able to adhere and penetrate through tight junctions from the apical surface to traverse the monolayer. This interaction with the host and subsequent transmigration is not without consequence as it can result in the enhanced response of neutrophils and T lymphocytes in HUVEC culture (136, 137). Evidence limited to *in vitro* studies indicate that *B. burgdorferi* has the ability to undergo intracellular invasion of human fibroblast, umbilical vein endothelial, synovial, neuronal, and glial cells without the loss of viability (49, 133, 138, 139). These findings lead to the speculation that borrelial cellular invasion is a mechanism for immune evasion and disease modulation, but the significance is unknown, as this has not been observed using an *in vivo* model system. Furthermore, an important effect of *B. burgdorferi* interacting with the host is the alteration of virulence determinants, including Dbp, OspA, and BBA64, which are important for mammalian infection (117, 140). The host–pathogen interactions and the resulting responses of *B. burgdorferi* and host tissue-specific pathways are important for the advancement of the spirochete into secondary colonization sites. The targeting of immunoprotective niches requires non-specific and specific vascular interactions, as described above, followed by transmigration that is likely mediated by several distinct borrelial adhesins.

B. burgdorferi-Elicited Transmigration in the Mammalian Host: The Role of P66

P66 (*bb0603*) is encoded on the borrelial chromosome and forms an OM β-barrel porin with adhesive capabilities to host integrins that contribute to mammalian infection (141–148). *B. burgdorferi* lacking P66 is able to persist in ticks through molting, but is unable to establish infection in the mammalian dermis and is cleared at the site of inoculation within 48 h following inoculation (149, 150). Expression of *p66* is not observed in the tick vector until exposure to a blood meal and continues well into mammalian infection providing further support for the importance of P66 during mammalian infection. P66 engages the innate and adaptive immune response in the murine host as it is required for infection of mice deficient in the innate immune signaling molecules MyD88 and TLR-4 and elicits a specific antibody response against *p66* expressing *B. burgdorferi*. A short-term infection model with intravenously infected Balb/c mice was used to evaluate the tissue localization of wild-type or *p66* mutant *B. burgdorferi* 1 h after inoculation and resulted in a P66-dependent tissue tropism for the ear and heart (94). Disseminated *B. burgdorferi* requires specific adhesins dependent upon the tissue for colonization of distal sites.

The requirement for infectivity could be due to one or both of P66 identified functions: porin and/or integrin-binding activity. P66 was initially identified as an integral membrane porin in *B. burgdorferi* and was further shown to possess channel conductance (141, 142). Recent work by Kenedy et al. characterized the β-barrel structure of P66 and found that it associates with lipoproteins OspA and OspB (143). The second identified function of P66 is its interaction with β_1 chain and β_3 chain integrins that are homodimeric proteins with the function of host cellular signaling and interactions with host cell matrices (147, 149, 151, 152). Traditional mutational analysis and a phage-display screen verified β_3 intergrin binding by P66 (147, 153). The specific binding region for integrin interaction was localized to amino acids 203–209 of P66 (151). Transcriptional analysis of human embryonic kidney cells and endothelial cells exposed to *B. burgdorferi* with or without *p66* indicated significant changes among host genes involved in several pathways including cellular interactions and actin arrangement indicating a potential role in pathogen invasion (152). Interaction of P66 with the host causing potential cellular arrangements may support the transmigration of the pathogen through tight junctions or possibly the intracellular invasion of *B. burgdorferi*. An *in vitro* gentamicin protection study demonstrated *B. burgdorferi* utilizes β_1 integrin to invade endothelial or fibroblast cells requiring the rearrangement of actin filaments and Src kinase activity (49). Internalized *B. burgdorferi* displayed the round body morphology previously observed when the pathogen is under stress conditions, but the change in shape could also be due

to spatial limitations within the host cell. At this time, the physiologic relevance of intracellular *B. burgdorferi* is unclear because evidence that this event also occurs during a natural infection has not been reported.

The requirement for borrelial P66 binding of host β_3 integrin for infection in the murine experimental model was assessed by Ristow et al. (154). Mice expressing or devoid of β_3 integrin had the same ID_{50} as wild-type *B. burgdorferi* and were also not infected by the *p66* mutant strain, indicating that β_3 integrin is not essential for infection and redundant integrin binding by borrelial cells may also support tissue invasion. Two *p66* integrin-binding targeted mutant strains were generated consisting of a regional deletion of amino acids 202 to 208 (BDel202-208) and double point mutation (BD205A,D207A). Phenotypes of the *p66* targeted mutant strains were characterized for infectivity, *in vitro* integrin binding, porin activity, and penetration of endothelial monolayers. BDel202-208 had an attenuated infectivity phenotype with significant reduced bacterial loads in the heart and tibiotarsal joint, while BD205A,D207A decreased in the heart relative to wild-type *B. burgdorferi* when mice were subcutaneously infected. Intravenous infection slightly alters the kinetics of borrelial infection and only BDel202-208 had a reduced bacterial burden in the heart. BDel202-208 and BD205A,D207A significantly reduced $\alpha_V\beta_3$ integrin binding relative to wild-type, but did not disrupt channel conductance and maintained porin activity (147, 154). In correspondence with integrin binding, BDel202-208 and BD205A-D207A had an impaired ability to cross endothelial monolayer when compared to the wild-type strain. It is well established that P66 and, more specifically, the region encompassing the integrin-binding region is important for *B. burgdorferi* infection and possibly invasion. The route of host interaction is possibly through β_1 integrin in addition to β_3 integrin. β_1 integrin could not be directly evaluated *in vivo* due to lethality associated with β_1 deficiency.

The potential that P66 was involved in the dissemination or invasion of *B. burgdorferi* was evaluated by *in vivo* IVM for vascular interactions and in a newly developed high resolution intravital transmigration assay (155). Previous studies utilizing *in vivo* IVM focused on vascular interactions in the skin (86–88). *Cd1d*$^{-/-}$ mice are a preferred strain to examine *B. burgdorferi* infection in the joint because the lack of invariant natural killer T cells (iNKT) increases the bacterial burden and the likelihood of visualizing spirochetal vascular interactions or transmigration (156, 157). Kumar et al. focused on the vascular interactions in joint proximal tissue of *Cd1d*$^{-/-}$ mice that were capable of recapitulating tethering, dragging, and stationary adhesion interactions of wild-type *B. burgdorferi* observed in murine skin (155). Spinning disk laser confocal microscopy captured a back and forth motility of *B. burgdorferi* as it transmigrates in the joint one day after high dose intravenous inoculation of *Cd1d*$^{-/-}$ mice (155). *B. burgdorferi* lacking *p66* showed no difference in tethering, dragging, or stationary adhesion relative to wild-type. Therefore, this adhesive porin is not involved in vasculature interactions. Transmigration and clearance from the bloodstream of *p66* mutants was dramatically reduced when compared to wild-type or complemented *p66* mutant strains raising the question whether the inability of *B. burgdorferi* to invade was due to clearance by the host immune response rather than the ability to cross the epithelial layer. Targeted *p66* mutant strains were also not able to transmigrate, but interestingly, bloodstream clearance is at a similar rate to wild-type indicating that P66 serves an active role in a borrelial transmigration mechanism. *In vivo* localization of β_3 integrin in the vasculature in relation to *B. burgdorferi* was determined that P66-β_3 integrin interaction associates with transmigration events at cellular junctions where stationary adhesion most often occurs. *B. burgdorferi* localized to areas in the vasculature with higher concentrations of β_3 integrin indicating this colocalization is taking place and contributing to transmigration. Studies with β_3 integrin-deficient mice showed *B. burgdorferi* was able to disseminate to various tissues, suggesting that β_3 integrin binding is not the sole mechanism for borrelial invasion. It is likely that the pathogen utilizes redundant invasion mechanisms through the binding of other integrins, such as β_1, or host receptors. Active transmigration in the joint occurs above background levels 24 h after inoculation, which may allow the time for activation of cell signaling pathways in endothelial cells and borrelial adaptation to support transmigration for successful invasion (158). The contribution of BBK32 to endothelial invasion, in addition to the previously noted vascular interactions, is determined not to be involved as the *bbk32* mutant and wild-type *B. burgdorferi* displayed similar rates of transmigration. The role of P66 porin activity in vascular interactions, transmigration, and survival following epithelial invasion is unknown at this time. Taken together, P66 is involved in a potential invade-to-evade mechanism in *B. burgdorferi* that may have additional pathogenic functions (154, 155). Further studies are needed to elucidate possible P66 initiated actin and ECM rearrangements and the impact on the inflammatory response characteristic of Lyme disease.

B. burgdorferi Protease HtrA Role in Invasion

The high-temperature requirement (HtrA) family of ATP-independent serine proteases serve as chaperones, support membrane integrity by degrading damaged or improperly folded proteins, and pathogenesis by processing virulence determinants or degrading components of the ECM in other bacterial pathogens (159, 160). *B. burgdorferi* chromosomally encodes a single HtrA protease (BbHtrA) at *bb0104* (13, 14). BbHtrA exhibits proteolytic activity and the potential of this protein to support borrelial pathogenesis and physiology was the subject of a MicroCommentary in Molecular Microbiology and a perspectives article in Frontiers in Cellular and Infection Microbiology (161–165). BbHtrA is a surface exposed protein that localizes to both the soluble and membrane fractions and can also form oligomeric structures (163, 164, 166). Production levels of BbHtrA are higher at 37°C relative to 34°C correlating with higher activity levels and induction during the stationary phase of *in vitro* cultivation conditions (167). It was the first HtrA in which proteolytic activity inhibited by zinc was described (166). Cell fission protein BB0323 is required for mammalian infection and proteolytically cleaved by BbHtrA demonstrating that *B. burgdorferi* is able to modify its own proteins (168). The *B. burgdorferi*

htrA mutant strain does not contain processed BB0323 at elevated temperatures validating the proteolytic function of BbHtrA *in vivo* (167). BbHtrA targets proteins for proteolysis that vary in function including cell fission (BB0323), chemotaxis (CheX), laminin binding (BmpD), and integrin binding (P66) (163, 168, 169). Additional borrelial proteins are bound by BbHtrA without undergoing degradation, indicating that BbHtrA may also function as a chaperone (163). Transcripts of *p66* in a borrelial strain overexpressing *BbhtrA* were reduced demonstrating another layer of regulation employed by this borrelial protease (169). The impact of BbHtrA on pathogenesis is widespread in regards its diverse mechanisms of regulation.

Another host target of borrelial proteolysis is aggrecan, which is a large aggregating proteoglycan found in cartilage with multiple functional domains consisting of two GAG-attachment domains and three globular domains (170). Behera et al. found that *B. burgdorferi*-induced aggrecanase 1 and 2 (ADAMITS 4 and 5) in human chrondrocyte cells causes the cleavage of aggrecan, thus possibly explaining cartilage damage associated with Lyme disease (171). The binding ability of *B. burgdorferi* aggrecan was attributed to borrelial GAG-binding protein (Bgp) (encoded by *bb0588*) and BbHtrA (164). The aggrecan interglobular domain (IGD), which resides between globular domains 1 and 2 (G1 and G2), is specifically cleaved by BbHtrA, thereby disrupting its ability to associate with GAG (164, 170). Proteolytic assays were performed to identify other host ECM proteins degraded by BbHtrA (165). The hyalectin family of chondroitin sulfate proteoglycans, including aggrecan, brevican, neurocan, and versican, were also degraded by BbHtrA (**Figure 3**). Additional BbHtrA degraded ECM proteins were identified as fibronectin, biglycan, and decorin. *B. burgdorferi* is known to encode specific adhesins with the ability to bind these proteoglycans. Cell junction protein E-cadherin was only moderately degraded while collagen II and tenascin C were unaffected by incubation with recombinant BbHtrA. Together, these findings indicate that BbHtrA also has target specificity for degradation of host ECM. A point mutation was generated in the active site of BbHtrA, designated BbHtrA^{S226A}, eliminating the protease activity and resulting in the loss of degradation of host ECM proteins (164, 165). The potential physiologic significance of BbHtrA proteolytic and chaperone activity was confirmed when Ye et al. who generated an *htrA* deletion mutant strain that was not recoverable by cultivation from murine tissues two weeks following inoculation (167). It cannot be determined if the loss of infectivity was due to lack of proper borrelial protein processing through degradation and chaperone activity, a reduction in host ECM degradation that may inhibit invasion, or a compounding effect of both functions. The absence of *htrA* results in *B. burgdorferi* cells with *in vitro* growth deficiencies, cell membrane blebbing, and clustering of cells at elevated temperature when BbHtrA has the highest level of activity (167). These described *in vitro* phenotypes of *B. burgdorferi* would significantly impair infection if also observed *in vivo*. BbHtrA is highly immunogenic causing the stimulation

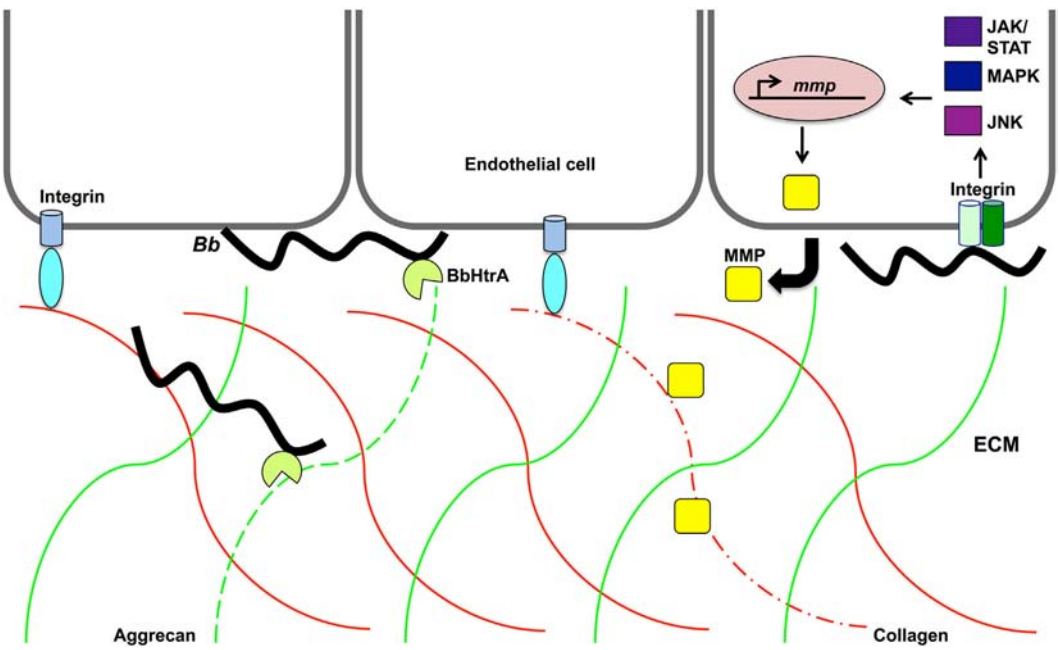

FIGURE 3 | Proteolytic activity may promote *Borrelia burgdorferi* invasion of the extracellular matrix. *B. burgdorferi* (*Bb*) protease BbHtrA cleaves the family of hyalectin proteoglycans that includes hyalectin family of chondroitin sulfate proteoglycans, including aggrecan, brevican, neurocan, and versican. Specifically, BbHtrA protease activity degrades aggrecan (green lines) as indicated by dotted green line. This activity may contribute to ECM degradation and allow invasion of the pathogen. The presence of *B. burgdorferi* induces host matrix metalloproteases (MMP) from endothelial cells, chondrocytes, neutrophils, lymphoblast, and keratinocytes. Collagen (red lines) is degraded (dotted red line) by MMP-1 that is transcriptionally regulated along with MMP-3 through the JNK, mitogen-activated protein kinase, and JAK/STAT pathways.

of inflammatory signaling and possibly causes further damage of the host ECM (164, 165). The immunogenicity of BbHtrA did not translate into protection against infection in the murine model (172). *B. burgdorferi* may utilize the multifunctional BbHtrA to promote invasion through its ability to regulate and/or process virulence determinants along with the degradation of the host ECM.

Induction of Host Proteases in Response to *B. burgdorferi*

Borrelia burgdorferi induction of aggrecanase activity was first observed in the synovial fluid of patients with Lyme arthritis and was thought to possibly be caused by host MMPs (173). MMPs are secreted zinc and calcium-dependent host proteases that act to remodel the ECM by specific degradation of aggrecan, collagen, elastin, fibronectin, and gelatin (174). MMPs aid in tissue growth, repair, and remodeling of healthy tissues. These host proteases also cleave chemokines, cytokines, and the accompanying receptors causing an inactivation or activation of these immunological molecules. The degraded ECM fragments released stimulate a localized inflammatory response similar to that observed in Lyme disease manifestations. *B. burgdorferi* induces a range of MMPs that are dependent upon the types of cell stimulated and infecting borrelial strain (173, 175–177). Human neutrophils, lymphoblast, and keratinocytes, secrete pro-MMP-9 when exposed to *B. burgdorferi* (175). HUVECs were able to release pro-MMP-9 and pro-MMP-2 under the same conditions. Borrelial infection induces the production of collagenase-1 (MMP-1), gelatinase-2 (MMP-2), stromelysin-1 (MMP-3), and gelatinase B (MMP-9) in synovial fluid from patients with erythema migrans in addition to aggrecanase activation (173, 178) (**Figure 3**). The aggrecanase activity observed in synovial fluid of Lyme disease patients was found not to be associated with the stimulated MMP-2, MMP-3, or MMP-9, but may have been due to the more recently characterized BbHtrA activity described above (173). Cartilage explants from rhesus monkey and bovine were used as an *in vitro* model of Lyme arthritis and incubation with *B. burgdorferi* resulted in cartilage degradation that was prevented in the presence of MMP inhibitors (173). This could provide an explanation for the damage observed with Lyme arthritis and shed light on mechanisms utilized by *B. burgdorferi* for invasion and modulation of disease. The *B. burgdorferi*-induced profile of MMPs is specific to the tissue type and host species (173, 175). It was shown that both similarities and differences exist in the types of MMPs induced by *B. burgdorferi* in mice and humans and could be the reason for differences in susceptibility to borrelial infection and the development of arthritis. Different MMPs activated in response to *B. burgdorferi* are regulated through distinct host pathways (179–181). Specifically, MMP-1 and MMP-3 are regulated through c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and Janus kinase/signal transducer and activation of transcription (JAK/STAT) pathway (179) (**Figure 3**). MMP-1, MMP-3, and MMP-9 regulation occurs through toll-like receptor 2 (TLR-2) (180, 181). MMP-1 and MMP-3 are also induced through the interaction of borrelial cells with α_3 integrins (181). *B. burgdorferi* may utilize MMPs to manipulate ECM remodeling to promote invasion of the pathogen for secondary colonization of tissues with the diversity of induced MMPs and regulatory pathways ensuring progression of disease.

The idea that MMPs could promote invasion was supported by the increased transmigration of *B. burgdorferi* through ECM components under *in vitro* conditions when incubated with MMP-9 (175). MMP-9 is able to cleave collagen type I and the resulting fragments chemoattract peripheral blood mononuclear cells; thus, this activity also elicits an immune response that could limit borrelial pathogenesis (182). The specific role of MMP-9 in *B. burgdorferi* invasion and inflammatory response under *in vivo* conditions is assessed by infecting MMP-9$^{-/-}$ mice and comparing to wild-type mice in regards to bacterial burden, arthritis, and carditis. Murine hearts and joints were colonized at similar levels of bacterial burden indicating that MMP-9 alone is not responsible for invasion. This observation does not completely rule out the involvement of MMP-9 in aiding *B. burgdorferi* invasion of tissues through ECM remodeling. It is likely redundant mechanisms that are utilized to guarantee successful pathogenesis, which may occur through other MMPs or BbHtrA. Infected MMP-9$^{-/-}$ mice develop less arthritis relative to wild-type mice with an unexpected similarity in cytokine and chemokine expression, indicating that the ability of MMP-9 to modulate an inflammatory response is independent of *B. burgdorferi* infection. Further investigation of other MMPs individually or in combination is needed to fully understand the contribution of MMPs to *B. burgdorferi* invasion.

Plasmin has been observed in multiple studies to be activated by *B. burgdorferi* and is able to degrade ECM components and activate MMPs (173, 175, 183). The PAS, also referred to as the fibrinolytic system, involves the induction of a series of proteases and accompanying regulatory inhibitors for the purpose of fibrin cleavage that support tissue remodeling, healing, and cell migration (129, 184). It was suspected that the activation of plasmin by the presence of *B. burgdorferi* could serve as a potential mechanism for spirochetal invasion. *B. burgdorferi* readily binds plasminogen and is converted to plasmin by host-derived urokinase plasminogen activator (uPA) (38, 185, 186). This tightly regulated process is kept in check by plasminogen inhibitors type 1 and 2 (PAI-1 and PAI-2). Plasminogen binds *B. burgdorferi* through numerous identified receptors and promotes the transmigration of the pathogen across HUVEC monolayers (187). MMP-9 induction was observed in the presence of plasminogen when human PMBC cells were co-incubated with *B. burgdorferi* (175). During a tick blood meal, plasminogen is activated, but is not required for borrelial transmission to a murine host (188). uPA is induced in monocytes of mice and humans in the presence of *B. burgdorferi* (189, 190). Hovius et al. demonstrated that borrelia induces the PAS and, more specifically, showed that the receptor of uPA controls bacterial burden and promotes the phagocytosis of *B. burgdorferi* by leukocytes (191). uPAR-deficient mice had increased *B. burgdorferi* colonization relative to the C56BL/6 wild-type mice. However, this increase was not observed in uPA, tPA, or PAI-1 knockout mice. *In vitro* phagocytosis assays display limited leukocyte phagocytosis in the absence of uPAR, but not the other members of the PAS system. Therefore, the host PAS is not hijacked

by *B. burgdorferi* for ECM remodeling leading to the conclusion that this system does not contribute to invasion. However, this conclusion does not rule out the contributions of tick salivary glands or other supporting pathogenic mechanisms in mediating transmigration.

SUMMARY

The success of *B. burgdorferi* as a pathogen involves dynamic adaptation and interactions of specific tissues of the mammalian host with the pathogen during each stage of disease. *B. burgdorferi* engages numerous adhesins that are important for mammalian infection, but the specific contributions of these adhesins for dissemination or invasion are not fully elucidated. The specific mechanisms known to contribute to borrelial dissemination in the form of vascular interaction and invasion by transmigration under *in vitro* conditions or during mammalian infection has been the focus of this review. It is clear that *B. burgdorferi* is a resilient bacterium as it relies on redundant functions for both dissemination and invasion to ensure progression of pathogenesis. This presents a challenge to clearly understand these mechanisms. Significant advances in our understanding of *B. burgdorferi* vascular interactions and invasion have been made through the development of *in vivo* imaging technologies allowing the identification of stages of interactions. Borrelial adhesins are responsible for interactions with the host that promote dissemination or invasion in a tissue specific manner. It is unclear the degree to which *B. burgdorferi* interaction with the endothelium can directly induce cellular rearrangement allowing for the invasion of host tissues. Furthermore, pathogen and host proteases combat a barrier to *B. burgdorferi* invasion by proteolytically cleaving the ECM.

AUTHOR CONTRIBUTIONS

The only contributor to this manuscript is JH.

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PERSPECTIVE
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A Universal Vaccine against Leptospirosis: Are We Going in the Right Direction?

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Leptospirosis is the most widespread zoonosis in the world and a neglected tropical disease estimated to cause severe infection in more than one million people worldwide every year that can be combated by effective immunization. However, no significant progress has been made on the leptospirosis vaccine since the advent of bacterins over 100 years. Although protective against lethal infection, particularly in animals, bacterin-induced immunity is considered short term, serovar restricted, and the vaccine can cause serious side effects. The urgent need for a new vaccine has motivated several research groups to evaluate the protective immune response induced by recombinant vaccines. Significant protection has been reported with several promising outer membrane proteins, including LipL32 and the leptospiral immunoglobulin-like proteins. However, efficacy was variable and failed to induce a cross-protective response or sterile immunity among vaccinated animals. As hundreds of draft genomes of all known *Leptospira* species are now available, this should aid novel target discovery through reverse vaccinology (RV) and pangenomic studies. The identification of surface-exposed vaccine candidates that are highly conserved among infectious *Leptospira* spp. is a requirement for the development of a cross-protective universal vaccine. However, the lack of immune correlates is a major drawback to the application of RV to *Leptospira* genomes. In addition, as the protective immune response against leptospirosis is not fully understood, the rational use of adjuvants tends to be a process of trial and error. In this perspective, we discuss current advances, the pitfalls, and possible solutions for the development of a universal leptospirosis vaccine.

Keywords: *Leptospira*, reverse vaccinology, genome mining, vaccine discovery, vaccine candidate, recombinant vaccine, subunit vaccine, animal model

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INTRODUCTION

Following the discovery of leptospirosis, it was primarily associated with rural populations (1). This disease is caused by pathogenic *Leptospira* spp. and can be transmitted by direct contact via infected animals or by indirect contact as leptospires can survive outside the host. Agricultural workers, mineworkers, veterinarians, or individuals that came into direct contact with infected animals or contaminated environments were the main at-risk groups. However, toward the end of the 20th century, there were reports of leptospirosis among the homeless in major cities in the USA (2) and in urban slum communities in developing countries (3). The WHO estimated that the global incidence

of leptospirosis more than doubled from approximately 500,000 cases in 1999 (4), to over a million cases in 2015 (5). Urban leptospirosis is now endemic in urban slums due to the lack of sanitation, rodent infestation, extreme poverty, and limited access to public health services in these communities.

Severe leptospirosis or Weil's disease (jaundice, acute renal failure, and bleeding) has a case fatality rate of >10%. However, leptospirosis-associated pulmonary hemorrhage syndrome (LPHS) is being increasingly reported in developing countries (6) and the fatality rate is >50% (7). Clinical diagnosis of leptospirosis is difficult due to its similarity with other hemorrhagic diseases, and laboratory diagnostic tests are inadequate in these settings (8). There remains an urgent need for point-of-care rapid diagnostic tests. Vaccination of at-risk populations remains the most viable strategy for the control of leptospirosis. Classical, inactivated, vaccines have been available for over 100 years and are used routinely for agricultural and companion animals, reviewed in Ref. (9). Some countries have approved their use in human at-risk populations, although due to the severe side-effects and perceived short-term immunity and lack of cross-protection, they have not been adopted by the global community (10).

Of the 22 known *Leptospira* spp., 15 are infectious and can cause disease with varying degrees of severity. The pathogenesis of leptospirosis is a multifactorial process that is poorly understood, see, e.g., Ref. (11). Serological classification of leptospires indicates the existence of at least 250 serovars distributed in 18 serogroups (12). All this genetic and phenotypic diversity of pathogenic *Leptospira* spp. is a major drawback for vaccine development. The idea of a universal vaccine capable of protecting against all infectious *Leptospira* spp. and serovars would appear to be farfetched. However, some progress has been made with other pathogens such as influenza (13, 14), dengue (15), and others (16, 17). This perspective focuses on current advances, limitations, possible solutions, and looks forward to the possibility of a universal leptospirosis vaccine.

EXPERIMENTAL RECOMBINANT VACCINES

LipL32 is the immunodominant protein in pathogenic *Leptospira* spp. (18), there are over 38,000 copies per cell (19), and it can comprise up to 75% of the protein content of the outer membrane (OM) (20). However, there is a doubt as to its cellular localization; the latest report suggests it may occupy a subcellular location on the inner leaflet of the OM (21). LipL32 is not required for virulence; an *Leptospira interrogans lipL32* knockout mutant could still infect hamsters (22). The biological function of LipL32 remains unknown, yet it is remarkable that such an abundant protein can be removed from the leptospiral OM with little or no effect on growth rate or OM structure. This is an example of the redundancy encoded in the *Leptospira* genome, as seen with other proteins, e.g., putative adhesins (23). There are over 20 publications on LipL32 and vaccine development. However, when rigorous statistical analysis is applied [e.g., Fisher's exact test (24)], only five demonstrated significant protection against leptospirosis, reviewed in Ref. (10, 25). In addition, problems with reproducibility, survival in the control groups, high challenge doses (septic shock or leptospirosis), and the subcellular location of LipL32 have complicated its candidacy for inclusion in a universal vaccine formulation.

The leptospiral immunoglobulin-like (Lig) protein family includes LigA, LigB, and LigC and is only found in pathogenic *Leptospira* spp. (26, 27). While LigA and LigB are highly conserved (28), only LigB is present in all pathogenic *Leptospira* spp. (29). LigA and LigB are virulence determinants that are upregulated during infection (30), play a role in host cell adherence (31), prevent blood clotting (32, 33), and inhibit complement (34, 35). However, as seen for LipL32, an *L. interrogans ligB* knockout mutant remained virulent in the hamster model (36). Nevertheless, the Lig proteins are the standout vaccine candidates to date, with high, reproducible, levels of protection in animal models of acute leptospirosis in over 15 scientific reports, although not all withstood rigorous statistical analysis (10). The C-terminal (non-identical) region of LigA is an accepted vaccine candidate, having been evaluated in subunit (37–41), DNA (42), encapsulated (43), lipidated (44), and carbon nanotube vaccine preparations (45). However, when evaluated in a hamster colonization model, LigA failed to prevent infection (46). There is less evidence in support of LigB, the N-terminal conserved (repeat) region conferred significant protection as a subunit vaccine preparation (47) and a DNA vaccine (48) in the hamster model. Our group found that the same LigB polypeptide (LigBrep) not only protected hamsters but also induced sterile immunity in survivors (manuscript submitted).

Using the classical approach to vaccine candidate discovery, approximately 30 leptospiral, non-LipL32, non-Lig, proteins have been evaluated (10, 25). Of these, 10 proteins conferred significant protection against challenge with *Leptospira* spp. when the data were reanalyzed using, when necessary, a more rigorous statistical analysis (Fisher's exact test) (10). The first report of protein-based protection came from studies of recombinant OmpL1 and LipL41 in the hamster model (49), and although only 1/3 experiments demonstrated significant protection, this provided the initial impetus for further research into protein-based vaccine candidates against leptospirosis. In an evaluation of three putative OMPs (Lp1454, Lp1118, and MceII), the subunit formulations failed to protect hamsters (50); however, when combined and encapsulated in liposomes, they conferred significant protection against challenge (51, 52). The putative lipoprotein LemA, identified using a reverse vaccinology (RV) approach (53), significantly protected immunized hamsters when administered as a DNA vaccine and protection increased using a prime-boost strategy (*lemA*/LemA) (54). In the most extensive study to date, 238 proteins identified using RV were evaluated as vaccine candidates (55). A hamster colonization model was used to evaluate pools of recombinant proteins (5 proteins/pool) and >70% were immunogenic. However, none of the recombinant protein pools conferred protection against colonization.

TARGET DISCOVERY

Cytoplasmic proteins, inner membrane proteins, and OM lipoproteins that are not exposed on the surface (i.e., those

attached to the inner leaflet of the OM) are likely to be ineffective recombinant vaccines. Antibodies induced by subsurface proteins would not be able to bind to infecting leptospires making the vaccine ineffective. Therefore, vaccine candidates should be surface exposed on the leptospiral cell. Equally important are the potential roles in pathogenicity and the immunogenicity of these proteins. Furthermore, it is doubtful that a protein-based vaccine candidate would be capable of inducing a protective immune response if the protein components of the vaccine lacked one or more of these characteristics.

Lipoproteins attached to the outer leaflet of the OM and transmembrane β-barrel proteins spanning the OM (OMPs) should be fully or partially surface exposed (**Figure 1**). The localization of LipL32 is still unresolved; there is experimental data for both surface (56–59) and subsurface locations (21). Leptospiral genomes encode OMPs such as LptD, BamA-like, TonB-dependent receptors, and several other porins that play crucial roles in bacterial survival and potential role in pathogenicity. These proteins are ideal targets and should be evaluated as potential vaccine candidates. RV was developed to identify surface-related proteins in the genome of pathogens using bioinformatics (60). RV has been used to analyze *Leptospira* genomes and there are several reports in the literature that have used *in silico* genome mining toward the identification of leptospiral vaccine candidates, reviewed in Ref. (61).

Recently, dozens of leptospiral proteins have been described as adhesins, reviewed in Ref. (11), and blocking the adhesion of leptospires is thought to impair their virulence. Similarly, several proteins have been described as host complement activation inhibitors, suggesting that leptospires evade the complement system, reviewed in Ref. (62). In many studies (63–67), the surface localization of the leptospiral antigens were determined by *in vitro* approaches including proteinase K digestion and a surface immuno-fluorescence assay (IFA) (68). These approaches have contributed to the controversy surrounding the localization of proteins such as LipL32. Another example is that of LIC13166, this protein was originally demonstrated to be an OMP exposed on the surface of the leptospiral cell by surface biotinylation, membrane affinity, and surface-IFA experiments (68). However, in a recent publication, it was shown that LIC13166 is, in fact, a flagellar protein, renamed FcpA, which is located in the periplasm (69). The subcellular location of adhesins, complement binding

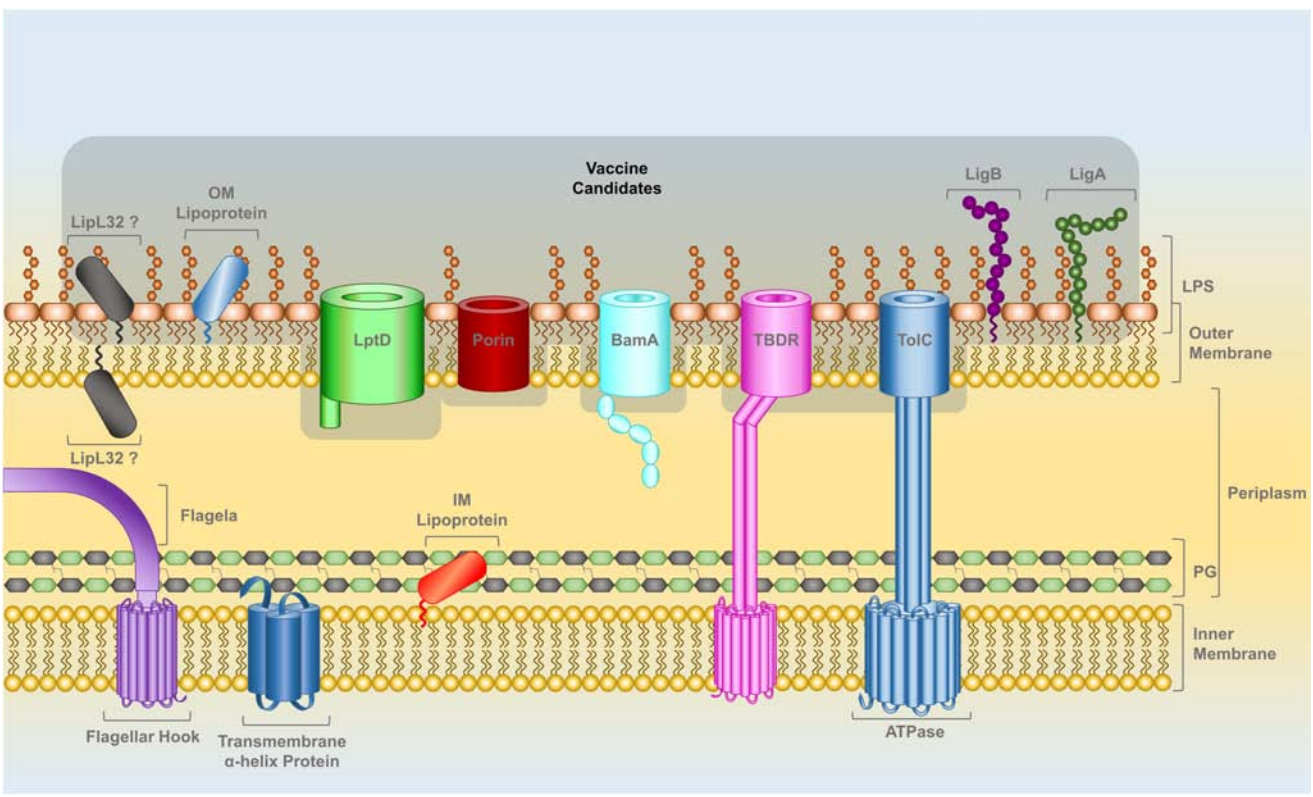

FIGURE 1 | The cell wall of *Leptospira* spp., a diderm bacteria with inner and outer lipid membranes, is the target for the identification of potential vaccine candidates. The outer leaflet of the outer membrane (OM) is composed of lipopolysaccharide. Lipoproteins can be attached by a lipid anchor to one of the leaflets of either membranes. The IM is spanned by alpha-helix transmembrane proteins while beta-barrel transmembrane proteins span the OM. Leptospiral motility is provided by two flagella that are attached to the IM and are located within the periplasmic space (PS). A peptidoglycan layer is also present in the PS. OM lipoproteins, such as LigA and LigB, as well as OM beta-barrel proteins, such as LptD, BamA, TolC-, TonB-dependent receptors, and other porins have at least a portion of their structure exposed on leptospiral surface and are prospective vaccine candidates, highlighted in gray. The localization of the lipoprotein LipL32 in the OM is controversial; the latest reports indicate that it has a subsurface location (see text).

proteins, and virulence factors described in knockout experiments should be properly investigated; otherwise the biological relevance of these findings will remain unclear. We are currently developing an alternative method to improve the identification of surface leptospiral proteins while maintaining the integrity of the leptospiral OM.

CROSS-PROTECTION

The perceived lack of cross-protection following immunization with a bacterin is another factor that has limited their widespread use. There are, however, several reports of bacterins conferring cross-protection against species-related serovars. An evaluation of bacterins reported 100% cross protection between *L. interrogans* serovars Canicola, Copenhageni, and *Leptospira borgpetersenii* serovar Ballum but not serovar Mozdok (70). A multivalent bacterin containing serovars from four different serogroups demonstrated cross-protection in a canine model of leptospirosis (71). Another study of two bacterins based on different serovars, but belonging to the same serogroup and species, reported species-related cross-protection, although 50% of the control group survived (72). It is likely that the protective antigens in these studies were proteins, as leptospiral lipopolysaccharide (LPS) does not induce cross-protection, even among species-related serovars (73). Rather, protein-enriched samples were responsible for cross-protection against species-related serovars in a gerbil model of lethal leptospirosis. This was further supported by a study using a live vaccine based on an LPS defective mutant. Species-related cross-protection was demonstrated, although the vaccine could not prevent colonization by a non-related serovar (74). Several studies of individual proteins have claimed to show cross-protection. An adenovirus construct containing *lipL32* conferred cross-protection against a species-related serovar, although >50% of the control groups survived (75). A treatment based on anti-LipL32 monoclonal antibodies protected hamsters challenged with a species-related serovar (76). Prime-boost strategies using LemA and LigBrep conferred cross-protection against a species-related serovar, albeit in one-off experiments (54, 77).

At least one strain for every known *Leptospira* spp. has been sequenced and new isolates are continually being sequenced and their genomes released on GenBank or other public databases, see, e.g., Ref. (29, 78–83), thereby providing a panoramic view of *Leptospira* pathogenomics, permitting the identification of orthologs and protein sequence similarity among infectious species. This has significantly contributed to the identification and selection of conserved vaccine candidates based on a simple *in silico* sequence analysis (**Figure 2**). Protein sequences are usually highly conserved among the same species regardless of the serovar or serogroup, while they can differ considerably when comparing the same serovar in different species. While, serological classification is unquestionably important for epidemiology and bacterin-based vaccine studies, it is of limited use for recombinant vaccine development. This is a problem associated with a leptospiral bacterin vaccine, the immune response is primarily directed against the leptospiral LPS and while it protects against infection by closely related serovars or serovars from the same *Leptospira* spp., leptospiral LPS does not stimulate memory B-cells (10). As there is no clear definition of cross-protection in the field of leptospirosis, this is a major drawback to vaccine candidate discovery and evaluation. Ideally, recombinant vaccine-induced cross-protection should be defined as cross-species protection rather than cross-serovar protection. A universal vaccine should therefore protect against all 15 infectious *Leptospira* spp. regardless of serovar. However, if this is not a viable option, it should be possible to identify the main circulating species and develop a region-specific recombinant vaccine rather than a universal vaccine. This could potentially allow the characterization of the protective immune response and establish standard protocols for the evaluation of cross-protection of recombinant vaccine candidates (**Figure 2**).

MODULATION OF THE IMMUNE RESPONSE

Several adjuvants and delivery systems have been used to enhance the immune response against leptospiral antigens. Aluminum hydroxide (alhydrogel) and Freund's adjuvant are by far the most common, although others including flagellin (84), CpGs (85), nanostructures (45), liposomes (43, 51, 52), xanthan (85) have been investigated. While Freund's adjuvant cannot be used in humans due to its high reactogenicity (86), it is the most potent commercially available adjuvant (87), is useful for the primary screening of vaccine antigens, and has been used successfully in vaccine formulations against leptospirosis (39, 41). To date, only partial protection has been demonstrated with vaccines using alhydrogel, the most widely used adjuvant in human vaccines. Recently, other adjuvants have become commercially available and have been approved for use in the formulation of human vaccines, comprising the adjuvants MF59 (squalene), AS01 [monophosphoryl lipid A (MPL), QS21], AS03 (α-tocopherol, squalene, and polysorbate 80), AS04 (MPL combined with alhydrogel), and virosomes (liposome/VLPs) (88, 89). These prospective adjuvants have not yet been evaluated as adjuvants for leptospirosis vaccines.

Rational modulation of the immune response is difficult to achieve for leptospirosis vaccines as little is known about the protective immune response that should be induced by a leptospirosis vaccine. Humoral immunity is believed to be responsible for protection; anti-LPS antibodies are protective in animal models and can be passively transferred between animals (90). As predominantly extracellular organisms, leptospires are most likely cleared from the bloodstream by phagocytosis followed by opsonization. However, at least in some hosts, e.g., cattle, induction of cellular immunity is equally important (90). Until recently, there were no published reports of correlation between antibody titer, induced by leptospiral recombinant vaccines and protection against challenge. However, an oral immunization strategy based on LigA found that survival was dependent on a minimum antibody titer being reached in a 2-week period following immunization (44), and if this can be reproduced, it will be an extremely important finding. The lack of immune correlates is a major limitation in target discovery using RV as

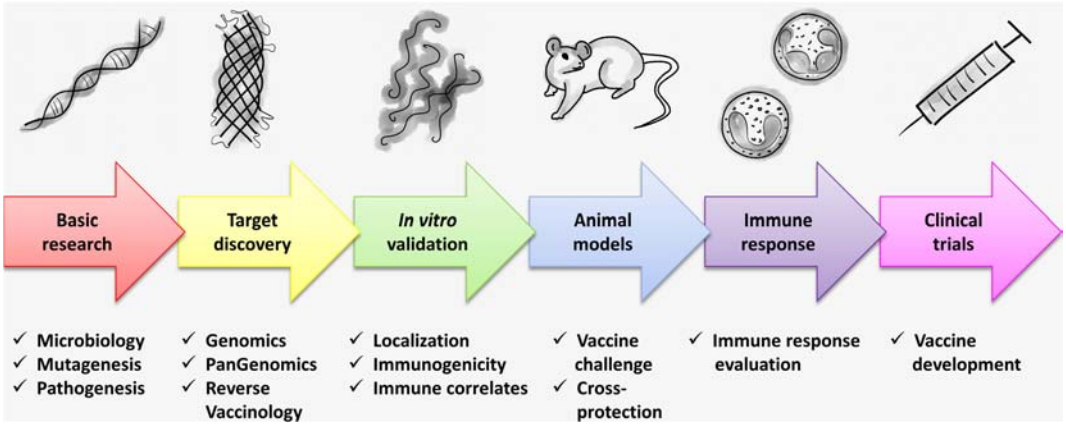

FIGURE 2 | A schematic representation of the development pipeline for a universal vaccine against leptospirosis. The basic research on *Leptospira* microbiology and molecular biology contributes to vaccine development. *Leptospira* mutagenesis is an example of basic research that has and will continue to further our understanding of pathogenesis and identification of virulence factors. Genomic and pangenomic studies are of central importance to the development of a universal leptospirosis vaccines, permitting the identification of potential vaccine candidates and the analysis of protein sequences among different *Leptospira* spp. RV has not been fully explored in leptospirosis and needs to be more thoroughly exploited. Once potential vaccine candidates are identified, an *in vitro* validation is required, particularly to confirm the localization of antigens on the surface of the leptospiral cell. At this stage, a prospective vaccine candidate can be assessed for immunogenicity. The lack of well-defined correlates of immunity for leptospirosis represents one for the major limitations for leptospirosis vaccine development and remains to be resolved. Therefore, surface-related, conserved (among infectious *Leptospira* spp.), and immunogenic leptospiral antigens must be evaluated in vaccine challenge experiments using animal models. Cross-protection, defined as cross-species rather than cross-serovar protection should be evaluated. In addition, as the protective immune response is not fully understood, continued research in this field is necessary. Finally, the long-term goal of this pipeline is to identify experimental vaccine preparations for evaluation in clinical trials.

they are essential for the *in vitro* screening of potential vaccine candidates, see, e.g., the bactericidal assay for *Neisseria meningitidis* (91) and the opsonophagocytosis assay for *Staphylococcus aureus* (92).

ANIMAL MODELS OF LEPTOSPIROSIS

The recommended animal model for acute leptospirosis is the Syrian hamster; this model replicates the human symptoms of the disease, including kidney failure, LPHS, and kidney, liver, and lung tissue damage, which result in death (93). Furthermore, the hamster model is the recommended model for potency testing of bacterin vaccines (94). The acute model is dependent on a virulent challenge strain and the lack thereof has had a major impact on protection studies. However, to date, no well-established correlates of immunity have been identified and, therefore, vaccinated hamsters must be challenged with a virulent *Leptospira* strain to demonstrate protection. Due to significant variation among the hamster models of acute leptospirosis, we recommend that the research community adopt a standardized model (see Supplementary Material). An alternative to the lethal model is the hamster colonization model, and this is the model of choice when evaluating vaccine candidates for agricultural animals including cattle, swine, and horses (55, 95). Unlike the acute model, the primary endpoint in this model is kidney colonization.

A major limitation of the hamster model is the lack of commercial reagents for characterization of the immune response, e.g., induction of cytokines and chemokines cannot be measured directly. Alternate models include the guinea pig and the gerbil, although there are few commercially available reagents for these models. Due to the wide range of commercially available reagents, the mouse model is attractive, reviewed in Ref. (96). Wild-type mice are naturally resistant to leptospirosis, although colonization is possible with some serovars (9, 97). Lethal leptospirosis has been demonstrated in C3H/HeJ (41), SCID, and Rag1 knockout mice (98). Maintenance host models of chronic infection have been developed using the Wistar strain of *Rattus norvegicus* (9, 99).

CONCLUSION

Alternatives to whole-cell inactivated leptospiral vaccines have so far failed to live up to their initial promise, and the concept of a universal leptospiral vaccine remains just that, a concept. Several reviews have highlighted the modest numbers (~30) of leptospiral proteins that have been tested using various vaccine strategies, including subunit, DNA vaccines, prime-boost, encapsulated, and live avirulent strains. Of these, less than a handful has been successful. However, the availability of multiple genome sequences, combined with advances in bioinformatics (e.g., RV) and the characterization of surface-exposed virulence factors, will improve the discovery of potential vaccine candidates. The next challenge is to develop *in vitro* assays based on correlates of immunity for the high-throughput screening of these vaccine candidates. While there are several animal models of leptospirosis, their standardization is necessary for the critical interpretation of protection data. Cross-protection is a priority for a universal vaccine and will require the identification of vaccine candidates that are conserved among the infectious *Leptospira* spp. Our

poor understanding of the (protective) immune response has hindered the intelligent selection of adjuvants for use in vaccine formulations. Finally, while the field is moving in the right direction, a universal vaccine for leptospirosis remains a long-term goal.

AUTHOR CONTRIBUTIONS

AG, JS, and AM wrote the manuscript. AG and JS created the figures, and all the authors contributed to and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00256/full#supplementary-material.

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Spirochetal Lipoproteins and Immune Evasion

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Spirochetes are a major threat to public health. However, the exact pathogenesis of spirochetal diseases remains unclear. Spirochetes express lipoproteins that often determine the cross talk between the host and spirochetes. Lipoproteins are pro-inflammatory, modulatory of immune responses, and enable the spirochetes to evade the immune system. In this article, we review the modulatory effects of spirochetal lipoproteins related to immune evasion. Understanding lipoprotein-induced immunomodulation will aid in elucidating innate pathogenesis processes and subsequent adaptive mechanisms potentially relevant to spirochetal disease vaccine development and treatment.

Keywords: spirochetes, lipoproteins, evasion mechanism, immune system, immunity

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INTRODUCTION

Spirochetes cause many human diseases such as syphilis, Lyme disease, and leptospirosis that pose major threats to public health (1). Epidemiological studies have shown that the incidence of Lyme disease (2–4), syphilis (5–7), and leptospirosis (8, 9) have increased, both within United States and globally (10, 11). However, the immunopathogenesis of spirochetal diseases remains unclear (12–14). Despite the apparent immune response generated following spirochete infection (i.e., tissue inflammation) (15), spirochetes are known to persist in their host (16) through a wide variety of mechanisms ranging from a dynamic outer membrane capable of antigenic variation in the presence of outer-surface proteins capable of inhibiting macrophage facilitated phagocytosis (17, 18).

A critical question is what cellular components can trigger the strong immune responses that are characteristic of spirochetal infections. Spirochetal membranes play a pivotal role in interacting with a host's immune system (19, 20). Bacterial components such as lipopolysaccharides (LPSs) often play a major role in the induction of inflammation in bacterial infections (21, 22). Interestingly, aggressive immune responses are often observed despite the lack of LPS (endotoxin) in particular spirochetes, such as *Borrelia burgdorferi* (19, 23–25). Certain spirochetes such as *Treponema pallidum*, the spirochete responsible for syphilis, rely greatly on their ability to express adhesins over the surface of their membrane as a tool with which they can invade various tissues (26). Lipids compose 25–30% of a cell's dry weight (19, 20). Detergent treatments of spirochetal membranes have confirmed that lipoproteins are the most abundant in number out of all proteins expressed by spirochetes (27–32) and are major integral spirochetal membrane proteins (27, 33). For example, *B. burgdorferi* species express >100 lipoproteins (34) and *Leptospira* spp. have >140 lipoprotein genes (35). Although numerous examples of spirochetal lipoproteins can be listed, a few prominent ones include OspA from *B. burgdorferi*, Tp47 from *T. pallidum*, and Lip32 from the *Leptospira* species (36–38). The number of bacterial lipoproteins that have been studied parallels the myriad of roles that lipoproteins play in bacteria such as envelope biogenesis, stress responses, pathogenicity, and nutrient transport (39–41).

However, there is limited evidence regarding the interplay between lipoproteins and human immune responses, partly due to the fact that *in vitro* studies do not accurately reflect human models. Understanding lipoprotein-induced immunomodulation will aid in elucidating innate pathogenesis processes and subsequent adaptive mechanisms potentially relevant to spirochetal disease vaccine development and treatment. In this article, we review the scientific evidence regarding the modulatory effects of spirochetal lipoproteins related to immune activation and evasion.

MODULATORY EFFECTS OF SPIROCHETAL LIPOPROTEINS RELATED TO ACTIVATION OF THE IMMUNE SYSTEM

Understanding the dualistic roles (activation vs inhibition) of lipoproteins in their interaction with the immune system is pivotal (42). Thus, before we explore mechanisms of spirochetal immune evasion, a better understanding of all the regulatory mechanisms (such as pro-inflammatory effects and immune activation) of spirochetal lipoproteins is needed. Better understanding of spirochetal lipoproteins and their regulatory mechanisms may provide insight into clinical outcomes arising from spirochetal infections. For example, spirochetal infections may increase the risk of Alzheimer's disease (43).

Spirochetal Lipoproteins Induce Pro-inflammatory Effects

One of the primary manifestations of spirochetal infection is tissue inflammation that is the mainstay of spirochetal diseases such as Lyme neuroborreliosis (22, 29). Spirochetal lipoproteins are known to induce strong pro-inflammatory responses in their hosts (27, 33, 34, 44–52) that comprise the initial innate immune response to the invading pathogen (49). Components of the inflammatory infiltrate include keratinocytes, macrophages, leukocytes, and cells capable of responding to the presence of lipoproteins (53–55). A better understanding of the modulatory effects of spirochetal lipoproteins in myeloid and non-myeloid immune cells is needed.

Spirochetal Lipoproteins Have Modulatory Effects on Neutrophils

Neutrophils have a major role in the immunopathogenesis of acute bacterial infections. Spirochetal lipoproteins, such as OspB, have been documented to inhibit neutrophil function and prevent oxidative burst in a variety of tissues, to prolong host infection (56–58). However, other lipoproteins can promote neutrophil activation. For example, OspA, even when presented at picomolar concentrations, has been seen to play a role in the activation of neutrophils and their chemotaxic capabilities (51, 59). Subsequent to neurophil activation, neutrophil tissue infiltration contributes to localized tissue inflammation that is pre-dominant in inflamed arthritic joints and in myocarditis (associated with spirochetal infections) (50, 51, 60). In addition to mediating inflammatory responses, spirochetes, such as *Leptospira*, may induce neutrophils extracellular traps, which are a relatively novel pathogen-killing mechanism for extracellular microbes independent of phagocytic uptake and degranulation (61). Thus, spirochetal lipoproteins can modulate the function of neutrophils that are recruited early in acute inflammatory responses.

Spirochetal Lipoproteins Have Pleotropic Modulatory Effects on Monocytes and Macrophages (M/M) That Are Mediated through Several Pathways

Except for neutrophils, M/M also play a major role in spirochetal immunopathogenesis. Lipoproteins bind CD14 in the membrane of M/M at the CD14 site that also interacts with LPS (62–64). This interaction activates the NF-κB pathway and induces pro-inflammatory responses (62, 63, 65). In addition, unlike the membrane-bound CD14, soluble CD14 also allows the activation of non-myeloid cells (66). Furthermore, the pro-inflammatory effects of spirochetal lipoproteins are often mediated by toll-like receptors (TLR) (67–69). TLR signaling leads to increased production of numerous cytokines that induce pro-inflammatory responses (25, 47). Interestingly, TLR-deficient mice had exacerbated inflammation and increased spirochetal burdens, both of which were attenuated by impairing T cell responses (70). As a bodily response to the vast amounts of pro-inflammatory cytokines produced upon spirochetal lipoprotein presence, monocytes have also been seen to produce IL-10 upon being presented with *B. burgdorferi* lipoproteins (71–75). IL-10, unlike cytokines such as IL-1 and IL-12, is known to reduce inflammation *via* TLR-pathway downregulation and can therefore assist in combatting the spirochetal infection as well as any possible chronic effects such as arthritis (76, 77). The above was confirmed in recent mice studies that utilized a TLR2 agonist, Pam3CSK4, to induce IL-10 production which attenuated inflammatory response to *Leptospira* (78). Thus, spirochetal lipoproteins exert their pro-inflammatory effects through several pathways including CD14, TLR, and NF-κB signaling and induce both pro-inflammatory (such as IL-1) and anti-inflammatory cytokines (IL-10) production in myeloid cells such as M/M.

Spirochetal Lipoproteins Induce Activation of Dendritic Cells

Similar to the activation of neutrophils, M/M, spirochetes also maintain the ability to activate other myeloid cells such as dendritic cells, key components in linking both the innate and adaptive immune system. Spirochetes activate cell adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), which then facilitate T-cell interactions and subsequent dendritic cell migration to lymph nodes for the mounting of an immune response (79, 80). In early stages of inflammation, lipoproteins in *T. pallidum* upregulate ICAM-1 and activate dendritic cells to mount immune responses (25, 46, 49, 81–84). Immune activation can also be induced upon spirochetal death or phagocytosis of spirochetes, both processes of which lead to further introduction of lipoproteins to the surrounding environment (80). The modulatory effects of spirochetal lipoproteins on dendritic cells are particularly important since dendritic cells play a major role in vaccine responses (discussed below).

Chronic Modulatory Effects of Spirochetal Lipoproteins and Effects on Adaptive Immunity May Drive Pathogenesis of Spirochetal Diseases

Spirochetal lipoproteins may also play a role in the transition from the acute immune responses to the more chronic effects that characterize spirochetal diseases such as arthritis, peripheral neuropathy, numerous neurologic manifestations, and the vascular endothelial damage thought to underlie a significant portion of the chronic symptoms in spirochetal diseases (85–89). Although the exact mechanism of transition may not be well understood, lipoproteins may activate B-cells and T-cells, both of which are known to play major roles in long-term adaptive immunity (46, 47, 49–52). Further understanding of the exact transition process has major potential in terms of possibly delaying, or inhibiting, many of the debilitating chronic effects characteristic of numerous spirochetal infections.

MODULATORY EFFECTS OF SPIROCHETAL LIPOPROTEINS RELATED TO FACILITATION OF IMMUNE EVASION

Spirochetes evade a host's immune system through mechanisms such as antigenic variation, which is capable of producing myriads of variants (90). Spirochetal interference of the innate immune system presents one more mechanism, in a list of many, to allow for the persistence of spirochetes in their host (16, 91). Spirochetes use multiple mechanisms of immune evasion that are related to spirochetal lipoproteins. Indeed, except for pro-inflammatory effects, lipoproteins are also responsible for modulatory effects such as immune evasion. Spirochetes may limit the expression of membrane lipoproteins and their access to antibodies (92, 93) or induce antigenic variation of surface lipoproteins (19, 90, 94–100). Spirochetal lipoproteins may also interact with, and inhibit, components of innate immunity such as the complement (63, 68, 88, 101–108), neutrophils, and serum lipoproteins (109). Major pathways of spirochetal immune evasion are discussed below (see also **Table 1** and **Figure 1**) (110–130).

TABLE 1 | Mechanisms of immune evasion of major spirochetal lipoproteins.

Bacteria	Role in immune evasion
Borrelia burgdorferi	Antigenic variation [VlsE proteins (118, 120, 131–134), OspC (135)]
	Evasion of complement-mediated lysis [OspE, Erp (136–138), CspA (139)]
	Impairment of neutrophil function (BBA57) (140)
Oral treponemes (*ex. Treponema denticola*)	C3b inactivation (various lipoproteins) (141)
Borrelia recurrentis	Antigenic variation (variable large and small protein genes and Vmp variants) (19, 110)
	Bind to complement regulatory proteins, i.e., CFH and CFHR-1 [FhbA, BhCRASP-1, and HcpA (142–145)]
Borrelia turicatae	Antigenic variation (variable large and small protein genes and Vmp variants) (19, 110)
	Inhibit C4bp and C1-Inh, the major inhibitors of the classical and lectin pathway of complement activation (CihC) (146)
	Binds to human complement regulators, Factor H, CFHR-1 (HcpA) (143)
Borrelia hermsii	Antigenic variation (variable large and small protein genes and Vmp variants) (19, 110)
	Bind to complement regulatory proteins, i.e., CFH and CFHR-1 [FhbA, BhCRASP-1, and HcpA (142–145)]
Leptospira interrogans	Impairment of neutrophil function (LIC11207) (147)
	Bind to complement regulators (LigA, LigB, Len A, Len B) (148)

Antigenic variation in borrelias may result from recombination of variable large and small protein genes. Lipoproteins may also impair mechanisms of innate immunity such as neutrophil function and complement activation. These mechanisms allow the spirochete to evade the host's immune response and persist in the mammalian host. BBA57, Borrelia burgdorferi A57 protein; BhCRASP-1, Borrelia hermsii complement regulator-acquiring surface protein 1; C1-Inh, human C1 esterase inhibitor; CihC, C1-inhibitor and C4bp-binding protein; C4bp, C4b-binding protein; CspA, complement regulator-acquiring surface protein-1; Erp, OspE-F-related lipoprotein; FhbA, complement factor H-binding protein; HcpA, human complement regulator and plasminogen-binding protein; LIC11207, L. interrogans serovar Copenhageni (LIC) protein 11207; LigA, leptospiral immunoglobulin-like protein A; LigB, leptospiral immunoglobulin-like protein B; OspC, outer-surface protein C; OspE, outer-surface protein E; VlsE, variable major protein-like sequence E; Vmp, variable major lipoprotein.

Differential Dynamics of Spirochetal Lipoprotein Expression As a Mechanism of Immune Evasion

The expression of lipoproteins on the outer leaflet of the membrane allows the spirochete to interact with tissues and the host's immune system (110). Naturally, the vast abundance of lipoproteins a given spirochete can express are not all necessary at a given time point, and their expression is time sensitive (111). Although more work is needed to elucidate the time-sensitive expression of surface lipoproteins, studies have hinted at the possibility of a temperature-sensitive mechanism to underlie expression patterns (112). For example, OspA in *B. burgdorferi* is not needed upon host infection and is therefore downregulated upon infection of a host *via* a temperature-sensitive alteration in membrane composition (111). Coupled closely with the need of a lipoprotein to be expressed on the exterior of the cell for interactions to occur, the lipoprotein must maintain its N-terminus as it has been documented that it is this region specifically to which immune system–spirochete interactions occur (113, 114). In line with the above statement, removal of the N-terminus disrupts the aforesaid interactions while synthesis of N-terminus analogs restored immune cell activation (114, 115). The limitation of outer-membrane lipoprotein expression in spirochetes may also act as a mechanism to facilitate host humoral defense evasion. Antibody recognizable lipoproteins may be scarcely expressed on the exterior leaflets, as opposed to the relatively more lipoprotein dense cytoplasmic leaflet (92, 93, 116). Further studies are needed to elucidate the role of differential dynamics of spirochetal lipoprotein expression in spirochetal immunopathogenesis.

Antigenic Variation of Surface Lipoproteins

Coupled with the limited expression of outer-membrane lipoproteins in spirochetes, antigenic variation is a major mechanism

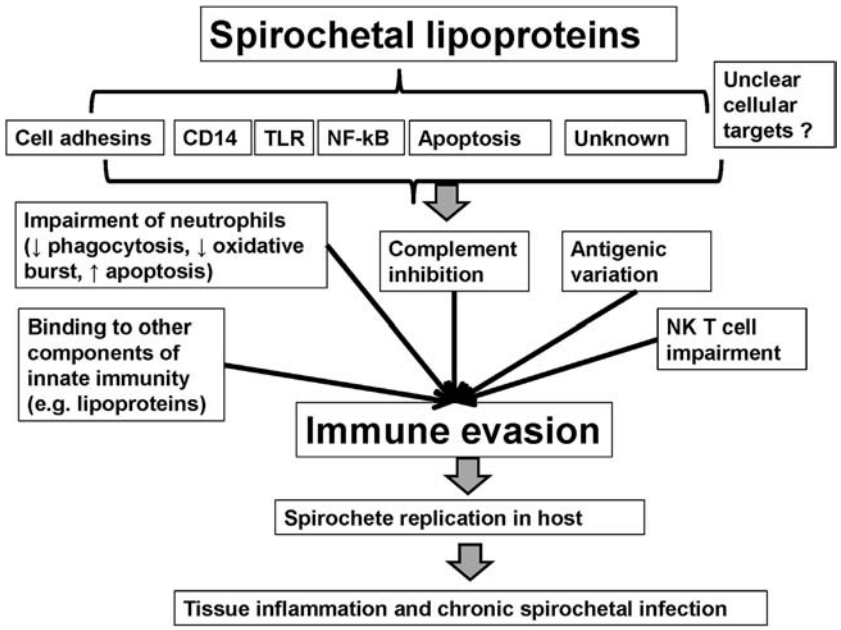

FIGURE 1 | **Mechanisms of immune evasion mediated by spirochetal lipoproteins**.

by which invading bacteria can evade the host immune response (117). Spirochetes also undergo a process of antigenic variation in terms of expressed outer-leaflet lipoproteins (96, 118). Studies in immunocompromised hosts have suggested that the host immune responses have a major role in producing spirochetal antigenic variants (96). Antigenic variation in borrelias may result from recombination of variable large and small protein genes (98) and the diversity of variable major lipoprotein lipoproteins allows these pathogens to evade the host immune response (19, 23, 119). Moreover, outer-leaflet lipoprotein variation also allows spirochetal adherence to a wide variety of host cells, as studies of *T. pallidum* TP0435 isoforms have recently shown (26). The antigenic variation of major surface lipoproteins is described in **Table 1** (19, 90, 94–100).

The ability to vary surface lipoprotein expression has been studied in *B. burgdorferi*, where it has been shown that prolonged infections are due to the embodiment of a *vls* locus that is capable of random segmental variation in the surface-exposed lipoprotein it encodes (118, 120). The *vls* locus variation specifically allows for the variation in the encoded variable major protein-like sequence lipoprotein which has been documented to allow for persistence of *B. burgdorferi* in its host (120). The antigenic variation of spirochetes leads to evasion of the immune system and ultimately to the phenomenon of host relapsing (121). Most interestingly, antigenic variation characteristic of *B. burgdorferi* is only seen during host infection. Spirochetal antigenic variation has not been described *in vitro*. Thus, the cross talk between host cellular responses and *B. burgdorferi* is needed for development of antigenic variation (perhaps through downregulation of OspA) (96). Elimination of the ability to undergo antigenic variation, as was done in *Borrelia hermsii*, may greatly reduce host infectivity/persistence (119). Understanding the exact mechanisms behind a spirochete's ability to elicit immune evasion *via* antigenic variation could set the basis for targeted interventions to inhibit infections (122).

Inhibition of Neutrophil Function by Spirochetes

Neutrophil-mediated phagocytosis of pathogens is a major host immune response to infection. Thus, spirochetes evade immune responses by inactivating neutrophil function (56). The most prominent examples of the above can be seen with the *B. burgdorferi* surface protein OspB, which may prevent phagocytosis of the spirochete and inhibit respiratory/oxidative burst in a variety of tissues, such as the skin (56–58). It should be noted that *B. burgdorferi* also contains outer-surface protein C which plays a role in inhibiting phagocytosis by macrophages (18). Similar to OspB that impairs neutrophil function, the novel lipoprotein *Leptospira interrogans* serovar Copenhageni (LIC) protein 11207 from *Leptospira*, promotes apoptotic pathways in neutrophils (123). Thus, spirochetal lipoproteins can both activate and impair neutrophils.

Lipoprotein Inhibition of Complement Activation

One of the major components of a host's innate immune system is the complement system that plays a role in the phagocytosis/elimination of a pathogen and is a target of spirochetes upon infection (124). Activation of the complement system is known to occur through the recognition of surface-exposed lipoproteins as well as other antigens such as oligosaccharides (124). The multi-stage process of complement activation presents spirochetes (such as *B. burgdorferi*) with the opportunity to attack at multiple phases. For example, *B. burgdorferi* binds and inhibits the C1 initiation

complex and accelerates C3b inactivation (91, 125). Furthermore, *B. burgdorferi* can bind either Factor H or FHL-1, two important complement regulators which upon being bound by CRASP-2 and CRASP-1 (*B. burgdorferi* membrane-bound lipoproteins), respectively, are inactivated and inhibit formation of complement system activation products (126, 127). *B. burgdorferi* also maintains the ability to bind factor H, *via* particular Osp, such as outer-surface protein E, accomplishing the same outcome as with CRASP-2 binding (128). Hijacking of the complement system is a conserved mechanism of immune evasion among numerous pathogens (such as *Plasmodium falciparum*) (129). Therefore, understanding the mechanisms behind complement hijacking in spirochetes could potentially contribute to understanding conserved pathways in other pathogens.

Lipoprotein Inhibition of Natural Killer T (NKT) Cells

Natural killer (NK) cells act to bridge the innate and adaptive immune responses to pathogenic infections; however, it is their ability to respond to a variety of lipid antigens that allows them to maintain a functional presence during combat of spirochetal infections (130). Spirochetes are capable of interfering with the NKT cells that respond to CD1d glycolipids on the surface of spirochetes such as *B. burgdorferi* (149). Although the exact biochemical pathway of interference is not well understood, patients with syphilis have been known to exhibit low NKT numbers (150). Further studies are needed to understand the possible interaction between spirochetal lipoproteins and NK cells.

UNDERSTANDING LIPOPROTEIN-MEDIATED PATHWAYS OF IMMUNE EVASION MAY PAVE THE WAY FOR DEVELOPMENT OF STRATEGIES TO TREAT SPIROCHETAL INFECTIONS

Understanding the pleotropic modulatory effects of lipoproteins may contribute to the development of new approaches to combat a plethora of diseases (151–154). Use of adjuvants in vaccines may enhance recognition of whole proteins by the adaptive immune system (151, 155). The immunopotent effects of spirochetal lipoproteins have hinted at the possibility for the development of vaccines that rely on the use of synthetic or derived lipopeptides (151, 155, 156). Spirochetal lipoproteins, such as OspA, can be expressed on the surface of outer-membrane vesicles to elicit an immune response similar to vaccines (157). Improvements in recombinant bacterial lipoprotein generation promise to make lipopeptide-based vaccines more feasible in the near future (158). The incorporation of numerous epitopes, such as lipoproteins, as adjuvants into vaccines can help target various diseases including cancer (155, 159). On the other hand, incorporation of a lipid moiety in peptide-based vaccines may induce TLR2 signaling in dendritic cells and subsequent protection against viral and bacterial infections (156). Finally, the use of lipopeptide-based antibiotics such as daptomycin, that can cause both immunomodulation (160) and also target spirochetes (161), remains to be studied as a therapeutic option for patients with spirochetal infections.

CONCLUSION

Lipoproteins play a significant role in the various stages of a spirochete's ability to infect a host and survive, through pleotropic effects involving transfer from vector to host, immune activation, or even immune evasion. Further studies are needed to understand the molecular basis and mechanisms that underpin the numerous modulatory effects (both acute and chronic) of spirochetal lipoproteins. The payout from such targeted research can be significant considering the sheer amount of spirochetal infections occurring on a yearly basis as well as the morbidity associated with chronic spirochetal infections in humans. Ultimately, the use of knowledge surrounding spirochetal lipoproteins can be put toward the development of vaccines or, perhaps shed light on the pathogenesis of other vector-based pathogens.

AUTHOR CONTRIBUTIONS

AC, AB, and TK contributed to writing of this manuscript.

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Discovery of Novel Leptospirosis Vaccine Candidates Using Reverse and Structural Vaccinology

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Leptospira spp. are diderm (two membranes) bacteria that infect mammals causing leptospirosis, a public health problem with global implications. Thousands of people die every year due to leptospirosis, especially in developing countries with tropical climates. Prophylaxis is difficult due to multiple factors, including the large number of asymptomatic hosts that transmit the bacteria, poor sanitation, increasing numbers of slum dwellers, and the lack of an effective vaccine. Several leptospiral recombinant antigens were evaluated as a replacement for the inactivated (bacterin) vaccine; however, success has been limited. A prospective vaccine candidate is likely to be a surface-related protein that can stimulate the host immune response to clear leptospires from blood and organs. In this study, a comprehensive bioinformatics approach based on reverse and structural vaccinology was applied toward the discovery of novel leptospiral vaccine candidates. The *Leptospira interrogans* serovar Copenhageni strain L1-130 genome was mined *in silico* for the enhanced identification of conserved β-barrel (βb) transmembrane proteins and outer membrane (OM) lipoproteins. Orthologs of the prospective vaccine candidates were screened in the genomes of 20 additional *Leptospira* spp. Three-dimensional structural models, with a high degree of confidence, were created for each of the surface-exposed proteins. Major histocompatibility complex II (MHC-II) epitopes were identified, and their locations were mapped on the structural models. A total of 18 βb transmembrane proteins and 8 OM lipoproteins were identified. These proteins were conserved among the pathogenic *Leptospira* spp. and were predicted to have epitopes for several variants of MHC-II receptors. A structural and functional analysis of the sequence of these surface proteins demonstrated that most βb transmembrane proteins seem to be TonB-dependent receptors associated with transportation. Other proteins identified included, e.g., TolC efflux pump proteins, a BamA-like OM component of the βb transmembrane protein assembly machinery, and the LptD-like LPS assembly protein. The structural mapping of the immunodominant epitopes identified the location

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Abbreviations: βb, β-barrel; βb-OMP, β-barrel transmembrane proteins; CDS, coding sequence; GO, Gene Ontology; Lig, Leptospiral immunoglobulin-like; LIC, *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130; MHC, major histocompatibility complex; OM, outer membrane; OMP, outer membrane protein; POTRA, polypeptide translocation-associated domain; RMSD, root-mean-square deviation; RV, reverse vaccinology; SP, signal peptide; SV, structural vaccinology; TBDR, TonB-dependent receptor; TMH, transmembrane α-helix.

of conserved, surface-exposed, immunogenic regions for each vaccine candidate. The proteins identified in this study are currently being evaluated for experimental evidence for their involvement in virulence, disease pathogenesis, and physiology, in addition to vaccine development.

Keywords: *Leptospira interrogans*, outer membrane protein, epitope prediction, bioinformatics, transport proteins, structural modeling, genome mining, diderm bacteria

INTRODUCTION

Leptospirosis is a zoonosis caused by spirochetes belonging to the *Leptospira* genus. More than 250 antigenically distinct serovars have been described for 15 infectious *Leptospira* spp. (10 pathogenic and 5 intermediate species) to date (1, 2). Leptospirosis is a neglected tropical disease with an estimated incidence of over one million severe cases in humans, resulting in ~60,000 fatalities (3). Furthermore, the disease has a major impact on the health of agricultural and companion animals, with serious economic consequences (4). Recombinant vaccine development is a major research focus because of the lack of effective control measures. Classical immunization strategies based on whole-cell, inactivated leptospires (bacterins), or cell wall components have been well documented (5). However, while bacterin vaccines are highly efficacious, they cause serious adverse reactions and confer short-term immunity that is restricted to the serovars used in the bacterin preparation (6).

Several research groups have used the classical approach for the identification of protein targets for use in recombinant vaccines with mixed results, reviewed in Ref. (7). The most promising targets to date are the leptospiral immunoglobulin-like (Lig) proteins. We recently showed that a LigB-based subunit vaccine protected hamsters against leptospirosis and induced sterile immunity (8). While these results will need to be confirmed by other research groups, protection conferred by LigA, with reports of up to 100% efficacy in the hamster model, has been consistently reproduced by different groups throughout the world. However, sterile immunity was not evident in LigA-vaccinated survivors (9–11). In addition, *ligA* is present in only three pathogenic *Leptospira* spp. (12), further limiting its ability to induce cross-protective immunity. Of note, LipL32, the immunodominant leptospiral lipoprotein, was extensively investigated as a vaccine candidate using different strategies (e.g., subunit, DNA vaccine, BCG, and adenovirus constructs) with inconclusive results; efficacy ranged from 12 to 87% (13–17). Another putative lipoprotein, LemA, was identified using reverse vaccinology (RV) and induced partial protection using a prime-boost strategy (18, 19). Finally, the putative outer membrane protein (OMP) OmpL37, perhaps one of the most promising antigens recently characterized, was not protective against lethal disease in the hamster model (20). The current status of leptospiral vaccine development shows that there is an unmet need for the discovery of new vaccine candidates and that further success will require reevaluation of the *Leptospira* genome (21).

The RV approach was first applied almost two decades ago and led to the discovery of protective vaccine candidates for several bacterial diseases (22). The best example is the recently licensed vaccine against meningococcal disease caused by *Neisseria meningitidis* serogroup B; the protein components of the vaccine were discovered by RV (23). However, while there are some examples of its partial application toward target discovery in the field of leptospirosis (18, 19), it has not been successfully implemented (24), reviewed in Ref. (25). RV targets are generally surface-related proteins that are recognized by the host immune system, thereby eliminating the bacteria and preventing disease. Ideally, these targets should play an important role during pathogenesis, increasing vaccine efficacy. In diderm bacteria, such as *Leptospira* spp. and Gram-negative bacteria, β-barrel transmembrane proteins (βb-OMPs) and some outer membrane (OM) lipid-anchored proteins (lipoproteins) are the only types of proteins that are surface exposed (26, 27). Proteins with a transmembrane α-helix (TMH) structure tend to be localized to the inner membrane and are rarely found in the OM, e.g., the Wza translocon for capsular polysaccharides in *Escherichia coli* (28). Even though various βb-OMPs and lipoproteins have been annotated in the *Leptospira* genome, many are still identified as hypothetical proteins. While some βb-OMPs and lipoproteins were characterized by means other than RV, we believe that a large number of these types of prospective vaccine antigens are yet to be discovered.

In addition to RV, recent advances in vaccine research using the structural information of antigens have led to the development of structural vaccinology (SV) (29). Based primarily on protein design for the optimization of antigen structure and consequently enhanced protection, SV is a combination of structural biology, immunology, and bioinformatics (30). Solving protein structures is time consuming, expensive, and sometimes difficult, especially for proteins such as the βb-OMPs (31, 32). Advances in structural bioinformatics has allowed reliable prediction of three-dimensional (3D) structural models of proteins based on the alignment of the query sequence to known-structure templates, which may be identified using sequence similarity searches (homology modeling) or fold recognition (threading) methods (31, 33). In contrast to homology-based methods, protein threading allows the prediction of structural models of proteins with low similarity to known proteins (no orthologs). This is attractive for leptospiral proteins, as there are no solved structures for leptospiral βb-OMPs. In the current study, we report the application of RV and SV toward the discovery of leptospiral vaccine candidates, i.e., βb-OMPs and OM lipoproteins, structural modeling, the *in silico* identification of major histocompatibility complex (MHC-II)-binding epitopes, and the selection of surface-related immunogenic epitopes.

RESULTS

Identification of βb-OMPs and OM Lipoproteins in the *Leptospira interrogans* Genome

Reverse vaccinology was employed for the identification of surface-exposed proteins, including βb-OMPs and OM lipoproteins in the genome of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (LIC). The bioinformatics workflow and total numbers of proteins identified by each bioinformatics algorithm (predictor) are shown (**Figure 1**). The predictors for subcellular localization (Cello, PSORTb, and Gneg-mPLoc) found 523 proteins (by 1 or more predictor) located in the OM of *L. interrogans*, 24 were identified by all three predictors. A total of 1,196 proteins were predicted to contain a signal peptide (SP) for translocation across the cytoplasmic membrane to, e.g., the OM. Of these, 72 proteins were identified by all three SP predictors (SignalCF, SignalP, and PrediSI). One or more of the transmembrane α-helix (TMH) predictors (Phobius, TMHMM, HHTOP, and MEMSAT) determined that 3,302 proteins did not contain a TMH, this was reduced to 2,929 proteins by all 4 predictors. The transmembrane β-barrel (βb) structure predictors (Bomp, HHomp, TMBETADISC-RBF, and MCMBB) identified 1,085 βb-OMPs, 20 of which were confirmed by all 4 predictors. Finally, 230 proteins were identified as lipoproteins by at least one of the predictors, 108 by both (LipoP and SpLip). A complete list of the proteins identified by each individual predictor is provided (Table S1 in Supplementary Material).

A total of 165 βb-OMPs were identified by at least 1 predictor, while only 1 protein (LIC10714) was identified by all predictors. In this study, a βb-OMP was defined as a protein predicted to contain a βb structure, an SP, and <2 TMHs. As an SP can be identified as a TMH, a protein containing a single, N-terminal, TMH was classified as a non-TMH protein. An OM lipoprotein was defined as a protein predicted to be located in the OM and that contained a lipobox. A total of 54 OM lipoproteins were identified by at least one predictor of each feature; however, no proteins were identified at the intersect between all the predictors for lipoproteins and cell localization. A list of the 165 βb-OMPs and 54 OM lipoproteins identified, the gene products as annotated in the LIC genome and the result for each predictor is provided (Table S2 in Supplementary Material).

Filtering Predicted Protein Features with Increased Confidence

Due to the particularities of each predictor, low agreement between the different bioinformatics tools was expected when identifying the same feature. To reduce the impact of a prediction when non-weighted (naïve) voting resulted in ambiguities (e.g., two negative and one positive result by three different predictors), we used an iterative weighted voting system. An in-house Python script was written to integrate the results and to identify those proteins with a high level of confidence in the consensus prediction among those selected by at least one predictor. The lists of 165 βb-OMPs and 54 OM lipoproteins were used as the script input and, after thousand iterations, each predictor received a final prediction weight (Table S3 in Supplementary Material). A final voting score >0.5 (0–1 scale) for each feature of interest (e.g., >0.5 for OM localization and >0.5 for lipoprotein prediction) was indicative of a high level of confidence in the prediction, resulting in the selection of 18 βb-OMPs and 9 OM lipoproteins (**Table 1**).

Conservation of Surface-Exposed Proteins among *Leptospira* spp. and Similarity to Mammalian Host Proteins

The genome sequences from 20 additional *Leptospira* spp. (Table S4 in Supplementary Material) were screened for orthologs of the 18 βb-OMPs and the 9 OM lipoproteins. Interestingly, all the pathogenic *Leptospira* spp. contained orthologs of the 18 βb-OMPs (**Table 2**), except for *L. kmetyi* that did not contain an ortholog of LIC10881. This protein was also absent from the intermediate and saprophytic *Leptospira* spp. Of the 9 OM lipoproteins, LIC12048 was absent in *L. borgpetersenii*, and LIC20172 was not found in *Leptospira kirschneri*, these proteins were, however, retained for further analysis as they were present in the majority of the pathogenic *Leptospira* spp. The OM lipoprotein LIC12690 was excluded from further analysis as it was only found in *L. interrogans*, *L. kirschneri*, and *Leptospira noguchii*. In addition, the βb-OMPs and OM lipoproteins were screened against selected mammalian proteomes for similarity to any of these potential vaccine candidates. No similarities were found among any of the leptospiral proteins and human, dogs, cattle, pig, horse, or sheep proteins (data not shown).

Multiple Sequence Alignments of *Leptospira* Surface-Exposed Proteins

A multiple sequence alignment was performed with each βb-OMP and OM lipoprotein and their respective orthologs. The alignments suggested that LIC10881 and LIC10964 were truncated sequences compared to their respective orthologs (Data Sheet S1 in Supplementary Material). An analysis of the genome region that contained the LIC10881 coding sequence (CDS) revealed the presence of a possible point mutation in the last nucleotide of LIC10881, that created a stop codon. When the point mutation was altered to a tryptophan codon (TG**A** → TG**G**), the LIC10881 and LIC10882 CDS were reassembled as a single CDS, LIC10881*, and the multiple alignment of LIC10881* was no longer truncated (Data Sheet S1 in Supplementary Material). Similarly, we identified a potential frameshift mutation in the LIC10896 CDS that was altered by the insertion of a cytosine at position 2,597. Reassembly of LIC10896 and LIC10895 identified a single CDS, LIC10896*. When the LIC10896* protein sequence was included in the multiple alignment, the sequence was no longer truncated (Data Sheet S1 in Supplementary Material). The modified LIC10881* and LIC10896* proteins were used for further analysis.

Functional Annotation Based on Protein Sequence

Eleven of the 18 βb-OMPs identified in this study were originally annotated as OMPs in the LIC genome, one was annotated

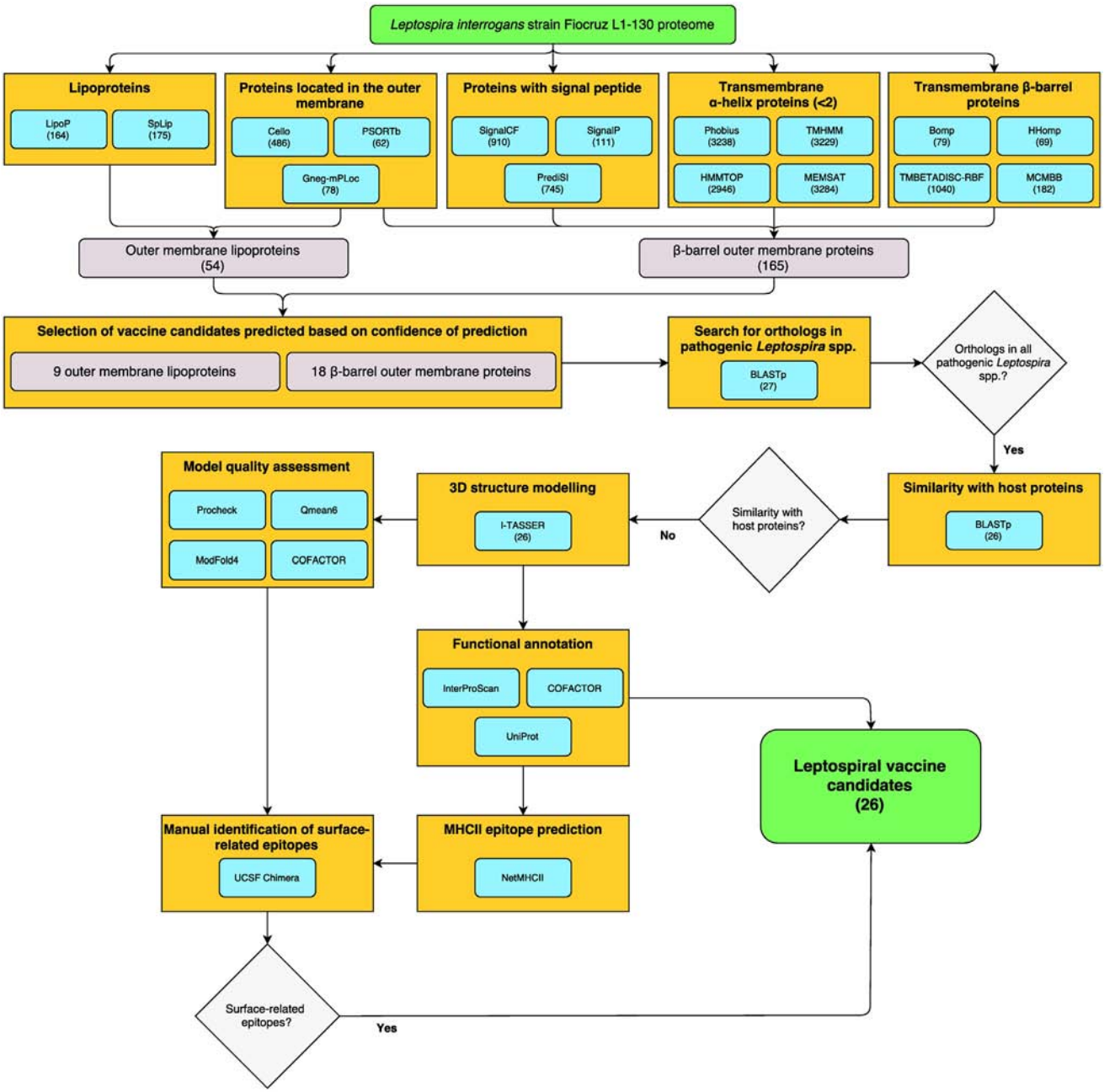

FIGURE 1 | Reverse vaccinology (RV)- and structural vaccinology (SV)-based bioinformatics workflow for the identification of leptospiral vaccine candidates. The complete LIC genome-derived proteome provided the input sequences for 16 bioinformatics programs for the prediction of βb-outer membrane proteins (OMPs) and outer membrane (OM) lipoproteins. The number of proteins selected by each program is shown in parentheses. High confidence predicted proteins were screened using a Python algorithm. Proteins conserved among pathogenic *Leptospira* spp. and with no similarity to host proteins were selected for the three-dimensional (3D) structure prediction by fold recognition modeling. Functional annotation was based on the primary structure and on the 3D models. Strong binder major histocompatibility complex-II immunogenic epitopes were predicted for the selected proteins and a structural approach was employed to map the surface-related immunogenic regions in the βb-OMPs. The final list of conserved βb-OMPs and OM lipoproteins and the surface-related epitopes are novel prospective leptospiral vaccine candidates.

as a cytoplasmic membrane protein, and six were annotated as hypothetical proteins (**Table 1**). Seven of the eight OM lipoproteins were annotated as hypothetical proteins or putative lipoproteins in the original genome annotation. The exception was LIC10024 that was annotated as an adenylate/guanylate cyclase (AGC). The KEGG database was used to identify any corresponding orthologs and their annotations in the genomes of the other pathogenic *Leptospira* spp. Of note, the genome

TABLE 1 | Functional annotation of βb-outer membrane proteins (OMPs) and outer membrane (OM) lipoproteins.

	Gene ID	Product/original annotation[a]	Uniprot protein name	KEGG orthologs	Interpro scan analysis
βb-OMPs	LIC10496	Conserved hypothetical protein	Uncharacterized protein	TolC-like OMP	OM efflux protein
	LIC10714	OM receptor protein	OM receptor protein (*smc*)	OM receptor for Fe^{3+}-dicitrate/TonB-dependent receptor (TBDR)	TBDR
	LIC10881	OMP, TonB dependent	OMP, TonB dependent	TBDR	TBDR [plug and β-barrel (βb) domains[b]]
	LIC10896	TonB-dependent outer membrane receptor	TonB-dependent outer membrane receptor (*fecA*)	TonB-dependent outer membrane receptor	TBDR
	LIC10964	TonB-dependent outer membrane hemin receptor	TonB-dependent outer membrane hemin receptor (*phuR*)	TonB-dependent outer membrane hemin receptor	TBDR
	LIC11086	Conserved hypothetical protein	Uncharacterized protein	Hypothetical protein	MetA-pathway of phenol degradation, putative
	LIC11211	Hypothetical protein	Uncharacterized protein	Hypothetical protein	None predicted
	LIC11268	Conserved hypothetical protein	Uncharacterized protein	Hypothetical protein	Alginate export domain
	LIC11458	OMP, porin superfamily	OMP, porin superfamily (outer membrane LPS export porin—lps-ep—family[c])	Hypothetical protein	None predicted
	LIC11506	OMP	OMP	Hypothetical protein	Predicted OMP, Leptospiraceae
	LIC11623	OMP	OMP	Hypothetical protein, Oma87-like OMP, BamA	Bacterial surface antigen (D15)/surface antigen variable number
	LIC12254	OMP	OMP	Hypothetical protein, Oma87-related protein, surface antigen (D15)	Bacterial surface antigen (D15)
	LIC12374	OMP, TonB dependent	OMP, TonB dependent (*btuB*)	TonB-dependent outer membrane receptor, obalamin receptor protein	TBDR, βb, plug domain
	LIC12575	Cytoplasmic membrane protein	Cytoplasmic membrane protein	Cytoplasmic membrane protein, TolC-like protein	Outer membrane efflux protein
	LIC13477	Conserved hypothetical protein	Uncharacterized protein	Hypothetical protein	None predicted
	LIC20019	Conserved hypothetical protein	Uncharacterized protein	Hypothetical protein	Putative porin/Porin 6
	LIC20087	OMP	OMP	Putative OMP, hypothetical protein	None predicted
	LIC20151	TonB-dependent outer membrane receptor	TonB-dependent outer membrane receptor	TonB-dependent hemin-binding protein	TBDR, plug and βb domains
OM lipoproteins	LIC10024	Adenylate/guanylate cyclase (AGC)	AGC	AGC	7TM-DISM receptor, extracellular domain, type 1; nucleotide cyclase; adenylyl cyclase class-3/4/guanylyl cyclase
	LIC10647	Conserved hypothetical protein	Uncharacterized protein	Hypothetical protein	None predicted
	LIC10713	Putative lipoprotein	Putative lipoprotein	Putative lipoprotein, peptidase M75	None predicted
	LIC11003	LipL71	LipL71	LipL71, peptidoglycan-binding protein LysM	Domain of unknown function DUF4398; LysM domain
	LIC11755	Conserved hypothetical protein	Uncharacterized protein	Hypothetical protein	None predicted
	LIC12048	Conserved hypothetical protein	Uncharacterized protein	Hypothetical protein	None predicted
	LIC13411	Putative lipoprotein	Putative lipoprotein	Hypothetical proteins	None predicted
	LIC20172	Lipoprotein	Lipoprotein	Hypothetical proteins	LruC domain; domain of unknown function DUF4842

[a]*As annotated in the LIC genome.*

[b]*Domains corresponding to LIC10881* (LIC10881 + LIC10882).*

[c]*Annotated based on results from Transporter Classification Database.*

TABLE 2 | Conservation of the 18 βb-outer membrane proteins (OMPs) and 9 outer membrane (OM) lipoproteins in 20 *Leptospira* spp.

	Gene ID	Pathogenic *Leptospira* spp.									Intermediate *Leptospira* spp.					Saprophytic *Leptospira* spp.					
		ale.	*als.*	*bor.*	*kir.*	*kme.*	*may.*	*nog.*	*san.*	*wei.*	*bro.*	*fai.*	*ina.*	*lic.*	*wolf.*	*bif.*	*mey.*	*ter.*	*van.*	*wolb.*	*yan.*
βb-OMPs	LIC10496	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	✓	✓	✓	✓
	LIC10714	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC10881*	✓	✓	✓	✓	–	✓	✓	✓	✓	–	–	–	–	–	–	–	–	–	–	–
	LIC10896*	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	✓	✓	✓	✓	✓	✓	✓	✓
	LIC10964	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	✓	–	–
	LIC11086	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC11211	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓	✓	✓	–	–	–
	LIC11268	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC11458	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC11506	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓	✓	✓	–	✓	✓	✓	✓	✓
	LIC11623	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC12254	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	–	–	–	✓
	LIC12374	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC12575	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC13477	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC20019	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC20087	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC20151	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	✓	✓	✓	✓	✓	✓	✓	✓	✓
OM lipoproteins	LIC10024	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	–	–	–
	LIC10647	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	–	–	✓	✓	–	–	–	–	–	–
	LIC10713	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	–	✓	✓	✓	✓	–
	LIC11003	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC11755	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC12048	✓	✓	–	✓	✓	✓	✓	✓	✓	–	–	–	✓	✓	✓	–	–	–	–	–
	LIC12690	–	–	–	✓	–	–	✓	–	–	–	–	–	✓	–	✓	✓	✓	✓	✓	✓
	LIC13411	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	LIC20172	✓	✓	✓	–	✓	✓	✓	✓	✓	✓	–	✓	✓	✓	✓	✓	✓	✓	✓	✓

The search for leptospiral orthologs was repeated using LIC10881 and LIC10896*.*

✓, conserved ortholog; –, no ortholog; ale., L. alexanderi; als., L. alstonii; bor., L. borgpetersenii; kir., L. kirschneri; kme., L. kmetyi; may., L. mayottensis; nog., L. noguchii; san., L. santarosai; wei., L. weilii; bro., L. broomii; fai., L. fainei; ina., L. inadai; lic., L. licerasiae; wolf., L. wolffii; bif., L. biflexa; mey., L. meyeri; ter., L. terpstrae; van., L. vanthielii; wolb., L. wolbachii; yan., L. yanagawae.

annotations of LIC10496, LIC11458, LIC11506, and LIC12575 differed substantially to those of their orthologs (**Table 1**).

A functional annotation was performed using InterProScan for the 18 βb-OMPs and 8 OM lipoproteins (**Table 1**). InterProScan identified OMP-related domains in 14 of the βb-OMPs. However, it failed to identify functional domains in four of the predicted βb-OMPs (LIC11211, LIC11458, LIC13477, and LIC20087) and five out of eight OM lipoproteins (LIC10647, LIC10713, LIC11755, LIC12048, and LIC13411). Of the remaining OM lipoproteins, two (LIC11003 and LIC20172) contained domains with an unknown function and LIC10024 was confirmed as an AGC protein.

Structural Modeling Improved the Prediction of OMP Function

For each protein sequence, the I-TASSER software predicted five 3D models and each model was ranked in order of quality by a *C*-score. The top-ranking models for 16 of the βb-OMPs contained a typical transmembrane βb structure (**Figure 2**). While the LIC11268 model that contained a transmembrane βb structure was ranked third, it was included for further analysis. The structural models of the eight OM lipoproteins are shown (Data Sheet 3 in Supplementary Material). The refined βb-OMP and OM lipoprotein models were analyzed by COFACTOR, a structure-based method for assigning biological function to protein molecules. A COFACTOR output includes the top 10 closest structures in PDB ranked by TM-score, Gene Ontology (GO) terms associated with the protein model, and the root-mean-square deviation of atomic position (RMSD) related to the best templates used for modeling. GO terms, including molecular function, biological process, and cellular location, associated with the protein 3D models were predicted based on the GOs assigned to the template structures and provided a functional insight into the selected leptospiral proteins. When more than one GO term per category was predicted for each protein, some were related to more distant templates (the last of the 10 closest structures), they were collapsed to the closest parent term on the AmiGO2 database and are shown (**Figure 3**) according to their frequency in the βb-OMPs and OM lipoproteins. The complete list of GO terms predicted for each protein structure is provided (Table S5 in Supplementary Material). As expected, most of the βb-OMP molecular function and biological process GO terms were related to transportation. This was supported by the cell location GO term, most of the βb-OMPs were classified as membrane or OM. The GO terms predicted for the OM lipoproteins were distributed among several different categories. Most of the GO terms for molecular function were for catalytic/binding activity and the

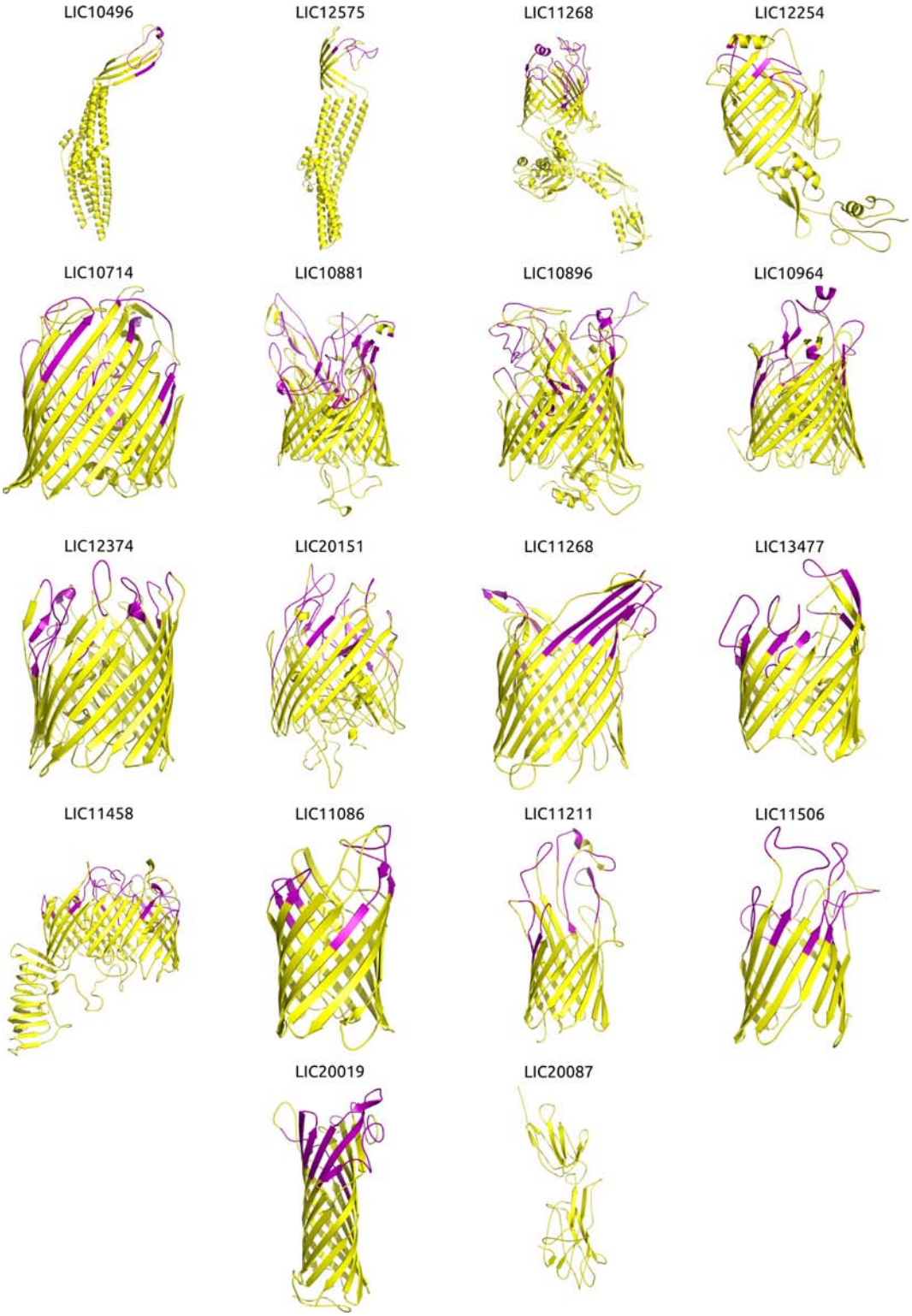

FIGURE 2 | Three-dimensional (3D) structures of the βb-outer membrane proteins (OMPs) and mapping of the immunogenic surface-related epitopes (SRE). Structural modeling was performed using I-TASSER, and the structures were visualized and the images generated using PyMOL. Proteins are orientated as following: the upper portion is surface-exposed on the OM and the lower portion is located in the periplasmic space. The orientation was derived from an interpretation of the orientation and structure of the matching PDB structure. Immunogenic major histocompatibility complex-II epitopes (strong binders) for 14 HLAs were predicted using NetMHCII and mapped onto the βb-OMPs structural models. Immunogenic SRE are indicated in purple in each βb-OMP structure.

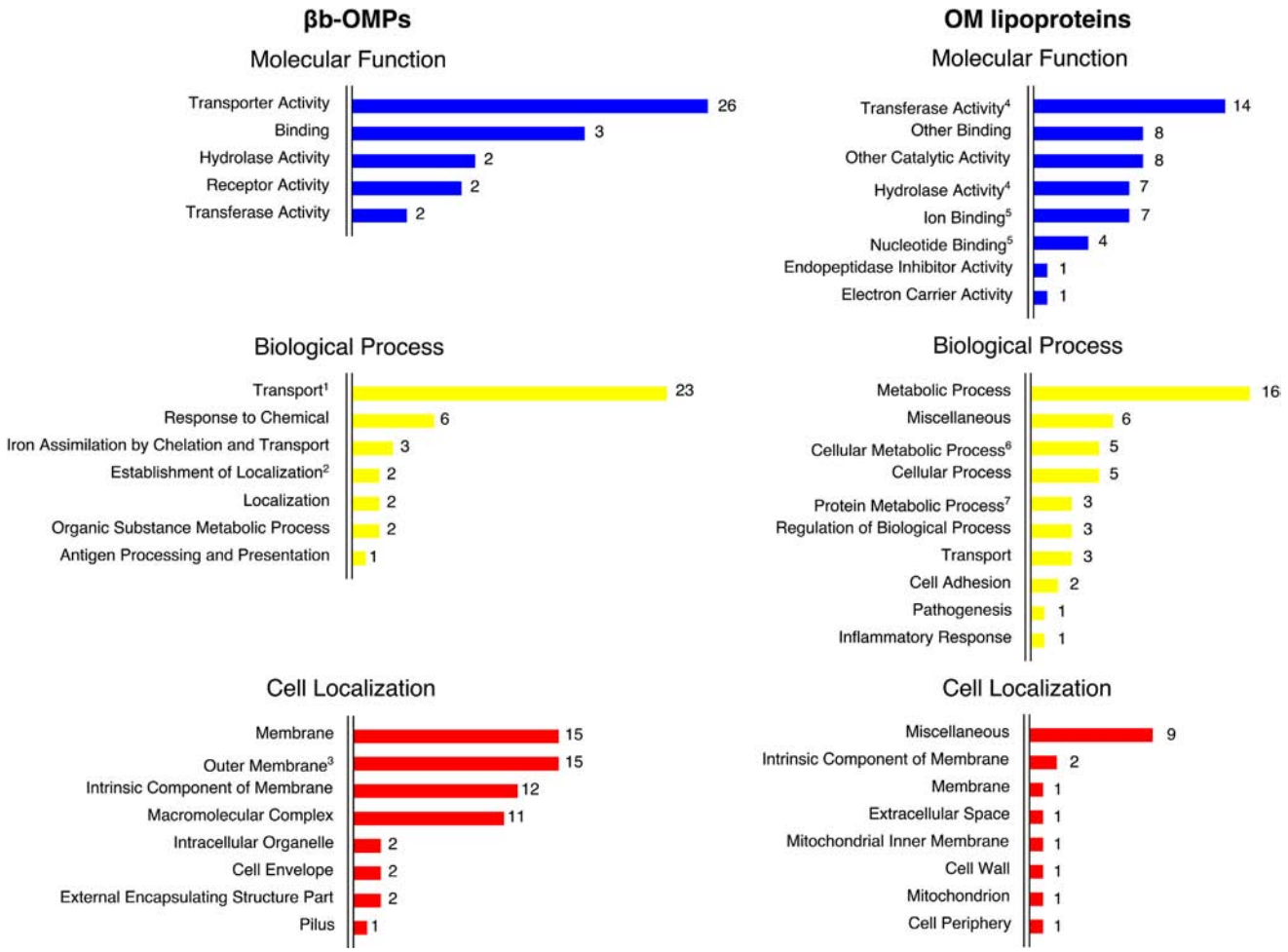

FIGURE 3 | The Gene Ontology (GO) terms predicted by COFACTOR were based on the structural models of the βb-outer membrane proteins (OMPs) and outer membrane (OM) lipoproteins. The GO terms were assigned for three domains: molecular function, biological process, and cell localization. COFACTOR usually predicted more than one term for each GO domain for the target protein structure. The GO terms were collapsed to the closest parent term (based on the AmiGO2 database) to facilitate the overall view of the GO terms. In some cases, related terms were maintained for convenience, as indicated: 1—*transport* is also a child term of *establishment of localization*; 2—*establishment of localization* is also a child term of *localization*; 3—*outer membrane* is also a child term of *membrane*; 4—*transferase activity* and *hydrolase activity* are also children terms of *catalytic activity*; 5—*ion binding* and *nucleotide binding* are also children terms of *binding*; 6—*cellular metabolic process* is also a child term of *metabolic process* and *cellular process*; and 7—*protein metabolic process* is also a child term of *metabolic process*.

predicted biological processes were related to metabolism. The predicted cell location GO terms were diverse (**Figure 3**; Table S5 in Supplementary Material).

Quality of Predicted Models

The closest PDB structure (PDB code, protein name, and organism of origin) for the predicted structural models and an estimation of the quality are shown (**Table 3**). The quality of each predicted model was evaluated using Procheck, QMEAN6, and ModFOLD4. Procheck was used to evaluate the stereochemical quality of each protein structure and the proportion of disallowed residues in the Ramachandran plot ranged from 0.7% for LIC10496 to 11.6% for LIC20151. When assessed by QMEAN and ModFOLD, most of the βb-OMPs models had good quality values for the overall structure.

Surface-Related Immunogenic Epitope Prediction

Due to the importance of phagocytosis in the clearance of leptospires during an infection, the presence of MHC class II-binding epitopes in the 18 βb-OMPs and 8 OM lipoproteins was evaluated. NetMHCII was used to predict 15 amino acid long peptides that can bind, at different affinity levels, to MHC class II molecules encoded by several HLAs. Predictions were made for 14 HLA-DRB alleles and only strong binder (SB) epitopes (IC50 <50 nM) were considered for analysis. Each predicted immunogenic epitope had a 9mer core that was aligned, and a consensus sequence was determined. The location of each SB epitope in their respective structural models was identified (**Figure 2**). The correct orientation of the βb structural models in the OM was deduced

TABLE 3 | Closest PDB structure and quality assessment for the βb-outer membrane proteins (OMPs) and outer membrane (OM) lipoproteins models.

	Gene ID	Closest structure on PDB (PDB code), organism of origin[a]	Quality assessment					
			TM-score[a]	RMSD[a]	Ramachandran disallowed residues (%)[b]	ModFold score	Confidence and *P*-value ModFold	QMEAN score
βb-OMPs	LIC10496	OMP TolC (1tqq), *Escherichia coli*	0.862	1.10	0.7	0.5390	HIGH: 3.324E−3	0.403
	LIC10714	TonB-dependent receptor (TBDR)—ferrichrome-iron receptor FhuA (1fl1), *E. coli*	0.840	1.10	8.3	0.6236	HIGH: 1.38E−3	0.327
	LIC10881*	TBDR—transferrin-binding protein A TbpA (3v89), *Neisseria meningitidis*	0.592	4.86	3.4	0.3089	MEDIUM: 3.63E−2	0.344
	LIC10896*	TBDR—ferripyoverdine receptor FpvA (2w78), *Pseudomonas aeruginosa*	0.719	2.25	5.0	0.3127	MEDIUM: 3.50E−2	0.197
	LIC10964	TBDR—Zn-transporter ZnuD (4rdr), *N. meningitidis*	0.837	1.37	6.3	0.5544	HIGH: 2.834E−3	0.326
	LIC11086	Protein involved in meta-pathway of phenol degradation-like protein, Pput2725 (4rl8), *Pseudomonas putida*	0.769	1.31	2.3	0.2233	LOW: 8.848E−2	0.231
	LIC11211	Toluene transporter TbuX (3bry), *Ralstonia pickettii*	0.873	2.28	3.1	0.0000	POOR: 9.017E−1	0.184
	LIC11268	TBDR—transferrin-binding protein A TbpA (3v89), *N. meningitidis*	0.914	1.65	4.2	0.0000	POOR: 9.017E−1	0.137
	LIC11458	LPS assembly protein LptD (4q35), *Shigella flexneri*	0.722	1.56	5.4	0.0777	POOR: 4.02E−1	0.254
	LIC11506	OM Porin OmpG (2iww), *E. coli*	0.660	3.33	3.1	0.1192	POOR: 2.613E−1	0.083
	LIC11623	OMP assembly factor BamA (4k3b), *Neisseria gonorrhoeae*	0.506	3.37	4.9	0.2569	LOW: 6.245E−2	0.349
	LIC12254	OMP assembly factor BamA lacking polypeptide translocation-associated domains 1–3 (4k3c), *H. ducreyi*	0.882	2.29	4.6	0.2076	POOR: 1.042E−1	0.177
	LIC12374	TBDR—vitamin B12 transporter BtuB (2gsk), *E. coli*	0.824	2.11	6.4	0.6066	HIGH: 1.646E−3	0.282
	LIC12575	OMP TolC (1tqq), *E. coli*	0.849	1.01	1.8	0.5207	HIGH: 4.02E−3	0.403
	LIC13477	Alginate production protein AlgE (3rbh), *P. aeruginosa*	0.804	1.81	3.3	0.2664	LOW: 5.653E−2	0.259
	LIC20019	Plasminogen activator Pla/coagulase/fibrinolysin (2x4m), *Yersinia pestis*	0.769	2.02	2.8	0.2117	LOW: 9.987E−2	0.279
	LIC20087	Major Pilin Protein (4s3l), *Streptococcus pneumoniae*	0.896	2.20	3.7	0.0000	POOR: 9.017E−1	0.246
	LIC20151	TBDR—vitamin B12 transporter BtuB (2gsk), *E. coli*	0.794	1.57	11.6	0.5631	HIGH: 2.588E−3	0.315
OM lipoproteins	LIC10024	Adenylate cyclase type 10 (4clf), *Homo sapiens*	0.509	3.15	7.9	0.2229	LOW: 8.883E−2	0.229
	LIC10647	Major fimbrial subunit protein (4q98), *Porphyromonas gingivalis*	0.828	2.89	3.0	0.0000	POOR: 9.017E−1	0.21
	LIC10713	Putative iron-regulated protein A (4ecg), *Parabacteroides distasonis*	0.743	2.02	3.6	0.5401	HIGH: 3.286E−3	0.438
	LIC11003	Cytoplasmic domain of bacterial cell division protein EzrA (4uxv), *Bacillus subtilis*	0.853	2.24	2.0	0.0226	POOR: 7.126E−1	0.31
	LIC11755	RNA-dependent RNA polymerase (3ja4), Cypovirus 1	0.923	1.54	4.3	0.1582	POOR: 1.741E−1	0.042
	LIC12048	Tc toxin/TcdB2/TccC3 (4o9x), *Photorhabdus luminescens*	0.890	2.48	4.5	0.0000	POOR: 9.017E−1	0.144
	LIC13411	Chalcone-flavanone isomerase family protein (4doo), *Arabidopsis thaliana*	0.669	2.46	2.1	0.0000	POOR: 9.017E−1	0.342
	LIC20172	Secretory component of immunoglobulin A (3chn), *H. sapiens*	0.799	2.64	4.9	0.0000	POOR: 9.017E−1	0.018

[a]*Obtained by COFACTOR analysis.*

[b]*Obtained by Procheck analysis.*

using the corresponding PDB structure as a template. The total number of 9mers and 15mers predicted for each βb-OMP and OM lipoprotein, as well as the number of surface-related 9mers and 15mers of the βb-OMPs is provided (**Table 4**). LIC20087 and the OM lipoproteins were not analyzed for surface-related epitopes (SREs) as they did not contain βb structures; therefore, it was not possible to determine their localization in the OM. Each immunogenic epitope (9mer core or consensus) was evaluated for conservation among the orthologous proteins. A representative set of the most conserved immunogenic SREs for each βb-OMP is shown (**Figure 4**). The SREs were relatively well conserved in all the βb-OMPs. The alignments of the βb-OMP immunogenic epitopes to the corresponding regions in the orthologs from pathogenic *Leptospira* spp. are highlighted in the alignment files (Data Sheet S1 in Supplementary Material). A list of the βb-OMP and OM lipoprotein SB epitopes for all MHC-II alleles is provided (Tables S6 and S7 in Supplementary Material, respectively).

DISCUSSION

The main principle of RV is to evaluate all the potential vaccine candidates encoded in the genome of the pathogen of interest and to reduce the number of vaccine targets to a number that can be reasonably tested in the laboratory (34). The initial screening is achieved by using bioinformatics to identify all surface-exposed proteins (potential vaccine candidates) and this typically reduces the number of targets 10-fold, from thousands to hundreds of proteins. Screening using *in vitro* assays further reduces the number of vaccine candidates and hence the number of laboratory animals required for efficacy testing. However, as no immune correlates for leptospirosis have been identified to date, the only way to screen the proteins identified as surface-exposed is to use a lethal animal model and look for protection (35). Therefore, the *in silico* identification of surface-exposed proteins must be sufficiently rigorous to screen out undesirable proteins, yet be sensitive enough to include all potential vaccine candidates.

In diderm bacteria, vaccine targets include transmembrane proteins (βb-OMPs), lipoproteins anchored to the outer leaflet of the OM, and possibly secreted proteins, particularly those that interact with the βb-OMPs. As there is no concrete evidence that *Leptospira* spp. secrete proteins, their role in pathogenesis is unclear, and therefore, there is no supporting data for their use as vaccine candidates. The prediction of the antiparallel transmembrane β-sheets that form βb proteins is usually achieved with a high degree of confidence (36). The identification of the SP, the βb secondary structure, the absence of TMHs, and homology to known proteins is a straightforward process. Lipoprotein prediction is based on the identification of a lipobox and localization to the OM is based on homology to known OM lipoproteins (37, 38). In the current study, 16 different bioinformatics programs were used to analyze the LIC proteome, one of the most studied *Leptospira* strains and one that is routinely used as the challenge strain in animal models of leptospirosis (7). A total of 165 βb-OMPs and 54 OM lipoproteins were identified in the first round of screening. However, the absence of experimental data for leptospiral proteins severely limits the use of protein sets with known subcellular localization and structures that can be used to verify the prediction based on true or false analyses. To overcome this limitation, we developed an algorithm that reduced the final target list of potential vaccine candidates to 18 βb-OMPs and 8 OM lipoproteins, each predicted with a high degree of confidence. The algorithm was intended to normalize, calculate a weight, and transform the data based on a measure of agreement among the predictors. This was achieved by giving higher weights to those predictors whose results were confirmed by other predictors and lower weights to those predictors with higher rates of disagreement; the result of the voting was expected to be more reliable, as confirmed in the current study. The bioinformatics pipeline and algorithm developed in this study, including the SV approach, can be applied to the identification of OMPs and vaccine candidates in any other diderm pathogen with a known genome sequence.

Structural vaccinology represents the cutting edge of vaccine target discovery and development. Several approaches have been investigated (29) although all are based on the common theme of protein structural data. However, most of these approaches use information from experimentally determined protein structures (e.g., X-ray diffraction), which it is time consuming and expensive, especially when compared to *in silico* modeling (39). In the last decade, protein structure modeling has improved enormously, as demonstrated by the critical assessment of protein structure prediction (CASP) experiments (40). I-TASSER, the modeling tool used for leptospiral βb-OMPs and OM lipoproteins structural modeling, was ranked number one for structure and function prediction in the most recent CASP experiments (40, 41). I-TASSER was shown to be as precise as structure solving by crystallography (42); it predicts structure models based on protein threading, even allowing model prediction for proteins with low sequence similarity to any other protein, as it considers sequence to structure fold recognition. Even though I-TASSER was expected to provide high-quality structural models, some of the models generated in the current study were considered poor quality, as expected for proteins with unknown functions (33). The overall quality of a protein structure prediction is indicative that it might not be similar to the template structures; however, the overall folding is representative of how a given protein folds. In the case of βb-OMPs, the most unreliable regions were the folded loops and plug domains (data not shown); however, the transmembrane β-sheets were well predicted, giving a spatial and structural insight into where the surface-exposed part of the protein is located. Therefore, poor quality models were included in the epitope prediction analysis and mapping of the SREs in the structure. Modeling was particularly important for uncharacterized proteins with no known functional domains and that had low or no similarity to other known proteins. Overall model quality can be improved by separating different domains and modeling them separately. However, this approach was not available for proteins with no functional domains and could have generated final models that did not represent the fully resolved structure (39). We generated structural models that included a βb for 17 of the 18 predicted βb-OMPs. LIC20087 was the exception and even though the structural model was highly similar to a pilin (43), it did not contain a βb structure. Although there

TABLE 4 | Number of predicted strong binder (SB) major histocompatibility complex-II (MHC-II) epitopes in each βb-outer membrane protein (OMP) and outer membrane (OM) lipoproteins for 14 HLAs.

	Gene ID	Number of surface-related epitopes (SRE) identified among total SB epitopes—surface-related 9mer (SRE9)/SRE15 (SB9/SB15)[c]													
		HLA-DRB 10101	HLA-DRB 10301	HLA-DRB 10401	HLA-DRB 10404	HLA-DRB 10405	HLA-DRB 10701	HLA-DRB 10802	HLA-DRB 10901	HLA-DRB 11101	HLA-DRB 11302	HLA-DRB 11501	HLA-DRB 30101	HLA-DRB 40101	HLA-DRB 50101
βb-OMPs[a]	LIC10496	3/17 (29/111)	0/0 (8/25)	1/5 (4/16)	0/0 (6/27)	1/5 (4/23)	0/0 (9/42)	0/0 (0/0)	1/3 (3/10)	0/0 (10/39)	2/12 (7/44)	0/0 (3/10)	1/5 (3/16)	0/0 (15/50)	0/0 (14/65)
	LIC10714	14/60 (44/171)	2/12 (5/23)	8/28 (14/46)	3/14 (11/39)	7/21 (11/35)	8/39 (21/74)	1/3 (3/11)	3/17 (7/30)	1/1 (9/35)	1/7 (5/30)	3/8 (8/38)	2/11 (6/35)	2/22 (8/31)	5/17 (19/68)
	LIC10881*	23/56 (49/157)	2/7 (8/20)	2/6 (8/16)	9/30 (16/57)	10/25 (14/38)	5/20 (20/97)	0/0 (0/0)	3/7 (6/15)	8/22 (17/51)	1/7 (8/38)	5/14 (14/41)	4/23 (8/44)	5/14 (6/15)	7/31 (18/89)
	LIC10896*	23/73 (68/251)	2/7 (9/33)	7/19 (17/59)	3/12 (10/37)	6/21 (24/73)	9/41 (30/132)	3/4 (8/23)	2/10 (12/53)	7/29 (28/94)	1/7 (6/34)	1/2 (14/54)	2/10 (6/29)	2/4 (8/21)	5/23 (27/94)
	LIC10964	9/33 (50/182)	2/7 (4/12)	3/8 (10/34)	0/0 (6/32)	5/21 (15/53)	6/35 (26/111)	0/0 (0/0)	4/10 (7/22)	1/4 (21/51)	2/13 (6/37)	2/5 (10/32)	0/0 (2/7)	1/3 (11/34)	5/28 (19/88)
	LIC11086	2/9 (26/79)	0/0 (1/4)	1/5 (3/14)	1/6 (2/7)	1/5 (4/13)	2/6 (11/41)	1/6 (1/6)	0/0 (9/26)	2/11 (9/36)	0/0 (6/26)	0/0 (7/33)	1/7 (1/7)	0/0 (1/6)	1/3 (11/43)
	LIC11211	5/13 (21/57)	1/3 (3/10)	0/0 (9/27)	1/6 (6/24)	1/2 (5/19)	1/4 (12/40)	0/0 (0/0)	0/0 (1/3)	1/2 (11/35)	0/0 (3/11)	1/5 (5/22)	0/0 (1/5)	0/0 (5/14)	0/0 (9/40)
	LIC11268	9/44 (26/105)	2/10 (4/14)	2/6 (6/24)	1/1 (7/23)	3/9 (13/42)	4/18 (10/42)	0/0 (0/0)	0/0 (0/0)	3/10 (6/19)	1/1 (4/13)	0/0 (5/17)	2/9 (9/37)	1/3 (3/9)	3/15 (6/25)
	LIC11458	16/56 (62/205)	1/3 (11/38)	7/30 (19/61)	4/19 (19/76)	7/26 (18/66)	4/21 (25/100)	0/0 (1/2)	3/12 (7/23)	6/23 (31/109)	2/13 (8/42)	2/9 (14/61)	1/7 (5/30)	1/7 (2/8)	3/20 (28/134)
	LIC11506	7/28 (27/96)	0/0 (0/0)	2/7 (7/21)	1/1 (4/12)	2/5 (8/26)	5/18 (20/78)	0/0 (0/0)	1/6 (6/19)	4/20 (8/37)	1/5 (2/12)	4/20 (12/47)	0/0 (4/18)	0/0 (1/6)	5/17 (12/36)
	LIC11623	16/65 (60/221)	1/6 (11/43)	4/16 (12/52)	0/0 (8/25)	2/9 (14/49)	5/24 (29/123)	0/0 (0/0)	1/5 (5/20)	6/16 (26/85)	3/14 (7/30)	1/1 (17/61)	2/12 (9/48)	5/18 (17/56)	2/7 (27/102)
	LIC12254	4/27 (24/89)	0/0 (3/15)	0/0 (2/9)	1/5 (8/42)	0/0 (6/17)	1/7 (10/48)	1/2 (3/6)	4/6 (9/18)	2/7 (10/37)	2/11 (8/30)	0/0 (5/29)	0/0 (8/25)	0/0 (0/1)	1/7 (11/56)
	LIC12347	12/40 (42/144)	1/3 (7/29)	2/11 (8/27)	3/13 (8/37)	2/7 (10/34)	5/21 (17/80)	0/0 (0/0)	0/0 (2/7)	2/7 (10/26)	1/4 (2/10)	1/7 (6/26)	1/7 (10/50)	0/0 (0/0)	2/6 (16/55)
	LIC12575	5/16 (32/98)	0/0 (5/19)	0/0 (1/6)	0/0 (5/13)	1/5 (5/27)	2/12 (9/48)	0/0 (0/0)	0/0 (3/9)	0/0 (9/31)	1/2 (5/21)	0/0 (4/14)	0/0 (3/14)	0/0 (7/25)	2/8 (11/46)
	LIC13477	9/27 (32/112)	0/0 (1/7)	1/2 (7/31)	0/0 (5/9)	1/5 (9/31)	1/12 (12/49)	0/0 (0/0)	2/8 (3/12)	2/3 (7/21)	1/2 (2/5)	2/4 (9/33)	1/5 (4/17)	0/0 (1/2)	2/11 (10/41)
	LIC20019	7/24 (21/70)	1/6 (1/6)	2/7 (5/16)	3/13 (4/19)	9/32 (10/38)	4/24 (13/50)	0/0 (0/0)	1/7 (1/7)	5/10 (11/33)	0/0 (2/2)	2/8 (7/19)	3/14 (5/21)	2/9 (3/15)	1/7 (8/27)
	LIC20087	–/– (17/94)	–/– (1/5)	–/– (4/15)	–/– (4/17)	–/– (11/32)	–/– (12/52)	–/– (1/7)	–/– (4/16)	–/– (7/22)	–/– (1/5)	–/– (4/17)	–/– (2/8)	–/– (1/6)	–/– (7/31)
	LIC20151	14/62 (44/157)	3/9 (5/16)	5/20 (10/35)	4/17 (7/24)	5/17 (15/43)	4/22 (24/109)	0/0 (0/0)	1/2 (5/16)	2/8 (10/36)	4/20 (9/43)	4/12 (12/43)	1/2 (5/27)	4/12 (8/21)	5/25 (15/66)

(*Continued*)

TABLE 4 | Continued

Gene ID		Number of surface-related epitopes (SRE) identified among total SB epitopes—surface-related 9mer (SRE9)/SRE15 (SB9/SB15)[c]										
		HLA-DRB 10101	HLA-DRB 10301	HLA-DRB 10401	HLA-DRB 10404	HLA-DRB 10405	HLA-DRB 10701	HLA-DRB 10802	HLA-DRB 10901	HLA-DRB 11101	HLA-DRB 11302	HLA-DRB 11501	HLA-DRB 30101	HLA-DRB 40101	HLA-DRB 50101
OM lipoproteins[b]	LIC10024	54/223	10/31	9/37	16/74	21/58	39/135	3/8	8/25	36/112	6/24	32/103	6/25	13/35	30/144
	LIC10647	24/88	0/0	10/31	6/27	10/39	14/57	0/0	1/5	8/24	1/5	10/30	2/9	1/1	15/74
	LIC10713	23/86	0/0	7/22	6/23	4/12	8/35	0/0	2/5	3/12	3/11	1/2	2/9	2/8	9/30
	LIC11003	12/36	1/2	0/0	0/0	1/3	4/18	0/0	0/0	4/16	3/12	0/0	1/1	5/17	8/21
	LIC11755	64/222	13/44	17/65	16/62	12/50	34/145	1/3	5/15	19/60	12/67	16/59	9/38	10/32	23/123
	LIC12048	102/331	13/54	20/75	23/85	27/85	38/142	3/4	8/31	18/61	18/69	15/62	10/54	8/28	19/77
	LIC13411	17/60	0/0	3/8	3/8	7/19	6/18	0/0	2/4	1/2	0/0	3/16	0/0	2/12	4/25
	LIC20172	22/101	3/9	7/26	10/31	4/16	10/32	0/0	1/2	8/30	8/30	1/1	3/12	3/8	7/32

[a]*The number of predicted surface-related 9mer (SRE9) and 15mer (SRE15) epitopes and the total number of strong binder 9mer (SB9) and 15mer (SB15) epitopes (including those that are not surface-related) are shown.*

[b]*For the OM lipoproteins, only SB9 and SB15 are shown.*

[c]*SB epitopes predicted by NetMHCII as CI50 <50 nM.*

were some low-quality scores for some of the structural models, all the predicted models had high TM-scores and low RMSDs and this allowed COFACTOR to predict function based on the structural model. Function prediction based on structure is a valuable alternative during an *in silico* investigation of protein function, particularly in cases where no other similarity was found.

Leptospiral βb-OMPs and OM lipoproteins represent ideal targets for vaccine development, they are surface-exposed, and therefore amenable to recognition by the host immune system. Substantial evidence supports the theory that a protective immune response is antibody-based (5); to date, only bovines have been shown to require a cellular immune response for protection (44). As leptospires are extracellular pathogens, phagocytosis plays an important role in the immune response, as pathogenic leptospires can resist complement-mediated killing (44). Following phagocytosis, proteins from the pathogen are processed by antigen-presenting cells and presented via MHC-II molecules to T-helper cells that can stimulate inflammation and activation of B-cells for antibody production (44). Therefore, we screened the novel βb-OMPs and OM lipoproteins for the presence of MHC-II SB epitopes. I-TASSER cannot predict protein structure in the context of the bacterial outer membrane. Therefore, the orientation of the OMPs models in the OM was determined by an interpretation of the orientation of the closest PDB structure from the COFACTOR analysis. We identified the location of the epitopes in the structural models of the βb-OMPs. These epitopes are likely to be exposed on the surface of the bacteria and therefore bind to MHC-II receptors, subsequently triggering an immune response. This approach is particularly relevant to βb-OMPs, these proteins usually contain long surface-exposed loops, but they also contain immunodominant regions that are not surface-exposed (**Figure 2**; **Table 4**; Table S6 in Supplementary Material). Therefore, recombinant vaccines should not use the entire βb-OMP in vaccine preparations to prevent stimulation of a non-protective immune response. The inclusion of only the surface-exposed MHC-II epitopes in the vaccine preparation would be more likely to stimulate a protective immune response. Each of the proteins included in the final target list will be discussed in detail in the following sections.

LIC10496 and LIC12575 Are TolC-Like Proteins

LIC10496 was originally annotated as a hypothetical protein and LIC12575 was annotated as a cytoplasmic membrane protein. However, their orthologs were annotated as TolC-like OMPs in the other pathogenic *Leptospira* spp. Furthermore, functional annotation by InterProScan predicted that they contained an OM efflux protein domain (**Table 1**), a property of TolC proteins, and a component of Type I secretion systems. In diderm bacteria, TolC is an OM efflux protein that forms a trimeric channel composed of a 12-stranded βb that spans the OM (4 transmembrane β-sheets per monomer) and long periplasmic α-helices that span the periplasm. The channel is connected to the cytoplasm by a TMH inner membrane protein channel. TolC transporters are involved in the transport of a variety of substrates, from small

FIGURE 4 | Sequence logos showing the frequency of amino acid residues at each position of the immunogenic surface-related epitopes (SREs) identified in the βb-outer membrane proteins (OMPs), according to multiple sequence alignment of the orthologs from pathogenic *Leptospira* spp. Only the most conserved SRE from each βb-OMPs is shown. The overall height of the stack indicates the level of sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each amino at that position.

molecules to protein secretion. TolC proteins from *Edwardsiella tarda* (45), *Listeria monocytogenes* (46), and *Salmonella paratyphi* (47) were reported to be protective vaccine antigens. The structural models of the leptospiral TolC proteins were highly similar to an *E. coli* TolC protein (48). Both were characterized as high-quality models, with high ModFOLD and QMEAN scores and the lowest number of disallowed residues on the Ramachandran plot (**Table 3**). Interestingly, the extracellular loops connecting the β-sheets in the βb were predicted to contain MHC-II epitopes. Both leptospiral TolC-like proteins were conserved in all *Leptospira* spp., except for *L. wolffii* that has no ortholog for LIC10496. Structure-based GO predictions for these proteins suggested a possible relation to copper ions, siderophores, or protein transport (Table S5 in Supplementary Material). A previous study linked LIC12575 to the leptospiral response when exposed to *in vivo* like conditions (49). These data support the idea that LIC10496 and LIC12575 are promising vaccine candidates.

LIC10714, LIC10881, LIC10896, LIC10964, LIC12374, and LIC20151 Are TonB-Dependent Receptors

A TonB-dependent receptor (TBDR) domain was identified in all these proteins and this was supported by the structural models that predicted TBDRs. All the leptospiral TBDR models were reliable in terms of quality, this was expected as there is a high degree of similarity among TBDRs even in different bacteria (50). TBDRs are a βb proteins comprised of 22 amphipathic β-strands, and a globular plug domain that is folded up inside the barrel. Indeed, this structural folding was observed for LIC10881* and LIC10896* that were originally partial CDSs, supporting our finding that LIC10882 and LIC10895 are not proteins but are instead part of the LIC10881 and LIC10896 CDS, respectively, as in seen in other *Leptospira* spp. Interestingly, LIC10881* was absent in saprophytic and intermediate *Leptospira* spp., suggesting a potential role in pathogenesis. Gram-negative bacteria contain variable numbers

of TBDRs, the *E. coli* genome contains 7 TBDRs (50); while other bacteria can contain up to 65 TBDRs (51). The LIC genome contains 13 genes that are annotated as TBDRs (52). While orthologs of LIC10714 in other *Leptospira* spp. genomes were annotated as TBDRs, LIC10714 was annotated as an OM receptor protein. LIC10714 contained the characteristic domains and the model included a 22-stranded βb with high structural similarity to FhuA from *E. coli* (53), even though it was suggested to be a leptospiral FecA (54). An ortholog of LIC10714 in *L. biflexa* was knocked out, resulting in a mutant with impaired ability to use iron citrate, iron chloride, iron sulfate, and aerobactin as iron sources, suggesting a role in iron metabolism (55). LIC10714 was also found to bind fibronectin (renamed as MFn2) and it was suggested that the extracellular loops could play a role in the bacterial adhesion process (56). The structural model of LIC10881* was similar to TbpA, a transferrin transporter in pathogenic *Neisseria* spp. (57). The LIC10896* 3D model was similar to FpvA, a pyoverdine-Fe transporter from *Pseudomonas aeruginosa* (58). LIC10964 was predicted to be a hemin transporter encoded by *phuR*, this gene is absent from all saprophytic *Leptospira* spp. except for *L. vanthielii* (**Table 2**). Of note, *phuR* was upregulated in the infection-mimicking model of leptospiral growth within dialysis membrane chambers (DMCs) implanted in the rat peritoneal cavity (59). The LIC10964 model was highly similar to the *N. meningitidis* zinc transporter, ZnuD (60). The LIC12374 and LIC20151 models were structurally similar to the *E. coli* BtuB cobalamin (vitamin B12) transporter (61). It has been suggested that pathogenic *Leptospira* spp. are autotrophic for vitamin B12 (12).

The actual molecule transported by a TBDR is difficult to predict by bioinformatics and should be determined experimentally. TBDRs play an important role in pathogenicity, they are conserved among the pathogenic *Leptospira* spp., and a significant portion of its structure was predicted to be surface-exposed. MHC-II epitopes were predicted for the TBDRs and several were located on the surface-exposed region of the protein. While TBDRs have been explored as vaccine candidates in other bacterial diseases (62–64), they have not yet been evaluated as experimental vaccines against leptospirosis. TBDRs transport essential molecules and if this is blocked, e.g., by antibodies, it would be a potentially lethal event.

Are LIC11268 and LIC13477 Alginate Transporters?

Although both proteins were originally annotated as hypothetical proteins, InterProScan found an alginate export domain in LIC11268 (**Table 1**). However, the structural model of LIC11268 suggested it was a βb-OMP with high structural similarity to the neisserial TbpA transferrin transporter, a known TBDR (57). LIC13477 was identified as an alginate transporter based on structural annotation (**Table 3**) and was structurally similar to the OMP AlgE from *P. aeruginosa* (65). AlgE contains 18-stranded β-sheets in the transmembrane βb and is different to other TBDRs. The predicted models of both LIC11268 and LIC13477 contained an 18-stranded βb. Although an alternative, low quality, model of LIC11268 contained 20-stranded β-sheets (data not shown). Alginate is a polysaccharide in biofilms (66) and both *L. biflexa* and *L. interrogans* contain a complete set of genes for alginate biosynthesis (52, 67). Nevertheless, alginate exporters have not yet been identified in *L. interrogans*. The *in silico* modeling of LIC11268 and LIC13477 suggested they may play a role in the transport of alginate to the extracellular milieu, but this will need to be confirmed experimentally. Both proteins were predicted to contain a βb structure, potentially spanning the leptospiral OM. Immunogenic epitopes were identified in the surface-exposed regions and both proteins were conserved among pathogenic *Leptospira* spp., suggesting they are promising vaccine candidates.

LIC11623 and LIC12254 Are BamA-Like βb-OMPs

Both proteins were originally annotated as OMPs, while in the current study, they were found to contain the bacterial surface antigen D15/Oma87 domains (**Table 1**). Of note, the orthologs of LIC11623 and LIC12254 in other *Leptospira* spp. were annotated as Oma87-like proteins. These BamA-like proteins are responsible for the assembly of βb-OMPs in Gram-negative bacteria. BamA is the OM component of the βb assembly machinery (Bam) complex and consists of a transmembrane βb and five polypeptide translocation-associated (POTRA) domains that extend into the periplasm. Both the βb and the five POTRA domains were identified in the LIC11623 model, and the closest PDB structure was BamA from *Neisseria gonorrhoeae* (68). Interestingly, although the LIC12254 model was similar to BamA, three of the POTRA domains (P1, P2, and P3) were missing. The closest PDB structure was *Hd*BamAΔ3 from *H. ducreyi*, it too lacks three POTRA domains (68). Immunization with recombinant D15/Oma87 induced protective immune responses against *Haemophilus influenza* (69) and *Pasteurella multocida* (70). In addition, LIC12254 was only found in two of the saprophytic *Leptospira* spp., suggesting a possible role in pathogenesis. Although LIC11623 was described as a BamA-like protein in *L. interrogans* (52, 54), neither of these proteins have been evaluated as a vaccine candidate.

LIC11458 Is an LptD-Like Export Porin

LIC11458 was identified as LptD/OstA, a member of the OM LPS export porin (LPS-ep) family (**Tables 1** and **3**). In diderm bacteria, LptD is responsible for LPS assembly in the OM (71). The structural model of LIC11458 was similar to the *Shigella flexneri* LptD, containing a transmembrane βb and a periplasmic β-jellyroll domain (72). The predicted transmembrane architecture of this protein forms a large 26-stranded βb with immunogenic surface-exposed epitopes. LPS is an essential virulence factor in pathogenic *Leptospira* spp. and, in contrast to the highly variable LPS molecule, LIC11458 was conserved among *Leptospira* spp. An immune response directed against LIC11458, as well as stimulating opsonizing leptospires, could potentially impair LPS assembly, ultimately killing the bacteria.

LIC11086, LIC11211, and LIC11506 Are Transport Proteins

LIC11086 was predicted to contain a MetA-pathway domain for phenol degradation. The structural model of LIC11086 was

highly similar to the crystal structure of Pput2725, a protein from *Pseudomonas putida* F1, a microorganism that can biodegrade hydrocarbons in the environment (73). Both structures contained a 12-stranded barrel with an N-terminal segment preceding the first β-strand that blocks the barrel. Proteins with this domain are predicted to transport hydrophobic molecules through the membrane, usually trichlorophenol and some are relatively well characterized (74, 75). LIC11211 was originally annotated as a hypothetical protein. The structural model of LIC11211 resembled TodX, an aromatic hydrocarbon transporter, also from *P. putida* (76). TodX is a 14-stranded βb, with an N-terminal flexible hatch domain. Both Pput2725 and TodX are members of the FadL family, a transporter of hydrophobic molecules in *E. coli* (77). *P. putida* and other biodegradation bacteria, such as *Ralstonia pickettii*, have intracellular pathways for the degradation of toxic hydrocarbons that enter the cells via FadL, with structural changes in the hatch domain (76). The role of hydrophobic molecule transporters in *Leptospira* spp. is unknown. LIC11506 was predicted to contain an OMP domain that is restricted to the Leptospiraceae (*Leptospira* and *Leptonema*). The structural model of LIC11506 was similar to *E. coli* OmpG, a βb with 14 antiparallel β-strands involved in the transport of carbohydrates into the cell (78). All three predicted leptospiral βb-OMPs have orthologs in all pathogenic strains; however, the LIC11506 and LIC11211 orthologs were absent in two species of intermediate pathogenicity (**Table 2**).

LIC20019 and LIC20087

Both proteins are encoded on chromosome II, LIC20019 was annotated as a hypothetical protein while LIC20087 was annotated as an OMP. However, functional annotation showed that while LIC20019 contained a putative OMP porin 6 domain, exclusive to *Leptospira* spp., no such domain was identified in LIC20087. Furthermore, LIC20019 was modeled as a perfect βb, while LIC20087 was the only protein among the βb-OMPs identified in the present study that did not contain a βb. The LIC20087 model displayed structural similarity to the Type II pilus protein PitB from *Streptococcus pneumoniae* (79). The presence of pili or their function in *Leptospira* spp. is unknown; however, it was predicted to be surface-exposed and represents a potential vaccine candidate. As it is not expected for pili to be intrinsic to the OM, LIC20087 was not evaluated for the presence of surface-exposed immunogenic epitopes. Whether LIC20087 was misidentified as a βb-OMP due to the presence of several β-sheets in the pilus subunit protein or due to a poor threading template model, will need to be further investigated. This protein was shown to be immunogenic during infection and a possible candidate for early leptospirosis diagnosis (80). LIC20019 was, however, modeled as an βb-OMP, with high structural similarity to the plasminogen activator Pla from *Yersinia pestis* (81). Pla is an OM protease (omptin) whose inactivation drastically reduces *Y. pestis* virulence (82). Omptins are widely distributed among Enterobacteriaceae and have several functions (83). Some leptospiral proteins have been reported to bind plasminogen, however, just a few were investigated for cellular localization. Besides an apparent redundancy in the extracellular component binding proteins in *Leptospira* spp., LIC20019 was predicted to be exposed on the bacterial surface and to be highly immunogenic, a potentially strong vaccine candidate.

The OM Lipoproteins

Leptospiral lipoproteins that were previously shown to be protective in the hamster model of leptospirosis, such as LigA (LIC10465), LigB (LIC10464), and LemA (LIC11058) were not selected among the list of nine predicted OM lipoproteins in this work. While these proteins were predicted by both LipoP and SpLip as lipoproteins (Table S1 in Supplementary Material), they were not consistently predicted to be located in the OM by the localization predictors. LemA was included in the list of the 54 OM lipoproteins (Table S2 in Supplementary Material) as it was identified by Cello as an OM protein. These observations are indicative that the list of 54 OM lipoproteins and 165 βb-OMPs included potential vaccine candidates that should not necessarily be excluded from future studies.

Of the eight OM lipoproteins selected, LIC10024 was the only lipoprotein originally annotated as an AGC. LIC11003 was annotated as LipL71 and the remaining six proteins were annotated as conserved hypothetical proteins or putative lipoproteins. In the current study, LIC11003 and LIC20172 were identified as peptidoglycan-binding proteins LysM (also known as LruA) and LruC, respectively. LruA and LruC were previously described as leptospiral recurrent uveitis-associated proteins A, B, and C (84, 85). LruC was experimentally demonstrated to be an OM lipoprotein but was not exposed on the bacterial surface, it was located in the inner leaflet of the OM (84). In contrast, LruA was exposed on the leptospiral surface, with a possible role in the modulation of interactions with human apolipoprotein A-I (ApoA-I), contributing to leptospiral virulence (86). Furthermore, LruA was shown to be essential for *L. interrogans* virulence in the hamster model (86). None of the Lru proteins have been evaluated as vaccine candidates and in order to do so, it will be necessary to exclude the regions that are responsible for leptospiral-related uveitis. LIC10024 was originally annotated as a membrane bound AGC, with an undefined cellular location. Another leptospiral protein (LA4008/LIC13201) with an AGC domain was shown to have host cell cAMP-elevating activity (87). The role of AGC proteins in leptospiral pathogenicity and the location of LIC10024 in the cell remain to be determined. Following *in vitro* analysis, LIC13411 was identified as a leptospiral adhesin that binds to VE-cadherin, an endothelial cell receptor (88). LIC13411 was also demonstrated to be present in the OM, supporting the findings of the current study. Unlike the βb-OMPs, the structural models of the OM lipoproteins could not be used to predict function or localization. The best matches to PDB structures included three eukaryotic proteins and a viral protein (**Table 3**). Most of the PDB structures identified were not bacterial surface-related proteins. This could be due to the absence of known conserved domains and low similarity to known protein structures. However, all eight lipoproteins were predicted to be OM lipoproteins and were highly conserved among *Leptospira* spp., including several immunogenic epitopes, suggesting they could be potential vaccine candidates.

CONCLUSION

We report the discovery of 26 new vaccine candidates using an innovative approach that represents the most extensive bioinformatics-based screening of vaccine targets in the field of leptospirosis and, to the best of our knowledge, is the first report using SV. The bioinformatics approach developed in this work can be applied to other diderm pathogens. The proteins identified in the current study are novel and have not yet been evaluated as vaccine candidates. We identified proteins that are likely to be functionally involved in diverse pathways, many related to pathogenesis, including iron and vitamin transport, OMP, and LPS assembly. The inhibition of these proteins by a host immune response will likely impair these essential pathways. Our group is currently evaluating a new experimental approach to confirm the subcellular location of these proteins in the leptospiral cell. Of the proteins identified in the present study, those that are surface-exposed will be evaluated as vaccine candidates in the hamster model of leptospirosis.

MATERIALS AND METHODS

Genome Sequence Retrieval

The genome-derived proteome (chromosomes I and II) of *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain Fiocruz L1-130 was downloaded from the *Leptospira* Genome Project website (http://aeg.lbi.ic.unicamp.br/world/lic) in FASTA format, corresponding to GenBank accession numbers AE016823.1 and AE016824.1, respectively. High-quality draft, improved high-quality draft, or complete genomes for 20 additional *Leptospira* spp. (Table S4 in Supplementary Material) were obtained from GenBank.

Prediction of Primary and Secondary Structure Features

The amino acid sequences corresponding to all 3,773 CDS in both LIC chromosomes were used as the input sequences for 16 bioinformatics programs for the identification of βb integral transmembrane proteins and OM-associated lipoproteins. Subcellular localization was predicted using PSORTb v. 3.0.2 (89), CELLO v. 2.5 (90), and GNeg-mPLoc v. 2.0 (91). The presence of a SP was predicted using SignalP v. 4.1 (92), Signal-CF (93), and PrediSi (94). TMHs were predicted using MEMSAT3 (95), TMHMM v. 2.0 (96), HMMTOP v. 2.0 (97), and Phobius (98). Proteins with more than one TMH (cut-off <2 TMH) were excluded from further analysis. Proteins with a single TMH, especially when located at the N-terminus, were included as a predicted SP can be confused with a TMH. The βb-OMPs were predicted using HHomp (99), BOMP (100), MCMBB (101), and TMBETADISC-RBF (102). Leptospiral lipoproteins were predicted using LipoP v. 1.0 (38) and SpLip (37). Each program was used with the default settings and when available the option for Gram-negative bacteria was selected. Sequence data and results retrieval for each predictor was automated using Python scripts whenever possible.

Filtering Predicted Protein Features with Increased Confidence

Low agreement is expected between different bioinformatics tools when predicting OMPs (103). To reduce the impact of a prediction when naïve (unweighted) voting would result in ambiguities (e.g., two negative results and two positive results), we used an iterative weighted voting system. A Python script was used to integrate the results, increasing confidence in the consensus prediction of a protein feature identified by more than one predictor. The Consensus by Voting with Iterative Re-weighting based on Agreement (CoVIRA) algorithm, script, and several examples are available on a GitHub repository (https://github.com/biopro/covira) and the algorithm followed the logic:

$$\mathrm{PtAS}_j = \sum_{i=1}^{\mathrm{NPd}-1} \left(V_i \times \mathrm{PdW}_i\right),$$

where PtAS_j, *Protein Agreement Score*—the value per protein by a given predictor.

$$j = 1, 2, \ldots, \mathrm{NPt}$$

NPt—the *total number of proteins* evaluated.

V_i, *Vote*, always 1 or 0—one (1) when both predictors were in agreement, zero (0) for no agreement.

$$i = 1, 2, \ldots, \mathrm{NPd}$$

PdW_i, *predictor weight*—the calculated weight of each predictor in the total number of votes per protein, varied by iteration.

$$i = 1, 2, \ldots, \mathrm{NPd}$$

NPd—total number of predictors used per protein feature.

In the first iteration, PdW was

$$\mathrm{PdW}_i = \frac{1}{\mathrm{NPd}}.$$

From the second to the 1,000 iteration, the PdW for the next iteration was

$$\mathrm{PdW}_i = \frac{\overline{\mathrm{PtAS}_i}}{\left|\max\left\{\overline{\mathrm{PtAS}_1}, \ldots, \overline{\mathrm{PtAS}_{\mathrm{NPd}}}\right\} - \min\left\{\overline{\mathrm{PtAS}_1}, \ldots, \overline{\mathrm{PtAS}_{\mathrm{NPd}}}\right\}\right|},$$

where $\overline{\mathrm{PtAS}_i}$—the arithmetic mean per protein per predictor:

$$\overline{\mathrm{PtAS}_i} = \frac{\sum \mathrm{PtAS}_j}{\mathrm{NPt}}.$$

A predictor with low accuracy was expected to generate a low agreement score and as accuracy improved, so would the agreement score. Finally, the last vote, based on the final weight of each predictor, was performed to identify those βb-OMPs and OM lipoproteins with a high confidence prediction. A final vote value >0.5 (0–1 scale) for each feature of interest (SP, OM localization, etc.) was considered as a confident prediction, and these proteins were selected for further analysis. The voting algorithm was validated using a set of proteins with and without an SP. The presence of an SP was evaluated using SignalP, PreDiSi, and SignalCF. The results were analyzed by naïve voting, each predictor had an

equal weight. Based on the same results, CoVIRA was executed to calculate a final score and prediction for each protein. The results were analyzed by a receiver operating characteristic (ROC) curve, and the CoVIRA prediction was improved compared to the naïve voting (see Data Sheet S2 in Supplementary Material).

Identification of *Leptospira* spp. Orthologs and Similar Mammalian Host Proteins

Orthologs to the selected proteins were identified in the leptospiral genome sequences (Table S1 in Supplementary Material) using the reciprocal best hit (RBH) method based on protein BLAST (BLASTp) searches. Protein sequences with >70% of similarity and >40% coverage that were also the best reciprocal hit were considered orthologs. A multiple sequence alignment was performed among orthologs using the online MUSCLE tool (MUltiple Sequence Comparison by Log-Expectation) (104). The βb-OMPs and OM lipoproteins with orthologs in other pathogenic *Leptospira* spp. were screened against the human, bovine, canine, equine, ovine, and swine genomes using the online Blastp server (taxIDs: *Homo sapiens* 9606, *Bos taurus* 9913, *Canis familiaris* 9615, *Equus caballus* 9796, *Ovis aries* 9940, and *Sus scrofa domesticus* 9825). Leptospiral proteins with >40% similarity to any host proteins were excluded from the final target list of potential vaccine candidates.

Structural Modeling of Predicted Surface-Exposed Leptospiral Proteins

The 3D structures of conserved βb-OMPs and OM lipoproteins were generated by protein threading. Prior to modeling, the SP sequence was manually removed from the final FASTA amino acid sequence. βb-spanning OM proteins and OM lipoprotein structures were predicted using the I-TASSER server (41, 42). I-TASSER predicted up to five alternative models and the model with the higher *C*-Score was refined using ModRefiner (105). Quality assessment of models was performed using ModFold4 (106), Qmean6 (107), and Procheck (108). ModFold and Qmean returned a score (0–1) that inferred the overall quality of the structure. ModFold also assigned a *P*-value and a degree of confidence (poor, low, medium, high, and cert) to the model. Procheck was used to analyze the stereochemistry of the refined model by evaluating the Ramachandran plot of each protein structure (109). The 3D structures were visualized using UCSF Chimera (110) and PyMol (111).

Sequence and Structural Functional Annotation

Functional annotation was performed using the InterProScan tool (112, 113). In addition, the UniProt Knowledgebase was screened using the locus tag (LIC number) to identify functional information and to access the annotation in other *Leptospira* spp. by KEGG database. In addition to the primary amino acid sequence analysis, a 3D structure-based functional annotation was performed using COFACTOR (114), to identify the PDB structures with the closest structure to the target protein model, and assigned GO terms for the protein model based on these PDB structures.

Epitope Prediction and OMP Structural Allocation

The presence of MHC-II linear epitopes in the amino acid sequence of the selected proteins was predicted using NetMHCII v. 2.2 (115, 116). Fourteen HLA-DRB alleles were used in the prediction of immunogenic epitopes for each protein, including the most frequent alleles in human populations. The location of SB immunogenic epitopes (threshold values of IC50 <50 nM) were determined in the 3D structures of the OMPs, those most likely located within the surface-exposed portion of the OMPs were selected. The orientation of the OMPs models in the OM was determined by an interpretation of the orientation of the closest PDB structure from the COFACTOR analysis. The predicted MHC-II epitopes were aligned to the corresponding region in the ortholog proteins from other pathogenic *Leptospira* spp. (**Table 2**) by multiple sequence alignment generated by MUSCLE, and sequence logos were generated for each epitope using WebLogo (117). Images of the structural models, highlighting the SREs were generated by PyMol.

AUTHOR CONTRIBUTIONS

AG and AM designed the study and wrote the manuscript. FK wrote the Python scripts and automated software input whenever possible. AG, FK, JDS, and JCS performed the bioinformatics analysis. AG and JDS created the figures and tables. AG, FK, JDS, JCS, and LP analyzed data. All authors contributed to and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00463/full#supplementary-material.

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Curcumin Promotes the Clearance of *Listeria monocytogenes* both *In Vitro* and *In Vivo* by Reducing Listeriolysin O Oligomers

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The pore-forming toxin listeriolysin O (LLO), an essential virulence factor that is secreted by *Listeria monocytogenes* (*L. monocytogenes*), is responsible for bacterial breaching at the phagosomal membranes and subsequent release into the cytoplasm; it cannot be recognized by the host immune system. The vital role that LLO plays in bacterial pathogenicity and evading host immune clearance makes this virulence a promising target for addressing *L. monocytogenes* infection. In this study, we hypothesized that curcumin, a polyphenol derived from turmeric that could effectively inhibit LLO pore-forming activity, might be useful in the prevention or treatment of *L. monocytogenes* infection. Thus, the *in vitro* protective effects of curcumin against *L. monocytogenes* infection by targeting LLO were assessed via hemolytic activity assays, cytotoxicity tests, intracellular growth assays, and confocal microscopy. Our results revealed that treating infected macrophages with curcumin can lead to a decrease in LLO-mediated bacteria phagosomal escape and limit the intracellular growth of *L. monocytogenes*. Moreover, results from animal experiments show that this natural compound effectively increases protection against bacterial infection and helps the host to clear the invading pathogen completely from an animal model, establishing it as a potent antagonist of *L. monocytogenes*. The results from our molecular modeling and mutational analysis demonstrated that curcumin directly engages with domains 2 and 4 of LLO, thereby decreasing the hemolytic activity of LLO by influencing its oligomerization. Taken together, these results suggest that, as an antitoxin agent, curcumin can be further developed into a novel therapy against *L. monocytogenes* infections by targeting LLO.

Keywords: anti-virulence, listeriolysin O, *Listeria monocytogenes*, molecular modeling, infection

INTRODUCTION

The Gram-positive opportunistic bacterium *Listeria monocytogenes* (*L. monocytogenes*) is recognized as a zoonotic pathogen that is facultative and intracytosolic, and it causes listeriosis. Clinical manifestations of human *L. monocytogenes* infections range from non-symptomatic intestinal carriage and mild febrile gastroenteritis to meningitis, abortion, sepsis, and even fetal infections. Notably,

despite the administration of current antibiotic therapy, which calls for high-dose antibiotics, the mortality rate of listeriosis can reach 30% among immunocompromised individuals, the elderly and pregnant women (1). With the continuing outbreak and prevalence of *L. monocytogenes* infections all over the world, and especially the antibiotic-resistant strains that have been isolated from humans and the environment, this bacterium is a major concern for public health (2–4).

Listeria monocytogenes is an invasive bacterium, and it expresses several virulence factors that are highly associated with cell invasion, intracellular bacterial survival, and cell-to-cell spreading. Following their internalization into target cells, including both phagocytic cells and diverse non-phagocytic cells, bacteria either are killed or end up escaping from the primary internalization vesicle into the cytoplasm (5). Once within the cytosol, the bacteria grow rapidly, and they utilize the host actin cytoskeleton by expressing a surface protein called ActA to form F-actin, which provides for bacterial motility and dissemination into neighboring cells. The pore-forming toxin listeriolysin O (LLO), in concert with PLCs (PI-PLC and PC-PLC), is the essential virulence factor that is required for destabilizing the vacuolar membrane and promoting the escape of the bacterium from the vesicle. The bacterium that resides in the host cell cytosol will undergo a novel round of proliferation. In this manner, bacteria are capable of completing their intracellular life cycle and avoiding exposure to the circulating components in the host immune system (6).

The early eradication of *L. monocytogenes* infection primarily relies on activated macrophages, neutrophils, natural killer (NK) cells, and T lymphocytes. Kupffer cells, the liver-resident macrophages, contribute primarily to trapping and destroying invasive bacteria by generating antimicrobial compounds (7). Busch et al. (8) have demonstrated that infecting mice with the indicated density of *L. monocytogenes* leads to the complete clearance of the bacteria from the spleen, which suggests that a low density of *L. monocytogenes* could lead to its complete clearance by the host immune system.

Throughout the intracellular life cycle, LLO, a member of the cholesterol-dependent cytolysin (CDC) family, is the critical virulence factor that is responsible for intracellular bacterial survival. This pore-forming toxin is secreted as a water-soluble monomer form that binds to a receptor on the organelles or the host plasma membrane, where the monomers oligomerize into a ring and through a sequential conformational change to form the membrane-inserting pore. The membrane insertion of LLO leads to a characteristic feature in which intracellular Ca^{2+} fluctuations lead to cell lysis from membrane perforation. Several studies have demonstrated that *L. monocytogenes* that lack LLO remain trapped within most cell types and are avirulent, and they display defects during intracellular bacterial growth in the host cell (9). Consistent with this finding, in comparison with wild-type strains, LLO-defective strains fail to cause mortality at a significantly lower bacterial burden in the murine model of systemic *L. monocytogenes* infection (10, 11). Taken together, these studies suggest that LLO can be a promising candidate for the development of a novel therapy against infections caused by *L. monocytogenes*.

In this work, we revealed a role for curcumin, a polyphenol derived from turmeric, which decreases LLO pore-forming activity by binding to the cleft between domain 2 and domain 4 of LLO, thereby interfering with LLO oligomerization. In this way, curcumin aids the host immune system in clearing bacteria by preventing them from escaping from the phagosomes. Additionally, we also observed that curcumin could strikingly inhibit the bacterial burden and protect mice from *L. monocytogenes* infection. Our results suggest that curcumin is a potent candidate against *L. monocytogenes* that acts by targeting LLO, and it may be a valuable alternative or adjunct to current antibiotic therapies.

MATERIALS AND METHODS

Bacterial Growth and Reagents

The *L. monocytogenes* strains used in this study were the wild-type strains ATCC19115, EGD, and the LLO deletion mutant EGDΔ*hly*. Tryptic soy broth (TSB; Qingdao Hope Biol-Technology Co., Ltd.) was used to grow *L. monocytogenes* and *Escherichia coli* strains with shaking at 200 rpm and 37°C. Curcumin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). When the experiment was finished, pathogens were sterilized by autoclave at 121°C for 30 min. Other chemical hazards were treated and disposed in accordance with the guidelines of Jilin University.

Preparation of LLO and Its Mutants

The DNA sequence of LLO was PCR amplified from *L. monocytogenes* genomic DNA with the following primers: 5′-GCGCCATATGGATGCATCTGCATTCAATAAAG-3′ (forward) and 5′-GCGCCTCGAGTTCGATTGGATTATCTA CTTTATTAC-3′ (reverse). The fragment was first cloned into a PET-21a expression vector and subsequently transformed into *E. coli* BL21 (DE3) cells for LLO expression.

The LLO mutagenesis was performed with a QuikChange Site-Directed Mutagenesis Kit. The primer pairs for these mutations were as follows: V100A forward, 5′ GATGGAAATG AATATATCGCGGTGGAGAAAAAGAAGAAATC 3′; V100A reverse, 5′ GATTTCTTCTTTTTCTCCACCGCGATATATTC ATTTCCATC 3′; L503A forward, 5′ GATGACCGGAACTTA CCAGCGGTGAAAAATAGAAATATCTCC 3′; and L503A reverse, 5′ GGAGATATTTCTATTTTTCACCGCTGGTAAGTT CCGGTCATC 3′.

Cultures of BL21 (DE3) cells harboring the vector that was cloned with the LLO, V100A, and L503A fragments were grown in TSB plus ampicillin at 37°C to an $OD_{600} = 0.6$. IPTG was then added to the TSB cultures at a final concentration of 0.5 mM with shaking for another 12 h at 16°C. The cells were then centrifuged and resuspended in an LLO lysis buffer (1× PBS, 1 mM DTT, and 1 mM PMSF) and subsequently broken by sonication. The supernatants were centrifuged at 4°C for 45 min, and the lysate was applied to a His-affinity column (GE Amersham). LLO and samples with specific mutations were eluted with 50 mM imidazole and concentrated in a storage buffer (35 mM Na_3PO_4,

125 mM NaCl, pH 5.5). The proteins were saved at −80°C for further studies.

Minimal Inhibitory Concentration (MIC) Determination Assay

The MIC was defined as the lowest concentration of the drug at which no visible bacterial growth was observed, and the MIC value of curcumin against wild-type *L. monocytogenes* EGD was determined by the broth microdilution method as previously reported (12).

Growth Curve Assay

For the growth curve assay, EGD was cultured in 120 ml of TSB at 37°C to an OD_{600} of 0.3 and aliquoted into six 50-ml Erlenmeyer flasks. Five of the cultures were supplemented with different concentrations of curcumin, which was pre-dissolved in DMSO at 0.25, 0.5, 1, 2, and 4 μg/ml. Then, the mixture samples were further grown at 37°C with shaking at 200 rpm, and the cell growth was monitored by reading the OD_{600} values every 30 min.

Hemolytic Activity Assay

The hemolytic activity was measured to assess the protective effect of curcumin against LLO or its variants during mediated cells lysis. In brief, 1 μl of the purified LLO or its mutants were pre-incubated with different concentrations of curcumin at 37°C for 15 min. Then, 25 μl of sheep erythrocytes (5×10^6 cells/ml) was added to each group. The mixture was incubated at 37°C for another 30 min, and then the erythrocytes were removed by centrifugation at 5,000 *g* for 1 min. The supernatant was measured at $OD_{543\ nm}$ by ultraviolet spectrophotometer. The curcumin-free cultures were used as 100% hemolysis controls, and the hemolysis was determined by comparing each sample to the control culture.

Cytotoxicity Test

The protective effect of curcumin on *L. monocytogenes*-infected cells was determined by lactate dehydrogenase (LDH) release assays, and J774 macrophages were plated at a density of 2.0×10^4 cells/well and grown overnight. The cells were infected with 200 μl of *L. monocytogenes* at moi = 5 with the indicated concentrations of curcumin, or they were treated with 10 ng of purified LLO, which was pre-incubated with different curcumin concentrations for 20 min at 37°C. After 5 h, the supernatants of each well were collected for analysis with a Cytotoxicity Detection Kit (LDH; Roche, Basel, Switzerland).

Intracellular Growth Assays

The J774 macrophage-like cells were cultured in DMEM high glucose (1×; HyClone) and supplemented with 5% fetal bovine serum (Biological Industries). In this assay, the cells were seeded onto cover slips at 3×10^5 cells/well on 13-mm cover slips with antibiotic-free medium overnight. The cells were incubated with 8 or 16 μg/ml curcumin and infected with bacteria at moi = 2.5 for 30 min. The cells were then washed five times with pre-warmed PBS and further incubated with 10 μg/ml gentamicin to kill extracellular bacteria. At arranged time points, the cover slips were placed in distilled water and vortexed violently for 5 min. The water containing the bacteria was then applied to solid TSB medium and cultured for 12 h.

MD Simulations

The LLO structure was taken from the X-ray crystal structure in the Protein Data Bank (PDB) using PDB code 4CDB. The free protein obtained from the PDB (4CDB) was first equilibrated with a 100-ns molecular simulation of the solute, which was used for molecular docking with the inhibitor. The curcumin geometries were optimized at the B3LYP/6-31G* level with a Gaussian 03 program. The standard docking procedures for LLO with curcumin were performed with AutoDock4 (13, 14). Subsequently, a molecular dynamics simulation of the complex systems was performed using computational biology methods that have been described in detail in previous reports (15–17).

The Molecular Mechanics/Poisson–Boltzmann Surface Area (MM-PBSA) decomposition process was used to analyze the interaction between curcumin and each residue in the LLO binding site in Amber 10. The binding interaction of each curcumin–residue pair includes three terms, namely, the Van der Waals contribution (ΔE_{vdw}), the electrostatic contribution (ΔE_{ele}), and the salvation contribution (ΔE_{sol}).

Confocal Microscopy

J774 macrophages were seeded on 13-mm cover slips and incubated with bacteria at moi = 2.5 with or without curcumin for 0.5, 3, or 5 h. The extracellular bacteria were killed with 10 μg/ml of gentamicin at 0.5 h after infection. The samples were fixed with 4% paraformaldehyde at 4°C for 0.5 h at each time point. Permeabilization and blocking were performed with 0.1% Triton X-100 and 5% BSA, and the samples were further incubated with a rabbit antibody (Abcam) against *L. monocytogenes* for 2 h at room temperature as the primary antibody and an Alexa Fluor 594-conjugated chicken antibody (Molecular Probes) for 1 h at room temperature as the secondary antibody to indicate the bacteria, while F-actin was stained with phalloidin coupled to Alexa 488 (Molecular Probes).

For the bacterial Live/Dead assay, J774 cells were grown in 100-mm dishes (Thermo Fisher Scientific, USA) at a density of 3.0×10^6 cells/dish overnight. The cells were treated with or without curcumin and infected with *L. monocytogenes* at moi = 2.5 for 5 h. At 0.5 h after infection, the extracellular bacteria were killed with 10 μg/ml of gentamicin. The cells were directly lyzed with PBS mixed with 0.2% Triton X-100 for 2 min at room temperature. The mixtures were centrifuged at 1,000 *g* for 10 min, and the supernatants were centrifuged at 10,000 × *g* for a further 20 min at 4°C. The viability of the bacteria in these cells was determined using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (L13152) according to the manufacturer's instructions.

The therapeutic effect of curcumin on cell survival, which was mediated by LLO or mutations, was also assessed by using the LIVE/DEAD (green/red) reagent (Roche) according to the manufacturer's instructions.

All the samples were observed under a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Electron Microscopic Observation

J774 cells were grown in 100-mm dishes at a density of 3.0×10^6 cells/dish overnight. The cells were infected with bacteria at moi = 2.5, and curcumin was added at a 16 μg/ml concentration. At 0.5 h after infection, the extracellular bacteria were killed by gentamicin. At various times after infection, the samples were collected and examined with an electron microscope according to previous studies (18).

Antibodies and Membrane-Binding Assay

A membrane-binding assay was conducted to determine whether curcumin had an effect on protein binding to target membranes. Sheep erythrocytes were lyzed in 20 mM $MgCl_2$, broken by sonication, and then, centrifuged at 3,000 × *g* for 10 min, and the supernatants were centrifuged at 20,000 × *g* for 2 h. Precipitate was added to the mixture containing pre-incubated LLO or mutants with various concentrations of curcumin for 30 min, following incubation for a further 15 min.

A total of 10 μl of each sample was loaded onto a 12% SDS-PAGE after boiling in Laemmli sample buffer. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Wako Pure Chemical Industries Ltd., Osaka, Japan) and blocked with 5% non-fat dried milk at room temperature for 2 h. To test the LLO expression, a rabbit antibody reactive to LLO (Abcam) was diluted at 1:2,000 and applied for 2 h for use as the primary antibody, and a horseradish peroxidase-conjugated antibody (Proteintech) at 1:3,000 was applied for another 2 h as the secondary antibody. Beta-actin (Proteintech) was used as an internal control in this assay, and it was used according to the recommended dilution.

The blots were detected using Amersham ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

Expression of Lysosome-Associated Membrane Protein-1 (LAMP-1)

J774 cells were plated in 100-mm dishes at a density of 3.0×10^6 cells/dish overnight. The cells were infected with bacteria at moi = 2.5, and curcumin was added at a concentration of 16 μg/ml. At 0.5 h after infection, the extracellular bacteria were killed by gentamicin. The expression of LAMP-1 protein at 3 and 5 h after infection was detected according to the instructions recommended by the manufacturer (Proteintech).

Oligomerization Assay

Listeriolysin O and its mutated forms were pre-incubated with curcumin at 37°C, and protein oligomerization was induced *in vitro*; the protocol was performed as previously described (11).

Animal Experiments

For all the following animal experiments, *L. monocytogenes* cultures were grown in TSB until reaching an $OD_{600} = 0.8$ and resuspended in PBS, following the use of PBS to adjust the bacterial concentration for different studies. Additionally, curcumin-treated mice were given 200 mg/kg curcumin subcutaneously at 2 h after infection and then at 8-h intervals until reaching defined time points.

Suspensions of 4×10^6 colony-forming units (CFUs) of bacteria were injected for mortality studies. The mice that were injected with PBS were used as the control group. The mortality analysis was monitored after 96 h. For other animal experiments, at the indicated times after infection, the mice were killed by cervical dislocation to avoid pain. Samples containing 1×10^6 CFUs of bacteria were injected for the bacterial loading assay and pathological analysis, and the data analysis was calculated by weighing and homogenizing the livers and spleens 48 h after infection. The organs were placed in 1% formalin for pathological analysis and stained with hematoxylin and eosin and then observed with a light microscope. The mice were injected with 4×10^5 CFUs of bacteria to determine the potential effect of curcumin on bacterial growth and clearance, and the mice were sacrificed 3 or 7 days postinfection, and their spleens and livers were homogenized in PBS; the bacterial load was calculated as mentioned previously.

Statistical Analysis

All the statistical analyses were performed using SPSS 13.0 software, defining differences to non-curcumin-treated groups as significant ($^*P < 0.05$ and $^{**}P < 0.01$).

RESULTS

Curcumin Antagonizes the Hemolytic Activity of LLO and Protects Cell from the Cytotoxicity Induced by *L. monocytogenes*

Previous studies in our lab demonstrated that some natural flavonoids with similar structures possess different inhibitory effects on the hemolytic activity of LLO by sharing similar mechanisms (19). Here, we found that curcumin (**Figure 1A**), a polyphenol, could also inhibit LLO-induced hemolysis. The result of the hemolysis assay revealed that the lysis of sheep red blood cells was significantly inhibited ($P < 0.05$) when curcumin was added at the indicated concentration of 0.5 μg/ml (**Figure 1B**). This result suggested that the significant reduction in the hemolytic activity of LLO probably occurred through a direct interaction of curcumin with this toxin and effectively antagonized its activity. Consistent with this finding, cocultured *L. monocytogenes* with curcumin at the indicated concentrations was enough to inhibit the hemolytic activity of LLO, and they had almost no effect on bacterial growth (**Figure 1C**). Taken together, curcumin at 0.5 μg/ml is sufficient to decrease the hemolytic activity of LLO, while it has almost no effect on the phagocytosis of *L. monocytogenes* under experimental conditions. Curcumin does not belong to the flavonoid family, and its structure was not similar to that of these compounds. Thus, we hypothesized that curcumin antagonizes LLO activity through a different mechanism.

Listeriolysin O is known as an essential virulence factor that mediates pathogen escape from the phagosome into the cytoplasm. We have shown the first evidence that curcumin could effectively inhibit the hemolytic activity mediated by LLO, and therefore, we hypothesized that curcumin is capable of blocking the escape of *L. monocytogenes*, resulting in a decrease in cytotoxicity caused

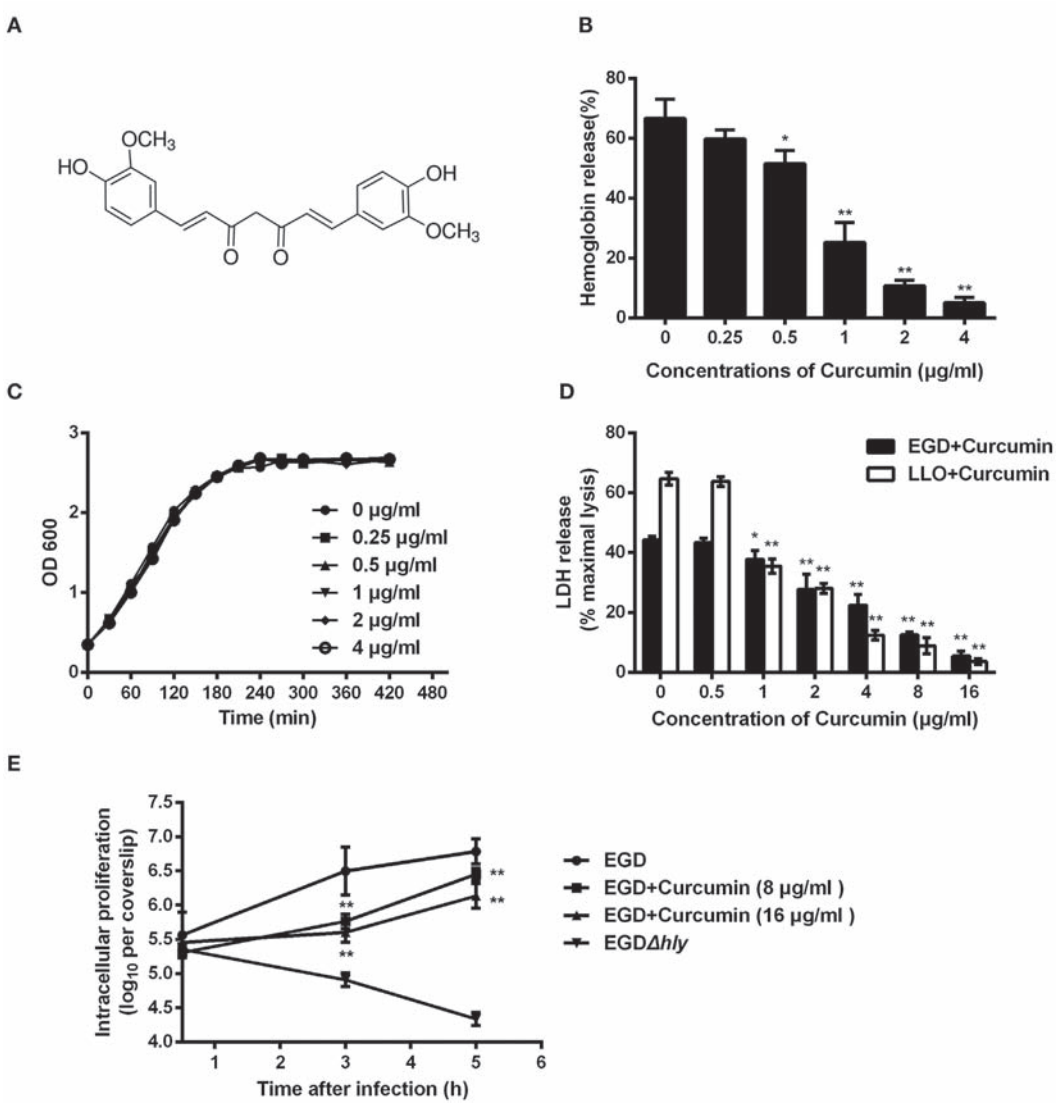

FIGURE 1 | **Curcumin inhibits *Listeria monocytogenes* (*L. monocytogenes*)-induced cytotoxicity by suppressing the hemolysis of listeriolysin O (LLO)**. **(A)** The chemical structure of curcumin. **(B)** Hemolytic activity of LLO pre-incubated with or without curcumin. The hemoglobin release is shown as a function of curcumin-antagonized, LLO-mediated hemolytic activity. **(C)** Growth curves for the *L. monocytogenes* strain EGD (WT) cocultured with different concentrations of curcumin. Similar results were obtained in two independent experiments. **(D)** The cytotoxicity of EGD and LLO on J774 cells in the presence of curcumin. The cytotoxicity was determined by relative lactate dehydrogenase (LDH) release 5 h after infection. **(E)** Time-course infection dynamics of EGD or EGDΔ*hly* after treatment with curcumin (8 or 16 μg/ml). The total colony-forming units (CFUs) of intracellular bacteria were determined by viable count measurements. In panels **(B**,**D**,**E)**, mean ± SD values for three independent experiments are shown. *P* values were calculated using one-way analysis of variance (ANOVA) (**P* < 0.05 and ***P* < 0.01).

by *L. monocytogenes*. The result in **Figure 1D** shows that the cytotoxicity caused by *L. monocytogenes* was significantly inhibited by curcumin, as evaluated by the release of LDH. Compared with the untreated group, 2 μg/ml curcumin significantly ($P < 0.01$) decreased the LDH release caused by *L. monocytogenes,* and cell death was reduced from 44.32 to 27.82%, suggesting that curcumin could protect cell membranes from damage caused by *L. monocytogenes*. Furthermore, to demonstrate that this protective effect is highly associated with curcumin, decreasing the hemolytic activity of LLO, we incubated LLO with different concentrations of curcumin, which were subsequently cocultured with J774 cells for 5 h; as expected, the cytotoxicity depended on the LLO. This result indicates that curcumin treatment reduced the *L. monocytogenes*-induced LDH release, which was the consequence of decreasing LLO membrane perforation. In addition, we tested the impact of curcumin on intracellular bacterial growth. As shown in **Figure 1E**, the *L. monocytogenes* lacking LLO (strain EGDΔ*hly*) display a defect in bacterial intracellular growth, and the wild-type strains (EGD) grow rapidly in J774 cells, while at the present curcumin concentration (16 μg/ml), the growth of

wild-type bacteria was decreased by 87.47 and 77.62% at 3 and 5 h, respectively.

Analysis of the Curcumin Binding Sites against LLO

For a stable LLO–curcumin structure, the standard molecular dynamics simulations were performed for the complex. As shown in **Figure 2A**, the stable structure of LLO with curcumin was given by the 200-ns MD simulation. It is shown that curcumin could be exactly embedded into the split between domains 2 and 4 in LLO via hydrophobic interactions. In detail, it is clear that the benzene ring on the left side of curcumin can form a strong interaction with Arg89 and Val100, which plays an important role in stabilizing the left side of curcumin. Moreover, Leu503, Val504, and Lys505 can also form a strong interaction with the right side of curcumin. In addition, the plane of the benzene ring on the right side of curcumin is parallel to the plane of the benzene ring in residue Tyr414. Then, a strong π–π interaction between this residue and curcumin most likely exists, leading to the stability of the right side of curcumin with LLO.

To validate the LLO–curcumin binding sites, the root-mean-square fluctuation (RMSF) of the residues around the LLO binding sites in the complex and free protein was calculated to explore the flexibility of these residues. As shown in **Figure 2B**, the flexibilities in the LLO binding sites in the presence and absence of curcumin are clearly different. The residues (80–100, 400–510) in the LLO binding sites that bind with curcumin show a small degree of flexibility with RMSF of <0.4 nm when compared with free protein, indicating that these residues seem to be more rigid as a result of their binding to curcumin. Through the above information, it was initially decided that the stabilization at the LLO–curcumin binding site was mostly due to residues Arg89, Val100, Lys412, Tyr414, Leu503, Val504, and Lys505, as shown in **Figure 2A**.

To confirm the LLO–curcumin binding sites, the contribution of the residues to the binding energy between curcumin and LLO was calculated using the MM-PBSA method. As shown in **Figure 2C**, Arg89, Val100, and Lys412 have strong appreciable binding energy contributions with values of <−1.0 kcal/mol, indicating the strong interaction between the LLO and the left part of the curcumin. Consistent with the results of the above analysis, Tyr414, Leu503, Val504, and Lys505 have strong interactions with curcumin, with values of ~−2.26, ~−2.00, ~−1.02, and ~−1.23 kcal/mol, respectively. In summation, it is verified that the key residues of the binding sites are Arg89, Val100, Lys412, Tyr414, Leu503, Val504, and Lys505.

To confirm the binding site in the LLO–curcumin complex, the same process of MD simulations was performed for the complex systems involving V100A-LLO and L503A-LLO mutants with curcumin, and the binding free energies of the two complexes were then calculated by using the MM-PBSA method. Subsequently,

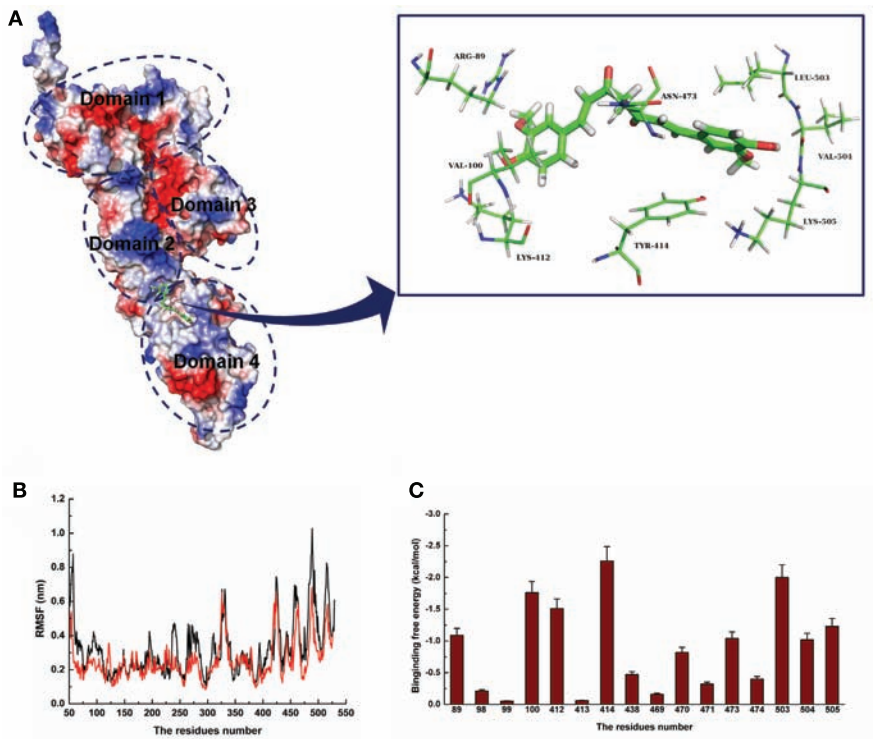

FIGURE 2 | The 3D structural determination of listeriolysin O (LLO) with a curcumin complex using molecular modeling method. (A) The structure of LLO–curcumin. **(B)** The root-mean-square fluctuation (RMSF) displayed by the protein during MD simulations of LLO–curcumin is presented. **(C)** The decomposition of the binding energy on a per-residue basis in the binding sites of the LLO–curcumin complex.

the binding free energies of curcumin with the two mutants were measured by the fluorescence spectroscopy quenching method. The total binding free energy of WT-LLO, V100A-LLO, and L503A-LLO with curcumin complexes and their detailed energy contributions are summarized in **Table 1**. The calculations of the binding free energy for the complexes revealed that the binding free energies of the mutants showed a decrease compared with the WT-LLO with the curcumin complex. However, according to the results from the fluorescence spectroscopy quenching method, the binding free energy between curcumin and the protein decreases in the following order: WT > V100A-LLO > L503A-LLO, which is consistent with the results of calculations based on the MD simulation. Thus, it is clear that the binding site of the LLO–curcumin complex is due to the residues Arg89, Val100, Lys412, Tyr414, Leu503, Val504, and Lys505.

Principal Component Analysis (PCA) of the Movement of LLO from the Complex

In this work, the most significant motions of the protein in a complex or an unliganded state were identified to explore the inhibition mechanism of curcumin by PCA on the basis of the MD trajectory of the free LLO and the LLO–curcumin complex. As shown in **Figure 3A**, there is an extended motion between domain 2 and domain 4 to the entire conformation of the free protein in the first principal component (PC1) (as represented by the dotted line in **Figure 3**), which is large enough to meet the requirement of the conformational transition for LLO from the monomer to the oligomer. Interestingly, curcumin could bind exactly in the split between domain 2 and domain 4 of the LLO based on the MD simulation, indicating that the motion of domain 2 and domain 4 in LLO could be influenced by the binding of curcumin. As we had expected, the extended motion between domain 2 and domain 4 is obviously weaker than that of the unliganded LLO due to the binding of curcumin with the split between domain 2 and domain 4, as shown in **Figure 3B**. Then, the motion of the conformation transition for LLO from the monomer to the oligomer is confirmed to be restricted by the binding of curcumin with LLO.

In summary, based on these findings, the following inhibition mechanism was realized: the binding of curcumin to the split between domains 2 and 4 in LLO blocks the transition in the conformational change from the monomer to the oligomer for LLO, leading to a decrease in the lytic activity of LLO.

TABLE 1 | The binding free energy (kcal/mol) of WT-LLO, V100A-LLO, and L503A-LLO systems based on the computational method and the values of the binding constants (K_A) based on the fluorescence spectroscopy quenching.

Proteins	WT-LLO	V100A	L503A
Binding energy	−8.8 ± 1.1	−5.4 ± 0.9	−4.7 ± 1.2
K_A (1 × 10^4), L·mol^{-1}	5.5 ± 1.5	4.7 ± 1.1	3.8 ± 0.9

Curcumin Decreases the Hemolytic Activity of LLO by Influencing Its Oligomerization

Since V100 and L503 were two amino acids that were predicted by molecular dynamics simulation as the key potential binding

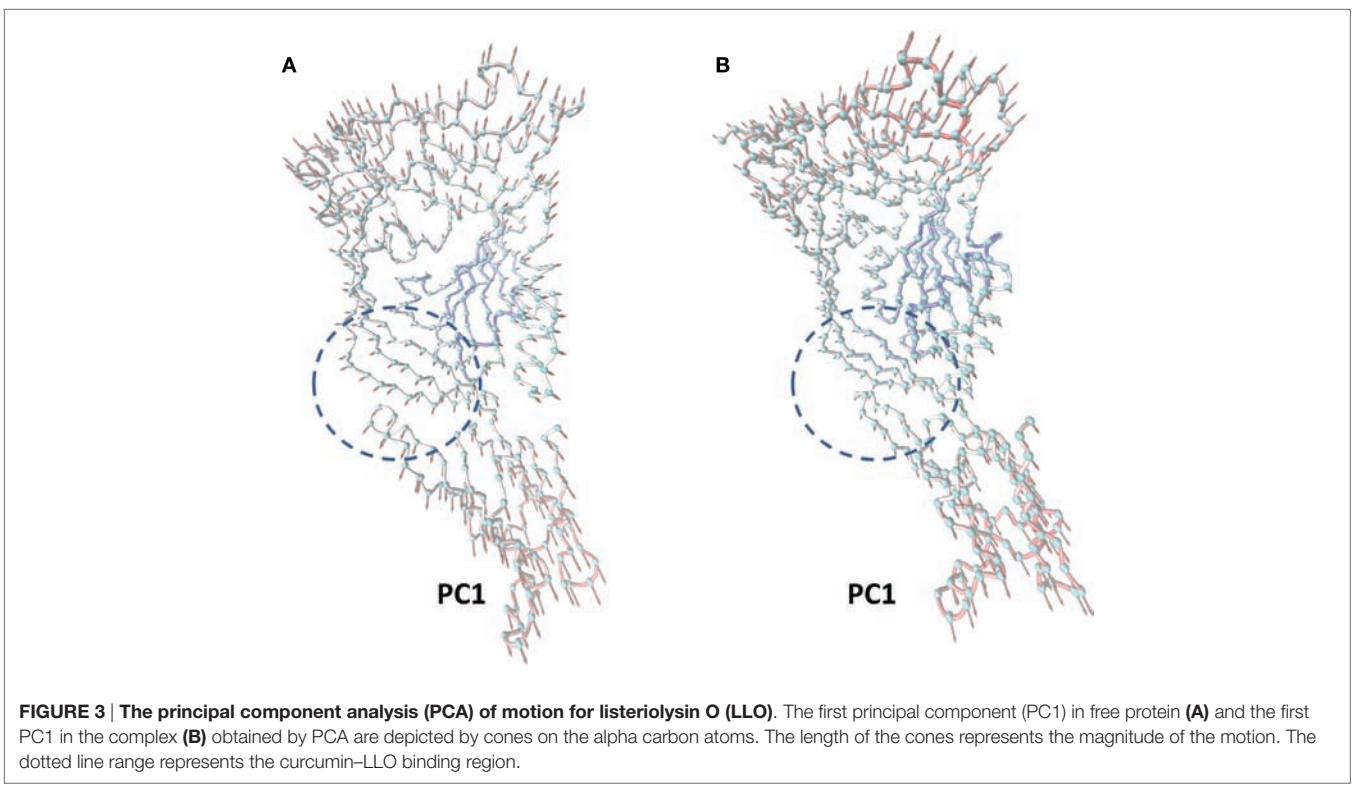

FIGURE 3 | The principal component analysis (PCA) of motion for listeriolysin O (LLO). The first principal component (PC1) in free protein **(A)** and the first PC1 in the complex **(B)** obtained by PCA are depicted by cones on the alpha carbon atoms. The length of the cones represents the magnitude of the motion. The dotted line range represents the curcumin–LLO binding region.

sites, a hemolysis assay was first developed to validate the above-mentioned results. The hemolytic assay showed that in comparison with WT-LLO, the protective effect of curcumin on LLO mutation-induced hemolysis in sheep blood cells significantly decreased by 8-fold and 16-fold in V100A and L503A (**Figure 4A**). Consistent with this result, the result from **Figure 4B** shows that compared with WT-LLO, the protective effects of curcumin on LLO mutations were 8- and 16-fold lower in V100A and L503A, respectively. Furthermore, the same results were observed in the live/dead and cytotoxicity assays, and the sensitivity of LLO to curcumin was more severely affected by the L503A mutation than the L100A mutation. Taken together, these results indicate that V100A and L503A were two important amino acids through which curcumin interfered with LLO (**Figure 4C**).

To express its cytolytic activity, the LLO monomers were first secreted by *L. monocytogenes*, subsequently binding to the target membrane, and then, oligomerization occurred to form pores and directly led to cytolytic activity. Thus, the influence of LLO and mutations on the membrane-binding assay was evaluated by Western blot assay, and no visual differences were detected between WT-LLO/V100A/L503A and the sample that was treated with curcumin, indicating that curcumin did not have an obvious impact on the binding of LLO/mutations to the target membrane (**Figure 4D**). Pretreating curcumin with LLO results in the significantly lower production of high molecular-weight LLO complexes, while this decreasing effect was less sensitive in LLO mutations, suggesting that this compound could directly reduce the oligomer formed by LLO (**Figure 4E**). Taken as a whole and consistent

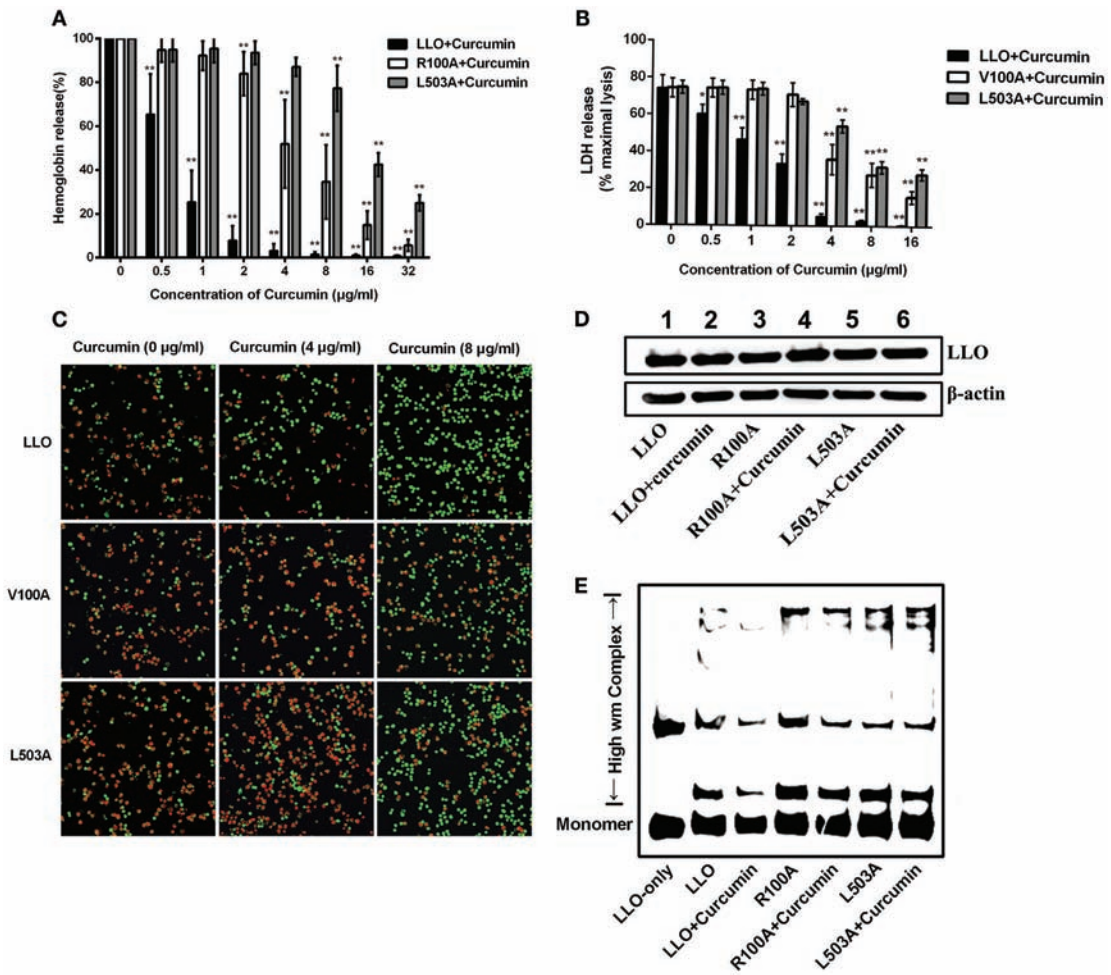

FIGURE 4 | **Curcumin interferes with listeriolysin O (LLO) oligomerization by interacting with V100 and L503**. **(A)** Comparison of hemolytic activity relative to LLO, followed by pre-incubation with curcumin. **(B)** The cytotoxicity of two LLO mutants and LLO with various concentrations of curcumin. The lactate dehydrogenase (LDH) release was detected as described in **Figure 1D**. In panels **(A**,**B)**, mean ± SD values for three independent experiments are shown. *P* values were calculated using one-way analysis of variance (ANOVA) (**P* < 0.05 and ***P* < 0.01). **(C)** Representative images of J774 cells that were challenged with LLO or mutants that co-incubated with curcumin. Cells with damaged membranes are shown in fluorescent red using ethidium homodimer-1 (EthD-1) and alternatively shown in fluorescent green using calcein-AM. **(D)** The impact of curcumin on the membrane-binding activity of wild-type LLO or the two mutants. The levels of the membrane-binding activity in different groups are visualized by Western blot, and the proteins of interest [LLO and β-actin (control)] were detected using specific antibodies. **(E)** The impact of curcumin on the oligomerization of LLO and two mutants. Oligomer formation was determined by Western blot using specific antibodies. In panels **(C**–**E)**, images are representative of three independent experiments.

with the predictions derived from the molecular modeling, these observations demonstrated that curcumin could decrease the activity of LLO by interfering with the oligomer formation of LLO.

Curcumin Reduces *Listeria* Growth in the Macrophage Cell Line (J774) by Interfering with LLO-Dependent Bacterial Phagosomal Escape

To gain insight into the mechanism through which curcumin prevents *L. monocytogenes* growth in J774 cells and clearly demonstrates which step of the intracellular life cycle was impacted, the cells were incubated with curcumin when infected with EGD or EGDΔ*hly* at the indicated time points (0.5, 3, and 5 h), washed, and fixed. The bacteria were labeled with red immunostaining, and F-actin was labeled with green immunostaining. The result at 0.5 h showed that no significant differences were observed between the EGD group or the curcumin-treated group (**Figures 5A,B**), suggesting that the concentration of curcumin (16 μg/ml) that inhibited intracellular bacterial growth has no significant influence on phagocytosis. Bacterial polymerization of actin is a typical feature associated with bacterial escape from the phagosome for inducing bacterial motility. We have demonstrated that curcumin could antagonize the hemolytic activity of LLO, suggesting that curcumin was able to limit the number of bacteria that would escape. As we expected, at 3 h after infection, and with EGDΔ*hly* as the negative control, the results were consistent with earlier studies showing that the LLO-deficient strains remained trapped in the phagosome and were incapable of recruiting actin. By contrast, ~56.71% of wild-type strains (yellow; merge) were labeled with both bacterial fluorescent antibody and F-actin fluorescent antibody (**Figure 6A**). The percentage of bacteria escaping into the cytoplasm and acquiring an actin tail was 23.94%, and it was strikingly lower in curcumin-treated macrophages (**Figure 6B**).

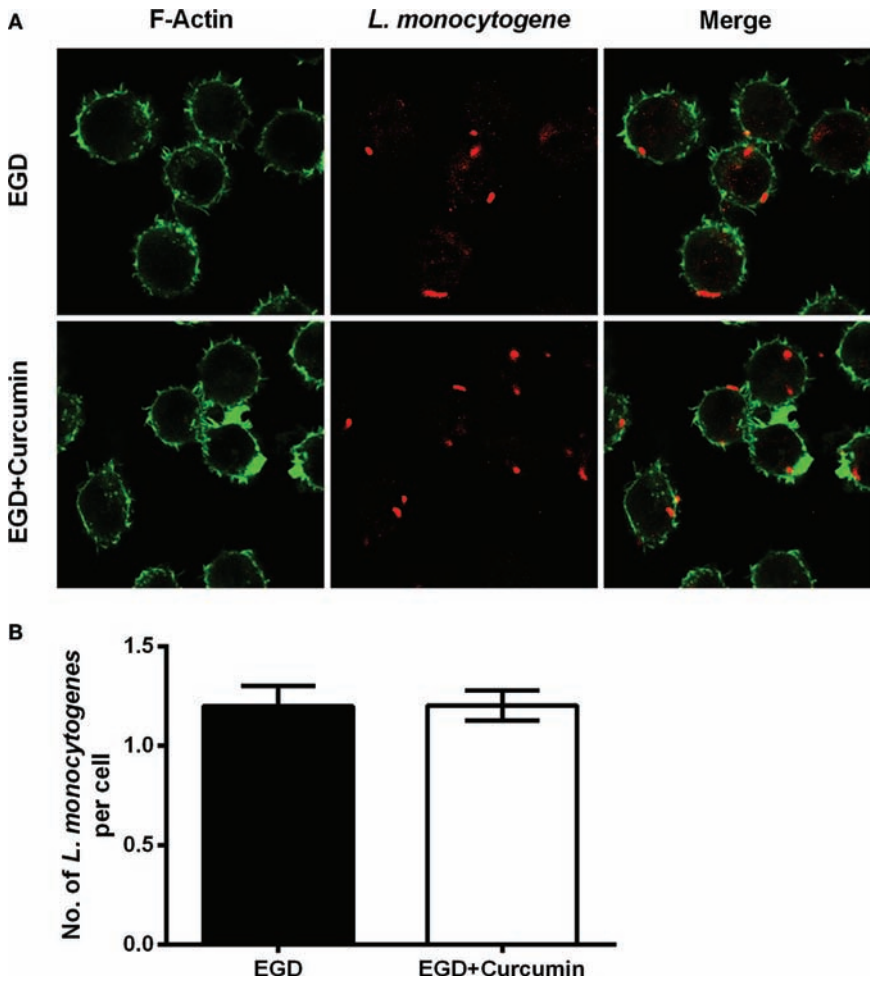

FIGURE 5 | Curcumin has no significant influence on phagocytosis in macrophages. **(A)** The representative images of cells that were infected with EGD for 30 min at moi = 2.5 in the presence or absence of curcumin. Images are representative of three independent experiments. *Listeria monocytogenes* (*L. monocytogenes*) stained red (rabbit anti-*L. monocytogenes* and Alexa Fluor 594-conjugated chicken anti-rabbit), and F-actin stained green (Alexa Fluor 488 phalloidin). **(B)** The number of intracellular bacteria per cell. The number of bacteria was automatically calculated 30 min after infection. Mean ± SD values for three independent experiments are shown (n = 200). P value was calculated using two-tailed Student's t-test.

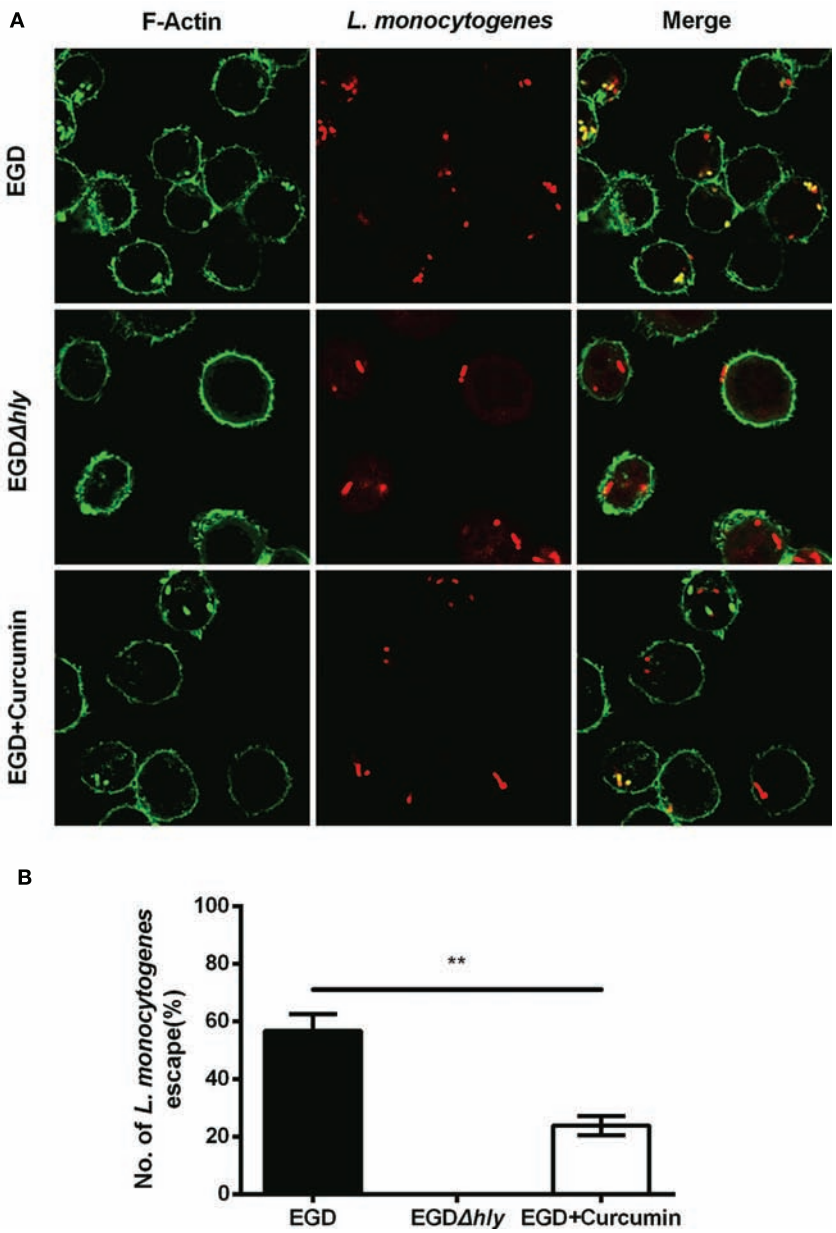

FIGURE 6 | **Curcumin inhibits *Listeria monocytogenes* (*L. monocytogenes*)-induced actin polymerization**. **(A)** Representative images of J774 cells infected with EGD or EGDΔ*hly* in the presence or absence of curcumin at 3 h after infection. Images are representative of three independent experiments. Bacteria are labeled in red, and F-actin is labeled in green as described in **Figure 5A**. **(B)** The number of escaping *L. monocytogenes*. The number of escaping bacteria (stained yellow) was automatically calculated. Mean ± SD values for three independent experiments are shown ($n = 200$). *P* value was calculated using two-tailed Student's *t*-test (**$P < 0.01$).

Taken together, these results demonstrated that curcumin effectively reduced bacterial multiplication by limiting the pathogen's LLO-mediated phagosomal escape.

Furthermore, intracellular bacterial replication was measured by immune staining for 5 h after infection (**Figure 7A**). In the macrophages that were infected with EGD, abundant amounts of bacteria were replicated and stained positive for F-actin, and this group served as the positive control (100% bacteria), whereas LLO-deficient *L. monocytogenes* remained confined in the phagosome with few bacteria in the cell (~8.39%). Treating the bacteria with 16 μg/ml resulted in a decreased percentage from 100 to 29.03 (**Figure 7B**). To assess whether the bacteria were alive or dead in the macrophages 5 h after infection, intercellular bacteria were collected and stained with PI or SYTO 9 (**Figure 7C**). The number of live bacteria in the curcumin-treated group decreased from 96.7 to 37.29% (**Figure 7D**), indicating that treating the macrophages with curcumin helped the cells to clear intracellular *L. monocytogenes*. Taken together, the results

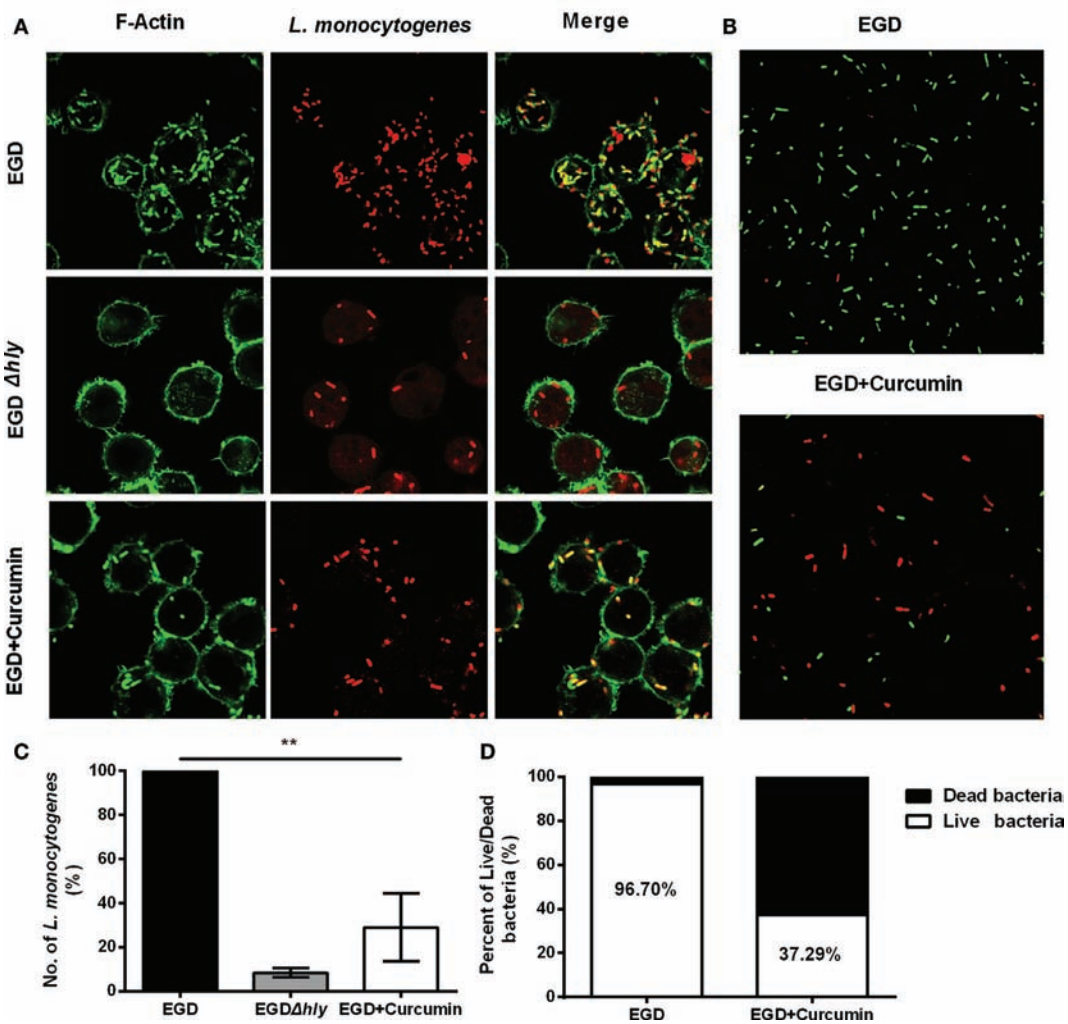

FIGURE 7 | **Curcumin decreases the intracellular replication of *Listeria monocytogenes* (*L. monocytogenes*)**. **(A)** Representative images of J774 cells infected with EGD or EGDΔ*hly* in the presence or absence of curcumin at 5 h after infection. Images are representative of three independent experiments. *L. monocytogenes* was stained red using *Listeria*-specific antibody (primary antibody) and Alexa Fluor 594-conjugated chicken antibody (secondary antibody). F-Actin was stained green using phalloidin coupled to Alexa 488. **(B)** The number of *L. monocytogenes* bacteria per cell. The EGD-treatment group was arbitrarily set as 100% as the WT treatment group. Mean ± SD values for three independent experiments are shown ($n = 200$). *P* value was calculated using two-tailed Student's *t*-test (**$P < 0.01$). **(C)** The impact of curcumin on the bactericidal activity of macrophages. Images are representative of three independent experiments. Intracellular bacteria were exposed to SYTO 9 (green; live bacteria) and propidium iodide (red; dead bacteria). **(D)** The percentage of live bacteria relative to dead bacteria was automatically calculated for 500 bacteria. Similar results were obtained in two independent experiments.

shown previously demonstrate that 16 μg/ml curcumin has no significant influence on phagocytosis but does inhibit the escape of *L. monocytogenes* by decreasing the LLO-mediated phagosome membrane perforation.

Curcumin Effectively Protects Mice from *L. monocytogenes* Infection

We then investigated whether targeting the LLO with curcumin could be an effective strategy for combating *L. monocytogenes* infections in the mouse model. Following an intraperitoneal administration of a sublethal dose of *L. monocytogenes* and the subcutaneous administration of curcumin at 200 mg/kg every 8 h for 48 h, the results showed that in the control group that was infected with bacteria, numerous spotty necroses were accompanied by inflammatory foci, congestion, and cell depletion as observed in the liver (**Figure 8A**), and a mass involving lymphocyte destruction with necrosis and congestion in the germinal centers of the spleen was also observed in the spleen (**Figure 8B**). In addition, the histopathology damage caused by *L. monocytogenes* in the liver and spleen was discernibly alleviated in the curcumin-treated group. Curcumin-treated mice displayed mild minor inflammatory lesions in their spleens and livers (**Figures 8A,B**). Curcumin has been shown to reduce bacterial burdens and mild histopathology damage during infection; the decreased bacterial load in the targeted tissues represented a significant ($P < 0.01$) reduction (**Figure 8C**). Moreover, with an

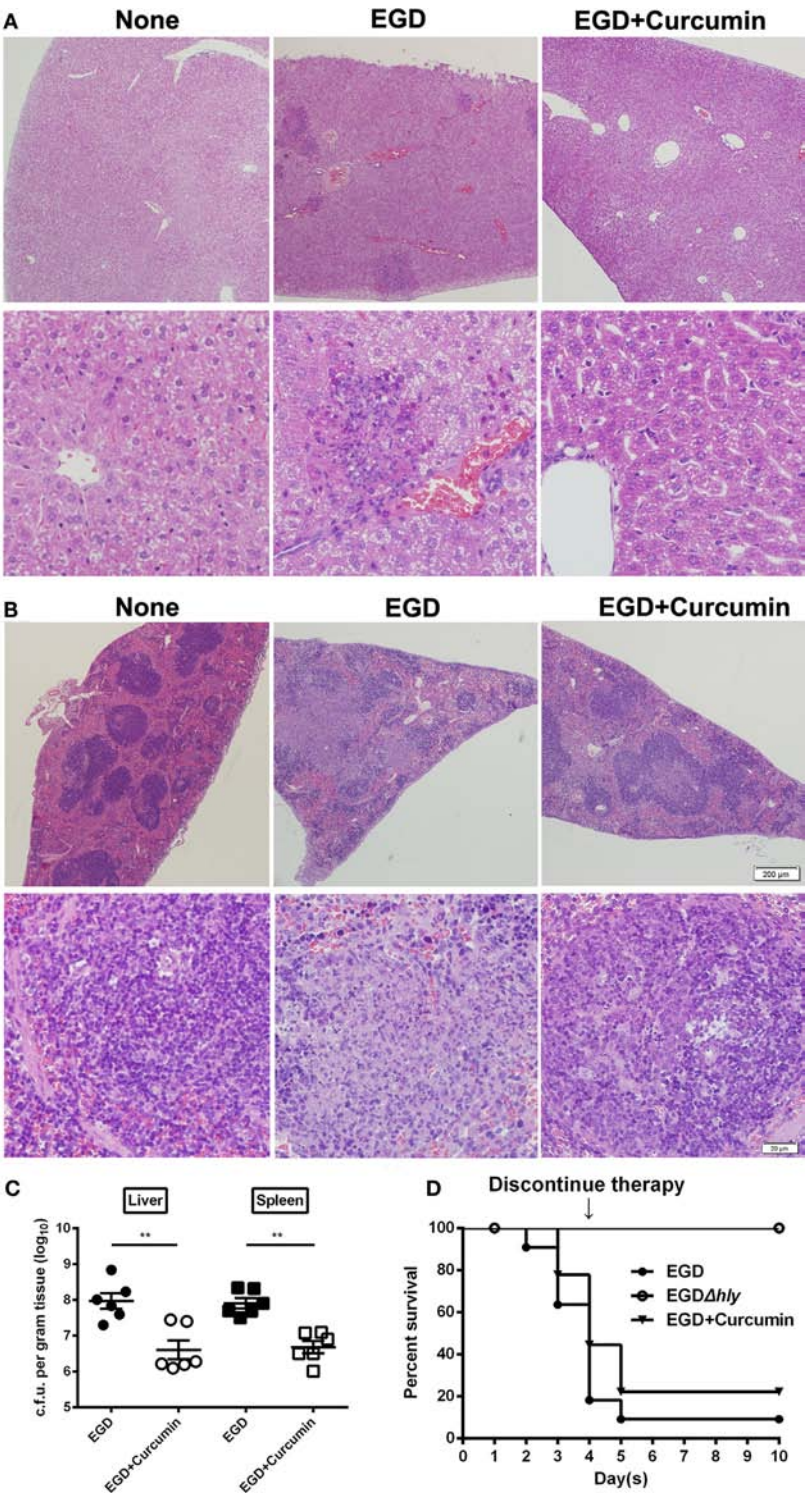

FIGURE 8 | **Curcumin protects mice against *Listeria monocytogenes* (*L. monocytogenes*) infection**. Histopathological analyses of livers **(A)** and spleens **(B)** in untreated mice infected with EGD and curcumin-treated mice were determined 48 h after infection. The images are from a representative stained section (original magnification, ×40 and ×200, respectively). **(C)** The bacterial burden in the livers and spleens was determined 48 h after infection in the control group (non-curcumin-treated group) and the curcumin-treated group. Data are expressed as the mean ± SEM. *P* values were calculated using one-tailed Mann–Whitney test (**$P < 0.01$). **(D)** Survival curves for mice. The survival rates for EGD (●), EGDΔ*hly* (○), and curcumin-treated (▼) mice after injection of 4×10^6 colony-forming units (CFUs) of bacteria/mouse are shown. The survival rate was assessed every day for 10 days. Each experimental group consisted of 6 **(A–C)** or 10 (**D**) mice. Similar results in **(A**–**C)** were obtained in two independent experiments.

intravenous inoculation of a lethal *L. monocytogenes* dose, the mice were partially protected, with 55.6% surviving compared to 80% dead controls 4 days after infection. Furthermore, curcumin-treated mice with 30% mice survival showed significantly longer survival times than the untreated group since therapy was discontinued. Altogether, these results suggest that after curcumin treatment, the mouse animal models were conferred with significant and effective protection against *L. monocytogenes* infection (**Figure 8D**). Together, our results established that the curcumin treatment systematically protected the mice from lethal infections of *L. monocytogenes*.

Curcumin Facilitates *L. monocytogenes* Clearance by Counteracting Bacteria-Induced Phagosomal Escape

It is well demonstrated that LLO grants wild-type *L. monocytogenes* the ability to escape before phagosomes fuse with the lysosome, which is an important step for macrophages to participate in the degradation of pathogens within phagosomes. Thus, in order to examine whether curcumin could aid macrophages in clearing bacteria by targeting LLO, electron microscopic observation was performed, and the expression level of LAMP-1 was evaluated. Transmission electron microscopy observations were performed at 3 h (**Figure 9A**) and 5 h (**Figure 9B**) after the infection of J774 cells by *L. monocytogenes* EGD. The results demonstrated that after curcumin treatment, most invading bacteria remained surrounded by an intact phagocytic membrane. In addition, unlike the EGD strain, the phagosome membrane was destroyed and bacteria spread intracellularly after 3 and 5 h, and *L. monocytogenes* were often found within lysosomal bodies, indicating that curcumin could help the phagolysosome to clear the EGD while reducing LLO activity and limiting the bacteria in the endosomal membrane. Since LAMP-1 is the marker that can be detected in the lysosome membrane, LLO-deficient bacteria have been shown to remain trapped in LAMP-1-positive

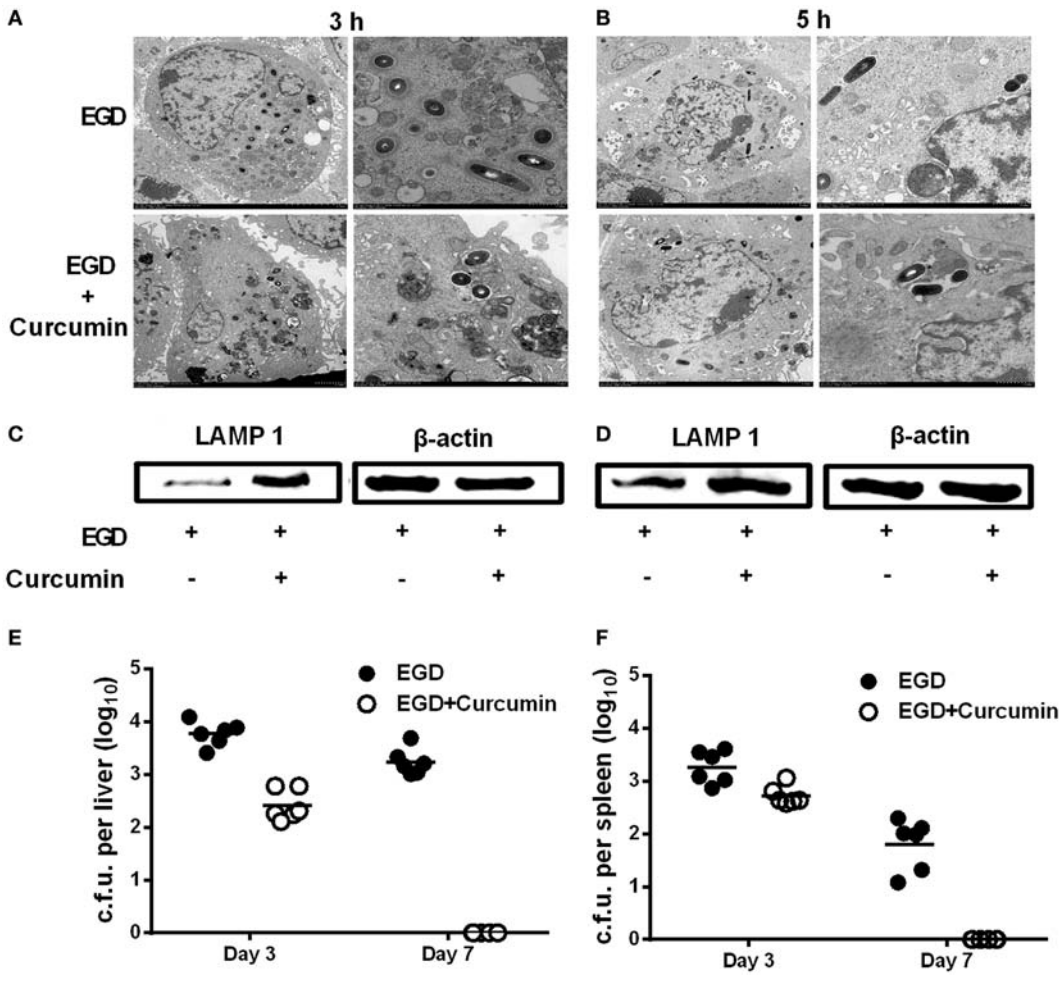

FIGURE 9 | Curcumin promotes the clearance of *Listeria monocytogenes* (*L. monocytogenes*) in macrophages and in mice. J774 cells were infected with EGD with or without curcumin for 3 h **(A)** and 5 h **(B)** after infection, and images were analyzed by transmission electron microscopy (TEM) at different magnifications (×1,200 and ×3,000, respectively). Lysosome-associated membrane protein-1 expression at 3 h **(C)** and 5 h **(D)** after infection with EGD was visualized by Western blot. On days 3 and 7 after inoculation [4×10^5 colony-forming units (CFUs) of EGD], quantification of viable bacteria in liver **(E)** and spleen **(F)** from BALB/c mice ($n = 6$) was determined. Images in **(A–D)** are representative of three independent experiments.

phagosomes. Thus, by using the Western blot assay, we evaluated LAMP-1 protein expression in the curcumin treatment group and the wild-type group. The results in **Figures 9C,D** show that more LAMP-1 protein expression was observed in the curcumin treatment group at both 3 and 5 h after infection, which suggested that more bacteria were confined with LAMP-1-positive J774 cells after curcumin treatment. This compound could facilitate the engulfment of more bacteria within the lysosome. Moreover, infection with a sublethal dose of *L. monocytogenes* is sufficient to trigger the immune system to clear the bacteria (8). Thus, we simultaneously investigated *L. monocytogenes* clearance in mice by establishing an *in vivo* model of intraperitoneal injection with a lower dose of bacteria. As shown in **Figures 9E,F**, mice infected with *L. monocytogenes* displayed a large number of bacterial colonies in their livers and spleens 3 days after infection, while the bacteria were still not cleared on day 7. Compared with this group, the curcumin-treated mice reflected higher resistance to bacterial infections because no *Listeria* colonies were detected in the spleens and livers on day 7, suggesting that curcumin effectively facilitates bacterial clearance depending on the host immune system.

DISCUSSION

Listeria monocytogenes is a facultative saprophytic bacterial pathogen that causes a high-mortality disease (30% mortality globally) called listeriosis (20, 21). Current clinical treatments for this pathogen largely rely on high doses of antibiotics, such as penicillin and gentamicin. In contrast to typical antibiotic-resistant bacteria, such as *Staphylococcus aureus*, most clinically isolated strains remain susceptible to these antibiotics (22). Due to limitations in antibiotic efficacy in terms of low outer membrane permeability in the host and due to intrinsic drawbacks of antibiotics (23, 24), *L. monocytogenes* infections have posed a severe challenge to public health (25, 26). To prevent *L. monocytogenes* from becoming another drug-resistant bacterium, or even worse, a new daunting bacterium equipped with multi-drug resistance in the post-antibiotic era, new therapeutic strategies are urgently needed to control *L. monocytogenes* infections.

Anti-virulence therapy has attracted great interest in recent years, and this strategy may afford an alternative choice that is effective against bacterial infections. Studies have shown that using natural compounds that decrease virulence activity could significantly affect intracellular infection with *L. monocytogenes* (27, 28). Moreover, most virulence factors are not essential for bacterial survival, suggesting that using this strategy might place bacteria under a milder pressure with lower chances of developing drug resistance. Supporting this idea, a previous study has demonstrated that after continuous exposure of *L. monocytogenes* to natural compounds, no development of drug-resistance strains was observed (29). Previous studies have demonstrated that LLO is critical for *L. monocytogenes* to escape from phagosomes, and LLO mutants do not cause any tissue damage or death in the mouse infection model, which renders this virulence an important drug target with which to combat *L. monocytogenes* infection (30–32).

In this study, we provide evidence that curcumin may be a potential anti-virulence agent that can be further developed against *L. monocytogenes* infection. *In vitro* studies showed that the effect of curcumin on LLO could clearly prevent *L. monocytogenes* from surviving in the cytoplasm and could reduce the colonization and toxicity of this bacterium in target cells, as well as facilitate the clearance of *L. monocytogenes* by macrophages. *In vivo* experiments suggested that curcumin could weaken the histopathological damage of the bacteria in an animal infection model, decreasing the mortality of the mice significantly. It has been shown that *L. monocytogenes* could be radically cleared by the host immune system after injection with a sublethal dose of bacterium (8). Consistent with this study, in the *in vitro* assay, compared with the untreated group, curcumin treatment could help the host to clear the same dose of bacteria more rapidly. Kohda et al. (33) showed that epigallocatechin gallate, the major tea catechin, inhibited the hemolytic activity of LLO, thereby inhibiting the escape of bacteria from the phagosome. Compared with that study, we performed more experiments to reveal the anti-virulence function of curcumin. Not only did we demonstrate that curcumin could decrease the LLO activity and restrict bacteria from escaping from phagolysosomes, we also showed that curcumin could facilitate the clearing of this bacterium in macrophages and in an animal infection model. Moreover, to our surprise, the effect of curcumin on inhibiting pore formation was not specific for LLO. We found that at similar concentrations, curcumin also exerted powerful effects in terms of attenuating the activity of other toxins in the CDC family, such as SLY and PLY (data not shown), suggesting that curcumin is a potential candidate against pore-forming toxins in the CDC family.

Previous studies in our lab have identified natural flavonoids without antimicrobial activity that could decrease the hemolytic activity of LLO. These compounds had similar structures and shared the same mechanism, directly engaging with loops 2 and 3 in domain 4, suggesting that this structure may be useful for the development of LLO inhibitors (11, 19). The structure of curcumin is quite different from these compounds but shows a powerful anti-hemolytic effect, which may offer another base structure for the development of new drugs. The results showed that by standard MD simulations, curcumin could bind specifically with the split between domains 2 and 4 of LLO by making strong contact with Val100 and Leu503, which is quite different from the results of previous reports. Principal component analysis showed that on the basis of the dynamic trajectory analysis, it was predicted that the engagement of curcumin with LLO could cause a conformational change in domains 2 and 4 of LLO. In free protein, it is obligatory that the extended motion between domains 2 and 4 to the entire conformation of the free protein could sufficiently meet the requirement of a conformational transition for LLO from a monomer to an oligomer. However, in the complex system, the extended motion between domains 2 and 4 was restricted, leading to a block in the conformational transition for LLO from the monomer to the oligomer. Owing to the block, the lytic activity of LLO in the complex is lower than that of the free protein.

Curcumin is a food-grade additive, and *L. monocytogenes* has the highest fatality rate among food-borne pathogens. Therefore, it may be possible to add curcumin to food to prevent *L. monocytogenes* infection (34). Unfortunately, the bioavailability of curcumin is low because it has intrinsically low water solubility (35). Thus, further investigations are required to translate this research into clinical applications to prevent or treat *L. monocytogenes*-related infectious diseases.

ETHICS STATEMENT

The 8-week-old BALB/c male mice used in this study were obtained from the Experimental Animal Center of Jilin University. All the animal experiments were approved by and conducted in accordance with the guidelines of the Animal Care and Use Committee of Jilin University. Moreover, all animal experiments were conducted according to the Regulations for the Administration of Affairs Concerning Experiment Animals.

AUTHOR CONTRIBUTIONS

XD, JW, and XZ conceived and designed the experiments. XZ, BZ, YC, SC, ZT, and GL performed the experiments. JW and XD contributed reagents/materials/analysis tools. XZ, JW, and XD wrote the paper.

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IFNγ Enhances CD64-Potentiated Phagocytosis of *Treponema pallidum* Opsonized with Human Syphilitic Serum by Human Macrophages

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Syphilis is a multi-stage, sexually transmitted disease caused by the spirochete *Treponema pallidum* (*Tp*). Considered broadly, syphilis can be conceptualized as a dualistic process in which spirochete-driven inflammation, the cause of clinical manifestations, coexists to varying extents with bacterial persistence. Inflammation is elicited in the tissues, along with the persistence of spirochetes to keep driving a robust immune response while evading host defenses; this duality is best exemplified during the florid, disseminated stage called secondary syphilis (SS). SS lesions typically contain copious amounts of spirochetes along with a mixed cellular infiltrate consisting of $CD4^+$ T cells, $CD8^+$ T cells, NK cells, plasma cells, and macrophages. In the rabbit model, *Tp* are cleared by macrophages *via* antibody-mediated opsonophagocytosis. Previously, we demonstrated that human syphilitic serum (HSS) promotes efficient uptake of *Tp* by human monocytes and that opsonophagocytosis of *Tp* markedly enhances cytokine production. Herein, we used monocyte-derived macrophages to study *Tp*–macrophage interactions *ex vivo*. In the absence of HSS, monocyte-derived macrophages internalized low numbers of *Tp* and secreted little cytokine (e.g., TNF). By contrast, these same macrophages internalized large numbers of unopsonized *Borrelia burgdorferi* and secreted robust levels of cytokines. Maturation of macrophages with M-CSF and IFNγ resulted in a macrophage phenotype with increased expression of HLA-DR, CD14, inducible nitric oxide synthase, TLR2, TLR8, and the Fcγ receptors (FcγR) CD64 and CD16, even in the absence of LPS. Importantly, IFNγ-polarized macrophages resulted in a statistically significant increase in opsonophagocytosis of *Tp* accompanied by enhanced production of cytokines, macrophage activation markers (CD40, CD80), TLRs (TLR2, TLR7, TLR8), chemokines (CCL19, CXCL10, CXCL11), and T_H1-promoting cytokines (IL-12, IL-15). Finally, the blockade of FcγRs, primarily CD64, significantly diminished spirochetal uptake and proinflammatory cytokine secretion by IFNγ-stimulated macrophages. Our *ex vivo* studies demonstrate the importance of CD64-potentiated uptake of opsonized *Tp* and suggest that IFNγ-activated macrophages have an important role in the context of early syphilis. Our study results also provide an *ex vivo* surrogate system for use in future syphilis vaccine studies.

Keywords: *Treponema pallidum*, human, macrophage, Fcγ receptor, phagocytosis, phagosomal signaling, vaccine model

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INTRODUCTION

Venereal syphilis is a multistage, sexually transmitted infection caused by the spirochetal bacterium, *Treponema pallidum* (*Tp*) (1, 2). Syphilis continues to be a major public health threat, affecting nearly 6 million people globally (3). In the United States, the rate of primary and secondary syphilis (SS) has more than tripled from 2.1 cases in 2000 to 6.3 cases per 100,000 in 2014 (4, 5). Syphilitic infection commences clinically with the appearance of an ulcerative lesion, known as a chancre, at the site of inoculation (2). However, even before the appearance of the chancre, treponemes begin to disseminate hematogenously, eventually giving rise to SS, a systemic inflammatory illness associated with diverse clinical manifestations, most commonly involving skin and mucous membranes (2). After weeks to months, the robust cellular and humoral responses elicited during SS gain control of the pathogen, driving down spirochetal burdens and ushering in the asymptomatic stage called latency (2). Approximately one-third of untreated patients will develop one of the recrudescent syndromes collectively known as tertiary syphilis (2). Considered broadly, syphilis can be conceptualized as a dualistic process in which spirochete-driven inflammation, the cause of clinical manifestations, coexists to varying extents with bacterial persistence.

Syphilitic lesions in all stages of disease contain a rich cellular infiltrate, composed primarily of lymphocytes, plasma cells and macrophages, accompanied by vasculopathic changes of varying severity (6, 7) capable of giving rise to a wide variety of histological patterns, including granulomata (8). Immunocytochemical analysis has revealed that the relative proportions of T cell subsets shifts from predominantly $CD4^+$ T cells in genital ulcers to a predominance of $CD8^+$ T cells in SS lesions (7, 9). Innate lymphocytes, more specifically NK cells, also have been identified in biopsies of skin rashes from SS patients (10). Transcriptional and immunofluorescent analyses have revealed that both primary and SS lesions contain classic T_H1 cytokines and that multiple lymphocytic populations ($CD4^+$, $CD8^+$, and NK cells) can be sources of IFNγ, the hallmark of a T_H1 response (10–14). The presence in human lesions of IFNγ and activated macrophages (9, 14) is consistent with a large body of literature from the experimental rabbit model suggesting that macrophages activated by IFNγ (15–18) are critical for spirochete clearance. Seminal *ex vivo* studies by Lukehart et al. (19–22) have demonstrated that opsonic antibodies present in immune rabbit serum markedly enhance phagocytosis and killing of *Tp* by rabbit peritoneal macrophages. Like their rabbit counterparts, human monocytes and macrophages also require syphilitic serum for efficient spirochetal uptake (10, 23). In addition to clearance, opsonophagocytosis of *Tp* also promotes a robust proinflammatory cytokine response which can be attributed to the release of spirochetal pattern-associated molecular patterns (PAMPs), most notably lipoproteins, from organisms degraded in phagosomes (10).

Despite the acknowledged central role of the macrophage in the immunobiology of syphilis, only recently have investigators begun to examine the interactions between *Tp* and human macrophages. We, like others, observed *Tp* infiltrating the epidermis by immunohistologic analysis of secondary syphilitic skin lesions (24, 25). Additionally, $CD68^+$ histiocytes were poised throughout the dermis, at the dermal-epidermal juncture and, in some cases, a few histiocytes were observed in the epidermis. $CD138^+$ plasma cells, a potential source of local opsonic antibodies, made up a portion of the cell-mediated inflammatory response (26). As in the recent communication by Marra et al. (23), herein we report that opsonic antibodies significantly enhance internalization of *Tp* by monocyte-derived human macrophages. However, we also note that IFNγ markedly enhances the expression of CD64, the primary receptor for opsonic uptake of treponemes, providing for the first time, mechanistic evidence for the linkage of spirochetal clearance with adaptive cellular responses *in vivo*. IFNγ plays a critical role in macrophage mediated responses by not only increasing opsonophagocytosis of *Tp* but also markedly broadening the inflammatory response of the macrophages following internalization of spirochetes, further emphasizing the interdependence of local innate and adaptive responses during syphilitic infection. However, even with IFNγ activation, uptake of *Tp* by macrophages was inefficient as determined by the substantial proportion of spirochetes that were not internalized despite a lengthy preincubation in high concentrations of the anti-treponemal antibodies present in human syphilitic sera (HSS). These latter results are in accord with prior studies showing that *Tp* presents a paucity of surface antigenic targets at the host-pathogen interface and that spirochete populations display a high degree of heterogeneity with respect to surface antibody (Ab) binding. Our findings not only illustrate the dualistic nature of syphilis but also demonstrate the utility of an *ex vivo* model for teasing apart its many components.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of the Institutional Review Boards at UConn Health, Farmington CT and Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) in Cali, Colombia. All study participants were provided written informed consent. All animal experimentation was conducted following the *Guide for the Care and Use of Laboratory Animals* (8th Edition) and in accordance with protocols reviewed and approved by the UConn Health Institutional Animal Care and Use Committee under the auspices of Animal Welfare Assurance A347-01.

Immunohistochemical (IHC) Analysis of SS Skin Biopsies

Four micrometer sections, cut from a 4-mm punch biopsy that were fixed in 10% buffered formalin and embedded in paraffin, were stained with hematoxylin and eosin (H&E) or labeled immunohistochemically with antibodies against CD4 (clone EP204, Epitomics, Cambridge, MA), CD8 (clone 4B11, Leica Biosystems Inc., Buffalo Grove, IL), CD56 (clone 56C04 Thermo-Scientific, Waltham, MA), CD68 (clone PG-M1, Dako, Carpinteria, CA) and CD138 (clone B-A38 Cell Marque, Rocklin, CA) using an automated immunohistochemistry staining platform (Bond Max, Leica-Microsystems, Buffalo Grove, IL). IHC detection of

Tp was performed manually as previously described (10) using a rabbit polyclonal anti-*Tp* Ab (Biocare, Concord, CA, USA). Skin specimens from healthy volunteers of the same socioeconomic background and conditions were not available for analysis. Tissue known to contain *Tp* were used for the positive control of *Tp* IHCs. Additionally, secondary alone controls were used to assess any non-specific Ab binding for each Ab used.

Bacterial Strains

Treponema pallidum (Nichols strain) was propagated by intratesticular inoculation of adult male New Zealand white rabbits and harvested in CMRL medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Atlantic Biologicals, Miami, FL, USA) at peak orchitis (12). Spirochetes were enumerated by dark-field microscopy on a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA, USA). Virulent *Borrelia burgdorferi* (*Bb*) strain 297 encoding green fluorescent protein on a cp32-based shuttle vector (27) was propagated in BSK-H medium containing 6% rabbit serum (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and 400 μg/ml of kanamycin (Sigma-Aldrich Chemical Co.). For macrophage incubation experiments, *Bb* temperature-shifted from 23 to 37°C were grown to late-logarithmic phase (~8×10^7 spirochetes/ml), washed twice with RPMI, and resuspended in RPMI to a final density of ~3×10^8 spirochetes/ml.

Macrophage Maturation and Activation

Peripheral blood was obtained from healthy donors determined to be seronegative for syphilis by Rapid Plasma Reagin test and/or Lyme disease by enzyme-linked immunosorbent assay (ELISA) performed in the clinical laboratory at John Dempsey Hospital. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep and SepMate-50 tubes in accordance with the recommendations of the manufacturer (STEMCELL Technologies, Vancouver, BC, Canada). To generate all macrophage phenotypes, 3×10^7 PBMCs were plated in 10 cm polystyrene Petri dishes (BD Falcon) and incubated for 2 h at 37°C with 5% CO_2. Adherent cells were washed thoroughly with ice-cold PBS to remove non-monocyte populations. Macrophage nomenclature used throughout follows the recommendations of Murray et al. (28). MΦ(−)s were generated by incubating the adherent monocytes in RPMI-1640 (RPMI) medium (Gibco, Thermo-Scientific) supplemented with 20% heat-inactivated (56°C for 30 min) normal human serum (NHS) (CORNING, Corning, NY, USA) and 1% penicillin–streptomycin (10,000 U/ml) (Gibco) for 10 days. MΦ(C)s were generated by incubating the adherent monocytes in RPMI, 20% heat-inactivated NHS, and 50 ng/ml of M-CSF (Peprotech, Rocky Hill, NJ, USA) (29) for 10 days. To generate MΦ(IFNγ)s, MΦ(C)s were divided on day 7 and 2.5 ng/ml of recombinant IFNγ (Roche Diagnostics, Mannheim, Germany) (30) was added to one portion for an additional three days of incubation. Media and cytokines were replenished every 3 days for a total of 10 days. Because *Tp* lacks endotoxin and would not be encountered by macrophages in syphilitic lesions, LPS, which is often used in macrophage differentiation protocols, was omitted from all culture media.

Human Syphilitic Sera

All *Tp* opsonization studies were performed using a pool of deidentified sera collected from five HIV-negative primary or SS patients seen at Parkland Memorial Hospital in Dallas, TX, USA (approved for use by the Institutional Review Board of the UConn Health) (31). Strong reactivity of the pool with *Tp* proteins was confirmed by immunoblot (Trinity Biotech, Carlsbad, CA, USA, Figure S2 in Supplementary Material).

Tp Opsonophagocytosis Assays

Freshly harvested *Tp*, adjusted to a final concentration of 3×10^8 treponemes/ml, were incubated with either 10% NHS or 10% pooled HSS for 2 h at RT prior to addition to macrophages. Macrophages, differentiated as described above, were plated at 1×10^5 cells in 500 μl of RPMI supplemented with 10% heat-inactivated FBS (Hyclone Laboratories, Inc., Logan, UT, USA) in an eight-well chamber microscopy slide (Millipore, Billerica, MA, USA). *Tp* were added at a multiplicity of infection (MOI) of 30:1 for 8 h at 37°C with 5% CO_2. All culture media and reagents were confirmed to be free of LPS contamination (≤10 pg/ml) by Limulus amebocyte lysate assay quantification (Cambrex, MA, USA). Following incubation, supernatants were removed and macrophages were prepared for IFA to evaluate binding and uptake of treponemes. Cells were fixed and permeabilized with 2% paraformaldehyde and 0.01% Triton X-100 for 10 min at RT. They then were rinsed with PBS, blocked with PBS containing 10% normal goat serum (NGS) for 1 h at RT, and then incubated with rabbit polyclonal anti-*Tp* (1:100, Abcam, Cambridge, MA, USA) in PBS 1% NGS for 1 h at RT. After four successive washes with PBS, the cells were then incubated with goat anti-rabbit immunoglobulin G (IgG)-Texas Red (1:500) in PBS 1% NGS for 1 h at RT. After staining for *Tp*, actin cytoskeletons were stained with Phalloidin-AF488 (1:20) (Life Technologies, Carlsbad, CA, USA) for 20 min at RT. The cells were then washed thoroughly with PBS six times, rinsed with deionized water to remove salt and allowed to air dry. Finally, Vectashield containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) was added and samples were sealed with a coverslip. To assess binding and internalization of *Tp*, images of 100–200 macrophages were acquired on an Olympus BX60 epifluorescence microscope equipped with a Retiga 2000R CCD camera (QImaging) or Ziess LSM 780 confocal microscope mounted on an inverted Axio Observer Z1. Similar numbers of macrophages were imaged for each experimental condition per biological replicate. Acquired images were processed with ImageJ (version 1.5.1 g) (NIH, USA) and uptake was quantitated in a blinded fashion. The percentage of macrophages with bound spirochetes was quantified by dividing the number of cells with surface bound spirochetes by the total number of macrophages imaged for each condition. The percentage of spirochete-positive macrophages were calculated by dividing the number of cells containing ≥1 internalized spirochetes by the total number of cells imaged. Phagocytic indices were calculated by dividing the number of degraded, internalized spirochetes by the total number of spirochete-positive macrophages for the same condition. To quantify the percentage of *Tp* remaining after the 8 h incubation period, 10 μl aliquots

from *Tp*-stimulated-macrophage supernatants were enumerated, in triplicate, by dark-field microscopy. Percentage of *Tp* recovered was calculated using a "time zero" spirochetal count.

Bb Phagocytosis Assays

MΦ(−)s, after maturation as described above, were plated at 1×10^5 cells in 500 µl of RPMI supplemented with 10% FBS in an eight-well chamber microscopy slide and incubated at 37°C with 5% CO_2 overnight to allow for attachment. Prior to incubation, medium was removed and replaced with fresh RPMI supplemented with 10% FBS. *Bb* were added at an MOI of 30:1 and incubated for 8 h. Following incubation, cells were fixed and permeablized and cytoskeletons were stained with Phalloidin-AF594 as described above. Binding and internalization of spirochetes was performed as described above.

Cytokine Analysis

TNF, IL-6, IL-1β, IL-10, IL-12, and IL-8 were measured in supernatants using a Human Inflammatory Cytokine Bead Array per the manufacturer's (BD Biosciences, San Jose, CA, USA) protocol. Data were collected on a MACSQaunt Analyzer (Miltenyi Biotec, Germany) and analyzed with FCAP Array™ Software version 3.0 (BD Biosciences).

Flow Cytometry

MΦ(C)s and MΦ(IFNγ)s were harvested from 10-cm polystyrene Petri dishes by replacing growth medium with PBS containing 1% FBS and gently lifting the cells with a scraper. The macrophages were washed once with PBS in 50 ml conical tubes by spinning at 300*g* for 10 min; the cells were decanted, resuspended in PBS 1% FBS, and dispensed into FACS tubes in preparation for staining. Cells were incubated for 10 min at 4°C with 10 µg/ml of purified human IgG (Sigma, St. Louis, MO, USA) for Fcγ receptor (FcR) blocking, followed by a 20 min incubation with fluorochrome-conjugated antibodies. Antibodies obtained from Biolegend (CD32-APC, CD64-PECy7, CD163-PerCP/Cy5.5, CD206-APC, anti-rabbit IgG-BV421), eBioscience (CD14-APC, CD16-PE, TLR2-FITC, TLR4-APC), Novus Biologicals (TLR7-PE, TLR8-AF647), BD Biosciences (HLA-DR-PE), Invitrogen (CD68-FITC), and Abcam [rabbit anti-human inducible nitric oxide synthase (iNOS)] were used at dilutions recommended by the manufacturers. For intracellular staining, the cells were surface-stained as described above, permeabilized in 250 µl of Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4°C, washed with PermWash buffer (BD Biosciences), stained with fluorochrome-conjugated antibodies (diluted in 50 µl of PermWash buffer) for 30 min at 4°C, and washed twice with PermWash and resuspended in FACS buffer. A minimum of 10,000 single cells events were acquired using a BD LSR-II flow cytometer and FACSDIVA™ software (BD Biosciences). Analysis of immunostaining was performed using FlowJo V10 for Mac (FlowJo LLC, Ashland, OR, USA). Mean fluorescence intensity (MFI) values were determined after subtracting background fluorescence (32).

Targeted Array Analysis

1×10^6 MΦ(C)s and MΦ(IFNγ)s were plated in 1 ml of RPMI containing 10% FBS in a 12-well tissue culture plate (Corning) and incubated with unopsonized or opsonized *Tp* for 8 h at an MOI 30:1. RNAs were extracted using a NucleoSpin RNA purification kit according to the manufacturer's (Macherey-Nagel Inc., Bethlehem, PA, USA) instructions and their concentrations determined using a Nanodrop spectrophotometer (Thermo-Scientific). cDNA synthesis was performed using the High Capacity cDNA Archive Kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. Transcripts were amplified using TaqMan® Human Immune Array (384-fluidics card) and TaqMan® Human Phagocytosis Array (96-well plate) per manufacturer's instructions (Applied Biosystems). Briefly, the Immune Array was performed in a 2 µ1 reaction volume containing 62.5 pg of cDNA, and 1 µ1 of gene expression master mix. The Phagocytosis Array was processed in a 20 µl reaction volume containing 5 ng of cDNA and 10 ml of gene expression master mix. Commercially available primers (Applied Biosystems) were used to amply transcripts for human *IRF3* (Hs01547283_m1), *IRF5* (Hs00158114_m1), *IRF7* (Hs00185375_m1), and *IFNB* (Hs00277188_s1) as described previously (33). Amplification reactions were performed using a 7900HT Fast Real Time thermocycler (Applied Biosystems) using the following conditions: 95°C for 20 min, and 40 cycles of 95°C for 1 s and 60°C for 20 s. Expression levels for all transcripts were normalized to *GAPDH* and the relative changes in gene expression between experimental groups were calculated using the $2^{-\Delta\Delta Ct}$ method (34). To identify genes differentially expressed in the presence of IFNγ and absence of *Tp*, the average fold changes for unstimulated MΦ(IFNγ)s were calculated relative to the unstimulated MΦ(C)s. To identify genes differentially expressed in the presence of *Tp*, relative fold changes were calculated based on the unstimulated MΦ(IFNγ)s condition, with a two-fold change and p-value < 0.05 threshold (Student's t-test) (35). Heat maps and graphs of the fold changes of transcript expression were generated using R statistical software, version 3.2.2 with package "ggplot2," function "heatmap.2." Fold change values displayed in the heat maps represent the average fold change compared to the corresponding unstimulated MΦ(IFNγ). No clustering analysis was performed.

Colocalization of CD64 and *Tp*

1×10^5 MΦ(IFNγ)s were plated in 500 µl of RPMI supplemented with 10% FBS in an eight-well chamber microscopy slide and incubated for 8 h with *Tp* at an MOI of 30:1. Following incubation, the cells, fixed and permeablized as above, were incubated with mouse anti-human CD64 (1:25, clone 10.1, Abcam) and rabbit polyclonal anti-*Tp* 1 h at RT, and then washed four times with PBS followed by incubation for 1 h at RT with goat anti-mouse IgG-OregonGreen488 (1:200, Invitrogen, Carlsbad, CA, USA) and goat anti-rabbit IgG-Texas Red (1:500, Life Technologies). Secondary Ab alone controls were evaluated for non-specific binding (Figure S6 in Supplementary Material). Images were acquired on a Zeiss LSM 780 confocal microscope mounted on an inverted Axio Observer Z1 and processed with ImageJ as described above. Colocalization of *Tp* and CD64 was visualized using the ImageJ plugin "Colocalization"; the coefficient of colocalization was determined using the plugin "JACoP."

FcγR Blocking Experiments

MΦ(IFNγ)s were pre-incubated with 10 μg/ml of mouse anti-human CD64 (clone 10.1, Abcam), mouse anti-human CD32 (clone 6C4, Affymetrix eBiosciences, San Diego, CA, USA), mouse anti-human CD16 (clone 3G8, Biolegend, San Diego, CA, USA), or 10 μg/ml each of all three antibodies for 1 h prior to adding spirochetes. Opsonophagocytosis assays were performed as described above.

Statistics

General statistical analysis was conducted using GraphPad Prism 6.0 h (GraphPad Software, San Diego, CA, USA). Phagocytic uptake/index, *Tp* recovery counts, cytokine concentrations, cytometric MFI ratio, and fold increase or decrease for each gene transcript assayed were compared among the different stimuli. Either a paired or unpaired Student's *t*-test (i.e., Mann–Whitney test or Wilcoxon test) was used for comparison across two groups. For the analysis of three or more conditions, we used a non-parametric statistical test for trend analysis (Friedman's test with a Dunnett's multiple comparisons post-test analysis). For each experiment, the standard error of the mean was calculated and a p-value < 0.05 was considered significant.

RESULTS

T. pallidum-Macrophage Interactions in SS Skin Lesions

To set the stage for our *ex vivo* studies, we assessed the distribution of macrophages, lymphocytes, and plasma cells in spatial relationship to *Tp* within representative skin punch biopsy specimens obtained from three HIV-negative SS patients. In line with prior studies (6, 7, 10, 36–39), IHC analysis of SS skin biopsies revealed a rich dermal-epidermal infiltrate (**Figure 1A**), which in addition to $CD4^+$, $CD8^+$, and $CD56^+$ lymphocytes (Figure S1 in Supplementary Material), had large numbers of $CD68^+$ macrophages (40) (**Figure 1B**). Abundant $CD68^+$ cells were present throughout the dermis, at the dermal–epidermal juncture, and, in some cases, surrounding vascular structures within the dermis (**Figure 1B**). Treponemal numbers and locations in the inflamed skin, as well as their spatial proximity with dermal macrophages, varied among samples (**Figure 1C**). In SS143 skin biopsy, a small cluster of $CD68^+$ macrophages was visualized in close proximity to a spirochete surrounding a blood vessel wall. In SS167 sample, substantial numbers of spirochetes were seen within the interstitium of the papillary dermis (**Figures 1B,C**). In SS133, large groups of spirochetes also were observed traversing the dermal–epidermal junction and within the stratum basale and stratum spinosum of the epidermis (**Figure 1C**), whereas very few macrophages were found within the epidermis (**Figure 1B**). Of particular interest, $CD138^+$ plasma cells were commonly observed in perivascular locations and throughout the papillary dermis (**Figure 1D**). In concert with previously published studies (2, 6, 7), our current results confirm the presence of macrophages in *Tp* infected tissue, and suggest that the cellular environment may be involved in shaping the phenotype of macrophages, in addition to providing a local source of opsonic antibodies needed for spirochetal recognition and clearance in *Tp* infected tissues.

Primary Human Macrophages Phagocytose *Tp* in the Presence of HSS but Exhibit a Blunted Cytokine Output

Having confirmed that the macrophage is an important cellular element of the inflammatory response to the syphilis spirochete *in vivo*, we next performed *ex vivo* experiments to directly characterize macrophage-*Tp* interactions. We began by maturing adherent human monocytes into macrophages using 20% heat-inactivated NHS without additional exogenous growth factors (e.g., M-CSF). We then assessed the ability of these macrophages [MΦ(−)] to bind and phagocytose spirochetes. As a phagocytosis control, we incubated MΦ(−)s with *Bb*, a spirochetal pathogen that is readily bound and internalized by primary human phagocytes (41, 42). As shown in **Figure 2A** and quantified in **Figure 2B**, *Bb* binds to MΦ(−)s and is internalized into phagosomal vacuoles (**Figures 2A,C**). We then assessed if macrophages were able to similarly bind and internalize *Tp* in the presence or absence of NHS or with HSS. For these experiments, we first confirmed that our pooled HSS reacted with spirochetal antigens by using a *Tp* IgG Marblot strip test (Figure S2 in Supplementary Material). The pooled HSS was determined to be highly reactive with many of *Tp*'s proteins, likely including some opsonic targets. It is important to note that we previously demonstrated that the same pooled HSS recognizes the Nichols strain variant of L4, an immunodominant extracellular loop in the β-barrel of BamA and opsonic target (31).

As shown in **Figure 2**, *Tp* binds to macrophages with or without NHS. The observed binding does not cause a concomitant increase in bacterial uptake, which suggests that *Tp* attaches to a non-phagocytic receptor yet to be defined on the macrophage's surface. On the other hand, addition of HSS caused a significant increase in spirochetal uptake (**Figures 2A,C**). In line with the increased uptake of opsonized *Tp*, fewer spirochetes were visualized bound to the macrophage surface (**Figure 2B**). Interestingly, while internalization of *Bb* resulted in copious secretion of TNF, opsonophagocytosis of *Tp* did not (**Figure 2D**). These findings confirm that HSS promotes opsonophagocytosis of *Tp* by human macrophages, but also raise the possibility that the milieu in which macrophages encounter spirochetes is an important determinant of macrophage activation and cytokine production following internalization of bacteria.

IFNγ Induces a Classically Activated Immunophenotype in Human Macrophages

The observation that cytokine production in *Tp* stimulated MΦ(−)s was unexpectedly low prompted us to generate a macrophage phenotype that more closely resembles macrophages present within an IFNγ rich syphilitic immune microenvironment *in vivo* (10, 14). To do so, human monocytes were differentiated into macrophages by adding M-CSF alone

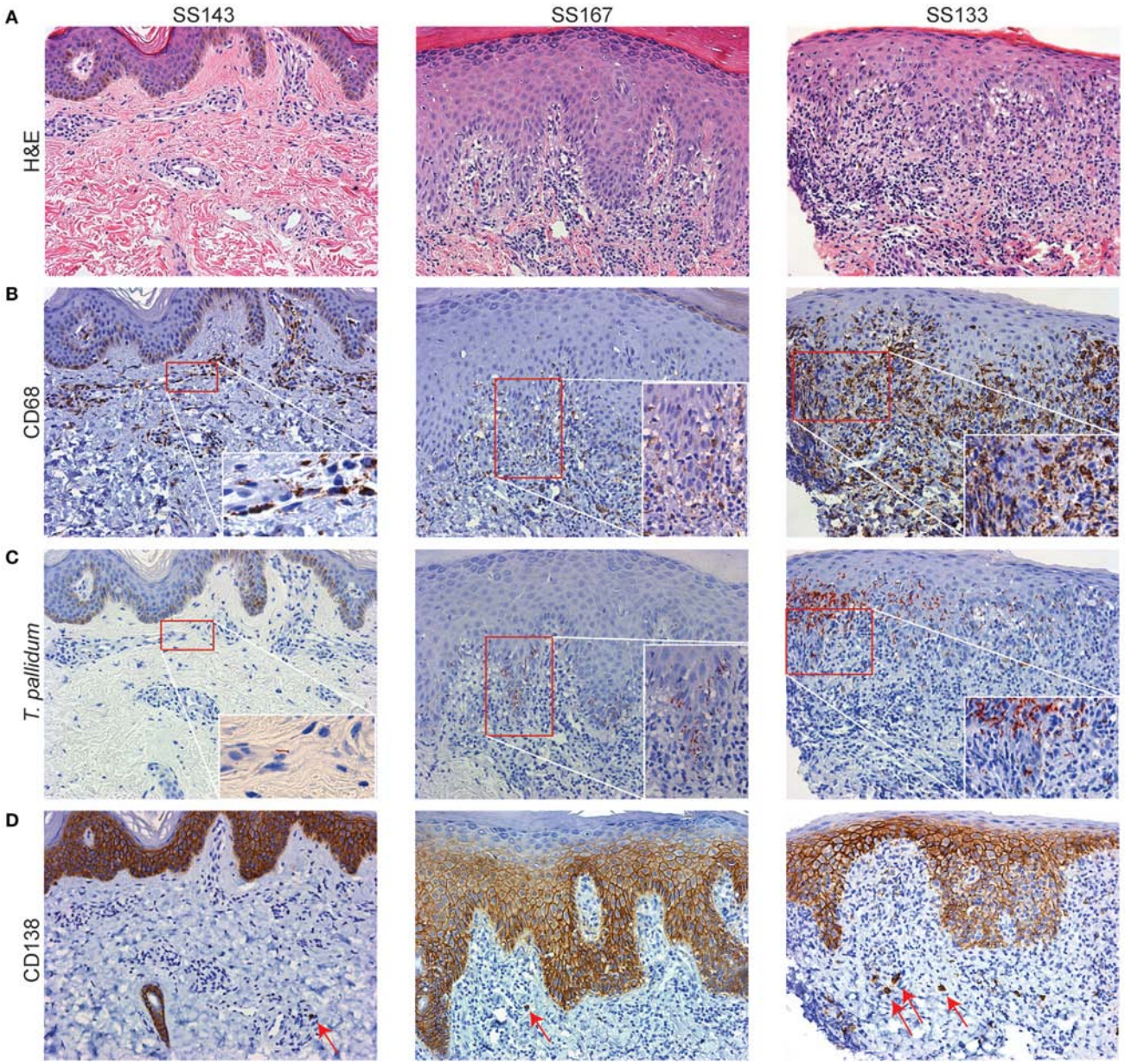

FIGURE 1 | Secondary syphilis (SS) skin lesions are enriched with dermal macrophages. Representative skin biopsies obtained from three SS patients' skin lesions were processed for hematoxylin and eosin (H&E) and immunohistochemical (IHC) analysis. **(A)** H&E-stained sections (10× magnification) reveal an extensive dermal–epidermal lymphohistiocytic immune cell infiltrate with perivascular inflammatory changes. SS167 reveals mild psoriasiform hyperplasia with basal vacuolar changes. SS133 exhibits a classic lichenoid reaction pattern with basal vacuolation. **(B–D)** IHC testing of sequential tissue sections **(B)** varying numbers of $CD68^+$ macrophages were observed in various locations throughout the dermis, but very few were present in the epidermis; **(C)** *Treponema pallidum* is shown in clusters and across the dermal–epidermal barrier (20× magnification). Insets depict higher magnifications of the red box areas in **(B,C)**. **(D)** $CD138^+$ plasma cells (red arrows) varied in frequency and location, including the perivascular space and throughout the papillary dermis. CD138 in basal epidermis has been documented but is non-contributory.

[MΦ(C)] or M-CSF with IFNγ [MΦ(IFNγ)] and then characterized by flow cytometry, gene transcript analysis, and cytokine responsiveness. Given that *Tp* is completely devoid of LPS (43), we omitted this potent endotoxin from our maturation protocols, whereas LPS is often used in macrophage stimulation experiments (29, 44, 45). To more clearly define the effect of IFNγ, we first had to measure the expression of several markers associated with either classically and/or alternatively activated macrophages (29, 46, 47). Expression of CD68, a well characterized macrophage marker, was unaffected by IFNγ

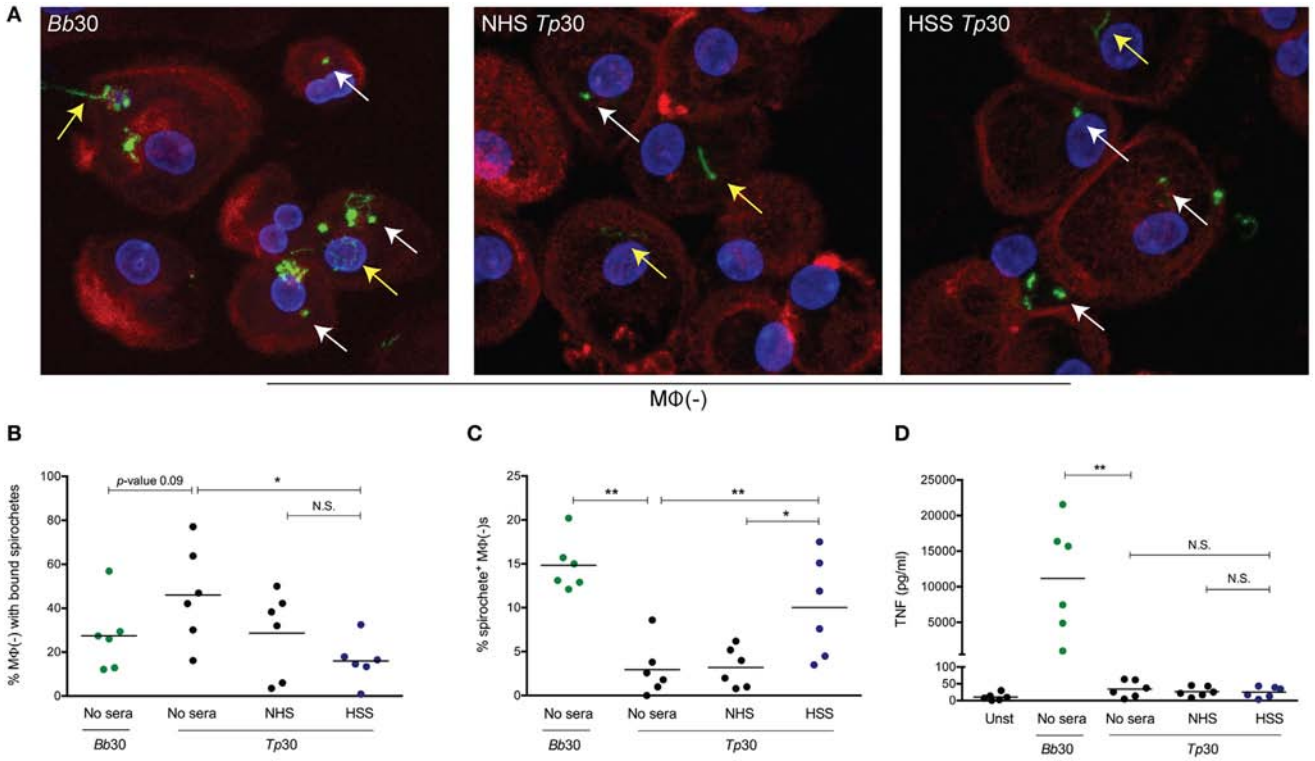

FIGURE 2 | Spirochetal uptake and cytokine secretion by primary human macrophages. Monocyte-derived macrophages [depicted as MΦ(–)], matured with 20% normal human serum (NHS) were stimulated with *Borrelia burdorferi* (*Bb)* or *Treponema pallidum* (*Tp)* at an multiplicity of infection (MOI) 30:1 for 8 h. *Tp* were incubated alone (no sera) or, where indicated, with 10% heat inactivated NHS or human syphilitic serum (HSS). **(A)** Macrophage actin cytoskeleton (red), nucleus (blue), and *Bb* or *Tp* (green) were labeled and imaged as described in Section "Materials and Methods." Representative confocal micrographs demonstrating binding (yellow arrow) and internalization (white arrow) of spirochetes are shown as 20 consecutive compressed Z-stack panels. Dot plot in **(B)** shows percentage of MΦ(–)s with surface bound spirochetes in four conditions, *Bb* alone and *Tp* with no sera, NHS, or HSS. Dot plot in **(C)** reveals the percentage of MΦ(–)s containing internalized *Bb* and *Tp* in the presence of HSS (blue circles) when compared to no sera and NHS (black circles). Dot plot in **(D)** reveals supernatant TNF concentration (pg/ml) for MΦ(–)s stimulated with *Bb* (green circles) and *Tp* under three different conditions. Statistical significance between *Bb* and no Sera *Tp* was determined by two-tailed Mann–Whitney test. The statistical significance between the three *Tp* conditions was determined by Friedman's test with a Dunnett's multiple comparison post-test analysis. N.S., not significant, **p*-value of <0.05, ***p*-value of <0.01.

(**Figure 3A**), while expression of the antigen presentation molecule HLA-DR was appreciably increased (48) (**Figure 3A**). CD14, a glycosylphosphatidylinositol-linked membrane glycoprotein that interacts with several pattern recognition receptors (PRRs), including TLR2 and complement receptor-3 (CR3) (42, 49–51), was upregulated by IFNγ (**Figure 3A**). iNOS, notorious for its vital role in antimicrobial activity as part of the oxidative burst of macrophages, was also significantly increased by IFNγ (**Figure 3B**). We also assessed macrophage expression of the scavenger receptor CD163 (52) and the C-type lectin CD206 (29), which are associated with alternative macrophage activation. CD163 and CD206 were for the most part unaffected by IFNγ (**Figure 3B**). We then examined the expression of TLR2, 7, and 8, which we have shown to be upregulated in SS skin lesions (10). Expression of TLR7 was similar between the two macrophage phenotypes. On the other hand, TLR2, which is essential for spirochetal lipoprotein recognition, and TLR8, an endosomal TLR involved in single-stranded spirochetal RNA recognition (53), were increased in response to IFNγ (**Figure 3C**).

We further examined transcriptional differences between unstimulated MΦ(C)s and MΦ(IFNγ)s by using a commercially available microarray panel. As shown in **Table 1**, several genes that code for macrophage activation markers, microbial PRRs, cytokines and chemokines, were significantly upregulated by IFNγ. Among them, the activation markers *CD40* and *CD80*, as well as *CD38*, a type II transmembrane glycoprotein involved in Ca^{2+} signaling during FcγR-mediated bacterial uptake (54) were transcriptionally increased. In line with the flow cytometry results shown in **Figure 3**, *TLR2* and *TLR8* were also upregulated in the arrays. *PTGS2,* the gene which codes for COX-2 and is associated with classically activated macrophages (44), and several inflammatory chemokines and cytokines (*CXCL11*, *IL1B*, *IL1A*, *IL8*, *IL15*) involved in cellular recruitment and activation were also transcriptionally upregulated. Of particular importance, all three FcγRs (CD16, CD32, and CD64) were upregulated in MΦ(IFNγ)s. In parallel stimulation experiments, we compared proinflammatory cytokine production in response to several TLR ligands (MMP, LPS, or R848) between MΦ(C)s and MΦ(IFNγ)s.

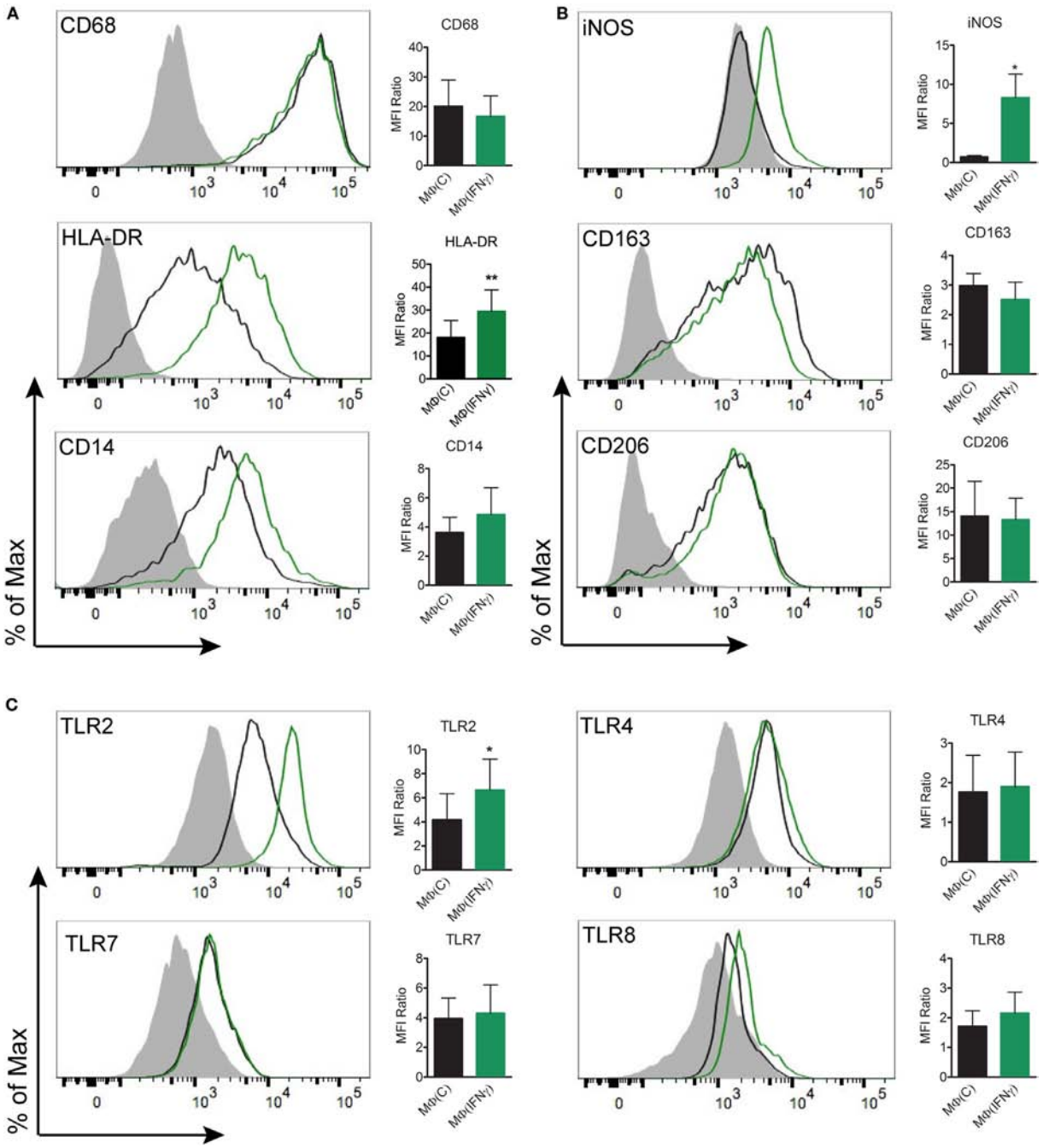

FIGURE 3 | Flow cytometric analysis of macrophage immunophenotypic profile. Monocyte-derived macrophages were generated from healthy controls by incubating monocytes with M-CSF [MΦ(C)] for 7 days and then a portion of the macrophages were activated with IFNγ [MΦ(IFNγ)] for the remaining 3 days. MΦ(C)s (black) and MΦ(IFNγ)s (green) from the same participant were stained for **(A)** macrophage markers: $CD68_{IC}$, HLA-DR, CD14, **(B)** phenotypic markers: $iNOS_{IC}$, CD163, CD206, and **(C)** Toll-like receptors: TLR2, TLR4, $TLR7_{IC}$, $TLR8_{IC}$; the filled gray histograms indicate the isotype control. Histograms are representative of a minimum of four independent experiments that were used to quantify the mean fluorescence index (MFI) ratio ± the SD as described under Section "Materials and Methods." Paired Student's *t*-tests (Wilcoxon test) was used to determine statistical significance between the two macrophage phenotypes, **p*-value of <0.05, ***p*-value of <0.01. IC, intracellular staining.

We observed that, independently of the TLR ligand used, IFNγ primed macrophages produced significantly more cytokines than non-primed MΦ(C)s (Figure S3 in Supplementary Material). Our combined observations thus confirm that M-CSF cultivated macrophages in the presence of IFNγ exhibit a classically activated macrophage phenotype.

TABLE 1 | Transcription profile of MΦ(IFNγ)s.

Gene transcript	Protein name	Average fold change
Activation markers		
CD38	CD38	97.7
CD40	CD40	2.0
CD80	CD80	3.0
HLA-DRA	HLA-DR α	14.8
HLA-DRB1	HLA-DR β1	8.0
Fcγ receptors		
FCGR1	CD64	1.9
FCGR2	CD32	1.4
FCGR3	CD16	1.8
Toll-like receptors		
TLR2	TLR2	2.0
TLR6	TLR6	2.9
TLR8	TLR8	2.7
TLR9	TLR9	3.1
TLR10	TLR10	4.3
Enzymes		
PTGS2	COX-2	5.7
Cytokines/chemokines/growth factors		
CSF2	GM-CSF	3.5
CSF3	G-CSF	9.8
CXCL11	CXCL11	5.9
IL15	IL-15	6.5
IL1A	IL-1α	4.7
IL1B	IL-1β	4.6
IL8	IL-8	8.4
TNF	TNF	2.1

Target array analysis of unstimulated MΦ(IFNγ)s vs. paired unstimulated MΦ(C)s (N = 6).

IFNγ Enhances HSS-Mediated Internalization of *Tp*, Inflammatory Immune Signature, and Cytokine Production by Human Macrophages

Having generated macrophages by closely considering the microenvironment of syphilitic skin lesions, we then asked how these cells would respond to opsonized *Tp ex vivo*. To address this question, we stimulated MΦ(C)s and MΦ(IFNγ)s with *Tp,* in the presence or absence of opsonic antibodies, and assessed their phagocytic potential and immune responsiveness by confocal microscopy and cytokine production, respectively. We observed binding of the spirochete to both macrophage phenotypes, irrespective of the presence of HSS (Figure S4A in Supplementary Material). Spirochetes were seen attached to the macrophages by their tips (**Figure 4A**, bottom left inset—yellow arrow) and in some cases across the entire length of the bacterial surface (**Figure 4A**, top right inset—blue arrow). The addition of HSS led to a marked increase in phagocytosis of *Tp* by both macrophage phenotypes (**Figures 4A,B**), although the phagocytic index was significantly greater in IFNγ stimulated cells (**Figure 4C**). In agreement with enhanced uptake of opsonized spirochetes, we recovered significantly fewer spirochetes when HSS was included in the stimulation experiments (Figure S4B in Supplementary Material). *Tp* stimulated MΦ(IFNγ)s secreted more TNF (**Figure 4D**) and IL-6 (**Figure 4E**) than their counterparts without IFNγ, but neither macrophage phenotype secreted IL-1β (**Figure 4F**). Overall, these findings confirm our hypothesis that elements present within the syphilitic tissue microenvironment, specifically IFNγ, alter macrophage uptake and responsiveness to opsonized *Tp*.

To further characterize the effect of opsonized *Tp* on the macrophage, we used targeted phagocytic and inflammatory transcriptional array analysis. For these experiments, the relative fold changes of gene transcripts between *Tp* stimulated and unstimulated MΦ(IFNγ)s were measured in three experimental conditions; *Tp* with no sera, with NHS and with HSS (**Figure 5**). We first compared genes induced in *Tp* stimulated MΦ(IFNγ)s with no sera or with NHS. Consistent with our findings above, where spirochetal uptake and cytokine secretion were similar between the two conditions in the absence of HSS, we observed that transcriptional profiles were also comparable, such that none of the genes met our differential expression threshold of two-fold change and p-value of <0.05 (Figure S5A in Supplementary Material). On the other hand, several genes were significantly upregulated in *Tp* stimulated MΦ(IFNγ)s in the presence of HSS when compared to similarly stimulated MΦ(IFNγ)s with NHS (Figure S5B in Supplementary Material). *CD38*, *CD40* and *CD80* were increased, whereas the activation marker *CD86* varied greatly between the individual participants (**Figure 5**). *TLR7* was markedly increased in 5/6 healthy volunteer macrophages, but this increase was not statistically significant. *TLR2* and *TLR8,* which have been shown to be important in spirochetal recognition (33, 53, 55) were significantly upregulated as a result of FcγR-mediated uptake of *Tp*. Given the significant upregulation of *TLR8* (**Figure 5**) and the importance of type I IFNs in response to spirochetes (33, 56), in parallel RT-PCR experiments, we also assessed the expression of *IFNB,* and three interferon regulatory factors (IRFs, *IRF3*, *IRF5*, and *IRF7*) by RT-PCR. We observed no change in expression of *IRF3* and *IRF5* (Figure S5C in Supplementary Material). *IRF7* was significantly upregulated, but we did not detect a substantial change in *IFNB* expression (Figure S5D in Supplementary Material).

We observed that many cytokine and chemokines involved in cellular recruitment as well as induction of other physiological responses, including enhanced phagocytosis, were differentially regulated in *Tp* stimulated MΦ(IFNγ)s in the presence of HSS. Among them, granulocyte macrophage colony-stimulating factor (GM-CSF), which is encoded by *CSF2* and has been described to trigger the differentiation and exiting of monocytes from the bone marrow was upregulated. There was a strong induction of chemotactic factors, including *CXCL10* and *CXCL11*, which are important for recruitment of monocytes/macrophages to the site of infection. *IL12B*, which is known to stimulate T cells and NK cells to secrete IFNγ, was robustly increased in 5/6 healthy volunteer macrophages. The transcript for *IL15* (**Figure 5**), a strong NK cell activating cytokine (57), was also elevated. Although IL-1β protein was not secreted by the macrophages following *Tp* stimulation (**Figure 4D**), *IL1B* was transcriptionally upregulated (**Figure 5**). The findings together suggest that neither *Tp* nor its PAMPs, escape the phagosome into the cytosol to induce caspase activation and cleavage of pro-IL-1β into the active cytokine in accord with the mechanism detailed by Netea et al. (58).

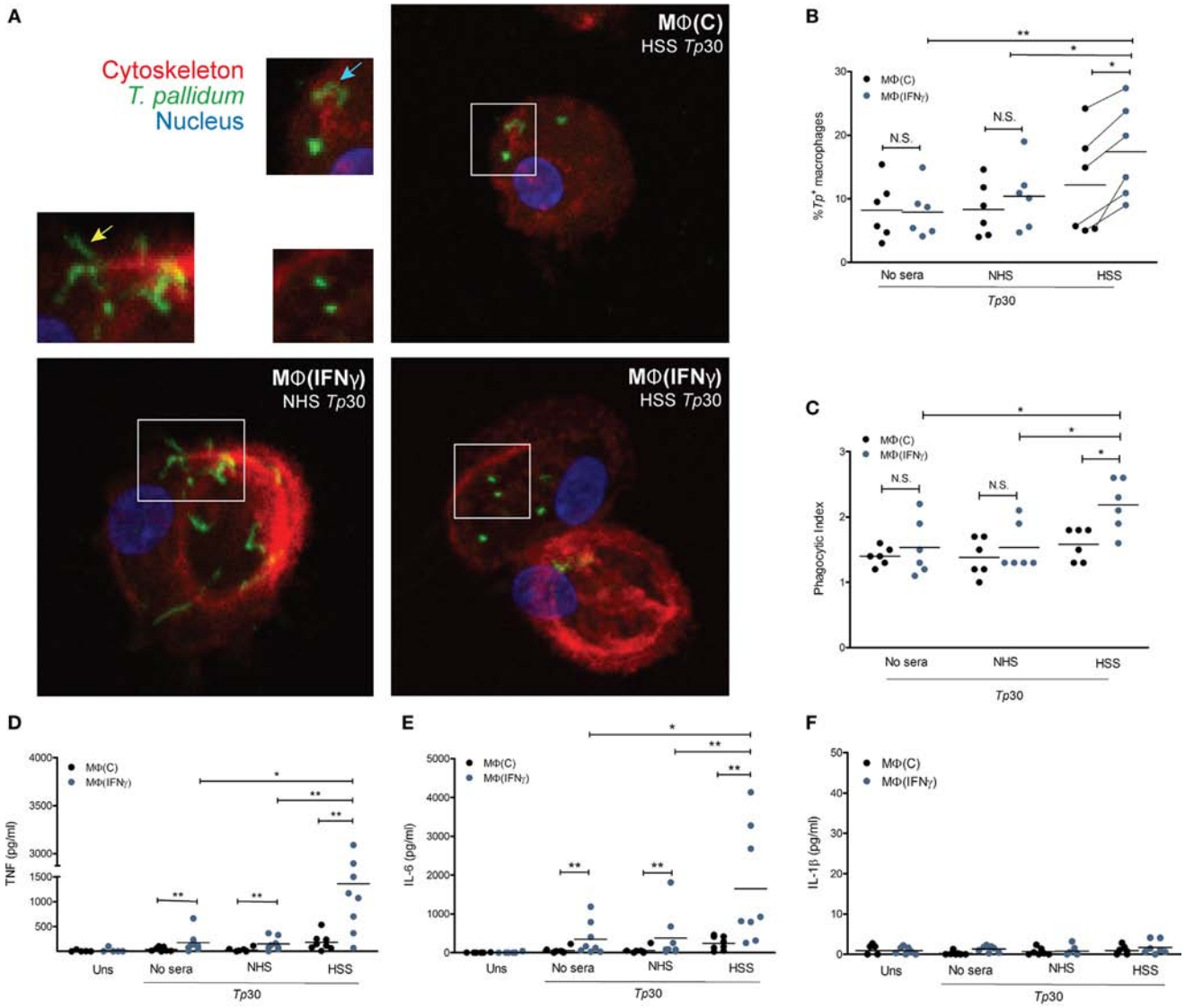

FIGURE 4 | *Treponema pallidum* (*Tp*) uptake by IFNγ activated MΦs. MΦ(C)s and MΦ(IFNγ)s were stimulated with MOI 30:1 of *Tp* for 8 h. *Tp* were either incubated alone (no sera) or where indicated with 10% heat inactivated normal human serum (NHS) or human syphilitic serum (HSS). **(A)** Following stimulation, macrophage cytoskeleton (red), *Tp* (green), and nucleus (blue) were labeled as described in Section "Materials and Methods." Representative confocal micrograph of NHS-*Tp* (Lower left panel, and left inset) show spirochetes binding by tip attachment as well as laying entirely against the cell surface. Confocal micrographs are a composite display of 20 consecutive Z-stack planes. Internalization of *Tp* was observed with HSS as shown in the two right panels. Quantification of spirochetal uptake was calculated by **(B)** % *Tp*+ macrophages and **(C)** phagocytic index. Phagocytosis of *Tp* was significantly enhanced by both HSS and IFNγ. Cytokine bead array was used to detect inflammatory cytokines in the culture supernatants following stimulation with *Tp*. **(D)** TNF and **(E)** IL-6 were significantly increased by MΦ(IFNγ)s following antibody-mediated uptake of *Tp*. **(F)** No IL-1β was detected in any experimental condition. The statistical significance between the *Tp* conditions was determined by Friedman's test with a Dunnett's multiple comparison post-test analysis. N.S., not significant, **p*-value of <0.05, ***p*-value of <0.01, ****p*-value of <0.001.

CD64 Is Primarily Responsible for Macrophage Driven Opsonophagocytosis of *Tp*

Fcγ receptors have been observed to be upregulated in syphilitic skin lesions by transcriptional analysis (10) and are important for binding of IgG with various receptor–ligand affinities (59). Based on our primary human macrophage data (**Figures 2A** and **4A**) and published studies (10, 12, 19, 23) demonstrating the importance of HSS in spirochetal uptake, we hypothesized that FcγRs are the major phagocytic receptors for *Tp*. To study this premise, we first assessed the effect of IFNγ on CD16, CD32, and CD64 expression by flow cytometry. Expression of CD16 (FcγRIII), which weakly binds IgG (59), was only mildly affected by IFNγ, while expression of CD32 (FcγRII), another low affinity IgG binder, was not affected (**Figure 6A**). Conversely, expression of CD64 (FcγRI), a high affinity receptor which binds IgG, specifically IgG_1 and IgG_3 (59), was significantly upregulated by IFNγ (**Figures 6A,B**).

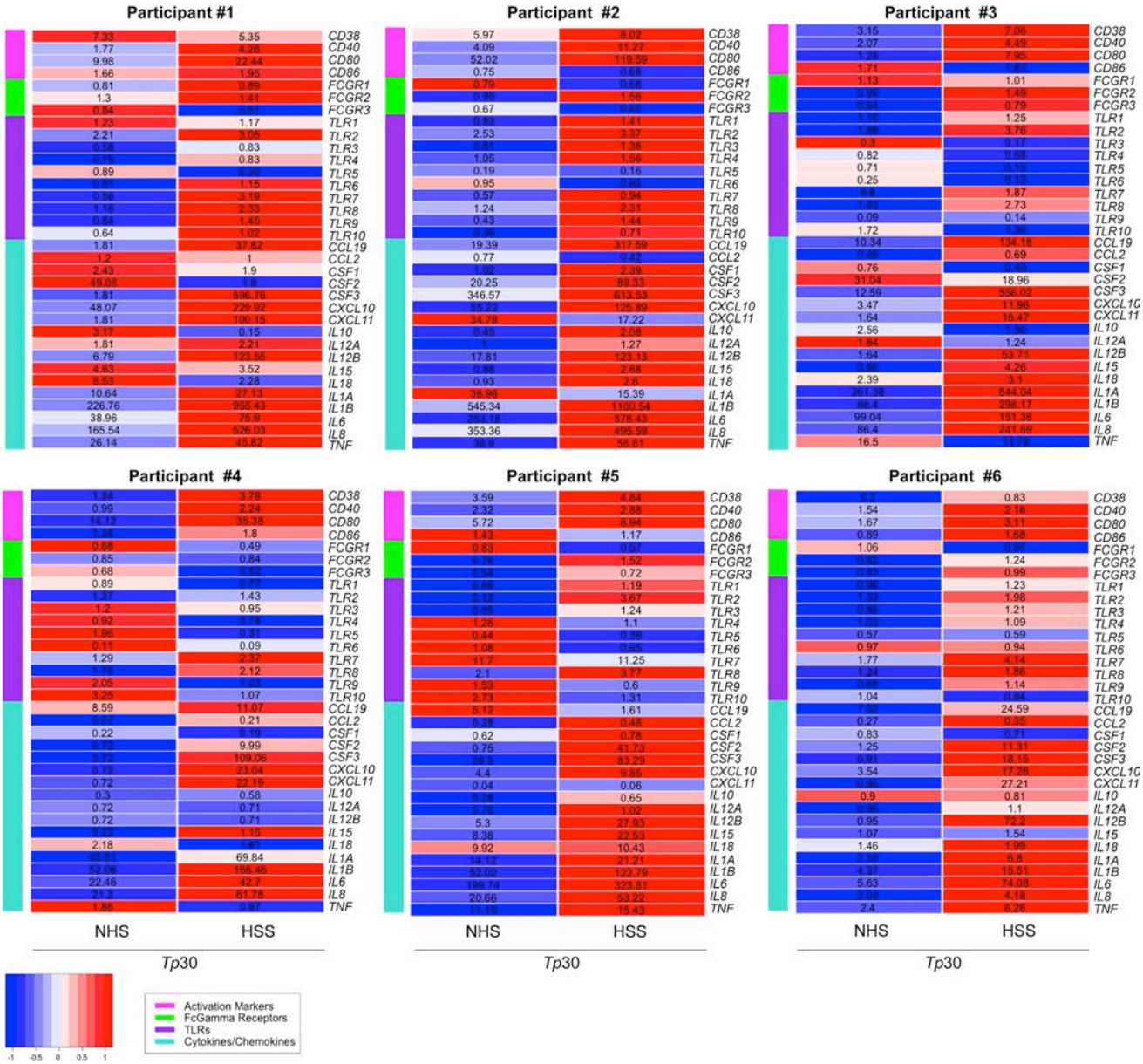

FIGURE 5 | Transcriptional profile of MΦ(IFNγ) stimulated with *Treponema pallidum* (*Tp*). Transcription profiles were determined by targeted array transcriptional analysis after stimulation of MΦ(IFNγ)s with unopsonized [no sera or normal human serum (NHS)] or opsonized [human syphilitic serum (HSS)] *Tp* (MOI 30:1) for 8 h in six-well tissue culture treated plates. Relative fold changes were normalized to the unstimulated MΦ(IFNγ)s control for each gene and then heat maps were generated based on gene categories of interest: activation markers, Fcγ receptors (FcγRs), TLRs, and cytokines/chemokines. Heat maps depict the *Z*-score of NHS-*Tp*30 and HSS-*Tp*30, based on comparing all stimulation conditions, with relative fold-changes values in corresponding gene panel. Each heat map represents an $N = 1$ for individual participants, from a total of six independent experiments.

Due to the significant increase in CD64 expression induced by IFNγ (**Figure 6A**) and importance of syphilitic serum in spirochetal uptake (**Figure 4C**), we then assessed the localization of CD64 with both unopsonized and opsonized *Tp* by confocal microscopy. CD64 could be seen localizing with unopsonized *Tp* (**Figure 7A**, middle right) however when the spirochetes were opsonized with HSS, the intensity of the colocalization was more robust and is most likely a result of FcγRs clustering to the site of treponeme attachment (**Figure 7A**, lower right). To determine the role of FcγRs in spirochetal uptake, we used monoclonal antibodies against human CD16, CD32, and CD64 to block the interaction between opsonized spirochetes and each of the three Fc receptors and then compared bacterial uptake and inflammatory cytokine production between blocked and unblocked MΦ(IFNγ)s. As shown in **Figure 7B**, there were no significant reductions in uptake by blocking CD16 or CD32.

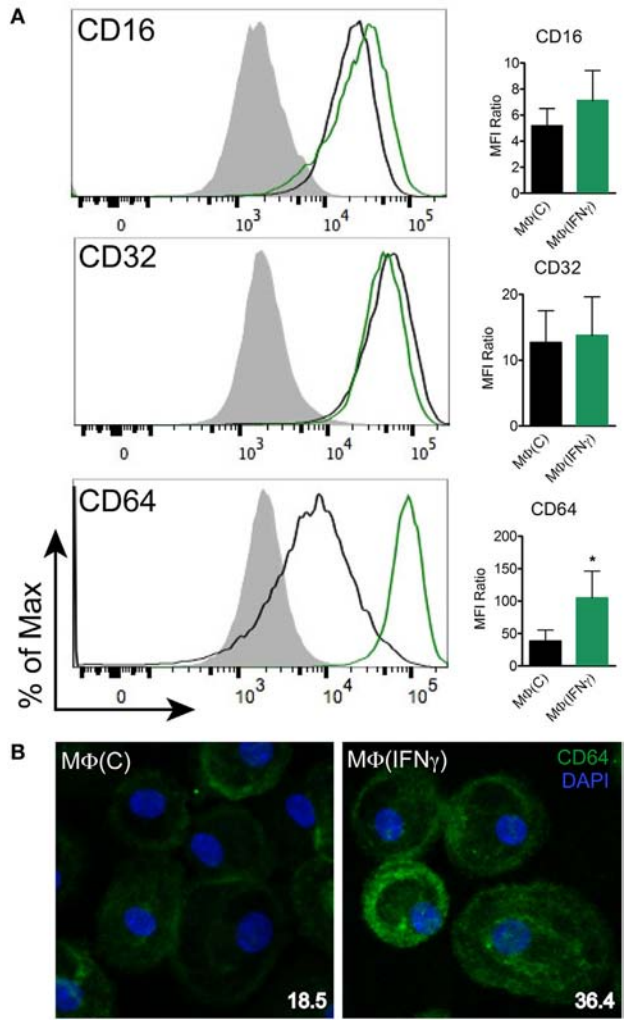

FIGURE 6 | CD64 expression on human macrophages. MΦ(C)s (black) and MΦ(IFNγ)s (green) from the same participant were assessed to determine the expression level of **(A)** Fcγ receptors (FcγRs): CD16, CD32, and CD64 by flow cytometry. The filled gray histograms indicate the isotype control. Histograms are representative of a minimum of four independent experiments. Data of the mean fluorescence intensity (MFI) ratio ± the SD were analyzed for statistical significance using the paired Student's *t*-test, **p*-value of <0.05. **(B)** MΦ(C)s and MΦ(IFNγ)s were incubated in eight-well chamber slides and expression of CD64 (green) was determined by IFA with mouse anti-human CD64 (clone 10.1) antibody. Representative confocal micrographs of CD64 expression levels shown as a composite of 10 consecutive Z-stack planes. CD64 MFI values (inset in bottom right) were calculated using ImageJ.

However, blockade of CD64 did cause a significant decrease in phagocytosis of opsonized spirochetes (**Figure 7B**), as well as TNF production (**Figure 7C**). This finding was not surprising because IgG1 and IgG3, the prominent IgG subclasses in syphilitic serum (60), are high affinity IgG subclasses known to bind CD64. Importantly, the use of monoclonal antibodies against CD64 alone, or in combination with CD16 and CD32, did not result in complete abrogation of phagocytosis (**Figure 7B**), suggesting that additional phagocytic receptors could have a role in *Tp* uptake. Taken together, these data show for the first time that CD64 acts as the primary phagocytic receptor for FcγR-mediated uptake of opsonized *Tp,* and that IFNγ-mediated enhanced expression of CD64 promotes Fc-receptor–ligand interactions.

DISCUSSION

Much of what is currently known about the role of the macrophage in syphilis pathogenesis emanates from several decades of *in vivo* and *ex vivo* studies using the rabbit model of infection (15, 16, 19, 20, 22, 61–63). Lukehart and Miller (19) demonstrated that chemically activated rabbit peritoneal macrophages phagocytose *Tp ex vivo* in the presence of syphilis immune rabbit sera. This landmark study was the first to underscore the importance of the macrophage in Ab-mediated uptake of the syphilis spirochete. Additional rabbit studies established that clearance of the spirochete *in vivo* temporally correlates with the influx of macrophages to infected tissues (15, 16, 64) and generation of a "lymphocyte factor" (65), now known to be IFNγ (18). Although the rabbit model has proved to be an important resource in syphilis research, humans are the obligate host for *Tp*. Thus, it is critical to develop an *ex vivo* human model to aid in understanding the immunological responses of the natural host evoked by the bacterium. Studies to understand the role of the macrophage in human syphilis have relied on a combination of transcriptional and IHC analysis of early syphilitic lesions and are only now being explored with an *ex vivo* macrophage system. Only a handful of studies have evaluated human monocyte/macrophage-*Tp* interactions *ex vivo* (10, 12, 23). In one such study, our group showed that HSS promotes spirochetal uptake by isolated human monocytes, leading to destruction of the bacterium within phagosomal vacuoles and enhanced secretion of proinflammatory cytokines (i.e., TNF) (10). Recently Marra et al. described that 20–47% of monocyte-derived-human macrophages internalized HSS opsonized spirochetes (23). However, the researchers did not assess the impact of the macrophage phenotype in phagocytosis or the inflammatory response elicited to the bacterium. The immunological environment provides critical information when developing a model system because macrophage phenotypic plasticity and polarization *ex vivo* are highly dependent on the cytokines and growth factors used in the differentiation protocols (28, 44, 52, 66). We elected to carefully model the immunologic niche where macrophage–*Tp* interactions are likely to occur under actual disease conditions for our system. We observed that the addition of IFNγ enhanced the macrophage's phagocytic capacity for opsonized spirochetes, as well as the secretion of inflammatory cytokines. Unlike the rabbit model, where 63–76% of the macrophages contained ingested opsonized spirochetes (19), the human system resulted in a much lower % of Tp^+ cells and confirms the variability of uptake described by Marra et al. (23). We feel this result observed in the *ex vivo* macrophage system more accurately reflects the true duality of the disease; this duality can be observed in **Figure 1**, where treponemes appear to be migrating to locations that are difficult for the immune cells such as $CD68^+$ macrophages to access, while increasing the odds for bacterial transmission.

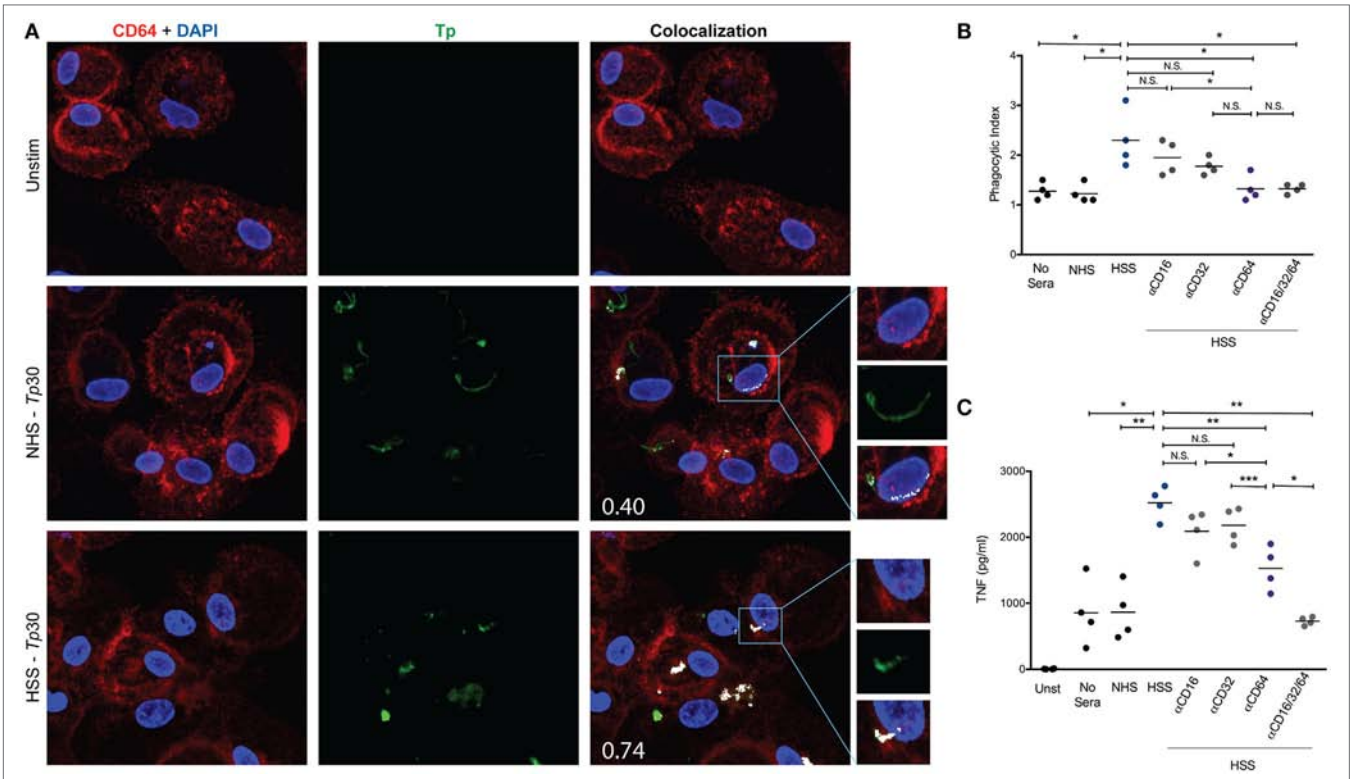

FIGURE 7 | CD64 is the primary phagocytic receptor. Mouse anti-human CD64 and polyclonal rabbit anti-*Tp* antibodies were used to identify locations of Fcγ receptors (FcγRs) and spirochetes on the cell surface by confocal microscopy. **(A)** Colocalization of CD64 (red pixels) and *Tp* (green pixels) were determined by ImageJ plug-in "JACoP" and are represented by white pixels. Mander's colocalization coefficient values (M2), shown in the lower left corner of colocalization images in A, are indicative of the proportion of the green signal overlapping with the red signal. **(B,C)** The blockade of FcγRs on the surface of MΦ(IFNγ)s was achieved by pre-incubating macrophages with 10 μg/ml of mouse anti-human CD16 (clone 3C8), mouse anti-human CD32 (clone 6C4), mouse anti-human CD64 (clone 10.1), or 10 μg/ml of each of the three antibodies for 1 h prior to *Tp*–macrophage stimulating at an multiplicity of infection (MOI) 30:1 with no sera, normal human serum (NHS), or human syphilitic serum (HSS) for an additional 8 h in an eight-well chamber slide. **(B)** Phagocytic index is shown and compared between each of the five conditions studied. **(C)** Cytokine bead array was used to detect inflammatory cytokine production following stimulation with *Tp* and shown in the dot plot for each of the five conditions studied. The statistical significance between the *Tp* conditions was determined by Friedman's test with a Dunnett's multiple comparison post-test analysis. N.S., not significant, **p*-value of <0.05, ***p*-value of <0.01.

Our study results reinforce our underlying hypothesis that macrophage differentiation is an important factor for optimal treponemal recognition. In the presence of M-CSF and IFNγ macrophages exhibited a "classically activated" phenotype (67) and responded to opsonized *Tp* by secreting large quantities of TNF. By comparison, while non-IFNγ stimulated macrophages were also capable of internalizing opsonized spirochetes, their cytokine responsiveness was markedly decreased. The difference in cytokine responses between the two macrophage phenotypes has several potential explanations. First, IFNγ induced expression of CD64 is likely to engender more efficient uptake of opsonized spirochetes, which translates into an increase in bacterial cargo available for signaling from within the phagosome. Second, upregulation of baseline TLR2 and TLR8 expression by IFNγ, as shown herein, could promote more opportunities for ligation of released spirochetal PAMPs (33, 53) and TLR signaling itself may lead to a more rapid maturation of the phagosome (68). Alternatively, as shown by Balce et al. (69) FcγR-mediated phagocytosis in association with IFNγ could facilitate phagosomal processing of proteins. IFNγ stabilizes MyD88 (70), in addition to modulation of the FcγR signaling cascade (59, 71), helping to enhance the recognition response to *Tp*. Intriguingly, a similar decrease in cytokine production was not seen in non-IFNγ primed *Bb*-stimulated macrophages. Several reasons likely account for the differential response between *Bb* and *Tp*. Although both spirochetes contain lipoproteins known to signal through TLR2 (33, 55, 72), *Bb* is larger in size than *Tp* and has a far richer repertoire of lipoproteins within its outer and inner membranes. In addition, *Bb*'s nucleic acid is far more abundant and complex than *Tp*'s, thus nucleic acid ligands are likely to have more opportunities to engage their cognate endosomal TLR receptors (i.e., TLR8). Ultimately, macrophages phagocytose *Bb* directly *via* the phagocytic receptor CR3 (42, 73). The process does not require the presence of complement or opsonic antibodies, leads to efficient uptake of the bacterium and activation of phagosomal receptors despite the absence of IFNγ.

Macrophage recognition of a bacterial pathogen is a very dynamic and complex process that involves a variety of receptors and is greatly influenced by the molecular composition and structure of the organism they encounter. Although *Tp* contains

an abundance of highly antigenic hydrophilic polypeptides, these molecules are tethered by covalently bound N-terminal lipids to the bacterium's periplasmic inner membrane leaflet and thus not available for innate immune receptor (i.e., TLR1/2) sensing (74–76). In addition, because *Tp* contains very few OMPs (77, 78), the bacterium provides limited binding sites for syphilitic opsonic antibodies (79). Nevertheless, our study demonstrates that HSS is able to opsonize *Tp* and lead to FcγR-mediated uptake by human macrophages, where CD64 acts as the primary phagocytic receptor. The *Tp*-specific antibodies generated by the host, are primarily comprised of IgG1 and IgG3 subclasses (60), are known to bind to CD64 with high affinity (59). Clustering of CD64, as demonstrated occurs in the presence of opsonized spirochetes (**Figure 7A**), initiates important signal transduction events that result in internalization of the pathogen (80). Although receptor dimerization can be sufficient for receptor activation, beads coated with low densities of IgG, similar to what occurs with *Tp* (77, 79), trigger inefficient receptor recruitment, thus slowing formation of the phagocytic cup (81). The heterogeneity of antibodies binding to spirochetal populations (79), in combination with the low density of OMPs (77) and fixed locations of the antigenic targets (78) may contribute directly to the slow phagocytic events of the treponemes previously described (82, 83). They may also explain why a large proportion of spirochetes are capable of avoiding opsonophagocytosis (Figure S3B in Supplementary Material). IFNγ-enhanced expression of CD64 most likely engenders more efficient uptake of opsonized spirochetes and inflammatory responses, which allow the human host to override the gridlock caused by the organisms unique OM structure and requirement of opsonic antibodies to eventually clear the pathogen.

Following opsonophagocytosis of a delicate pathogen, such as *Tp*, the acidic environment of the phagosomal vacuole disrupts the spirochete's OM (12). Liberation of once concealed spirochetal PAMPs makes them accessible to interact with their cognate receptors and initiate downstream inflammatory signals. Herein, we demonstrate that in addition to NF-κB mediated cytokine production, opsonized *Tp* induces transcription of type I interferons, several chemokines, and many other inflammatory mediators. Among them is IFN-β, a type I interferon known to be present in SS skin lesions (10), which we believe is triggered similarly to *Bb*, *via* a TLR8-IRF7 pathway (53). The chemokines CCL19, CXCL10, and CXCL11, are chemokines associated with recruitment of DCs, antigen engaged B cells, NK cells, and activated T cells to infected tissues (84–86) were differentially transcribed following uptake of opsonized *Tp*, and were upregulated in SS skin lesions (10). IL-12 and IL-15, two cytokines secreted by macrophages and associated with activation of T cells and NK cells (57), were also transcriptionally modulated. Interestingly, activated NK cells and $CD8^+$ T cells, known for production of IFNγ in response to *Tp* (12, 14), are typically associated with degranulation in response to viral infection and tumor cells (87, 88) but *Tp* is an extracellular pathogen. The immunological functions elicited by $CD8^+$ T cells and NK cells in the syphilitic lesions are unclear, with the

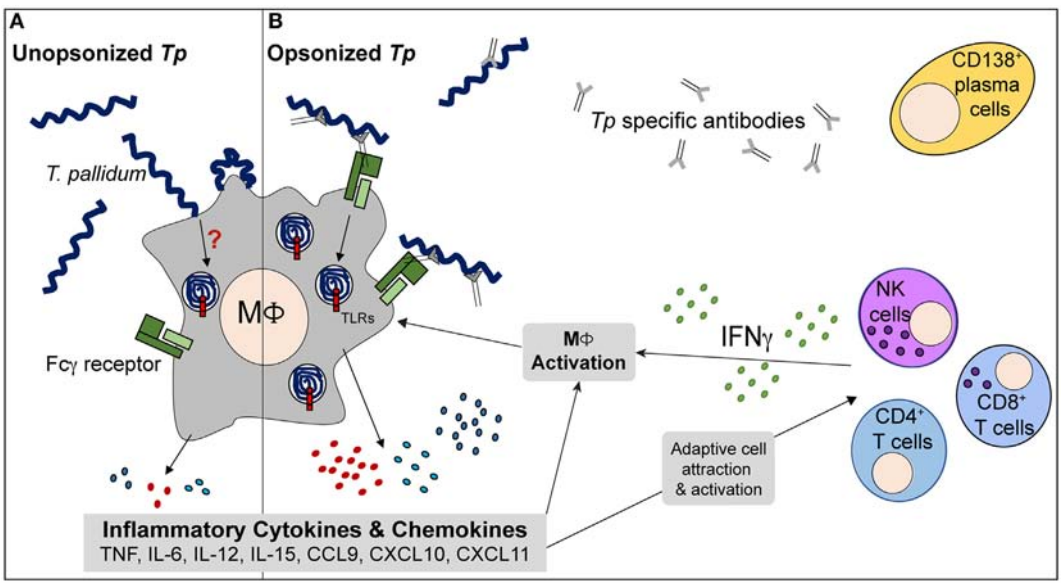

FIGURE 8 | Proposed model of immune interplay in secondary syphilis (SS) lesions. **(A)** During early response to *Tp*, human macrophages (MΦs) are able to bind unopsonized spirochetes but the cells phagocytose very limited numbers and many *Tp* are able to escape. The phagocytic process is inefficient and results in minimal inflammatory cytokine production. **(B)** Following antigen presentation by phagocytes, lymphocytes such as $CD4^+$ T cells, $CD8^+$ T cells, and NK cells produce IFNγ locally. T cell activation can aid in B cell maturation and *Tp* specific Ab production. The antibodies are directed to various targets of the treponemes, including the spirochete's rare outer membrane proteins. MΦs increase expression of Fcγ receptors (FcγRs) in response to the IFNγ rich microenvironment of the infected tissue and allow for enhanced phagocytosis of opsonized treponemes. Within the MΦs' phagosomal compartment, the spirochetes' fragile outer membrane is degraded, liberating the once concealed pattern-associated molecular patterns (PAMPs) and ultimately resulting in interaction with TLRs, such as TLR2 and TLR8. The activated MΦs produce elevated levels of inflammatory cytokines (TNF, IL-12, and IL-15) and chemokines, resulting in an important positive feedback loop that acts on both branches of the immune system. Ultimately, with aid from the adaptive immune system, the macrophage plays a central role in clearance of *Tp*.

exception of sourcing IFNγ for macrophage activation. It is also possible that macrophages are presenting antigen to T cells *via* a cross presentation mechanism and that other lymphocytes are involved in macrophage-independent mechanisms of clearance in the tissues. In short, it is clear that in the context of phagosomal signaling, the macrophage plays a fundamental role in generating proinflammatory signals in response to *Tp*, and also modulates innate and adaptive immune responses.

Venereal syphilis can be considered a battle between the ability of *Tp* to circumvent immune recognition and the proficiency of the host's innate and adaptive immune responses to search and destroy the spirochetal pathogen (38, 39, 89). Our results reinforce the importance of the human macrophage as a contributor in the innate immune response to *Tp*. They also offer new evidence that the balance between phagocytic uptake of the spirochete and the bacterium's ability to evade immune recognition by the macrophage is significantly influenced by the emergence of anti-treponemal opsonic antibodies, as well as the immune microenvironment where the macrophage resides. More specifically, our findings provide unequivocal confirmation that HSS markedly enhances uptake of spirochetes by human macrophages *in vitro*. Of particular noteworthiness, our study is the first to show that in human macrophages CD64 is the primary receptor during FcγR-mediated phagocytosis of *Tp*. Results from this study also substantiate the importance of IFNγ-mediated macrophage activation as a beneficial immune event that tips the balance of the battle with the spirochete in favor of the host. These findings allow us to build upon the model initially proposed by Lukehart (90) for the role of the human macrophage in the immunologic events that take place in the context of early syphilitic infection in humans. As shown in the model (**Figure 8**), phagocytosis by macrophages is central to the immune response against *Tp* and greatly influenced by Ab production to specific antigenic epitopes and production of IFNγ. The model proposes that following a prolonged period of time, and many struggles to gain control of the spirochete, macrophage-dependent and -independent immune responses ultimately lead to clearance of *Tp* from infected tissues. Our study also indicates the potential challenges faced in the development of a vaccine against a unique bacterium that avoids host recognition. Comprehensive analysis of confirmed OMPs such as TprC, TprD, and Tp0326 by our group (75, 76, 91) in addition to other proteins identified by other researchers (92–94), will be critical to better understand the surface-exposed antigenic loops of *Tp* that antibodies utilize for opsonization. Lastly, our *ex vivo* human macrophage model will provide researchers with the first human system to assess surrogate markers for syphilis vaccine candidates.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Review Boards at UConn Health, Farmington CT and Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) in Cali, Colombia. All study participants were provided written informed consent. All animal experimentation was conducted following the Guide for the Care and Use of Laboratory Animals (8th Edition) and in accordance with protocols reviewed and approved by the UConn Health Institutional Animal Care and Use Committee under the auspices of Animal Welfare Assurance A347-01.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception, design, and analysis of experiments. KH, AC, CLV, ML, LR, and DM performed the experiments in the manuscript. KH, JR, and JS wrote the manuscript. All authors critically reviewed the manuscript for intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01227/full#supplementary-material.

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