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Lipid exchange between *Borrelia burgdorferi* and host cells

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Jameson Thatcher Crowley

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Jameson Thatcher Crowley

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

acceptance of this dissertation.

Jorge L. Benach, PhD. (Advisor) Distinguished Professor
Department of Molecular Genetics and Microbiology

David G. Thanassi, PhD. (Chair) Professor
Department of Molecular Genetics and Microbiology

Martha B. Furie, PhD. Professor
Department of Pathology

Howard B. Fleit, PhD. Associate Professor
Department of Pathology

Erwin London, PhD. Professor
Department of Biochemistry and Cell Biology

This dissertation is accepted by the Graduate School

Charles Taber
Interim Dean of the Graduate School

Abstract of the Dissertation

Lipid exchange between *Borrelia burgdorferi* and host cells

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This dissertation examines the mechanism of transfer of *Borrelia burgdorferi*-derived antigenic lipids to non-phagocytic eukaryotic cells and how the mechanism of transfer of foreign antigenic bacterial lipids could contribute to the pathogenesis of Lyme disease. Lipid rafts are structural subdomains of the plasma membrane that are primarily found in eukaryotic cells due to the presence of cholesterol and glycosphingolipids. Interestingly, the presence of these membrane subdomains is also shared with the spirochetal prokaryote *B. burgdorferi.* The presence of free cholesterol and cholesterol-glycolipids in *B. burgdorferi* creates an opportunity for lipid-lipid interactions with constituents of the lipid rafts in eukaryotic cells. To investigate possible lipid-lipid interactions between *B. burgdorferi* and eukaryotic cells, fluorescent cholesterol analogs were utilized to label not only the entire membrane of these cells, but also the individual cholesterol-glycolipids of the spirochetes. Using transfer assays that examined the cells in a population and at the single-cell level, it was observed that there is a two-way exchange of lipids between *B. burgdorferi* and epithelial cells. In investigating these transfer events, free cholesterol and the antigenic cholesterol-glycolipids of *B. burgdorferi* were transferred to epithelial cells through direct contact and/or through outer membrane vesicles. Using the same

fluorescent cholesterol analog, cholesterol was also shown to be acquired by the bacteria from the plasma membrane of the epithelial cells. Given the limited biosynthetic capabilities of *B. burgdorferi,* acquisition of available cholesterol from the host appears to be essential for the spirochetes' growth. The two-way transfer of lipids could play a critical role in the pathogenesis of spirochetoses. One manifestation that the transfer event could contribute to is the generation of antibodies that cross-react with both bacterial and host molecules. In addition, the transfer of the antigenic glycolipids to the host cells could be a mechanism whereby the inflammatory and immune response of the host could recognize cells bearing spirochetal antigens, leading to the development of neurological and joint manifestations of Lyme disease.

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INTRODUCTION

Since being identified as the causative agent of Lyme disease, *B. burgdorferi* has been the focus of intense scientific and medical evaluation [1,2]. These spirochetes are unique when compared to other prokaryotic pathogens. Specifically, the structure and physical makeup of these organisms, as well as the fact that they can cause long lasting chronic infections, separates them from many other prokaryotes. The reasons or mechanisms for the chronic condition are not entirely understood. It is the unique aspects of *B. burgdorferi* and Lyme disease that are responsible for driving many different bacteriological and immunological studies.

One aspect of Lyme disease research that has recently been examined is how the unique composition of the *B. burgdorferi* membrane contributes to pathogenesis. Of particular interest to our laboratory has been the fact that *B. burgdorferi* is the only prokaryote known to have eukaryotic-like lipid rafts in its outer membrane (OM). The OM lipids of *B. burgdorferi* are unusual when compared to other Gram-negative bacteria because they contain cholesterol [3-5]. Free cholesterol and cholesterol-glycolipids constitute 40% of the total membrane lipids [3]. The presence of cholesterol in the *Borrelia* OM results in the formation of eukaryotic-like lipid raft domains [6]. Furthermore, the cholesterol that is in the lipid rafts is essential for growth and survival of the *Borrelia* because the spirochetes do not have the biosynthetic ability to synthesize cholesterol required for growth [7]. During a Lyme disease infection, the ordered raft-like domains may be important for sorting or organizing proteins and antigens. Ordered eukaryotic-like raft domains in *B. burgdorferi* could be important for functions such as vesicle production or direct interaction with host cells.

For this dissertation, an examination into the direct interaction between *B. burgdorferi* and eukaryotic cells was carried out. It is our hypothesis that *B. burgdorferi* lipid antigens are

transferred to host cells by both a contact-dependent and -independent mechanism of lipid complexes that contributes to the pathogenesis of Lyme disease, and that *B. burgdorferi* acquires cholesterol directly from cells through attachment. The two-way exchange of lipids from host to spirochete (cholesterol acquisition) and from spirochete to host (deposition of antigenic cholesterol-glycolipids) is the primary focus of this research. Given that there is transfer of antigenic material, we also examine the cross-reactivity of antibodies specific for the lipids of *B. burgdorferi* to determine if transfer of antigenic material to host cells could be an important event throughout the course of infection, resulting in immune responses that could damage the antigen-bearing cells.

CHAPTER 1 – BACKGROUND

1.1 BIOLOGY OF SPIROCHETES

B. burgdorferi are the tick-transmitted spirochetes that cause Lyme disease. The species of *Borrelia* responsible for causing disease in humans include *B. burgdorferi* sensu stricto [1,2], *B. afzelii* [8], and *B. garinii* [9] (Table 1.1). Other species of *Borrelia*, *B. japonica* [10], *B. andersoni* [11,12], *B. turdi* [13], *B. takunii* [13], *B. bissettii* [14], *B. valaisiana* [15], *B. sinica* [16], and *B. lusitaniae* [17], have been identified, but their role in human disease remains unclear (Table 1.1). While Lyme disease is the most commonly reported vector-borne disease in the United States [18], the substantial number of species worldwide highlights how many different species of *Borrelia* could potentially cause human disease.

1.1.1 Morphology

Spirochetes from the genus *Borrelia* have a spiral morphology and a cellular structure that is similar to Gram-negative bacteria [7,19]. The *Borrelia* cellular structure consists of a protoplasmic cylinder that is surrounded by an inner membrane, a thin cell wall (peptidoglycan), and an outer membrane [7,19]. Between the inner and outer membranes is an area called the periplasmic space [7,19]. *Borrelia* are different from other Gram-negative bacteria in that they have unique organization and components in the periplasmic space [7,19]. One aspect of *Borrelia* not present in the majority of other Gram-negative bacteria is the presence of bundles of periplasmic flagella [7,19]. These flagella are located in the periplasm, just under the outer membrane [7]. They are responsible for the spirochete's spiral morphology, wavelike motion, and movement [7]. These flagella are never exposed to the extracellular environment unless the outer membrane of the bacteria has been compromised [6,20,21]. Another difference in the

organization of *Borrelia* compared to other Gram-negative bacteria is the location of the cell wall. With the periplasmic flagella located under the outer membrane, the peptidoglycan cell wall is more closely associated with the inner membrane of the spirochete [7].

In addition to differences in overall organization of the *Borrelia* outer envelope, spirochetes also have significant differences in the composition of the outer membrane compared to other Gram-negative bacteria. *Borrelia* do not have lipopolysaccharide (LPS) as a major component of the outer membrane [22,23]. Instead, cholesterol and cholesterol-glycolipids constitute a significant portion of the *Borrelia* outer membrane [24,25]. Cholesterol in the outer membrane of *B. burgdorferi* appears to contribute to the increased membrane fluidity of the spirochetes [19,26]. *The presence of cholesterol in the outer membrane may contribute to the pathogenicity of B. burgdorferi. In this dissertation we will describe how the spirochetes could acquire cholesterol from the host. Additionally, we will examine how cholesterol and cholesterol-glycolipids might contribute to the formation of auto-reactive antibodies*

1.1.2 Motility

As stated in 1.1.1, *Borrelia* are unique from most other Gram-negative bacteria in that they have periplasmic flagella. In addition to contributing to the spiral appearance of the *B. burgdorferi,* the periplasmic flagella are essential for motility. Without this motility, the spirochetes would be unable to invade and disseminate throughout a mammalian host. Three distinct motions have been observed for spirochetes, which are translational motion, rotation about their longitudinal axis, and flexing [27,28]. Motility of the spirochetes is important for pathogenicity, because without functioning flagella to drive motion, the spirochetes are unable to penetrate cell layers *in vitro* and are noninfectious *in vivo* [29-32]. *B. burgdorferi* were shown to

be most motile in environments similar to interstitial fluid and move towards chemoattractants like serum and also avoid chemicals, like hydrogen peroxide, produced by host immune cells [33]. Furthermore, more recent research has elucidated a two-component chemotaxis system and signal transduction pathway in *B. burgdorferi* used to coordinate chemotaxis and motility [32-36]. This system is essential for chemotaxis and motility [34]. *The chemotaxis and motility of B. burgdorferi is essential for establishing infection. The motility of spirochetes is essential for acquisition of nutrients or factors like cholesterol and avoidance of hazardous environments.*

1.1.3 Outer Membrane Proteins

As an extracellular pathogen, the major antigens on the spirochetes that are recognized by the host immune system are surface-exposed lipoproteins found in the outer membrane of the spirochetes [37]. In *B. burgdorferi,* the outer surface proteins (Osps) comprise a large portion of the surface-exposed lipoproteins [38-42]. To reach the external surface of the outer membrane, all *Borrelia* lipoproteins must cross both the inner and outer membrane of the bacteria. Similar to those of other Gram-negative bacteria, *Borrelia* outer membrane proteins, which include the Osps, are synthesized with a signal peptide at their N-terminus [43]. This signal peptide initiates export of the protein by the Sec system and is then cleaved after translocation across the inner membrane [43]. For the lipoproteins to associate with membranes, it is necessary to be modified by a hydrophobic lipid moiety[43]. *Borrelia* lipoproteins contain a lipid modification at the amino-terminal cysteine [43]. Proteins with the consensus sequence, [L,V, I]-3[A, S, T, G]-2[G, A]-1C+1, are cleaved by signal peptidase II to expose the amino-terminal cysteine [43]. With the free amino-terminal cysteine, the tripalmitoyl-S-glyceryl-cysteine lipid modification is added to the protein [43]. *Lipoproteins, specifically the Osps, are major antigens on Borrelia that are*

recognized by the hosts. In addition to being significant antigens, the OspA and OspB lipoproteins through their lipid modifications associate with lipid rafts of B. burgdorferi. In this dissertation, we will demonstrate that the Osps are significant protein components in the outer membrane vesicles (OMV) of B. burgdorferi. Furthermore, we will use the Osps as tools to label the spirochetes in fluorescent microscopy.

The Osps of *B. burgdorferi* have been proposed to contribute to persistence of the spirochetes through differential expression of proteins and antigenic variation. This topic will be discussed in section 1.2.3. Other evidence exists that suggests the Osps have additional functions. OspA and OspB contribute to adherence of the tick midgut by the spirochete [44,45]. However, they also appear to have a role in late or chronic infections. During late Lyme disease, OspA and OspB expression increases in the spirochete. This is supported evidence that OspA is upregulated and can bind plasmin/plasminogen in the host [46]. Another role for OspA that has been proposed is that it could provide antigenic shielding of other antigens on the surface during tick feeding [47]. Other Osp proteins, OspC and OspEF related proteins (Erps), also were shown to be important for infection because they bind host plasmin/plasminogen [48,49]. *The Osps of B. burgdorferi are a group of outer membrane antigens that have been shown to contribute to spirochetal survival in the tick and in the dissemination and establishment of Lyme disease. However, their exact functions during infection remain largely unknown.*

1.1.4 Lipids of *Borrelia burgdorferi*

The presence of free cholesterol or cholesterol-glycolipids is unusual in the membrane of prokaryotes. In addition to *Borrelia*, cholesterol has been documented in the membrane compartment of only five other prokaryotes: *Helicobacter* [50-53], *Mycoplasma* [54], *Ehrlichia*

[55], *Anaplasma* [55], and *Brachyspira* [56]. Cholesterol is essential for growth and survival of *Borrelia*. *B. burgdorferi* does not have the biosynthetic ability to synthesize cholesterol or any long-chain saturated and unsaturated fatty acids that are required for growth [7]. As a result, the lipid composition of *B. burgdorferi* has been shown to reflect that of the culture medium or host animal fluids or tissues [7]. The presence of a cholesterol glucoside in spirochetes was first identified in *B. hermsii* [25], an agent of relapsing fever. Later, lipids and glycolipids of *B. burgdorferi* were isolated and characterized [24]. However, these analyses failed to definitively identify free cholesterol and the cholesterol-glycolipids as constituents of the *B. burgdorferi* lipids [24]. It was not until recently that free cholesterol and cholesterol-glycolipids containing cholesterol (cholesteryl galactoside) were identified in *B. burgdorferi* [4].

The cholesterol and cholesterol-glycolipids constitute a significant portion of the spirochete's outer membrane. The lipid fraction of *B. burgdorferi* is composed of glycolipids and phospholipid. Two phospholipids, phosphatidylcholine and phosphatidylglycerol, account for 46.8% of the total lipids in *B. burgdorferi* [3,24]. The cholesterol-containing lipids, which include free cholesterol, cholesteryl-esters and cholesterol-glycolipids, constitute a significant portion, 40% [3], of the total lipid content [4,5,24,57-61]. The two cholesterol-containing glycolipids of *B. burgdorferi* have been identified as *B. burgdorferi* glycolipid I [Bb-GL-I] or acylated cholesterol galactopyranoside (ACGal) and cholesteryl-β-D-galacto-pyranoside (CGal) [3-5,24,58,62,63] (Figure 1.1). ACGal has multiple names because this glycolipid was identified and named by two different research groups [3-5,24,58,62,63]. Furthermore, depending on the acyl chain modification, the chemical name for ACGal can also differ. Published chemical names for ACGal are cholesteryl 6-O-acyl-β-D-galactopyranoside, or cholesteryl 6-O-palmitoyl-β-D-galactopyranoside [3-5,24,58,62,63]. In both ACGal and CGal, the cholesterol molecules

are linked to the galactose sugars by a β1-3 linkage [64]. The other known glycolipid in the membrane of *B. burgdorferi* does not contain cholesterol. This glycolipid is mono-α-galactosyl-diacylglycerol (*B. burgdorferi* glycolipid II [Bb-GL-II] or MGalD) [3,5,24,58,62,63] (Figure 1.1). MGalD accounts for 12.8% of the total membrane lipids [3]. *The presence of free cholesterol and the cholesterol-glycolipids in the lipid fraction of B. burgdorferi is unique. These glycolipids are major constituents of the total lipids of in B. burgdorferi.*

1.2 FEATURES OF *BORRELIA BURGDORFERI* THAT CONTRIBUTE TO THE TRANSFER OF ANTIGENIC GLYCOLIPIDS DURING LYME DISEASE

1.2.1 Lipid Rafts in *Borrelia burgdorferi*

The presence of cholesterol in prokaryotes is unusual so making prokaryotic membrane composition and structure not similar to eukaryotic membranes. However, using ultrastructural, biochemical, and biophysical analysis, we previously determined that the cholesterol and cholesterol-glycolipids in the outer membrane of *B. burgdorferi* are constituents of eukaryotic-like lipid raft domains (Figure 1.2) [6]. Since the first identification of eukaryotic-like lipid rafts in a prokaryote by our laboratory [6], microdomains that do not have cholesterol were identified in another prokaryote, *Bacillus subtilis* [65]. *B. subtilis* does not have cholesterol, this indicates that other factors, such as proteins, are maintaining the structure of the microdomains. Furthermore, the extent of the analysis was limited to identification and analysis of detergent-resistant domains, and did not include other biochemical analyses. *The lipid rafts of B. burgdorferi are unique in the prokaryotes in that their formation is dependent on the presence of cholesterol.*

In eukaryotic cell membranes, lipid rafts are microdomains that are rich in sterols, sphingolipids, and phospholipids with saturated acyl tails that allow for tight packing of these lipids into ordered domains [66,67]. These cholesterol-rich domains segregate from the disordered membrane domains that contain mostly unsaturated lipids [66,68]. In addition to the enrichment of specific lipids, lipid-anchored proteins such as glycosyl phosphatidylinositol (GPI) proteins and proteins covalently linked to saturated acyl chains are targeted to lipid rafts [69]. Lipid rafts for eukaryotes have been shown to be important for the segregation of plasma membrane proteins [66-68,70-73]. They have been identified as important dynamic platforms in cell signaling [68]. In addition to cell signaling and protein segregation, cholesterol and lipid rafts in membranes contribute to endocytosis, exocytosis, vesicle formation, and budding [74-78].

Lipid rafts appear to play an important role in the host's immune response to infection with *B. burgdorferi*. In *B. burgdorferi*, it was determined that the presence of cholesterol and therefore intact lipid rafts was necessary for the activity of complement-independent bactericidal antibodies. When the cholesterol was depleted and lipid rafts were disrupted, the bactericidal activity of the antibodies was abrogated [6]. Furthermore, in a previous study to visualize the lipid rafts at an ultrastructural level on the spirochetes, we used anti-asialo-GM1 antibodies. The lipids enriched in lipid rafts, the cholesterol-glycolipids, have a β1-3 linkage to the galactose. This β1-3 linkage is shared with ganglioside GM1 [79] and allowed us to use the anti-asialo-GM1 antibody to detect the *B. burgdorferi* cholesterol-glycolipids. In addition to using the anti-asialo-GM1 antibodies as a tool, we also have evidence that generation of cross-reactive antibodies is at least partially due to the shared β1-3 linkage at the galactose [6,79]. *The presence of free cholesterol and cholesterol-glycolipids with saturated acyl chains in B.*

burgdorferi results in the formation of eukaryotic-like lipid raft domains in the outer membrane. Unlike in most other prokaryotes, the lipid rafts in B. burgdorferi allow the membrane of this prokaryote to resemble that of higher eukaryotic organisms, which could facilitate interaction or transfer of material through lipid-lipid interactions. The lipid rafts of B. burgdorferi contain unique cholesterol-glycolipids, and in this dissertation we will demonstrate that lipid components enriched in the lipids rafts are transferred to eukaryotic cells.

1.2.2 Outer Membrane Vesicles of *Borrelia burgdorferi*

Shedding of outer membrane vesicles from Gram-negative bacteria is a constitutive process that occurs during normal growth. Several models of OMV biogenesis have been studied and proposed [80,81], but the exact mechanisms have not been fully determined. One mechanism of OMV biogenesis suggests that OMV production is not due to random membrane instability, but instead is a result of normal processes such as cell growth and division [81].This model would explain how OMV release is a process conserved by most Gram-negative bacteria [81]. Other mechanisms that have been proposed for natural release of OMV are centered on instability of the outer membrane of the Gram-negative bacteria that results in blebbing. These mechanisms that (i) a lack of peptidoglycan-associated lipoproteins results in faster expansion and blebbing of the outer membrane; (ii) accumulation of peptidoglycan in the periplasm induces blebbing; and (iii) imbalance of charges on the outer membrane results in repulsion and membrane blebbing [80]. *OMV release is a constitutive process shared by most Gram-negative bacteria. It is likely that increased membrane fluidity contributes to OMV release. In this dissertation, we will show that B. burgdorferi release OMV and that these released vesicles are important because they can transfer the antigenic cholesterol-glycolipids to host cells.*

Recently, there has been a marked increase in research on the pathogenicity of OMVs and virulence in the host [81-101]. The vesicles released by Gram-negative bacteria consist of outer membrane and periplasmic proteins, phospholipids, lipopolysaccharide, and glycolipids [85,100]. Other bacteria with similar membrane composition to *B. burgdorferi, Treponema* and *Helicobacter,* have specifically been shown to also release OMVs that are implicated in virulence [102-118]. The ability of OMV from pathogenic bacteria to participate in host-pathogen interactions as virulence factors has been well documented [100]. Furthermore, there is evidence that OMV from other bacteria can fuse with the cell membrane [119-121]. *Released OMV can carry virulence factors as cargo, or act as virulence factors themselves. We will demonstrate that OMV of B. burgdorferi are enriched with the antigenic cholesterol-glycolipids and that they can either attach or fuse with the plasma membrane of eukaryotic cells.*

Borrelia produce OMVs that have a role in pathogenesis. Compared to other Gram-negative bacteria, limited research has been conducted to understand the OMV of *Borrelia*. To date, *Borrelia* OMVs have been isolated and visualized from culture preparations [57,122-126]. The antigenic cholesterol-glycolipids have been shown to be significant components of the *Borrelia* OMV [127]. In addition, the *Borrelia* OMV have been studied to understand different protein complexes in the vesicles [128], studied *in vitro* to understand the interactions between vesicles with B or endothelial cells [129,130], identified from *in vivo* tissue samples [131], and investigated as possible vaccines [132,133]. *Bacteria of similar morphology and membrane composition release vesicles that have been implicated in virulence. We will show that the OMV of B. burgdorferi contain the appropriate outer membrane proteins and the antigenic cholesterol-glycolipids. Furthermore, these OMV are enriched with the cholesterol-glycolipids.*

We will show in this dissertation that there is a mechanism by which host cells acquire these antigenic spirochetal lipids via released OMV [6,125,127]. Direct interaction of vesicles containing antigenic lipids with eukaryotic cells during Lyme infections could contribute to pathogenesis.

1.2.3 Dissemination of *Borrelia burgdorferi*

For *B. burgdorferi* to disseminate from the local area of infection in the skin and colonize distant tissues, it is necessary for the bacteria to interact with and bind various host proteins, extracellular matrices (ECM), and cell types. Degradation of ECM can contribute to the inflammatory aspects of Lyme disease. One mechanism utilized by *B. burgdorferi* to degrade ECM and disseminate is by binding plasminogen. Plasminogen is a zymogen present throughout the mammalian host and is cleaved to become the active enzyme plasmin. *B. burgdorferi* can bind plasmin/plasminogen using lysine residues on spirochetal proteins [134,135]. Active plasmin on the surface of the spirochetes can allow for penetration through cell layers by degradation of multiple components of the ECM [134,136,137].

As the spirochetes disseminate, they bind and interact with multiple cell types and a variety of host ECM molecules. *B. burgdorferi* associates with collagen fibers [138,139], integrins [140,141], fibronectin [142,143], decorin, which is a collagen-binding proteoglycan [144,145], and binds to other non-decorin glycosaminoglycans [146-149].
Additionally, *B. burgdorferi* has also been shown to adhere to many different non-phagocytic cells in the mammalian host. *B. burgdorferi* adhere to endothelial cells [142,143,150-152], epithelial cells [145,146,153,154], and cells of neural origin [155,156]. *We will show that in addition to dissemination, adherence to eukaryotic cells and possibly ECM serves as an*

important mechanism for invading B. burgdorferi to acquire essential nutrients. We specifically demonstrate that cholesterol, a lipid that the spirochetes cannot synthesize but is necessary for growth and survival, is acquired through direct contact with epithelial cells. Furthermore, we will demonstrate that through attachment to eukaryotic cells, the spirochetes transfer antigenic glycolipids to host cells.

1.2.4 The Enzymatic Synthesis of the Cholesterol-Glycolipids

The mechanism by which the spirochetes acquire and synthesize the cholesterol-glycolipids using host-derived lipids is not understood. In another similar bacterium that contains cholesterol and cholesterol-glycolipids, *Helicobacter pylori,* the glycosyltransferase *hp0421* was shown to be the enzyme responsible for synthesis of the cholesterol-glycolipids by incorporating the cholesterol molecule [64]. Mutants lacking *hp0421* are unable to develop the appropriate glycolipids [64]. In another study, it was proposed that the cholesterol-glycolipids could be enzymatically synthesized by *H. pylori* as the bacteria directly interacted with the host cell [157]. Through the direct interaction of prokaryote-derived cholesterol-glycolipids at the contact sites, the raft and membrane organization in the eukaryotic cells were disrupted which allowed for optimal activity of bacterial secretion systems [157]. *These studies provided evidence that similar events of a two-way transfer of cholesterol could be occurring when B. burgdorferi adheres to host cells throughout infection.*

Genetic analysis of *B. burgdorferi* and *H. pylori* indicates that these bacteria have several homologous glycosyltransfereases. A homolog to the *H. pylori* glycosyltransferase in *B. burgdorferi, bb0454,* was studied for similar activity to *hp0421* [61]. However, unlike the *H. pylori* enzyme, *bb0454* is not responsible for incorporation of cholesterol into the cholesterol-

glycolipids [61]. Instead, *B. burgdorferi bb0454* was shown to contribute to synthesis of MGalD and is specifically responsible for glycosylation of 1,2-diacylglycerol [61]. It has been hypothesized that in addition to the activity of galactosyltransferase *bb0454*, other uncharacterized spirochetal transferases could be responsible for constructing the cholesterol-glycolipids [61]. *Cholesterol acquisition is a necessary process in B. burgdorferi pathogenesis that is not well understood. Multiple mechanisms to obtain essential lipids could exist. We will show that one mechanism for cholesterol acquisition by invading spirochetes is to obtain cholesterol directly from the plasma membrane of eukaryotic cells. This acquisition occurs in a contact-dependent mechanism that is similar to that of H. pylori [64]. In addition to cholesterol acquisition, we will provide evidence that supports a two-way transfer of cholesterol where the spirochete also transfers free cholesterol and cholesterol-glycolipids to host cells.*

1.3 PATHOGENESIS OF *BORRELIA BURGDORFERI*

1.3.1 Lyme Disease

Since its discovery, Lyme disease has become recognized as the most prevalent vector-borne infection in the United States [18,158,159]. The causative agent of Lyme disease, *B. burgdorferi*, is transmitted to mammals by hard-bodied ticks of the *Ixodes* genus (Figure 3.1) [1,2,160,161]. Humans are incidental hosts in the three-stage life cycle of the *Ixodes* ticks (Figure 1.3). For efficient transmission of the spirochetes, the tick must feed for approximately 48 hours. After transmission of spirochetes to the human host and an incubation period of 3-32 days, the most common manifestation, erythema migrans, develops [63]. The erythema migrans

is a reddish, bullseye-like skin lesion at the site of the tick bite [158,159]. Other manifestations include fatigue, fever, headache, arthralgia, and myalgia [158,159]. *B. burgdorferi* has tropism for tissues that include the joints, heart, and nervous systems [162]. In the mammalian host, the observed tissue damage is thought to be caused by the inflammatory response to the spirochetes [63]. The genome of *B. burgdorferi* is not known to encode toxins or any machinery to secrete virulence factors that could cause the inflammation [63]. However, not all infections have noticeable manifestations, as it has been shown that in endemic areas asymptomatic or subclinical infections can occur [163]. If untreated, *B. burgdorferi* is capable of causing long-lasting infection in humans [2,160,161].

The exact mechanism by which the spirochetes maintain the chronic infection is not well understood. In untreated individuals, *B. burgdorferi* may cause chronic neuroborreliosis and arthritis [162,164]. Possible manifestations of neuroborreliosis include lymphocytic meningitis with episodic headache and mild neck stiffness, cranial neuropathy that results in facial palsy (Bell's palsy), encephalopathy, axonopathy in the peripheral and central nervous system, cerebellar ataxia, and myelitis [162,165-170]. In most individuals, Lyme arthritis can be treated successfully with antibiotic therapy [164]. However, persistent, long-lasting arthritis has been shown to still exist in up to 10% of individuals treated with antibiotics [171]. One of the proposed hypotheses to explain persistent arthritis [162] and neuroborreliosis [165,172-174] is infection-induced autoimmunity. The significant amount of antibodies elicited by *Borrelia* infection could indicate that antibodies are at least partially responsible for the neurological and arthritic manifestations of Lyme disease [165,175]. This implicates a subset of antibodies specific for the cholesterol-glycolipids that have been shown to develop in late Lyme disease as a possible source of autoreactive antibodies [3,5]. *There seems to be a role for autoantibodies in*

chronic Lyme disease that has not yet been completely elucidated. The presence of cholesterol throughout eukaryotic cells and also in the antigenic glycolipids of Borrelia provides evidence that antibodies against the spirochete cholesterol-glycolipids could cross- react with host cells. Also, the antibodies specific for these glycolipids are only observed during late infections. Timing of this antibody production also corresponds with some of the noted manifestations of chronic Lyme disease. Thus, antibodies against the Borrelia cholesterol-glycolipids could contribute to autoimmunity and some of the clinical manifestations observed in chronic Lyme disease.

1.3.2 Antigenic Variation and Lyme Disease

One of the proposed contributors to chronic Lyme disease is antigenic variation. Over the course of infection, differential Osp by the invading *B. burgdorferi* occurs and the Osps appear to have different roles in the different stages of the disease [44]. In an unfed tick, *B. burgdorferi* largely expresses OspA and OspB, which are believed to be important in adherence to the tick midgut by the spirochete but are down-regulated in the mammalian host [44,45]. What remains unclear is why OspA and OspB expression increases again later in chronic infections, but it is not clear why. This increased expression can be partly explained by the finding that OspA can bind plasmin/plasminogen [46]. When the tick begins to feed, the spirochetes migrate from the midgut to the salivary glands where they are transmitted to the mammalian host. As the tick is feeding and the spirochetes are transmitted, the Osp expression dramatically changes. OspC is expressed at high levels, while OspA and OspB are no longer observed to be the predominant Osp on the surface of the spirochete [44,45]. OspC could be important during the migration from the midgut to the salivary gland as it has been shown to

bind to the tick salivary protein Salp15 [176]. Furthermore, like OspA, OspC can bind plasmin/plasminogen, which indicates that it could play a role in the mammalian infection [48]. *Changing the expression of the Osps, predominant surface antigens of B. burgdorferi, could serve to subvert the host immune response and contribute to chronic Lyme disease.*

In addition to the Osps, another surface-exposed antigen is the Vmp-like surface exposed (VlsE) antigen [177]. VlsE is similar to the variable major proteins (Vmps) of relapsing fever *Borrelia* that have been shown to be responsible for antigenic variation in these spirochetes. The VlsE is believed to contribute to chronic infections with *B. burgdorferi* by providing a mechanism of antigenic variation. The Vmps lead to peaks of spirochetes that are predominantly composed of spirochetes of one serotype. However, the antigenic variation mechanism of VlsE differs from the Vmps. The recombination of cassettes in VlsE antigenic variation does not cause one predominant serotype. Instead, different cassettes and different numbers of cassettes are randomly replaced during this antigenic variation mechanism, which leads to thousands of different variants at a given time [178,179]. VlsE has been shown to be targeted by the mammalian host immune system [180,181] and appears to be critical for infection [182]. The antigenic variation provided by VlsE could be an important mechanism that allows the *B. burgdorferi* to persist in the mammalian host for long periods of time.

1.3.3 Autoimmunity and Lyme Disease

Autoimmunity has long been believed to be a major contributor to pathogenesis of chronic infections. Due to the significant host antibody response during a chronic *Borrelia* infection, autoimmunity has been proposed to contribute to some of the manifestations of Lyme disease [183,184]. Infection with *B. burgdorferi* can lead to both acute and chronic

neuroborreliosis with diverse manifestations in patients [165-170,185]. The documented manifestations do not appear to be caused directly by the spirochetes themselves as *B. burgdorferi* are rarely observed in nervous tissue biopsies of patients in the nervous system in the mouse model [183,186]. This observation suggests autoimmunity may contribute to some of the clinical manifestations [183,186]. Furthermore, patients with neurologic manifestations of Lyme disease demonstrate cross-reactive antibodies to peripheral nerve axons [175] and other components of the central nervous system [187]. Also, intrathecal antibodies of multiple isotypes, IgG, IgM, and IgA, collected from cerebral spinal fluid were shown to be auto-reactive [187,188] Autoantibodies have been further implicated in contributing to pathology of the disease as IgM cross-reacts with gangliosides and antigenic non-protein or lipid fractions of *B. burgdorferi* [60,165,172,173]. Another example of cross-reactive antibodies during an infection with *B. burgdorferi* is that anti-OspA antibodies react with neurons found throughout the nervous system [189]. *One aspect that could contribute to the autoimmunity is the cross-reactivity observed between antibodies and the non-protein fraction of B. burgdorferi that is now known to be the cholesterol-glycolipids. Therefore, it is possible that antibodies to the cholesterol-glycolipids could contribute to the pathology of Lyme disease. Though it remains unclear, there seems to be a role for cholesterol-glycolipid-specific autoantibodies in the mechanism that contributes to chronic Lyme disease. In this dissertation, we demonstrate that the antibodies made against the lipids (non-protein antigens) of B. burgdorferi do react with eukaryotic cells in a cross-reactive manner.*

Approximately 10% of Lyme arthritis patients have a condition called Treatment-Resistant Lyme Arthritis (TRLA). In this condition, patients have continuous joint inflammation that is not associated with an active *B. burgdorferi* infection [190-192]. In TRLA, there is

evidence that antibodies specific to the invading spirochete's Osps, OspA, OspB, and OspC, are actively produced in chronic Lyme infections and can cross-react with endogenous molecules [156,165,174,175,183,187,189,193-197]. *Autoimmunity has been considered a contributor to the pathogenesis of chronic infections with B. burgdorferi. The significant antibody response to a Borrelia infection combined with cross-reactive antibodies indicates that autoimmunity could play a role in pathogenesis of Lyme disease. It is possible that the cholesterol-glycolipid-specific antibodies contribute to conditions like TRLA.*

1.4 IMMUNITY TO *BORRELIA BURGDORFERI* INFECTIONS

1.4.1 *Borrelia burgdorferi* and the Innate Immune Response

B. burgdorferi encounters the first host immune cells in the skin at the site of the tick bite. Recognition and elimination of the invading bacteria are normally carried out by both innate and acquired immune responses. Phagocytic cells and the complement system are two key parts of the innate immune response that clear a bacterial infection. *B. burgdorferi* directly interacts with professional phagocytes of the host immune system. As the spirochetes disseminate and establish infection, *B. burgdorferi* are opsonized by antibodies and ingested by macrophages and polymorphonucleaer leukocytes (PMNs) [198-201]. Antibodies were shown to mediate phagocytosis through their fragment crystallizable (Fc) region binding to Fc receptors on phagocytes [198,201]. *B. burgdorferi* are also ingested by these phagocytic cells independent of opsonization [198,199,201-203]. Once ingested, both oxygen-dependent and –independent mechanisms destroy the spirochetes [198-200] indicating that *B. burgdorferi* do not have any

mechanisms for survival within phagocytes. *It is clear that the B. burgdorferi come into direct contact with cells that make up tissues of the host and cells of the immune system as the bacteria disseminate throughout the host. Importantly, to demonstrate that transfer of antigenic cholesterol-glycolipids occurs and could contribute to the pathogenicity and persistence of the spirochetes, we used non-phagocytic cells because even without the presence of antibodies, B. burgdorferi are quickly ingested by macrophages and PMNs.*

Another arm of the innate immune response, complement, has three pathways, the classical, alternative, and mannan-binding lectin pathways. While each pathway's initiation events are different, they all result in either the formation of the membrane attack complex (MAC), opsonization, or inflammation of local tissue [204]. Complement-dependent antibody-mediated killing of *B. burgdorferi* was observed *in vitro,* and antisera from infected animals cannot mediate bacterial killing without the presence of complement *in vitro* [205-213]. However, complement itself was shown to not be a critical component of the innate immune response to *B. burgdorferi*, as clearance occurs in mice deficient in C3, C5, or C1q [214]. Furthermore, the spirochetes have been shown to utilize many different mechanisms that can inhibit complement deposition [215-223]. *With B. burgdorferi possessing multiple methods of avoiding the effects of complement, the antibody response against B. burgdorferi has greater significance in the resolution of a Lyme disease infection.*

1.4.2 *Borrelia burgdorferi* and the Adaptive Immune Response

1.4.2.1 The Role of B cells

As part of the humoral immune response, B cells play an important role in controlling Lyme disease. The B1 and B2 B cells, and their respective subsets, are the two cell types that are critical in the host response to *Borrelia* [204,224]. The B1cell subsets, B1a and B1b, are self-renewing, T cell-independent, and responsible for natural IgM production [204,224,225]. Follicular (FO) B cells, a subset of B2 B cells, are the classical B cell type which is dependent on T cell help, specifically T_H2 help, for production of antibodies [204,226]. The other B2 subset, marginal zone (MZ) B cells, are similar to the B1 subsets in that they are T cell-independent and secrete IgM; however, they are not self-renewing [204,227-232]. Over the course of a *Borrelia* infection, the B cell response is both T cell-dependent and T cell-independent.

In the T_H2 response, B cells and antibodies play an important role in resolution of the infection, as well as protection. In the human infection, $CD4^+$ T cells contribute to the T_H1 response [233-237]. Infected C3H/HeN mice develop a strong T_H1 cell-mediated immunity where the predominant cytokine elicited is IFN-γ accompanied with severe, long lasting arthritis [238-240]. In BALB/c mice, T cells elicit an interleukin-4 (IL-4) response (responsible for class switching, somatic hypermutation, and affinity maturation) with less severe disease that is usually resolved, consistent with a T_H2 immune response [238,239,241]. Additionally, the antibody subclass response that these two strains of mice produce is different. In BALB/c mice there is a higher ratio of IgG1 over IgG2a, when compared to C3H/HeN mice [239-242]. Despite the fact that there is still a cellular response acting as a first line of defense in BALB/c mice, the FO B cell subset is the subset responsible for elimination of the spirochetes [238,243].

Mice which elicit a T_H2 immune response, such as BALB/c mice, manage *Borrelia* infections more effectively than C3H/HeN mice, which utilize the T_H1 response. This observation indicates the ability and importance of antibodies to protect against and resolve Lyme disease.

T cells are needed for B cells to produce antibodies during an immune response to a *Borrelia* infection; yet, B cells alone can successfully resolve a Lyme disease or relapsing fever infection in the absence of T cell help. Evidence of B-cells providing protection against a *B. burgdorferi* infection was observed by presensitized B-cells having the ability to protect a severe combined immunodeficiency (SCID) mouse [244]. A single exposure to *B. burgdorferi* sonicate in mice was enough to confer protection against subsequent infection challenges. The cytokines elicited from the subsequent challenge did not include IL-4, a T_H2-associated cytokine [245]. Additional evidence was shown in mice which are unable to carry out T cell help [246]. These mice showed no difference in arthritis or remission when compared to control mice [246]. Furthermore, sera from these mice were able to protect SCID mice [246,247] . Also, mice deficient in all T cells were shown to have no defect in resolving arthritis and carditis when compared to immunocompetent controls and sera from the these mice were able to protect naïve mice [248]. The significance of T cell-independent B cell responses in a *Borrelia* infection was further observed through the prevalence of different levels of response of B cell subtypes. An infection with *B. burgdorferi* caused a related expansion of MZ B cells as well as an increase in IgM plasmablasts, but neither B2 subset, which are both dependent on T cell help, were responsive [249]. Similar to the MZ B cells, the B1a subset, the source of natural IgM, and the B1b subset, believed to be the source of IgM that confers long-term immunity, also expand during *B. burgdorferi* infection [249]. *B cells have an important role in clearance of infections*

with B. burgdorferi, as T cells are not required for resolving manifestations like arthritis and carditis.

1.4.2.2 The Role of Antibodies

As an extracellular pathogen, *B. burgdorferi* is susceptible to antibodies either directly or through opsonization. The first demonstration of the importance of antibodies on clearance of *B. burgdorferi* was the ability of passively transferred serum from an immune rabbit to confer protection [250]. Since then, mouse studies have shown that antibodies alone could protect SCID mice [251-255]. Furthermore, monoclonal antibodies and immune sera against different *Borrelia* antigens are protective [252-260]. Other bactericidal antibodies directed at the surface of the spirochetes, the Osps in *B. burgdorferi,* have been investigated [205,207,208,212,261,262]. In these studies, the OspA, OspC, Omp66, and DbpA antigens caused the production of protective antibodies [263-275]. Furthermore, a Lyme disease OspA subunit vaccine was developed, but rather than provide immunity in the host, the antibody targeted the spirochetes by blocking their transmission to the mammalian host when the blood meal was taken up by the tick [263,276,277]. In addition to understanding the bactericidal antibodies and the immunity they provide, it is important to recognize the essential role antibodies play in the process of opsonization and phagocytosis of the spirochetes [198,201,278-285]. This process is of great importance in clearing an infection because *Borrelia* has no known mechanisms to evade phagocytes.

Complement-independent bactericidal antibodies against *B. burgdorferi* are a unique feature of the host response to this infection. CB2, of the IgG1 antibody subclass and H6831 of the IgG2a subclass, kill spirochetes in the absence of complement [261,262,286]. Fragment

antigen-binding (Fab) regions of CB2 and H6831 were bactericidal *in vitro* demonstrating that agglutination was not necessary [261,287]. The CB2 antibody causes damage to the spirochete that includes increased polar membrane blebbing, destabilization, and destruction of the outer membrane leading to lytic death of the spirochete [261,288]. The mechanism whereby CB2 causes lytic death is dependent on the cholesterol and cholesterol-glycolipids in the outer membrane [6,20]. Without the free cholesterol and cholesterol-glycolipids CB2 is no longer bactericidal [6]. *Given the unique requirement for the cholesterol-glycolipids in the mechanism of complement-independent bactericidal killing by CB2, we decided to investigate other roles for these lipids during infection. We will show that in addition to their role in CB2-mediated killing, the antigenic cholesterol-glycolipids are transferred to eukaryotic cells.*

1.4.2.3 Antibodies to the Cholesterol-Glycolipids

Throughout a *B. burgdorferi* infection, the humoral arm of the host immune system generates antibodies against not only protein antigens, but also lipid antigens of the invading pathogen. The cholesterol-glycolipids and lipids in the outer membrane of *B. burgdorferi* are antigens recognized by the host immune system. These antigens are recognized either in the whole bacteria or as released OMV. Development of anti-glycolipid antibodies is a feature of late-stage Lyme disease [3,5,58,62,289]. These antibodies against nonprotein components of *B. burgdorferi* were first detected in thin-layer chromatography analysis of lipid or glycolipid fractions of the bacteria [60]. The glycolipids of *B. burgdorferi* have been shown to elicit a strong antibody response [3-5,58,60,289]. ACGal, a major component of the *B. burgdorferi* lipid fraction, was shown to be the target of highly specific antibodies in 80% of patients in the late stages of Lyme disease [3,4,62]. Furthermore, chemoenzymatic synthesis and immunoblotting

of a glycolipid library based on the structure of ACGal was used to determine the epitope for these antibodies [290]. It was demonstrated that a galactose, a cholesterol, and a fatty acid with a minimal chain length of four carbon atoms is the essential structure for recognition by antibodies [290]. This indicates that the epitope is most likely a three-dimensional conformation or structure in the molecule. In addition to ACGal, other glycolipids of *B. burgdorferi* are antigenic. However, another antigenic *Borrelia* glycolipid, MGalD, was not as strong as ACGal because only 20% of patients with late stage Lyme disease develop reactive antibodies [3]. Development of antibodies to glycolipids in chronic Lyme disease also results in IgM antibodies that are cross-reactive against gangliosides [60,172,184]. *The cholesterol-glycolipids found in the membrane are important antigens that influence the host immune response. Antibodies to the Borrelia glycolipids appear to be important during late infection. Because the antigenic cholesterol-glycolipids contain cholesterol, a sterol found in eukaryotic cells, we believe this similarity could elicit cross-reactive antibodies during Lyme disease. We will show in this dissertation that the antigenic cholesterol-glycolipids are deposited in or on the surface of eukaryotic cells. The antigenic glycolipids on the surface of eukaryotic cells could serve to directly influence host immune factors to target "self" or elicit cross-reactive antibodies that are specific for components of the surface of host cells.*

1.4.3 iNKT Cells At The Immune Crossroads

Invariant natural killer T cells (iNKT cells) influence the host response to *B. burgdorferi*. iNKT cells are a subset of lymphocytes that straddle the innate and humoral immune systems, and their role in the host response to invading pathogens is not well understood. These cells express surface receptors that are characteristic of T and NK cells, but are unable to develop

immunological memory. iNKT cells express a T cell receptor (TCR) that only reacts with lipid or glycolipid antigens that have been presented by the major histocompatibility complex (MHC) class 1-related molecule CD1d. Activation of iNKT cells is accomplished by engaging its TCR with the glycolipid antigens presented in the CD1d molecules. Also, iNKT cells are activated through an indirect pathway by which activation of antigen-presenting cells occurs through toll-like receptors or cytokine signaling in conjunction with endogenous glycolipid antigens presentation by the CD1d molecule [291-293]. Within hours following antigen presentation, iNKT cells become activated and secrete many cytokines which include IL-2, IFN-γ, and IL-4 and are capable of driving a T_H1 or T_H2 immune response [293-298]. Additionally, rapid proliferation of iNKT cells has been observed following activation [293]. Secretion of IFN-γ by iNKT cells can lead to strong activation and recruitment of macrophages and neutrophils [293]. This activation is not exclusive to cells which contribute to the inflammatory response, for in chronic experimental infection models iNKT cells also activate conventional T lymphocytes which leads to a T_H2 bias in the host immune response [293]. Therefore, iNKT cells are important cells that mediate the crosstalk between the innate and adaptive immune systems. However, because the iNKT cells are similar to other innate immune cells, they cannot form a memory response [293]. Instead, during subsequent experimental challenges, the secondary response of iNKT cells to experimental antigens is significantly weaker than the primary response[293]. The iNKT cells become anergic and display hyporesponsiveness for up to 2 months, which is manifested by a blocking of proliferation and cytokine production [293].

MGalD bound to CD1d directly activates iNKT cells [296-298] resulting in proliferation and secretion of IL-4 and IFN-γ [296-298]. However, in murine *B. burgdorferi* infection models, the number and activation of iNKT cells vary, a phenomenon that is not well understood. CD1d

$^{-/-}$ C57BL/6 mice have increased bacterial burden and joint inflammation, which implicates iNKT cells in mediating host defense against *B. burgdorferi* [299]. Also, in C57BL/6 mice, the iNKT cells were activated by macrophages and localized to the heart [300]. The iNKT cells secreted IFN-γ and decreased the severity of the Lyme carditis [300]. However, BALB/c mice that were deficient in iNKT cells exhibited increased persistent joint inflammation, impaired bacterial clearance, and elevated *Borrelia*-specific antibody titers [301]. *It is clear that iNKT cells play a significant role in influencing the balance of the host response, T_H1 or T_H2, to invading pathogens. However, the role of these cells during a Borrelia infection, particularly in the context of antigen presentation of cholesterol-glycolipids and modulation of the immune response, has yet to be completely characterized. Although we have not worked with iNKT cells in this dissertation, their role in recognizing and mounting an immune response to antigenic glycolipids could be a major area of future research.*

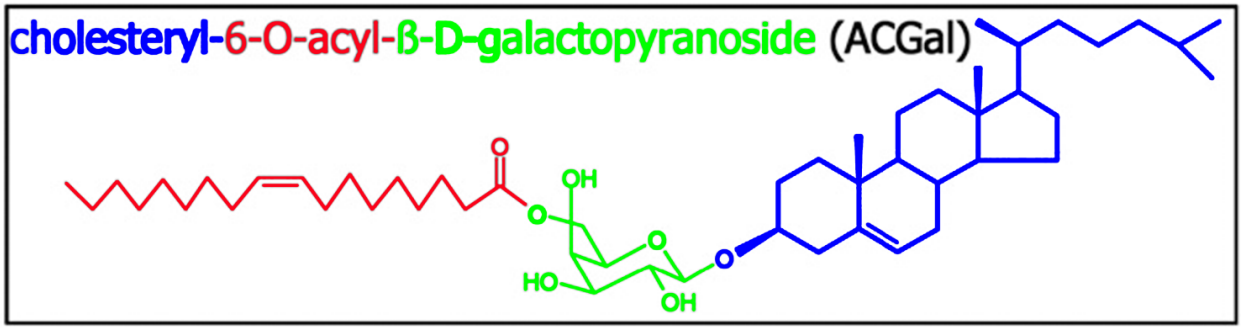
1.5 HYPOTHESIS AND RATIONALE

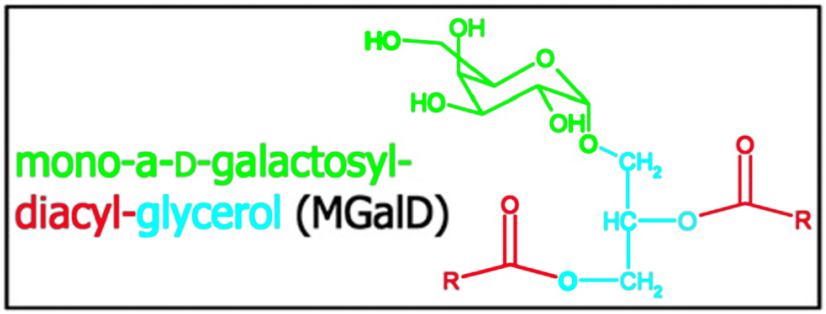
For this dissertation, the exchange of lipids between *B. burgdorferi* and eukaryotic cells was examined. This project was undertaken with the hypothesis that *B. burgdorferi* lipid antigens are transferred to host cells by both contact-dependent and - independent mechanisms of lipid complexes that contributes to the pathogenesis of Lyme disease. Therefore, this project and its results were broken down into two sections that consider these important events. The first section, **Chapter 2**, examines the two-way exchange of lipids between *B. burgdorferi* and eukaryotic cells. In this chapter we specifically examine the how the spirochete-derived lipid antigens are deposited on the membrane of eukaryotic cells. Transfer of the antigenic

cholesterol-glycolipids is of particular interest in these studies as they have been previously implicated in Lyme disease [3,5]. In this section we also consider the other side of the exchange that delves into how *B. burgdorferi* acquires cholesterol. Section 2 (**Chapter 3**) examines the cross-reactivity of antibodies made to the glycolipids of *B. burgdorferi.* Here, we assess the ability of these antibodies specific for prokaryotic antigens to cross-react with eukaryotic cells. *These antibodies could be directed at lipid antigens on the spirochetes directly, or lipid antigens transferred to eukaryotic cells. Together, these results identify mechanisms that contribute to pathogenicity of B. burgdorferi and allow the spirochetes to persist in the host. These mechanisms had previously not been considered, but fit with both the unique characteristics of B. burgdorferi as a pathogen and clinical manifestations in the host.*

1.6 FIGURES AND TABLE

Figure 1.1: The glycolipids of *B. burgdorferi.* Different components of the glycolipids are color coded for identification: cholesterol molecules are depicted in **dark blue**, galactose in **green**, acyl chains in **red**, and glycerol in **light blue.**


cholesteryl-6-O-acyl-ß-D-galactopyranoside (ACGal)
OH
O
HO
OH


mono-a-D-galactosyl-
diacyl-glycerol (MGalD)
HO
OH
O
HO
OH
CH2
HC
O
R
R
O
CH2

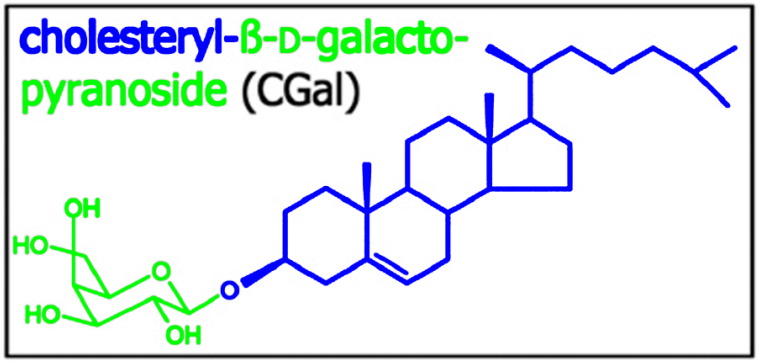

cholesteryl-ß-D-galacto-
pyranoside (CGal)
OH
HO
O
HO
OH

Figure 1.2: Lipid rafts in *B. burgdorferi.* *B. burgdorferi* lipid rafts composed of cholesterol-glycolipids (6 nm colloidal gold) at 33˚C. A rabbit polyclonal antibody to GM1 was used to detect the cholesterol-glycolipids of *B. burgdorferi.* Colloidal gold was coupled to a goat anti-rabbit secondary antibody which was used to detect anti-GM1. Scale bar equals 100 nm. Reprinted with permission from [6].

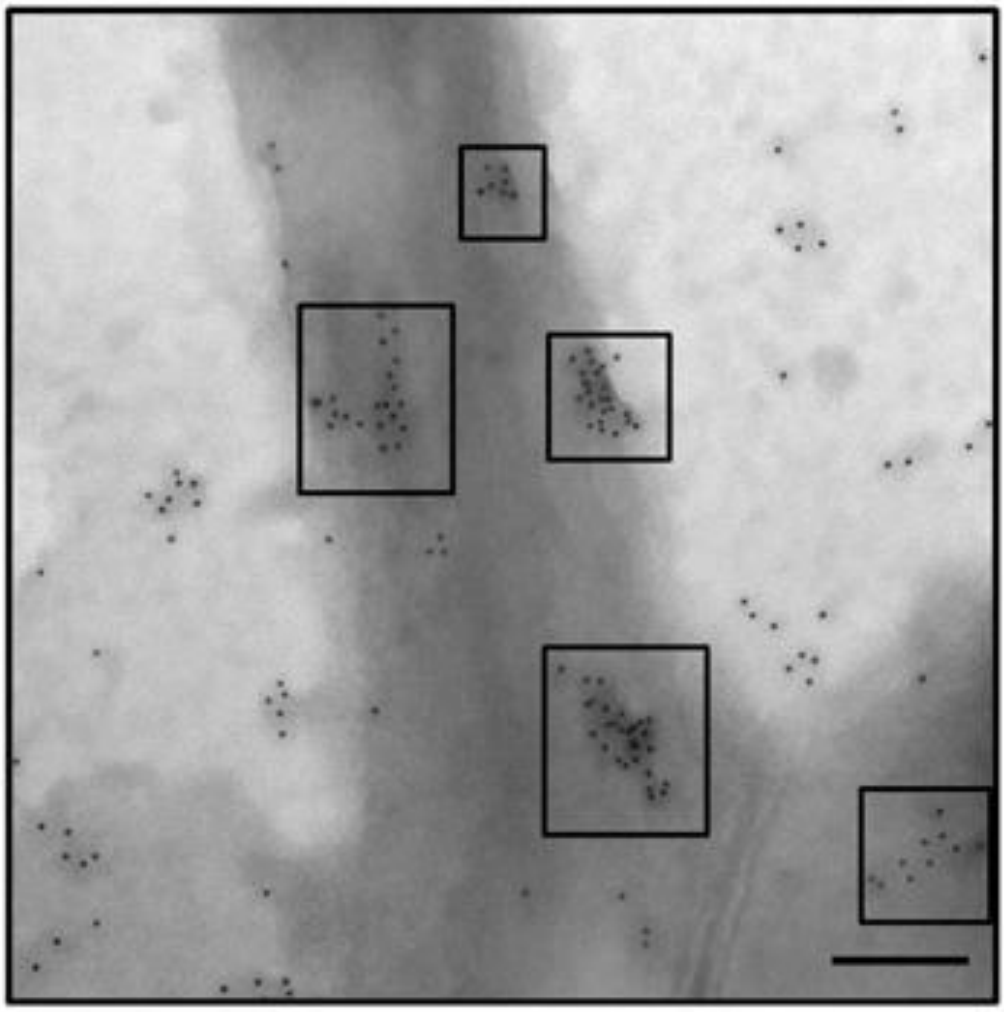


Figure 1.3: The enzootic cycle of *B. burgdorferi.* *I. scapularis* has a three-stage life cycle where the tick has one blood meal per stage. Larva and nymphs feed on small rodents while deer are important hosts for the adult ticks. Humans are incidental hosts and are not required to complete the life cycle of the tick. Humans are mostly infected through the bites of nymphs in the spring or summer months. Adult ticks can also transmit Lyme disease bacteria, but they are much larger and more easily discovered. Reprinted with permission from [63].

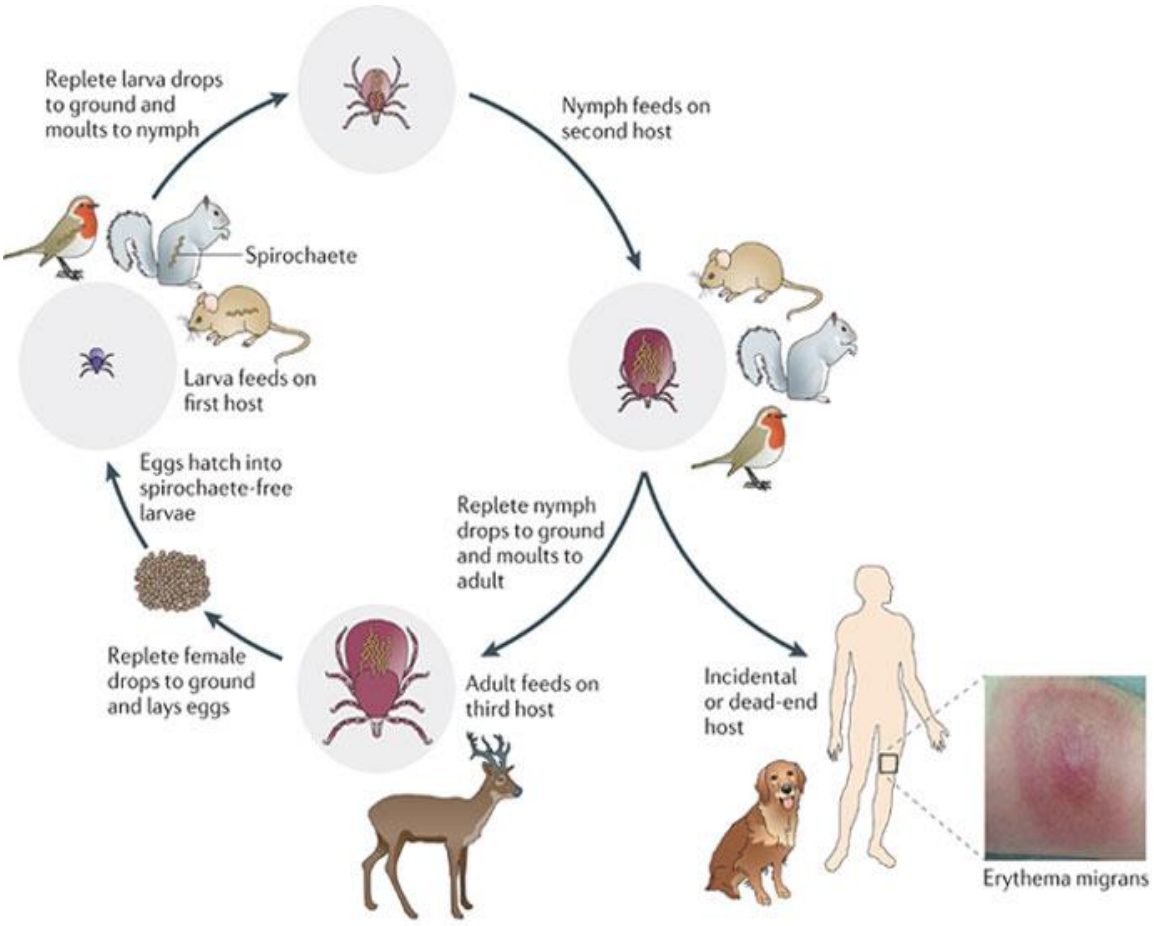


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Table 1.1: Species of *Borrelia* that cause Lyme disease throughout the world. Shown are the tick-borne agents of Lyme disease, the specific tick vector, geographical location, and pathogenicity.

	Species	Tick Vector	Location
Pathogenic	*Borrelia burgdorferi*	*Ixodes scapularis*	North America
		Ixodes pacificus	North America
		Ixodes ricinus	Europe
	Borrelia garinii	*Ixodes ricinus*	Europe
		Ixodes persulcatus	Asia
	Borrelia afzelii	*Ixodes ricinus*	Europe
		Ixodes persulcatus	Asia
Minimal/Non Pathogenic	*Borrelia andersonii*	*Ixodes dentatus*	North America
	Borrelia bissettii	*Ixodes spinipalpis*	North America
		Ixodes pacificus	North America
	Borrelia valaisiana	*Ixodes ricinus*	Europe and Asia
	Borrelia lusitaniae	*Ixodes ricinus*	Europe
	Borrelia japonica	*Ixodes ovatus*	Asia
	Borrelia tanukii	*Ixodes tanukii*	Asia
	Borrelia turdae	*Ixodes turdus*	Asia
	Borrelia sinica	*Ixodes persulcatus*	Asia

CHAPTER 2 – MATERIALS AND METHODS

Bacteria, Cultures, and Loading of Fluorescent and Radioactive Cholesterol Analogs

B. burgdorferi strain B31 were grown in microaerophilic conditions in BSK-II medium [302] supplemented with 6% rabbit serum (Sigma) at 33˚C. For the incorporation experiments, BSK-II without free cholesterol was made by using cholesterol free CMRL-1066 (Invitrogen). Removal of cholesterol from the CMRL-1066 eliminated the free cholesterol found in BSK-II (0.2 µg/mL). When the BSK-II was supplemented with 6% rabbit serum, the final concentration of cholesterol in the cholesterol-depleted BSK-II was 0.78 µg/mL. The environment sensitive fluorescent cholesterol analog 22-(*N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino)-23,24-Bisnor-5-Cholen-3β-Ol (NBD-cholesterol, Invitrogen), or 23-(dipyrromethene boron difluoride)-24-norcholesterol (TopFluor Cholesterol or BODIPY-cholesterol, Avanti Polar Lipids), was added to BSK-II without free cholesterol for 6 hrs at a concentration of 0.2 mg/L, 2.0 mg/L, and 4.0 mg/L. After the 6 hr incubations, the bacteria were washed three times with Hank's balanced salt solution (HBSS, Gibco). Fluorescence readings were calculated using a SpectraMaxM2 (ex/em - 490/504). The fluorescently labeled *B. burgdorferi* were used immediately for all assays. Spirochetes were also assessed for their ability to replicate after treatment with BODIPY-cholesterol. Labeled *Borrelia* were reintroduced into BSK-II medium and allowed to grow. Spirochetes were enumerated every 24 hrs by dark field microscopy to assess their ability to replicate.

B. burgdorferi were also labeled with ^{3}H-cholesterol (American Radiolabeled Chemicals, Inc.). Spirochetes were washed three times with HBSS and the endogenous cholesterol was depleted using 10 mM methyl-β-cyclodextrin (MβCD). These conditions were previously optimized [6]. After the depletion, the cholesterol was replaced by incubating the spirochetes

with 4.0 mg/L of cholesterol and 10.0 µCi of ^{3}H-cholesterol in HBSS. *B. burgdorferi* were washed three times with HBSS and were immediately used for all assays.

Lipid Extraction, Purification, and Analysis

B. burgdorferi were washed three times in large volumes of HBSS. The lipids were extracted using the Bligh and Dyer method [303]. The lipid extracts were concentrated under constant nitrogen gas stream. Lipid extracts were resolved by thin-layer chromatography on Si250 High Performance Thin Layer Chromatography (HPTLC) silica plates (J.T. Baker) with a chloroform/methanol (85/15) mobile phase. The components of the lipid extract were visualized with iodine vapor staining or exposure to UV light. Lipid extracts from samples containing ^{3}H-cholesterol were separated by HPTLC and stained with iodine vapor using a similar protocol. However, the spots on the plate were scraped and the silica containing the radioactive cholesterol was analyzed by liquid scintillation counting. For direct visualization of the HPTLC plate, the plate was first sprayed with EN^{3}HANCE Spray (DuPont), exposed using BioMax MR Film (Kodak) for 4 and 14 days at -80˚C, and developed using a Medical Film Processor Model SRX-101A (Konica).

Optimization for Fluorescent Labeling of *B. burgdorferi* Membrane

B. burgdorferi labeled with BODIPY-cholesterol were analyzed by the SpectraMax M2 plate reader (ex/em - 490/504) for incorporation of fluorescence. The viability, morphology, and motility of the BODIPY-cholesterol-treated *B. burgdorferi* were assessed by dark-field enumeration and fluorescence microscopy. The lipids of *B. burgdorferi* labeled with BODIPY-cholesterol were isolated and resolved on an HPTLC Si250 silica plate with chloroform-

methanol (85/15) and exposed to UV light and stained with iodine vapor for visualization of the lipids. Standards were known R_f values from identical solvent systems [3,5,6]. *B. burgdorferi* incubated with BODIPY-cholesterol were also analyzed for their ability to grow or recover following incorporation of the fluorescent label. Labeled *B. burgdorferi* were washed in HBSS, and resuspended in BSK-II medium, and growth was assessed by dark-field enumeration.

B. burgdorferi Lipid Transfer Assay

HeLa cells were maintained in DMEM medium (Gibco) with 10% fetal calf serum (Pel-Freez). The HeLa cells were grown on glass coverslips in T175 tissue culture flasks (Falcon). Cells were infected with *B. burgdorferi* labeled with BODIPY-cholesterol at a multiplicity of infection (MOI) of 20:1 for 2 hrs. Conditioned medium and negative control cell-free wash supernatant were also added to the coverslips for 2 hrs. Conditioned medium supernatants were generated by incubating *B. burgdorferi* labeled with BODIPY-cholesterol in BSK-II for 2 hrs. The spirochetes were pelleted by centrifugation at high speed for 15 min and the supernatants (cell-free) were examined by dark-field and fluorescent microscopy to ensure that intact organisms were not present. The supernatants were added directly to the HeLa cells for 2 hrs. The cell-free wash supernatant was included as a negative control to ensure that the BODIPY-cholesterol is not loosely associated with the OM or released nonspecifically from *B. burgdorferi* as there was no observable transfer of label. The negative control or cell-free wash supernatant was generated by resuspension of the labeled *B. burgdorferi* pellet after the final wash in BSK-II. This control was included to account for spontaneous, quick release of the probe and lysis of the bacteria during the spin because at higher speeds, *B. burgdorferi* can be significantly disrupted. The coverslips were washed three times in phosphate buffered saline (PBS, Gibco), fixed in

2.5% paraformaldehyde and blocked with 1% bovine serum albumin (Sigma) in PBS for immunofluorescence staining. The remaining *B. burgdorferi* that were attached to the HeLa cells were detected with CB2, a murine monoclonal antibody to OspB [261] followed by an Alexa Fluor 594 goat anti-mouse IgG (Invitrogen).

The samples analyzed for BODIPY-cholesterol colocalization with the cis-Golgi complex were probed with a monoclonal rabbit GM130 antibody (Abcam) followed by a Texas Red goat anti-rabbit IgG (Abcam). Samples were imaged by confocal laser microscopy using a Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope System. Additional approaches were used to detect lipid exchange between *B. burgdorferi* and cells. Relative fluorescence intensity (RFI) of the HeLa cells from 10 microscope fields of vision were calculated using Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope System Software. The mean geometric fluorescence was calculated from samples that were analyzed by a FACScan/Calibur flow cytometer for BODIPY fluorescence.

For experiments using radiolabeled *B. burgdorferi,* HeLa cells were incubated with *B. burgdorferi* labeled with 10.0 μCi ^{3}H-cholesterol at a MOI of 40:1 for 2 hrs. The HeLa cells were extensively washed to remove the spirochetes. HeLa cells were lifted from the tissue culture flasks using 0.05% Trypsin/EDTA (Gibco). Transferred radioactivity to the HeLa cells was detected by extracting the lipids using the Bligh and Dyer solvent extraction method. Lipids were separated on a HPTLC plate using chloroform-methanol (85/15). The HPTLC plate was stained with iodine and spots on the plate were scraped. In addition, incorporation of isotope into the lipids was measured by scraping the silica from the HPTLC plate, analyzed by liquid scintillation using a Beckman LS 6500 Liquid Scintillation Counter and reported as disintegrations per minute (DPM).

To control for *B. burgdorferi* that remained attached to HeLa cells after washing, *B. burgdorferi* that constitutively expressed green fluorescence protein (GFP) [304] was utilized. The GFP-expressing *B. burgdorferi* were incubated with HeLa cells at a MOI of 40:1 for 2 hrs. The HeLa cells were extensively washed to remove the spirochetes. HeLa cells were lifted from the tissue culture flasks using 0.05%Trypsin/EDTA (Gibco). Using the SpectraMaxM2, the amount of GFP fluorescence (*B. burgdorferi* still attached to HeLa cells) was measured in the HeLa cell pellet.

For experiments designed to determine if actin polymerization was required for the transfer of the *B. burgdorferi* derived lipids, the HeLa cells were treated with cytochalasin D (Sigma). The pretreatments were for 1 hour at 50 μg/mL, 10 μg/mL, and 1μg/mL. The cells were washed with PBS and the transfer assay was conducted. To assess levels of cytotoxicity caused by the cytochalasin D treatment or the transferred lipids, a lactate dehydrogenase (LDH) Release Assay (Promega) was used following the manufacturer's instructions.

Quantification of Released BODIPY-cholesterol from *B. burgdorferi*

For detection of released membrane material, labeled *B. burgdorferi* and unlabeled *B. burgdorferi* were incubated in HBSS for 2 hrs. The supernatants were collected at 1 hr and 2 hrs. The supernatants were labeled with 1 μg/ml of the hydrophobic-sensitive, fluorescent, lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (Invitrogen) for 30 min at 33˚C. After 20 min, the supernatants were analyzed for fluorescence in a SpectraMax M2 plate reader using an excitation of 360 nm and emission of 430 nm.

To measure the levels of overall sterol release from the labeled spirochetes, BODIPY-cholesterol was added to cholesterol free medium at concentrations of 0.2 μg/mL or 4.0 μg/mL

which are the concentrations of documented free cholesterol in BSK-II and 20X the amount of cholesterol in BSK-II, respectively. *B. burgdorferi* were labeled with 0.2 µg/mL or 4.0 µg/mL of BODIPY-cholesterol for 4 hrs. After the labeling, the spirochetes were incubated in HBSS, and the supernatants were collected at 1 hr and 2 hrs. BODIPY-cholesterol fluorescence of the supernatants was measured using the SpectraMax M2 plate reader. The same supernatants were analyzed for total cholesterol using the fluorometric Amplex Red Cholesterol Assay Kit (Invitrogen). The supernatants from the *B. burgdorferi* labeled with 4.0 µg/mL BODIPY-cholesterol were compared against supernatants from bacteria labeled with 0.2 µg/mL BODIPY-cholesterol.

Vesicle Isolation and Purification

B. burgdorferi labeled with BODIPY-cholesterol or unlabeled *B. burgdorferi* in the late-log phase of growth were pelleted by centrifugation, and resuspended in fresh BSK-II medium. To keep similar incubation times as the transfer assay, the labeled and unlabeled spirochetes were incubated for 2 hrs at 37°C to collect released vesicles. Following the removal of spirochetes, the supernatants were filtered twice using 0.22 µm-pore-size Steriflip filters (Millipore). Crude membrane and outer membrane vesicles (OMV) in the filtered supernatant were concentrated by ultracentrifugation for 1 hr at 100,000 x g. To purify the OMV, the membrane pellet was resuspended in 60% Optiprep (Axis Shield). A discontinuous gradient was made following the manufacturer's instructions. The discontinuous gradient was centrifuged for 16 hrs at 100,000 x g. The OMV were concentrated to form a white band that floated to the interface between the 20% layer and the 25% layer of the gradient. The gradient fractions were collected in 1 mL volumes (two 1 mL fractions for each Optiprep gradient percentage) from the

top of the tube with the least dense faction being collected first. The OMV from fraction 15%, 20%, and 25% were pooled based on similar protein contents to maximize the amount of OMV collected [125]. The isolated OMV were concentrated and removed from the Optiprep solution by centrifugation for 1 hr at 100,000 x g. The pelleted, purified OMV were resuspended in 20 mM HEPES (pH 7.5). The OMV were immediately used for vesicle transfer assays. Confirmation of the incorporation of BODIPY-cholesterol was conducted by measuring the fluorescence using the SpectraMax M2. Protein analysis of the isolated vesicle fractions was also conducted. Vesicle fractions were run on 12.5% acrylamide SDS-PAGE. For analysis of total protein content, the gels were stained with Coomassie Brilliant Blue R-250. Other samples were transferred to 0.4 5µm nitrocellulose membranes (GE Healthcare), and probed with CB2 (anti-OspB) [261], CB10 (anti-OspA), CB1 (anti-flaB) [261], CB312 (anti-dnaK), polyclonal rabbit anti-OspC, and monoclonal antibody 240.7 (anti-lp 6.6) [305]. Samples were detected using the appropriate secondary antibody – goat anti-mouse IgG (H+L) IRDye 800CW (Rockland) or goat anti-rabbit IgG (H+L) IR Dye 700CW (Rockland) and scanned using the Odyssey infrared scanner (LI-COR Biosciences) in the 800 and 700 channels.

B. burgdorferi Outer Membrane Vesicle Transfer Assay

HeLa cells were grown on glass coverslips in 24 well tissue culture plates. Cells were incubated with 40 µg (based on protein content) of vesicles purified from BODIPY-cholesterol labeled *B. burgdorferi* for 2 hrs. Protein content was determined by bicinchoninic acid (BCA) Assay and a Coomassie Plus Assay (Pierce). The HeLa cells were washed with HBSS to remove the vesicles. To label the plasma membrane of the HeLa cells, the coverslips were then incubated with Vybrant Cell Labeling Solution DiI (Invitrogen) following the manufacturer's

instructions. The coverslips were fixed with 2.5% paraformaldehyde and were imaged by confocal laser microscopy using a Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope System.

B. burgdorferi Cholesterol Extraction from Epithelial Membranes

HeLa cells were grown on glass coverslips in 24 well tissue culture plates. The HeLa cells were preloaded with 5 µg/mL of BODIPY-cholesterol for 2 hrs. To remove excess BODIPY-cholesterol, the cells were washed twice with 5 mg/mL of methyl-β-cyclodextrin (MβCD, Sigma) and one final time with BSK-II. HeLa cells labeled with BODIPY-cholesterol were incubated in BSK-II for 1 hr, before the supernatants and cells were measured for BODIPY-cholesterol fluorescence using the SpectraMax M2.

To observe the cholesterol extraction from HeLa cell membranes, the cells were infected at an MOI of 40:1 for 1 hr, washed 3 times with PBS, and fixed with 2.5% paraformaldehyde for 15 min. *B. burgdorferi* were detected by incubation with CB2 hybridoma supernatants [261] followed by an Alexa Fluor 594 goat anti-mouse IgG (Invitrogen). The cells were imaged by confocal laser microscopy using a Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope System.

Flow Cytometry

Flow cytometry was conducted at the Stony Brook Flow Cytometry Facility using a FACScan/Calibur flow cytometer using the 488nm laser. A minimum of 10,000 gated events were used in calculation of the mean geometric fluorescence for each replicate.

Transmission electron microscopy

To detect the native organization of cholesterol glycolipids and OspB in the *B. burgdorferi* OMV, the OMV were adhered to transmission electron microscopy (TEM) grids, fixed in 1% glutaraldehyde, washed, and blocked as described previously [6,20]. The cholesterol glycolipids were detected by adding anti-asialo GM1 in 1% BSA diluted 1:50 to the grids for 1 h followed by washing [20]. Immediately after the washes, the primary anti-asialo GM1 antibody was detected with an anti-rabbit IgG conjugated to 6 nm colloidal gold (Jackson Immunochemicals) in 1% BSA (1:250). This antibody was incubated with the grid for 1 h. On the same grids, OspB was detected by placing the grids in CB2 diluted 1:100 for 1 h followed by washes and incubation for 1 h with anti-mouse IgG conjugated to colloidal gold diluted 1:250 (18 nm, Jackson Immunochemicals). The OMV were stained with phosphotungstic acid (PTA) [20]. The grids were analyzed using a FEI BioTwinG^2 Transmission Electron Microscope.

Preparation of *B. burgdorferi* for Lipid Extraction

B. burgdorferi strain B31 were grown in microaerophilic conditions in BSK-II medium [302] supplemented with 6% rabbit serum (Sigma) at 33˚C. The cultures were grown to approximately stationary phase to maximize the amount of spirochetes in culture. *B. burgdorferi* were removed from the BSK-II by washing 5X in high volumes (50 mL) of cold PBS. After the washes, the bacterial pellet was resuspended in 1 mL of PBS. The lipids were extracted using the Bligh and Dyer solvent extraction method [303].

Protein Quantification and Analysis

To analyze protein levels in *B. burgdorferi* lipid extracts, the samples were dried under constant nitrogen stream. The lipid pellet was resuspended in 100 μL of PBS. Analysis of the proteins was conducted by the BCA Protein Assay Kit (Thermo Scientific) and the Coomassie Plus Protein Assay (Thermo Scientific) following the manufacturer's instructions. All but 10 μL of the remaining lipids were subjected to SDS-PAGE analysis on a 20% acrylamide gel and Coomassie staining to detect contaminating protein. The final 10μL of lipid extract was sent to the Stony Brook Proteomics Facility for mass spectrometry analysis. The OMV were analyzed for protein content using a combination of two-dimensional liquid chromatography and tandem mass spectrometry (MS/MS) or multidimensional protein identification technology (MudPIT).

Protease Treatment of Lipids

To remove protein from the lipid extract, the lipid extract was treated with a protease. The ability of the proteases to digest bovine serum albumin (BSA) (the contaminating protein) was assessed. Immobilized TPCK Trypsin (Thermo Scientific) and proteinase K (Sigma) were added to 200 μg, 100 μg, 50 μg, and 25 μg of the contaminating protein, BSA (Sigma) following the manufacturer's instructions. The reducing agent dithiothreitol (DTT) (Sigma) was added to the reactions at a final concentration of 15 mM. DTT was added to reduce the 17 disulfide bonds in BSA. The reactions were incubated overnight at 37˚C on an orbital rotor. Digestion of BSA was analyzed by SDS-PAGE and Coomassie staining as well as western blot.

Proteinase K was examined to see if it disrupted the lipids of *B. burgdorferi.* The lipids were extracted using the Bligh and Dyer solvent extraction method [303]. The lipids were dried under constant nitrogen stream and resuspended in PBS. The *B. burgdorferi* lipids from a 600

mL culture were treated with 1 mg/ml of proteinase K overnight at 37˚C on an orbital rotator. After proteinase K treatment, the lipids were run on HPTLC using the chloroform/methanol (85:15) mobile phase and stained with iodine to determine if the lipids were altered by the protease treatment.

Generation of Lipid Inoculum and Inoculation Schedule

A large culture (600 mL) of *B. burgdorferi* was grown to stationary phase. The spirochetes were pelleted and washed 5X in large volumes with PBS (50 mL each tube). After the last wash, the final bacterial pellet was resuspended in 1 mL PBS and subjected to Bligh and Dyer extraction. The mass of the lipid extract was measured by a laboratory scale. The lipid extract in the final solvent phase that was collected from the Bligh and Dyer procedure was added to the pre-weighed tube. The sample was dried under constant nitrogen stream. The weight of the lipids was determined by reweighing the glass tube with the dried pellet. The dried pellet was resuspended in 0.9 mL PBS and warmed in a 55˚C water bath with periodic vortexing. Proteinase K (final concentration 100μg/mL in 1 mL) was added to the lipids in PBS and incubated overnight at 37˚C on an orbital rotator. After the proteinase k treatment, a second Bligh and Dyer extraction was conducted to remove the proteinase K and also to obtain a final mass of the lipids. A similar procedure to measure the mass of the lipids was completed, and the lipids were resuspended in 1 mL PBS. This final lipid extract that would serve as the inoculum had a concentration of glycolipids in PBS of 7.0 mg/ml with a protein concentration was 202μg/mL. Despite the presence of measurable protein in the sample, the ratio of lipid to protein by mass was determined to be 35:1.

The animals were inoculated by the subcutaneous route without adjuvant. The rabbit received 100 μg/mL in 200 μL and each of the 10 mice received 50 μg/mL in 100 μL. The animals were injected weekly for six weeks. The reactivity of the antibodies to the *B. burgdorferi* lipids was assessed weekly starting at \week 3. The animals were sacrificed after 7 weeks.

Assessment of Antibody Reactivity

The reactivity of the antibodies was assessed using SDS-PAGE with western blot in conjunction with enzyme-linked immunosorbent assays (ELISAs). *B. burgdorferi* lysate (10 μg) and the lipid extract used as the inoculum (~200 μg/well) were used for the analysis. The serum from the animals was used as the primary antibody. As a positive control, the rabbit α-asialo GM1 antibody (Abcam) was included. Primary antibodies were detected using the appropriate Odyssey secondary antibodies (goat anti-mouse IgG (H+L) IRDye 800CW (Rockland) or goat anti-rabbit IgG (H+L) IR Dye 700CW) (Rockland). The immunoblots were scanned using the Odyssey infrared scanner (LI-COR Biosciences) in the 800 and 700 channels.

In addition to western blots, we also conducted ELISAs to quantify the reactivity of the antibodies raised against the lipids of *B. burgdorferi*. For the ELISA, four experimental conditions were used: (i) lipid extracts in the form of small unilamellar vesicles (SUV), (ii) lipid extracts in a solvent, (iii) *B. burgdorferi* lysates, and (iv) whole *Borrelia.* The ELISA plates were coated with *Borrelia* lysates and whole *Borrelia* samples using a traditional 50 mM carbonate buffer (pH 9.5). The protein content of *Borrelia* lysate was quantified and each well was coated with 5 μg. Each well was coated with 100,000 spirochetes which is ~ 0.5 μg. To coat the wells of the ELISA plate with the lipid extracts, we dissolved the lipids in methanol and 5.0μg of lipids

was dried out in each well. The SUVs were made [6], but were coated differently than the lipid extracts. Instead of using a carbonate buffer, we added 5µg of the SUVs to PBS (pH 7.4) coating buffer. Again, α-asialo GM1 (Abcam) antibody was included as a positive control. All primary antibodies were added at an optimized dilution of 1:100. The secondary antibodies, goat anti-mouse alkaline phosphatase (Sigma) and goat anti-rabbit alkaline phosphatase (Sigma), were reconstituted following the manufacturer's instructions and added to the assay (1:1000). The ELISAs were read on the SpectraMax M2 using optical density (OD) wavelength 405.

Generation of SUVs from *B. burgdorferi* lipid extracts.

For generation of lipid vesicles, a 100 µM concentration of the lipids is used to make SUV. In the case of *B. burgdorferi* lipid extracts, the concentration utilized is 500 µM. This concentration is used because the average molecular weight of *B. burgdorferi* lipids are 760 g/mol. The appropriate volume of lipids was added to a glass tube. The lipids were dried with liquid nitrogen. The dried lipids were incubated for 10 min at 70˚C in a water bath. At the completion of the incubation 18 µL of ethanol was added to the dried pellet. This mixture was vortexed and then 1 mL of warmed PBS (70˚C) was added to the tube. The mixture is vortexed again and incubated in at 70˚C for 10 min.

Fluorescence Microscopy Using the Rabbit Polyclonal Anti-*B. burgdorferi* Lipid Antibody

HeLa, MEB4, and GM95 cells were maintained in DMEM medium (Gibco) with 10% fetal calf serum (Pel-Freez). The cells were grown on glass coverslips. For fluorescent microscopy experiments, the cells were seeded at a density of $1.0 \times 10^5/cm^2$ and allowed to grow up to a density of ~ $2.0 \times 10^5/cm^2$. To grow the MEB4 and GM95 cells, poly-lysine coverslips

(BD) were required. Cells were infected with *B. burgdorferi* labeled with BODIPY-cholesterol at a MOI of 20:1 for 2 hrs or were untreated. The coverslips were washed three times in phosphate buffered saline (PBS, Gibco) and fixed in 2.5% paraformaldehyde. Blocking agents utilized were 5% BSA (Sigma) and 5% human serum, or no blocking agent. The rabbit polyclonal antibodies and rabbit anti-asialo GM1 were added to the coverslips. For HeLa cells, the dilution was optimized to be 1:100 while for MEB4 and GM95 the dilution of antibodies was titrated from 1:250 to 1:1000. The coverslips were washed 3X in PBS. A secondary goat anti-rabbit conjugated to fluorescein isothiocyanate (FITC) (Abcam) diluted 1:1000 was applied to the coverslips. The samples were observed using a Zeiss Eclipse E600 fluorescence microscope.

B. burgdorferi were detected using the polyclonal rabbit-anti *B. burgdorferi* glycolipid antibody. Spirochetes from a 6mL culture growing at 33˚C were collected, washed 3X in HBSS and added to fluorescence glass slides (Fisher Scientific). They were dried and fixed for 10 min in methanol. The polyclonal rabbit-anti *B. burgdorferi* glycolipid antibody was added at an optimized dilution of 1:100. The slide was washed 3X in PBS, and the spirochetes were detected with a secondary goat anti-rabbit conjugated to FITC (Abcam) diluted 1:1000. The samples were observed using a Zeiss Eclipse E600 fluorescence microscope.

Statistics

Statistics were calculated using GraphPad InStat 3 (GraphPad Software) All results were from three independent experiments all performed in triplicate unless otherwise noted. The statistical test used was the One-way ANOVA unless otherwise noted. Significance is reported only where data met a minimum significance of $p<0.05$.

CHAPTER 3 – THE TWO-WAY TRANSFER OF CHOLESTEROL AND ANTIGENIC CHOLESTEROL-GYLCOLIPIDS BETWEEN *B. BURGDORFERI* AND EUKARYOTIC CELLS

3.1 CHAPTER SUMMARY

The fluorescent cholesterol analogs, BODIPY-cholesterol and NBD-cholesterol, were utilized to label *B. burgdorferi* in culture. The fluorescent cholesterol, specifically BODIPY-cholesterol, labeled the membrane of the spirochetes and some of the individual cholesterol-glycolipids. This labeling was shown to not be cytotoxic to the spirochetes over the time points that the experiments were conducted. The *B. burgdorferi* labeled with BODIPY-cholesterol allowed for us to directly assess if antigenic glycolipids from spirochetes could be transferred to non-phagocytic eukaryotic cells Through the use of immunofluorescence (at the single cell level and across a population) in conjunction with radioactive labeling, we demonstrated transfer of cholesterol and antigenic cholesterol glycolipids from *B. burgdorferi* to HeLa cells. This transfer was dose-dependent and could be influenced by incubation time and incubation temperature. In addition to the transfer of spirochete-derived lipids to eukaryotic cells, we also used HeLa cells labeled with BODIPY-cholesterol to show that *B. burgdorferi* can acquire cholesterol from the plasma membrane of eukaryotic cells. These results together indicate that there is a two-way exchange of cholesterol between *B. burgdorferi* and eukaryotic cells.

3.2 RESULTS

The fluorescent cholesterol analog NBD-cholesterol was incorporated into the outer membrane of *B. burgdorferi* and was not cytotoxic.

To investigate a possible role for cholesterol in *B. burgdorferi* pathogenesis, fluorescent cholesterol analogs were tested for their ability to incorporate into the bacteria without toxic

effects. With multiple fluorescent cholesterol analogs available, 22-(*N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino)-23,24-Bisnor-5-Cholen-3β-Ol (NBD-cholesterol) was utilized because it is an environment-sensitive probe and most importantly has an unmodified hydroxyl group attached to C-3 that would allow for possible synthesis of the *B. burgdorferi* cholesterol-glycolipids. To investigate the behavior of NBD-cholesterol, we initially determined the incorporation level and relative toxicity of the fluorescent cholesterol for the spirochete. To incorporate the NBD-cholesterol into the *B. burgdorferi*, a cholesterol free BSK-II medium was utilized. In this medium, the most accessible source of cholesterol comes from the CMRL-1066 supplement. Thus, using cholesterol-free CMRL-1066 as a component of BSK-II eliminated the free cholesterol found in BSK-II (0.2 mg/L). However, to grow *B. burgdorferi* under laboratory conditions, it is also necessary to supplement the BSK-II medium with 6% rabbit serum. The rabbit serum provides a source of cholesterol (approximately 0.78 mg/L final concentration) bound to apolipoproteins to form lipoprotein complexes [306]. As a result, total removal of free cholesterol was not possible. Nonetheless, one of the two concentrations of fluorescent cholesterol used in the experiments (4.0 mg/L) is greater than the 0.78 mg/L of cholesterol derived from rabbit serum in BSK-II, and the labeled cholesterol is not bound to apolipoproteins. This suggests that the primary and most readily available source of cholesterol to the spirochetes is the fluorescent cholesterol analog.

Acquisition of the NBD-cholesterol probe by the spirochetes was demonstrated by spectrophotometry and fluorescent microscopy, and the cytotoxicity was determined by dark field enumeration of the experimental cultures at the conclusion of the experiment. The spirochetes incorporated NBD-cholesterol in a dose- and time-dependent manner (Figure 3.1A). At both concentrations measured, the NBD-cholesterol was quickly introduced into the

membranes of the spirochetes (Figure 3.1A). Spirochetes grown in dimethyl sulfoxide (DMSO) (which is the diluent for NBD-cholesterol) do not autofluoresce. At the concentrations and time periods used, NBD-cholesterol was not cytotoxic as there was no decrease in the numbers of *B. burgdorferi* during the 6 hr incubation period (Figure 3.1B). Because the fluorescence of the 4.0 mg/L condition is not cytotoxic and reaches a plateau at 2 hrs, we determined that incubation at this concentration and for 4 hrs was sufficient for maximal spirochetal labeling. Using these optimized conditions, the viability of the spirochetes was further assessed by fluorescent microscopy. *B. burgdorferi* labeled with NBD-cholesterol visually fluoresced green and did not exhibit any morphological defects (Figure 3.1C).

Exchange of NBD-cholesterol from *B. burgdorferi* to Eukaryotic Cells

The clustering of cholesterol into lipid rafts is a characteristic that is shared by both the eukaryotic host and *B. burgdorferi*. There is evidence that *H. pylori* attaches to, interacts with and can disrupt detergent-resistant regions (lipid rafts) of the host cell [157]. We hypothesized that the presence of cholesterol-rich lipid rafts in both the host and pathogen could serve as an ideal platform for interaction between the host and the spirochetes because lipid rafts have numerous roles in membrane organization, membrane trafficking, and membrane dynamics. One aspect of interaction were interested in studying was possible lipid-lipid interactions due to the presence of cholesterol in both organisms. To determine whether a direct molecular interaction exists between *B. burgdorferi* and host cells, we performed preliminary experiments to observe and quantify if there is any exchange of lipids. We chose to incubate the fluorescently labeled *B. burgdorferi* with HeLa cells in these experiments because *B. burgdorferi* come into contact with epithelial cells during dissemination and infection, and because they are not phagocytic. The

HeLa cells were grown on glass coverslips and exposed to *B. burgdorferi* labeled with NBD-cholesterol using a MOI of 20:1 for 30 min, 1 hr, and 2 hr intervals at 37°C. Confocal microscopy images of HeLa cells incubated with labeled *B. burgdorferi* show that the *B. burgdorferi*-derived NBD-cholesterol is transferred to the epithelial cells (Figure 3.2). Furthermore, we determined that lowest levels of transfer occur in the shorter incubation times of 30 min, but transfer levels increase over time (Figure 3.2). However, it was also observed over the course of the experiments that even with the addition of an anti-fade solution, the NBD fluorescent group photo-bleached quickly, resulting in images that were hazy and not sharp (Figure 3.2). It was determined that other fluorescent cholesterol analogs similar to NBD-cholesterol would have to be tested and used to improve the quality of the microscopy and overall fluorescence experiments. This would preserve the quality of the data and allow for other experimental techniques to be utilized in addition to confocal microscopy.

The fluorescent cholesterol analog BODIPY-cholesterol was incorporated into the outer membrane in a similar manner to NBD-cholesterol.

The fluorescent cholesterol analog 23-(dipyrromethene boron difluoride)-24-norcholesterol (BODIPY-cholesterol) was selected as a potential replacement for NBD-cholesterol because like NBD-cholesterol, BODIPY-cholesterol is an environment-sensitive, lipophilic probe that fluoresces in hydrophobic, but not aqueous, environments [307-309]. Furthermore, like NBD-cholesterol, BODIPY-cholesterol has an unmodified hydroxyl group attached to C-3 that would allow for synthesis of the *B. burgdorferi* cholesterol-glycolipids. We initially examined three concentrations (0.2 mg/L, 2.0 mg/L, and 4.0 mg/L) of BODIPY-cholesterol to determine the optimal amount to incorporate into the spirochetes. It is important to

note that similar to the amounts of NBD-cholesterol, two of the three concentrations of BODIPY-cholesterol used in the experiments (2.0 mg/L and 4.0 mg/L) are greater than the 0.78 mg/L of cholesterol derived from rabbit serum in BSK-II. This suggests that the primary source of cholesterol to the spirochetes is the fluorescent cholesterol analog.

Acquisition of the BODIPY-cholesterol in cholesterol free BSK-II by the spirochetes was demonstrated by spectrophotometry, by flow cytometry, and by fluorescent microscopy. The spirochetes incorporated BODIPY-cholesterol in a dose- and time-dependent manner (Figure 3.3A). Similar to the NBD-cholesterol, at all concentrations measured, the BODIPY-cholesterol was quickly introduced into the membranes of the spirochetes (Figure 3.3A). The BODIPY-cholesterol appeared to have much higher levels of fluorescence, but the background was also increased. After 6 hrs of incubation with the fluorescent cholesterol analog BODIPY-cholesterol, we observed increased variability in the levels of incorporation increased (Figure 3.3A). It is possible that at this time, there may be some self-quenching of the BODIPY fluorophore. At the concentrations and time periods examined, BODIPY-cholesterol was not cytotoxic as there appeared to be no significant decrease in the numbers of *B. burgdorferi* during the 6 hr incubation period (Figure 3.3B). Taken together, we determined that the most reproducible, fluorescence incorporation level was derived from growing the spirochetes for 4 hrs in the presence of 4.0 mg/L BODIPY-cholesterol. It is important to point out that all our incorporation experiments were done at, maximally, 4 hr time periods.

Using these optimized conditions, we observed the labeled spirochetes using fluorescent microscopy to assess the fluorescent labeling and viability. When labeled with BODIPY-cholesterol, the *B. burgdorferi* were visually green and did not exhibit any morphological or motility defects (Figure 3.4A). They maintained their spiral shape and wave-like motion which

was demonstrated by several blurry (moving) bacteria in the static image (Figure 3.4A). We also measured the mean geometric fluorescence of *B. burgdorferi* labeled with BODIPY-cholesterol compared to unlabeled *B. burgdorferi* by flow cytometry. *B. burgdorferi* grown in the presence of BODIPY-cholesterol were significantly more fluorescent when compared to unlabeled bacteria (Figure 3.4B). Spirochetes grown in DMSO (which is the diluent for BODIPY-cholesterol) do not autofluoresce.

BODIPY-cholesterol is utilized as a substrate by *B. burgdorferi* in the synthesis of the cholesterol-glycolipids.

Since we had demonstrated BODIPY-cholesterol can be incorporated by *B. burgdorferi,* we next examined whether the fluorescent cholesterol analog could serve as a substrate for synthesis of cholesterol-glycolipids. One of the main reasons that BODIPY-cholesterol was selected when compared to other fluorescent cholesterol analogs is the fact that the hydroxyl group attached to C-3 was unmodified. This could allow for *B. burgdorferi* enzymes to synthesize the cholesterol-glycolipids using the labeled cholesterol. *B. burgdorferi* were labeled by incubating the spirochetes with the optimized labeling conditions: 4.0 mg/L of BODIPY-cholesterol for 4 hrs in BSK-II medium lacking free cholesterol. Once labeled, the bacteria were washed and the lipids were extracted using the Bligh and Dyer solvent extraction method [303]. To resolve the individual lipids we used a chloroform-methanol (85/15) mobile phase on a HPTLC plate. Staining of the HPTLC plate with iodine demonstrated that the BODIPY-cholesterol spirochetes maintain their typical profile and possess free cholesterol, ACGal, MGalD, CGal, and the two phospholipids (Figure 3.5) [3,5,6]. UV excitation of the same chloroform-methanol HPTLC plate was used to identify cholesterol-glycolipids that were

synthesized using BODIPY-cholesterol in place of free cholesterol (Figure 3.5). Bands on the plate that were identified by UV excitation corresponded to free cholesterol and cholesterol-glycolipids (ACGal and CGal) (Figure 3.5). The glycolipid that does not contain cholesterol, MGalD, and the phospholipids did not have any incorporation of BODIPY-cholesterol and therefore were not observed when the plate was exposed to UV light (Figure 3.5). Further evidence that the spirochetes are actively synthesizing the cholesterol-glycolipids using BODIPY-cholesterol as a substrate is demonstrated by HPTLC and UV excitation analysis of *B. burgdorferi* lipid extracts at the beginning of the experiment (no incubation). At the 0 min incubation time, the fluorescent cholesterol probe has the same migration profile as cholesterol and is not incorporated into the cholesterol-glycolipids as was observed after 4 hrs of incubation with the probe (Figure 3.5). It is important to note that in these experiments the levels of CGal labeled with BODIPY-cholesterol appeared to be elevated above what was expected. It is possible that CGal could serve as a building block or an intermediate species in the synthesis of ACGal. In these experiments, we could be observing an intermediate step in the synthesis of ACGal because complete synthesis of ACGal is not achieved. Nonetheless, this data shows that BODIPY-cholesterol was used as a substrate for cholesterol-glycolipid synthesis in a similar manner to unmodified cholesterol by *B. burgdorferi,* thus making the individual cholesterol-glycolipids fluorescently labeled.

Incubation and incorporation of BODIPY-cholesterol into the membranes of *B. burgdorferi* does not affect the spirochetes' long term viability.

The evidence that *B. burgdorferi* can synthesize the cholesterol-glycolipids using BODIPY-cholesterol in place of free cholesterol led us to assess the cytotoxicity and long-term

growth of spirochetes labeled with fluorescent cholesterol. Spirochetes were labeled with BODIPY-cholesterol following the optimized protocol. After the 4 hr incubation, the spirochetes were washed and returned to normal BSK-II. The long-term viability of *B. burgdorferi* after being labeled with BODIPY-cholesterol was determined by dark-field enumeration. Following incubation and labeling with BODIPY-cholesterol, we did not observe significant delays in the growth of the labeled spirochetes when compared to controls (Figure 3.6A). In addition to analyzing the possible cytotoxic effects of labeling *B. burgdorferi* with BODIPY-cholesterol, we also assessed if the fluorescent cholesterol analog could serve as the only source of sterol in BSK-II (Figure 3.6B). Spirochetes did not replicate when grown in in BSK-II where the only source of cholesterol was BODIPY-cholesterol (no free cholesterol and no rabbit serum) (Figure 3.6B). However, when viewed using dark-field microscopy the spirochetes remained alive for up to 96 hrs. This same result was also observed when the only source of sterol was non-fluorescent cholesterol (no BODIPY-cholesterol and no rabbit serum) (Figure 3.6B). This experiment indicates that for survival of the spirochetes, these two different types of cholesterol molecules are interchangeable because in all conditions the spirochetes are alive. However, growth of the spirochetes only occurs in BSK-II that has been supplemented with 6% rabbit serum. Together these experiments indicate that long-term incubation with BODIPY-cholesterol is not cytotoxic to *B. burgdorferi* and that in our experimental system the fluorescent cholesterol is similar to the non-fluorescent cholesterol. We conclude that BODIPY-cholesterol is a useful cholesterol analog in *B. burgdorferi*, and that its behavior will likely reflect that of unlabeled cholesterol.

Exchange of lipids derived from *B. burgdorferi* to eukaryotic cells.

The presence of lipid rafts in both eukaryotic host cells and *B. burgdorferi* led to the hypothesis that these cholesterol-rich lipid rafts on both the host and pathogen could serve as an ideal platform for lipid-lipid interactions. Preliminary evidence presented using *B. burgdorferi* labeled with NBD-cholesterol indicated that there was a transfer of spirochete-derived lipids to eukaryotic host cells. To more decisively determine whether a direct molecular interaction exists between *B. burgdorferi* and host cells, we performed experiments using spirochetes labeled with BODIPY-cholesterol to observe and quantify if there is any exchange of lipids. Similar to the experiments with NBD-cholesterol, HeLa cells were grown on glass coverslips and exposed to *B. burgdorferi* labeled with BODIPY-cholesterol. The *B. burgdorferi* labeled with BODIPY-cholesterol were incubated with the HeLa cells at a MOI of 20:1 for 2 hrs at 37°C. The coverslips were washed to remove unattached spirochetes and were labeled for analysis by confocal microscopy. Confocal micrographs taken at multiple depths along the Z-axis indicate that over the 2 hr coincubation, the *B. burgdorferi* labeled with BODIPY-cholesterol transfer fluorescent spirochete-derived lipids to the unlabeled HeLa cells (Figure 3.7). The fluorescent lipids are located on the outer edge of the HeLa cells, presumably the plasma membrane, and also throughout the cells with concentrations near the nucleus (Figure 3.7). Furthermore, the *B. burgdorferi* labeled with BODIPY-cholesterol make contact and attach to the HeLa cells (Figure 3.7I-T). To follow the trafficking of BODIPY-cholesterol after incorporation, and determine where the *B. burgdorferi* derived lipids are being trafficked, we used the cis-Golgi marker GM130 (Figure 3.8). Labeled cholesterol trafficked to the Golgi apparatus within 2 hrs because there was a colocalization of the GM130 and the internalized BODIPY-cholesterol (Figure 3.8). This indicated that the perinuclear localization of the BODIPY-cholesterol was actually the

fluorescent probe in the Golgi-apparatus and also showed that the eukaryotic cells processed the *B. burgdorferi*-derived cholesterol in the same manner as cholesterol from other sources.

The exchange of lipids derived from labeled *B. burgdorferi*, specifically BODIPY-cholesterol and the cholesterol-glycolipids labeled with BODIPY-cholesterol, strongly suggests that the antigenic glycolipids are transferred from the spirochete to the host cell. However, from the experiments outlined in Figure 3.7 using the fluorescent cholesterol, we were unable to determine if the transferred lipids were in the form of the antigenic glycolipid. To confirm that the transfer of *B. burgdorferi* lipids to HeLa cells was linked to a specific process and not an artifact created by the addition of the BODIPY fluorophore to the cholesterol molecule, we performed lipid transfer experiments using ^{3}H-cholesterol. The increased sensitivity of radiolabeling relative to fluorescence also allowed us to investigate whether cholesterol-glycolipids were transferred to HeLa cells. Additionally, ^{3}H-cholesterol does not have a bulky fluorophore and more closely mimic the unlabeled cholesterol. In order to analyze the level of incorporation of the ^{3}H-cholesterol into *B. burgdorferi,* the lipids were extracted using the Bligh and Dyer solvent method [303] and analyzed by HPTLC. The silica from the lipid spots was identified by iodine staining. Each spot was scraped from the HPTLC plate and analyzed by liquid scintillation to quantify the amount of ^{3}H-cholesterol incorporated into *B. burgdorferi.* As an additional control, silica between the observed bands of free cholesterol and ACGal was also scraped to confirm that each band was distinct. When incubated with ^{3}H-cholesterol, *B. burgdorferi* incorporated the radioactive cholesterol into their membrane fraction (Figure 3.9A). The incorporation of ^{3}H-cholesterol was also similar to the incorporation of BODIPY-cholesterol, in that the spirochetes utilized the ^{3}H-cholesterol as a substrate for synthesis of the cholesterol-glycolipids (Figure 3.9A). Based on the liquid scintillation counts, the ratio of ^{3}H-

cholesterol, ^{3}H-cholesterol labeled ACGal, and ^{3}H-cholesterol labeled CGal in the spirochete was determined to be between 53.5:1:1 and 42.6:1.25:1 (Figure 3.9A). Furthermore, there was no radioactivity detected above background levels in the unlabeled controls or in lipids that do not contain cholesterol: MGalD, phosphatidylcholine, or phosphatidylglycerol (Figure 3.9A). Additional evidence to support the incorporation of ^{3}H-cholesterol into the lipid compartment of *B. burgdorferi,* specifically the cholesterol-glycolipids, was observed by exposing the radioactive HPTLC plates to BioMax MR Film (Figure 3.9B). The film depicting the radioactive bands on the HPTLC plate supports the fluorescence data already presented in Figure 3.5, because the cholesterol is incorporated into the bacterial cholesterol-glycolipid fractions and not the other lipids of *B. burgdorferi* (Figure 3.9B).

To initially demonstrate transfer of lipids from spirochetes to HeLa cells, *B. burgdorferi* labeled with ^{3}H-cholesterol were incubated with the cells at different MOIs. The radiolabeled spirochetes were added to the HeLa cells at a MOI of 40:1, 20:1, 10:1, and 1:1 for 2 hrs. The HeLa cells were washed three times and lifted from the tissue culture wells. The cells from each experimental condition were measured by liquid scintillation to quantify the levels of transfer (Figure 3.9C). Significant DPM were observed in the higher MOI in a dose-dependent manner (Figure 3.9C). These observations that there is a transfer of *B. burgdorferi* lipids to the host eukaryotic cells agree with data obtained using BODIPY-cholesterol. To confirm transfer of individual antigenic cholesterol-glycolipids, the spirochetes were (i) removed from the HeLa cells by multiple washes with PBS, (ii) the HeLa cells were lifted and the lipids were extracted by Bligh and Dyer lipid extraction, and (iii) the lipid profiles of the HeLa cells were analyzed by HPTLC (Figure 3.9D). Evidence that the cholesterol-glycolipids are transferred to the membrane of the HeLa cells comes from identification of the individual cholesterol-glycolipids

in the HeLa cell lipid extracts on the HPTLC plate (Figure 3.9D). Experimental HeLa cell lipid extracts were run on an HPTLC plate in parallel with a reference control *B. burgdorferi* lipid extract. The HPTLC plate was stained with iodine and the bands on the HeLa cell extract samples that corresponded to the known glycolipid bands from the *B. burgdorferi* control lipid extract were scraped for liquid scintillation analysis. The ratio of the counts of transferred ^{3}H-cholesterol, ^{3}H-cholesterol labeled ACGal, and ^{3}H-cholesterol labeled CGal was between 56:4:1 and 54.67:5.33:1 (Figure 3.9D). One important piece of evidence to indicate this is a specific transfer is that the ratio of ACGal to free cholesterol in the HeLa cells was higher than what was measured in the spirochetes. This suggests that ACGal transfers more efficiently than cholesterol, and this observation would not be expected if the HeLa cell-associated radioactivity was due to residual spirochetes attached to the HeLa cells. We also included an additional control to account for the contribution of spirochetes that remain attached to the cells after washing to the total DPMs. A GFP-labeled *B. burgdorferi* strain [304] was used to quantify the percentage of bacteria that did not dissociate from the cells. The HeLa cells were analyzed by the SpectraMax M2 to calculate GFP fluorescence levels. This control disclosed that 2.2 ± 0.4% of the GFP fluorescence (~61% of the total signal of 3.5 ± 0.9%) could be derived from spirochetes that remained attached. Despite this, however, ~39% of the signal of ^{3}H- cholesterol and cholesterol-glycolipid was associated with cells. Furthermore, we observed that the fraction of radioactive ACGal in HeLa cells after transfer (between 56:4:1 and 54.67:5.33:1 [cholesterol:ACGal:CGal]) was 3 to 5-fold times higher than ACGal derived from radioactively labeled *B. burgdorferi* alone (between 53.5:1:1 and 42.6:1.25:1 [cholesterol:ACGal:CGal]). This indicates that the amount of ACGal bound to the HeLa cells is 6-fold higher than that expected if it was just derived from *B. burgdorferi* that had not washed off the HeLa cells.

In this chapter we present four different methodologies (fluorescence confocal microscopy, single cell RFI analyses, flow cytometry, and isotope incorporation) to demonstrate lipid exchange from *B. burgdorferi* to cells. Of these four, isotope incorporation was the most sensitive, but was difficult to control for unbound spirochetes. However, it was essential to provide evidence for lipid exchange of the antigenic ACGal through differential incorporation of label and through HPTLC. Thus, we conclude that there is a transfer or uptake of both cholesterol and the antigenic cholesterol-glycolipids by eukaryotic host cells *in vitro*, and this could have important implications for the pathogenesis of Lyme disease.

Exchange of lipids from *B. burgdorferi* to HeLa cells is also accomplished by conditioned media collected from BODIPY-labeled *Borrelia*.

Based on our previous work presented in Figure 3.7 that showed not all of the spirochetes adhered to the HeLa cells, we had a good indication that the mechanism of transfer of *B. burgdorferi* lipids was not mediated by direct contact only. To address specifically other mechanisms of transfer, we asked if something was being released from the bacteria that could contribute to the transfer by a contact-independent mechanism. To investigate this, possibility we incubated a cell free conditioned medium that had been collected from *B. burgdorferi* labeled with BODIPY-cholesterol with the HeLa cells. The conditioned medium was added to the HeLa cells after incubation with and removal of spirochetes labeled with BODIPY-cholesterol. In addition to the conditioned medium, a cell-free wash supernatant (as a negative control) was used to control for nonspecific release of the BODIPY-cholesterol by the labeled *B. burgdorferi.* Images of HeLa cells incubated with the conditioned medium from BODIPY-cholesterol-labeled *B. burgdorferi* captured by confocal microscopy show that transfer of *Borrelia*-derived lipids can

also be conducted without the spirochetes making direct contact with the eukaryotic cells (Figure 3.10A-D). It was also observed that the cell-free wash supernatant did not confer any BODIPY-cholesterol labeling (Figure 3.10E-H). This indicates that the transfer of lipids is not nonspecific or spontaneous (Figure 3.10E-H).

In addition to visually observing the transfer of *B. burgdorferi*-derived lipids by confocal fluorescence microscopy we sought to use other methods to document this transfer. To provide further evidence of the transfer, we examined this event at the single-cell level and at a larger scale of all the HeLa cells in the tissue culture plate well. Using the confocal microscope RFI measurements of the HeLa cells from 10 microscope fields, it was observed that the BODIPY-*Borrelia* and the BODIPY-*Borrelia* conditioned medium samples had significantly higher transferred fluorescence than the negative control (Figure 3.11A). We also observed similar trends when studying the transfer event on a larger scale. After incubating the different experimental and control preparations with the HeLa cells, the HeLa cells in the tissue culture wells were washed and analyzed by flow cytometry for transferred BODIPY-cholesterol fluorescence from the spirochetes (Figure 3.11B). Both the BODIPY-*Borrelia* and the BODIPY-*Borrelia* conditioned medium conditions had significantly higher fluorescence indicating higher levels of transfer when compared to the controls (Figure 3.11B). One trend that was observed in both experiments was that the transferred fluorescence from the BODIPY-*Borrelia* condition was higher than that of the BODIPY-*Borrelia* conditioned medium (Figure 3.11A, B). This suggested that when whole *B. burgdorferi* are incubated with the HeLa cells, the transferred fluorescence could be the additive result of both contact-dependent and contact-independent mechanisms.

The transfer of *B. burgdorferi*-derived lipids is time- and temperature-dependent.

To further analyze the transfer of *B. burgdorferi*-derived lipids to host cells, effects of the amount that time and temperature on the transfer of *B. burgdorferi* lipids to eukaryotic cells was examined. Again, HeLa cells were grown on glass coverslips and exposed to *B. burgdorferi* labeled with BODIPY-cholesterol at a MOI of 20:1 at four time intervals at 37°C. We included as experimental conditions the labeled spirochetes, the conditioned medium (after removal of spirochetes labeled with BODIPY-cholesterol), and the cell free wash supernatant. These preparations were incubated with the HeLa cells for 15 min, 30 min, 1 hr and 2 hrs. Images of HeLa cells incubated with labeled *B. burgdorferi* and the conditioned medium from BODIPY-cholesterol labeled *B. burgdorferi* captured by confocal microscopy show that the lowest levels of transfer occur in the shorter incubation times of 15 min (Figure 3.12A-F) and 30 min (Figure 3.12G-L). There appears to be a threshold in the amount of incubation time because between the 30 min and 1 hr incubation times because the fluorescent labeling is most robust and highest at the longer incubation times of 1 hr (Figure 3.12M-R) and 2 hrs (Figure 3.12S-X). Over all the tested incubation times, little or no transfer was observed in the in the negative control cell-free wash supernatant (Figure 3.12E,F,K,L,Q,R,W,X). Using the same techniques outlined earlier in Figure 3.11, we confirmed the time dependency of the transfer of lipids from the spirochetes and conditioned medium to the HeLa cells by RFI analysis (Figure 3.13A) and flow cytometry (Figure 3.13B).

The transfer of *Borrelia*-derived lipids from the spirochete to the epithelial cells is also temperature-dependent. An identical experiment using the transfer assays was conducted using a coincubation time of 2 hrs, at different temperatures between 4˚C and 37˚C. Confocal microscopy provided visual evidence that at 4˚C there was little or no transfer of bacterial derived lipids from labeled spirochetes or conditioned medium (Figure 3.14A-F). The levels of transfer observed at 4˚C were lower when compared to images taken at higher temperatures, 25˚C (Figure 3.14G-L) and 37˚C (Figure 3.14M-R). Furthermore, we observed that the highest levels of transfer of the bacterial-derived lipids from labeled spirochetes and conditioned medium occurred at 37˚C (Figure 3.14M-R). The negative control cell-free wash supernatant did not show any visible transfer at any temperature (Figure 3.14E,F,K,L,Q,R). The RFI measurements (Figure 3.15A) and the flow cytometry analysis (Figure 3.15B) supported the confocal microscopy data that indicated at the higher temperatures, the levels of bacterial derived lipids being transferred to the HeLa cells was significantly higher. Together, these experiments suggest that the transfer of bacterial-derived lipids from *B. burgdorferi* to HeLa cells is a process that is influenced by both time and temperature.

Transfer of the BODIPY-cholesterol derived lipids does not require actin polymerization.

To further understand the mechanism of transfer beyond altering the temperature and incubation time, we investigated a possible mechanism by which the eukaryotic cell acquires the *B. burgdorferi*-derived lipids. One of the well-characterized mechanisms of internalization of bacteria and bacterial components is through actin polymerization. It is possible that one of the mechanisms by which the HeLa cells acquire the *Borrelia* derived lipids is through actin polymerization. To gain further insight into the transfer mechanism, experiments were conducted

to inhibit actin polymerization. Cytochalasin D is a highly utilized inhibitor of actin polymerization. To investigate the role of actin in the transfer mechanism, the HeLa cells were pre-treated with cytochalasin D for 1 hr. After the pre-treatment, the HeLa cells were washed and then incubated with the BODIPY-cholesterol-labeled *B. burgdorferi* or the BODIPY-*Borrelia* conditioned medium for 2 hrs at 37˚C. Confocal microscopy images of the HeLa cells after the coincubation indicated that cytochalasin D at all the concentrations tested (50 μg/mL, 10 μg/mL, and 1 μg/mL) did not inhibit the transfer of bacterial-derived lipids to eukaryotic cells (Figure 3.16). Similar levels of fluorescence were observed when the cytochalasin D-treated samples were compared to the untreated samples (Figure 3.16). The confocal microscopy data was further supported by RFI analysis at the single-cell level that showed the amount of fluorescence in the HeLa cells was similar to untreated cells, and did not appear to be inhibited by the cytochalasin D treatment (Figure 3.17).

One of the side effects of cytochalasin D treatment that cannot be overlooked is that treatment can result in elevated cytotoxicity. To ensure that the results in Figure 3.16 and Figure 3.17 were not due to an artifact caused by dying cells, LDH release assays were conducted to quantify the amount of cytotoxicity in cytochalasin D treated cells. We did not observe any significant cytotoxicity related to the cytochalasin D treatment at any of the concentrations utilized (Figure 3.18 "treatment only"). However, some cytotoxicity, that was not significant, was observed when the HeLa cells were treated with cytochalasin D and then incubated with the *B. burgdorferi* labeled with BODIPY-cholesterol or the BODIPY-*Borrelia* conditioned medium (Figure 3.18).

B. burgdorferi labeled with BODIPY-cholesterol specifically release fluorescent cholesterol probe in the form of OMV

Given that HeLa cells acquired BODIPY fluorescence from conditioned medium in Figure 3.10A-D, we sought to determine whether free BODIPY-cholesterol was being released nonspecifically or was associated with OMV. *B. burgdorferi* labeled with BODIPY-cholesterol were incubated in HBSS for 2 hrs. This time point was used because it was equal to the amount of time the *B. burgdorferi* labeled with BODIPY-cholesterol were incubated with the HeLa cells in earlier experiments. After centrifugation, fluorescence of the pellets and supernatants was measured in the SpectraMax M2. Over the 2 hr incubation time in the HBSS, the fluorescence of the spirochetes decreased while the fluorescence of the supernatant increased (Figure 3.19A). Furthermore, this release of BODIPY-cholesterol fluorescence occurred without the spirochetes being in the presence of the HeLa cells and this indicated that the release of lipids from *B. burgdorferi* does not require the cells. To identify released OMV in the supernatants, we used a lipophilic probe, DPH. DPH fluoresces in hydrophobic environments such as membranes, but not in aqueous environments, and has a linear response with membrane bilayer concentration [310]. By probing the supernatants from BODIPY-cholesterol labeled and unlabeled spirochetes with 1 µg/mL of DPH, we were able to determine if fragments of membrane were being released from the labeled spirochetes and how it compared to release by unlabeled *B. burgdorferi*. Labeled *B. burgdorferi* released intact membrane in amounts similar to unlabeled *B. burgdorferi* over the course of the experiment (Figure 3.19B), suggesting that the membrane release by the labeled spirochetes is a natural process and not aberrant or nonspecific release.

Although the BODIPY-cholesterol at 4.0 mg/L does not appear to be causing the spirochete to shed or release abnormal amounts of membrane, this experiment did not distinguish

whether adding excess or more cholesterol than is found in BSK-II, in the form of BODIPY-cholesterol, caused the spirochete to release increased amounts of cholesterol. To determine if having he spirochetes in medium with more cholesterol than is found in BSK-II causes the bacteria to release more sterols into supernatants, BODIPY-cholesterol, at concentrations of 0.2 μg/mL (1X concentration of free cholesterol in BSK-II) or 4.0 μg/mL (20X concentration of free cholesterol in BSK-II), was incorporated into *B. burgdorferi* for 2 hrs. The bacteria were then incubated in HBSS for 2 hrs, and the supernatants were collected each hour during that time. The fluorescent sterol released by the spirochetes was measured by the SpectraMaxM2 (Figure 3.20A), and the amount of cholesterol released into the supernatants was measured by Amplex Red Quantification Kit (Figure 3.20B). We did not observe any significant difference in the amount of fluorescent cholesterol released between the two labeling conditions of 0.2μg/mL or 4.0μg/mL of BODIPY-cholesterol (Figure 3.20A). Furthermore, there was no significant difference in released total cholesterol when 0.2 μg/mL or 4.0 μg/mL of BODIPY-cholesterol was added to the spirochetes (Figure 3.20B). Together these experiments indicate that adding relatively high fluorescent cholesterol to the spirochetes does not cause abnormal release of probe or free sterols.

Since DPH labeling cannot distinguish if membrane release is in the form of an intact OMV, we analyzed the released membrane by transmission electron microscopy (TEM). The negative-stain TEM micrographs show released OMV. The isolated vesicles from both BODIPY-cholesterol-labeled and unlabeled *B. burgdorferi* show similar spherical structures and larger branch-like structures (Figure 3.21). In both OMV preparations, we used double immunogold labeling to detect protein and the cholesterol-glycolipids. The OMV of labeled and unlabeled *B. burgdorferi* contain OspB (18 nm colloidal gold) and the cholesterol-glycolipids (6

nm colloidal gold) (Figure 3.21). The TEM images indicate that both labeled and unlabeled *B. burgdorferi* release OMV that are similar in morphology, protein content, and glycolipid content. Many gold particles appear to not be associated with the OMV. Previous work has shown that these are associated with very small pieces of membrane [6].

To further prove that that the membranes released from *B. burgdorferi* labeled with BODIPY-cholesterol are OMV and are similar in protein content to the unlabeled controls, we used a discontinuous Optiprep density gradient to purify OMV from labeled and unlabeled *B. burgdorferi*. To compare protein content, we performed SDS-PAGE using 11 purified OMV fractions from the labeled and unlabeled *B. burgdorferi*. We observed that both preparations had similar distributions of protein across the 11 gradient fractions (Figure 3.22A and 3.22B). Furthermore, the unlabeled and labeled vesicles had similar protein content in the fractions where OMV are expected to partition (15-25% fractions) (Figure 3.22A and 3.22B). This indicates that even with the addition of BODIPY-cholesterol to the spirochetes, the OMV are representative of naturally forming vesicles. Individual proteins in the OMVs were also identified. We determined by SDS-PAGE and western blot that OspA, OspB, and lp 6.6, some of the most abundant lipoproteins in the OMV, are found in both labeled and unlabeled *B. burgdorferi* (Figure 3.23A and 3.23B). Other periplasmic proteins like DnaK and FlaB are not as abundant in the OMV (Figure 3.23A and 3.23B). These electrophoretic and immunoblot data were also supported by previous mass spectrometry data from our laboratory that showed outer membrane proteins were the most abundant in the OMV [125] and that the BODIPY-labeled OMV are representative of naturally forming vesicles.

In addition to analyzing the protein content of labeled and unlabeled OMV, we also studied the lipid content of the vesicles. To examine the lipids found in *B. burgdorferi* OMV, we

pooled fractions 15-25% of both the BODIPY-cholesterol-labeled and unlabeled vesicles. The lipids were isolated using the Bligh and Dyer lipid extraction method and resolved on a HPTLC plate using a chloroform-methanol (85/15) solvent phase. From the HPTLC plates it was determined that the cholesterol-glycolipids are the major glycolipid components in the vesicles (Figure 3.24). BODIPY-cholesterol-labeled and unlabeled OMV have phosphatidylcholine, phosphatidylglycerol, cholesterol, ACGal, and CGal (Figure 3.24). They do not appear to contain significant amounts of the *B. burgdorferi* glycolipid that does not have cholesterol, MGalD (Figure 3.24). To resolve fluorescent glycolipids, the same HPTLC plate was exposed to UV light. The vesicles derived from BODIPY-cholesterol labeled *B. burgdorferi* have the fluorescent cholesterol analog BODIPY-cholesterol (Figure 3.24). The cholesterol-glycolipids were not detected on the plate. This was most likely due to the limit of detection of low amounts of lipids on the HPTLC plate.

Significant evidence has been presented that the BODIPY-cholesterol labeled vesicles fluoresce green. To confirm that the vesicles have significantly higher fluorescence compared to unlabeled vesicles, we measured the fluorescence of the BODIPY-cholesterol-labeled using the SpectraMaxM2. Compared to unlabeled vesicles, the BODIPY-cholesterol-labeled vesicles had significantly more fluorescence in the 20% fraction (Figure 3.25). This is the fraction where vesicles have been shown to migrate in the Optiprep gradient (Figure 3.22 and 3.23). Other fractions had detectable fluorescence in both the unlabeled and labeled vesicles, but this could be caused by the background interference by the vesicles in suspension (Figure 3.25). Nonetheless, this is further evidence that the vesicles collected from *B. burgdorferi* labeled with BODIPY-cholesterol fluoresce green.

B. burgdorferi release OMV that contribute to the transfer of bacterial derived lipids to HeLa cells

We have previously shown that the cell-free conditioned medium is partially responsible for the transfer of *B. burgdorferi*-derived lipids. It was hypothesized that the OMV in this conditioned medium were specifically responsible for the transfer. To test for the possibility that the OMV released by *B. burgdorferi* contribute to the transfer of bacterial antigens to the HeLa cells, we Optiprep purified vesicles from *B. burgdorferi* labeled with BODIPY-cholesterol. The HeLa cells were washed with PBS and incubated with Vybrant Cell-Labeling Solution DiI to label their plasma membranes (Figure 3.26A). It was observed that when fluorescent OMV were incubated with HeLa cells in the transfer assay, there was a colocalization between the BODIPY-cholesterol probe and the Vybrant Cell Labeling Solution DiI probe on the surface of the HeLa cells (Figure 3.26B and 3.26C). We observed this colocalization at the single cell level (merged cross section, Figure 3.26B) and in a merged 3D composite image of multiple cells on the coverslip (Figure 3.26C). In addition to colocalization on the surface, we also observed internalization in the form of free BODIPY-cholesterol fluorescence inside of the HeLa cell. This internalization of the BODIPY-cholesterol fluorescence was represented by diffuse staining inside the plasma membrane of the composite images and was similar to the transfer assays conducted with the whole bacteria. These confocal images show that OMV can contribute to the exchange of bacterial-derived lipids from *B. burgdorferi* to eukaryotic cells.

B. burgdorferi attach to HeLa cells and acquire cholesterol.

We have previously hypothesized that the presence of cholesterol-rich lipid rafts in both the host and pathogen could serve as an ideal platform for lipid-lipid interactions and showed

that there is a deposition of antigenic glycolipids onto the surface of eukaryotic cells by *B. burgdorferi*. This key event could contribute to pathogenesis, but could also serve to be beneficial for the spirochete's overall fitness. We sought to determine if *B. burgdorferi* acquires cholesterol through direct contact with HeLa cells using the BODIPY-cholesterol. To visualize the acquisition of the BODIPY-cholesterol, we labeled the HeLa cells with the fluorescent cholesterol probe and added unlabeled spirochetes to the experiment. The *B. burgdorferi* were incubated with HeLa cells labeled with BODIPY-cholesterol at a MOI of 40:1. Confocal microscopy images depicted a colocalization (yellow) between the BODIPY-cholesterol and *B. burgdorferi* outer membrane protein OspB (Figure 3.27). This colocalization was observed in single 0.5-µm Z-slices and showed the uptake of cholesterol by adherent *B. burgdorferi* on the spirochete and at the point of attachment with the HeLa cell (Figure 3.28). Furthermore, BODIPY-cholesterol labeling extended outward from the point of attachment along the length of the spirochete (Figure 3.28). Negative controls demonstrated that acquisition of BODIPY-cholesterol is not detected at the start of the experiment (Figure 3.28, 0 min panels). Therefore, *B. burgdorferi* most likely acquired BODIPY-cholesterol directly from the labeled HeLa cells over the incubation time and not from release of cholesterol by the HeLa cells into the supernatant. Together these data provide evidence that *B. burgdorferi* not only transfers cholesterol-glycolipids to the eukaryotic cells, but also can acquire cholesterol from the epithelial cells.

3.3 DISCUSSION

The host antibody response to *B. burgdorferi* has been studied extensively, but the understanding of the role of the unique cholesterol-glycolipids in pathogenesis is limited. The

presence of cholesterol in the membrane of a prokaryote is uncommon. Free cholesterol and cholesterol-glycolipids in the *B. burgdorferi* are required and allow for the formation of eukaryotic-like lipid rafts [6]. Lipid rafts and similar structural organization of the membrane creates an opportunity for lipid-lipid interactions between the constituents of the lipid rafts in eukaryotic cells and *B. burgdorferi*. In investigating the lipid-lipid interactions or transfers from *B. burgdorferi* to eukaryotic cells, we developed methods to incorporate labeled cholesterol into the spirochetal membranes. This is important for several reasons. We demonstrated that addition of a labeled cholesterol analog is tolerated by the spirochetes. Labeling of *B. burgdorferi* with NBD-cholesterol or BODIPY-cholesterol is not cytotoxic and the spirochetes are able to replicate when reintroduced into complete BSK-II medium. Not only is the membrane of *B. burgdorferi* labeled when the fluorescent cholesterol is added to the spirochetes, but the labeled cholesterol is also used as a substrate for synthesis of the cholesterol-glycolipids. Therefore, the labeling is not just limited to the overall membrane of the spirochetes because the individual cholesterol-glycolipids are labeled with the cholesterol probes.

Antibody responses to these cholesterol-glycolipids have been detected throughout the course of Lyme disease [3,5,62,289]. However, additional roles of these cholesterol-glycolipids in pathogenesis have not been elucidated. We demonstrate that the cholesterol and cholesterol-glycolipids are transferred to epithelial cells. After incubation with epithelial cells, confocal microscopy images suggested that *B. burgdorferi*-derived fluorescent cholesterol and possibly the fluorescently labeled cholesterol-glycolipids are transferred to the plasma membrane. To further support the confocal microscopy experiments that illustrated a transfer of *B. burgdorferi*-derived lipids to eukaryotic cells, we used several different techniques to demonstrate lipid exchange, including spectrophotometry, flow cytometry, and quantifiable fluorescence

microscopy (RFI) at the level of the single cell. These multiple techniques support a mechanism of transfer by direct contact between the spirochete and the plasma membrane and through released OMV in conditioned medium. One notable trend observed throughout all the experiments is that there were the highest levels of exchanged fluorescence in the condition where whole spirochetes were incubated with the epithelial cells. Based on these experiments, we believe that the transfer mechanism is additive, because the contact-dependent and -independent mechanisms are being quantified in this experimental condition. In addition to the transfer assay, confocal microscopy was used to show that the transferred lipids were observed in the Golgi apparatus. The presence of the fluorescent cholesterol in the Golgi apparatus indicates that the eukaryotic cells are processing and trafficking all or part of the exogenous BODIPY-cholesterol in a manner similar to that used for cholesterol derived from other sources [308].

Our confocal microscopy experiments demonstrated that *B. burgdorferi*-derived lipids are transferred to non-phagocytic eukaryotic cells. To further support our original findings, we also altered the experimental conditions (time and temperature) to characterize the contact-dependent and contact-independent transfer mechanism. We demonstrated that both methods of transfer were influenced by coincubation time and coincubation temperature. Significantly lower levels of transfer were observed at shorter incubation times (15 mins and 30 mins) and lower incubation temperatures (4˚C). Furthermore, the contact-dependent and - independent transfer and subsequent internalization of the spirochete-derived lipids was not dependent on eukaryotic cell actin polymerization. Inhibition of actin polymerization using cytochalasin D had no effect on the overall transfer. That the transfer of *Borrelia* derived lipids was not dependent on actin polymerization by the eukaryotic cells suggests that the transfer is accomplished by a lipid-lipid interaction at the surface of the plasma membrane. Significant levels of LDH release were not

observed over the course of the cytochalasin D treatments. Together, these results support a model where eukaryotic cells obtain *B. burgdorferi*-derived lipids through the additive results of contact-dependent and-independent transfer mechanism.

In these experiments, we demonstrated that lipids can be transferred from *B. burgdorferi* to eukaryotic cells. Unfortunately, even when using multiple experimental techniques, these experiments using fluorescent cholesterol are limited. We were unable to determine if the antigenic cholesterol-glycolipids are being transferred to eukaryotic cells. To bolster the results from the fluorescence experiments, we also used radioactive cholesterol to demonstrate lipid exchange. With the higher sensitivity of radiolabeled cholesterol, we were able to establish that not just labeled cholesterol, but also the two antigenic cholesterol-glycolipids, can be transferred to epithelial cells and are present in the eukaryotic membrane. Using radiolabeled spirochetes, we were able to detect low but significant DPM values of ACGal and CGal in the epithelial lipid extracts, suggesting that there is an exchange of the antigenic cholesterol-glycolipids into the HeLa cells**.** Regardless of the amount of transfer, together, our results demonstrate that a spirochetal cholesterol-based antigen is transferred to and present in the membrane of the epithelial cells.

The transfer experiments indicate that transfer of *B. burgdorferi*-derived lipids to HeLa cells can occur without direct contact using conditioned medium. Therefore we chose to investigate how OMV can contribute to the transfer of lipids between the spirochetes and host cells. *Borrelia* OMV have been isolated [122-124] and extensively studied [126,128,129,131,132,311]. We collected OMV from live organisms in BSK-II, thus replicating the same experimental conditions as would be found in the conditioned medium. It is important to note that *B. burgdorferi* is a fastidious organism and over the course of the experiment we

were capturing naturally forming vesicles and not vesicles released by the process of cell division. We demonstrated using spectrophotometry that the BODIPY-cholesterol labeling conditions does not elicit nonspecific release of membrane or cholesterol. Furthermore, TEM confirmed that the membranes being released into the conditioned medium by the labeled spirochetes consisted of intact OMVs. Similar to other species of *Borrelia* [122], the isolated OMV from both labeled and unlabeled spirochetes were also rich in OM proteins. Fluorescently labeling the spirochetes allowed us to collect fluorescently labeled OMV that were studied for their ability to be transferred to eukaryotic cells. In line with idea that the conditioned medium can transfer *B. burgdorferi*-derived lipids, we demonstrated that the transfer can occur through OMV released from the spirochetes, and the vesicles attach to or are incorporated into the epithelial plasma membrane.

While completing the transfer experiments, it was of interest to investigate how *B. burgdorferi* might acquire the cholesterol necessary for synthesizing the cholesterol-glycolipids. As an extracellular pathogen, for *B. burgdorferi* to persist in the host, the process of nutrient acquisition from the immediate environment is essential. Using BODIPY-cholesterol to label HeLa cells, we provide evidence that *B. burgdorferi* can extract cholesterol, an essential membrane lipid, from eukaryotic cells. Using confocal microscopy we observed that, *B. burgdorferi* attach to epithelial cells and incorporate cholesterol from the plasma membrane. In the confocal microscopy images, colocalization of the BODIPY-cholesterol from the HeLa cells and the lipoprotein OspB at the point of attachment demonstrated that the spirochetes acquired the eukaryotic cell derived lipid. The colocalization was observed at the point of attachment and throughout the spirochete, which extended away from the cell. Together, these findings represent a mechanism by which *B. burgdorferi* acquires cholesterol during infection. Cholesterol

acquisition is not exclusive to *B. burgdorferi* and has been shown to also be an important process for other extracellular pathogens. Using similar techniques to ours, a prokaryote with similar lipid requirements and composition, *H. pylori*, was shown to attach to cholesterol-rich domains and acquire cholesterol from eukaryotic membranes [64,157].

These results fit into the model which proposes that there is a two-way exchange of lipids between *B. burgdorferi* and epithelial cells. Given the limited biosynthetic capabilities of *B. burgdorferi* to make cholesterol, the process of cholesterol extraction from host cells is very important for the bacteria to persist and establish infection. The process of cholesterol acquisition from the host is likely to be more biologically significant early in infection, because acquisition of the cholesterol is crucial for the replication of the bacteria. Once the bacteria have disseminated throughout the host and infection is established, it is likely that the second part of the two-way exchange, transfer of antigenic lipids from the host cells becomes more significant. Whether inserted directly into the plasma membrane of eukaryotic cells, or attached to the surface of these cells, even at low levels, the presence of foreign antigens with similar composition and structural characteristics to eukaryotic sterols could have multiple consequences for the host immune response. It is also possible that these transferred lipids could contribute to heightened inflammation and arthritis. This issue of the host response, specifically antibodies, to the cholesterol-glycolipids will be addressed in **Chapter 3**. Furthermore, if the immune response were to recognize cells with transferred lipid antigens, the cells themselves could become targets of immune effectors.

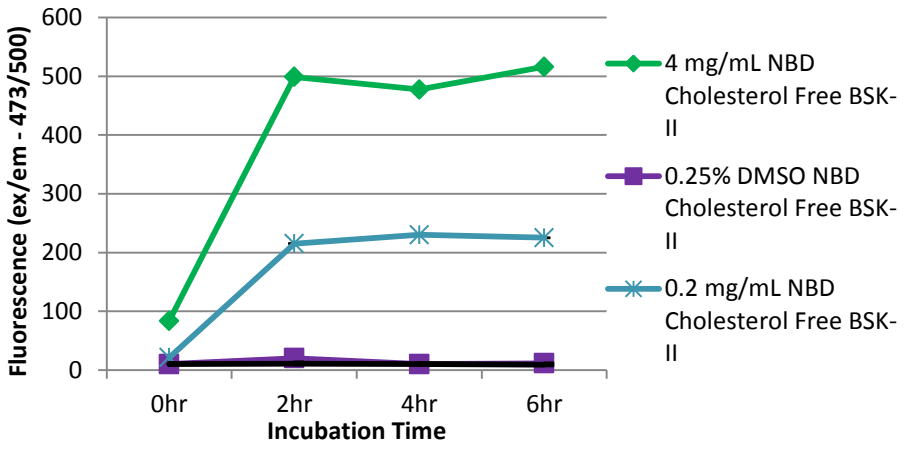
In Chapter 3, we show that there is a two-way exchange of lipids between *B. burgdorferi* and eukaryotic cells. The first part of this exchange focused on defining how antigenic

cholesterol-glycolipids were trafficked from the spirochete to the epithelial cell. We demonstrated that that this exchange is accomplished through direct contact with the spirochete. We also demonstrated that this transfer of *Borrelia*-derived lipids could be accomplished through contact with OMV. The second part of the exchange studied in this chapter addressed one possible mechanism by which *B. burgdorferi* obtain cholesterol. Through the use of a fluorescent cholesterol analog, we demonstrated that when the *B. burgdorferi* attach to the surface of an epithelial cell, the spirochetes can acquire cholesterol from the membrane of eukaryotic cells.

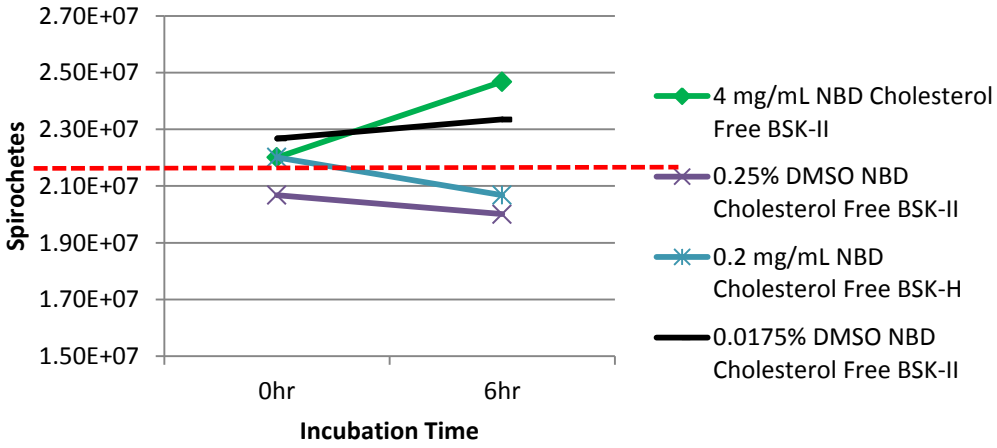
3.4 FIGURES

Figure 3.1. ***B. burgdorferi*** **incorporates NBD-cholesterol into their outer membrane. A.** Spirochetes were grown for 6 hrs in BSK-II medium lacking cholesterol and their fluorescence was measured by spectrophotometry. 4.0 mg/L NBD-cholesterol in BSK-II without free cholesterol (green); 0.25% DMSO in BSK-II without free cholesterol (purple); 0.2 mg/L NBD-cholesterol in BSK-II without free cholesterol (light blue); 0.0125% DMSO in BSK-II without free cholesterol (black). **B.** Spirochete viability was assessed by counting. Spirochetes grown with NBD-cholesterol or the control DMSO were analyzed by dark-field microscopy enumeration for cytotoxicity. 4.0 mg/L NBD-cholesterol in BSK-II without free cholesterol (green); 0.25% DMSO in BSK-II without free cholesterol (purple); 0.2 mg/L NBD-cholesterol in BSK-II without free cholesterol (light blue); 0.0125% DMSO in BSK-II without free cholesterol (black). Spirochetes (1.25×10^7) were used in the culture at the beginning of each experiment (red dotted line). **C.** Spirochetes that were grown for 6 hrs in BSK-II medium lacking cholesterol supplemented with 4.0 mg/L NBD-cholesterol fluoresce green when analyzed by fluorescent microscopy. Size bar = 20 μm. Error bars represent the mean ± standard error of the mean from three separate experiments.

A


600
500
400
300
200
100
0
Fluorescence (ex/em - 473/500)
0hr
2hr
4hr
6hr
Incubation Time
4 mg/mL NBD Cholesterol Free BSK-II
0.25% DMSO NBD Cholesterol Free BSK-II
0.2 mg/mL NBD Cholesterol Free BSK-II

B


2.70E+07
2.50E+07
2.30E+07
2.10E+07
1.90E+07
1.70E+07
1.50E+07
Spirochetes
0hr
6hr
Incubation Time
4 mg/mL NBD Cholesterol Free BSK-II
0.25% DMSO NBD Cholesterol Free BSK-II
0.2 mg/mL NBD Cholesterol Free BSK-H
0.0175% DMSO NBD Cholesterol Free BSK-II

C

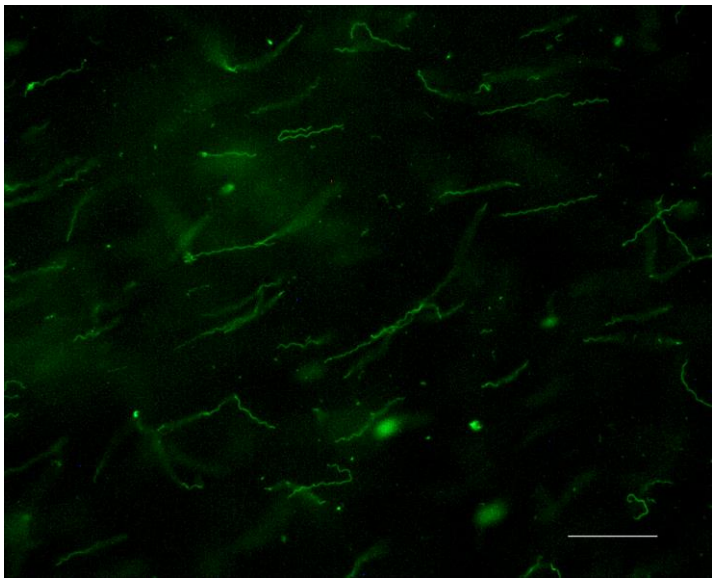


Figure 3.2. ***B. burgdorferi*** **incubated with NBD-cholesterol fluoresce green and transfer spirochetal derived lipids to eukaryotic cells.** Spirochetes labeled with NBD-cholesterol for 30 min, 1 hr, and 2 hrs appear to transfer *B. burgdorferi*- derived lipids in a time-dependent manner. Size bar = 2 0μm.

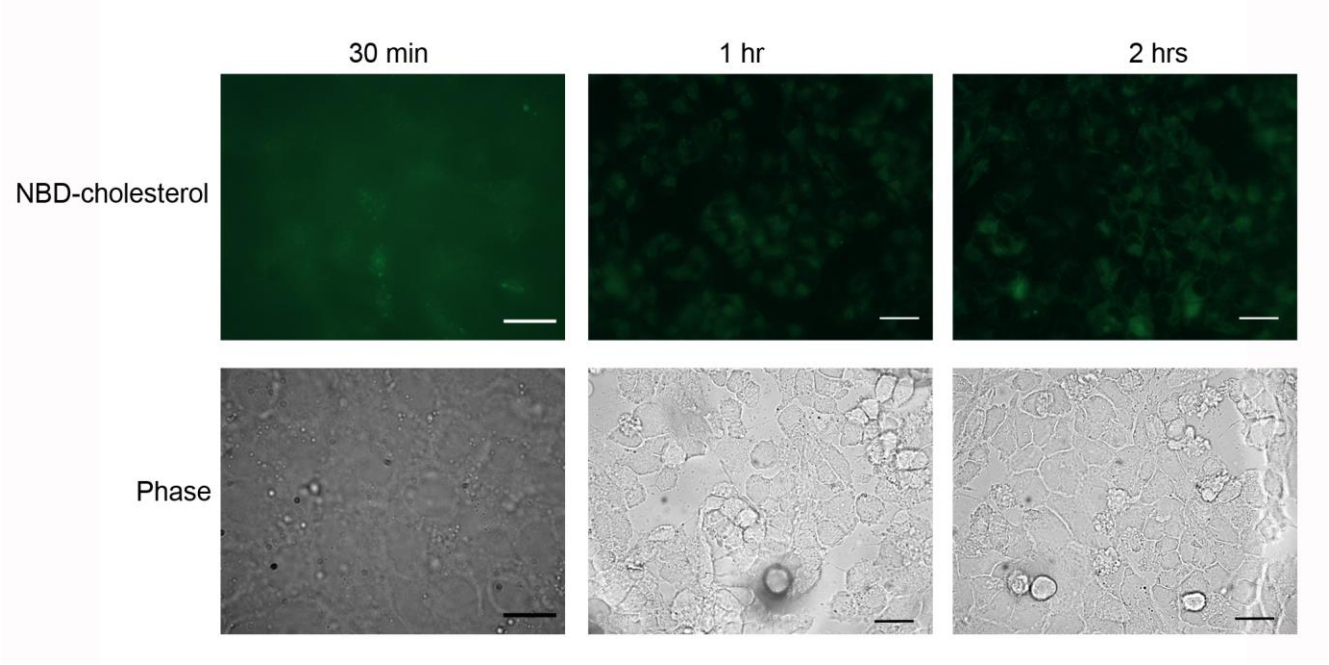
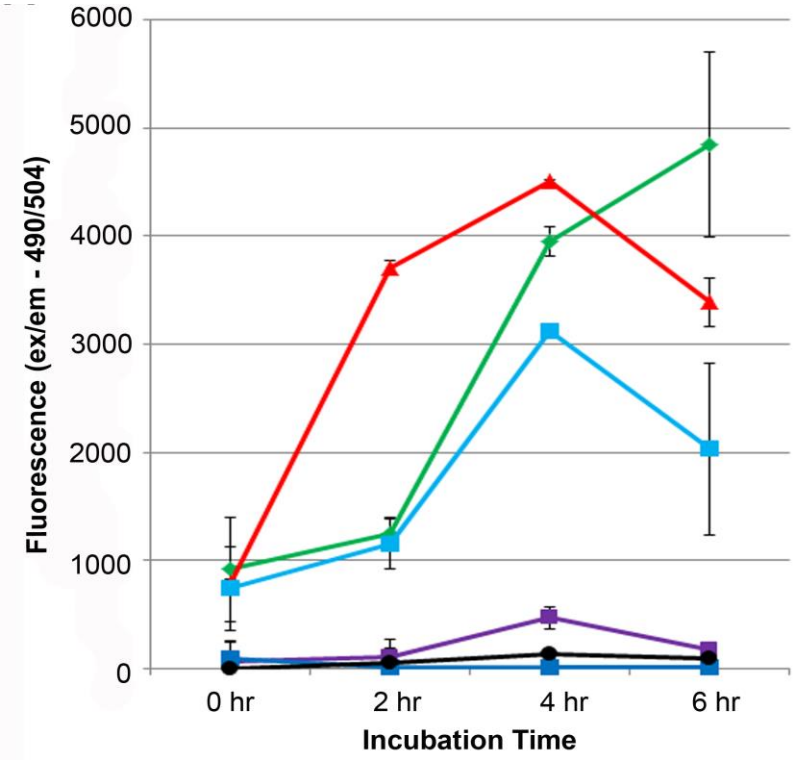

30 min
1 hr
2 hrs
NBD-cholesterol
Phase

Figure 3.3. ***B. burgdorferi*** **incorporates BODIPY-cholesterol into their outer membrane. A.** Spirochetes were grown for 6 hrs in BSK-II medium lacking cholesterol and their fluorescence was measured by spectrophotometry. 4.0 mg/L BODIPY-cholesterol in BSK-II without free cholesterol (green); 0.25% DMSO in BSK-II without free cholesterol (purple); 2.0 mg/L BODIPY-cholesterol BSK-II without free cholesterol (red); 0.175% DMSO in BSK-II without free cholesterol (dark blue); 0.2 mg/L BODIPY-cholesterol in BSK-II without free cholesterol (light blue); 0.0125% DMSO in BSK-II without free cholesterol (black). **B.** Spirochete viability was assessed by counting. Spirochetes grown with NBD-cholesterol or the control DMSO were analyzed by dark-field microscopy enumeration for cytotoxicity. 4.0 mg/L BODIPY-cholesterol in BSK-II without free cholesterol (green); 0.25% DMSO in BSK-II without free cholesterol (purple); 2.0 mg/L BODIPY-cholesterol BSK-II without free cholesterol (red); 0.175% DMSO in BSK-II without free cholesterol (dark blue); 0.2 mg/L BODIPY-cholesterol in BSK-II without free cholesterol (light blue); 0.0125% DMSO in BSK-II without free cholesterol (black) showed no significant cytotoxicity. 1.25×10^7 spirochetes were added in the culture at the beginning of each experiment (red dotted line). Mean ± standard error of the mean from three separate experiments.

A


6000
5000
4000
3000
2000
1000
0
Fluorescence (ex/em - 490/504)
0 hr
2 hr
4 hr
6 hr
Incubation Time

B

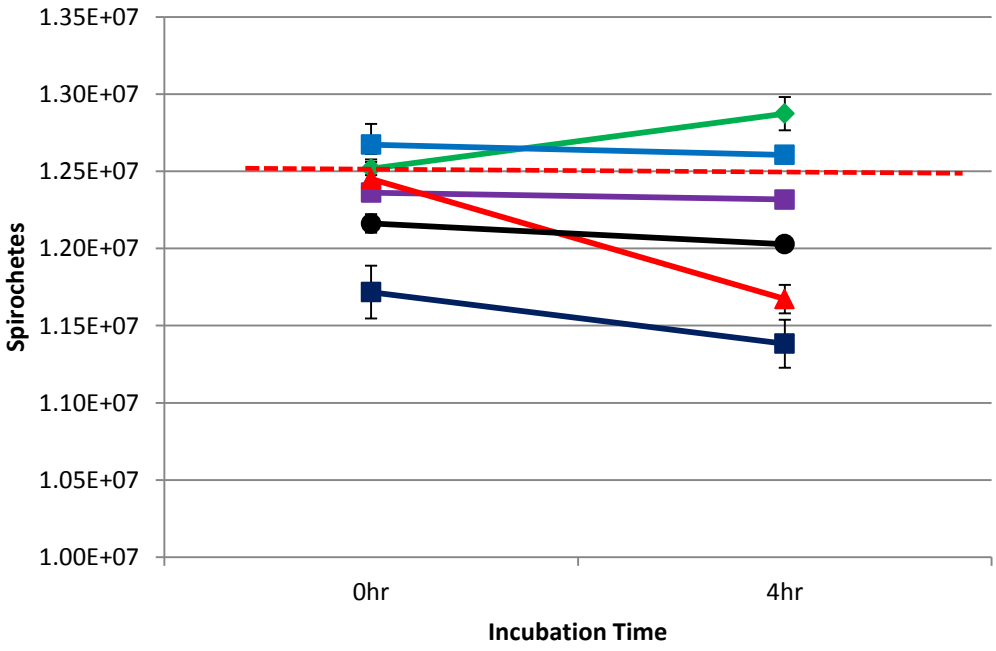
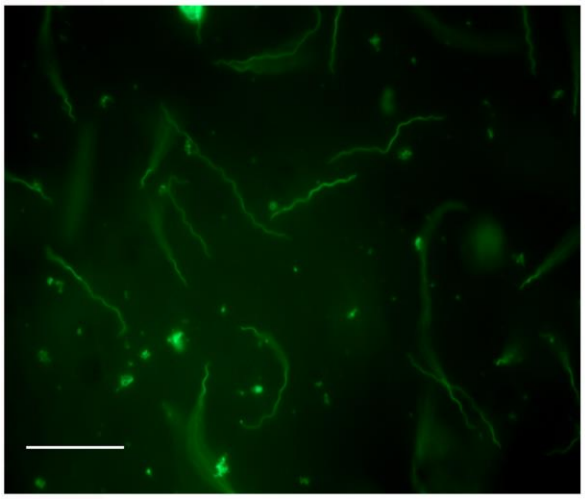

1.35E+07
1.30E+07
1.25E+07
1.20E+07
1.15E+07
1.10E+07
1.05E+07
1.00E+07
Spirochetes
0hr
4hr
Incubation Time

Figure 3.4. ***B. burgdorferi*** **incorporates BODIPY-cholesterol into their outer membrane, remain viable, and are visually fluorescent. A.** Unfixed, live spirochetes grown in the presence of 4.0 mg/L BODIPY-cholesterol for 4 hrs were analyzed by fluorescence microscopy. The spirochetes did not exhibit morphological or motility defects. Scale bar = 20 μm. **B.** *B. burgdorferi* incubated with and without BODIPY-cholesterol were analyzed by flow cytometry. ANOVA ***p<0.001.

A



B

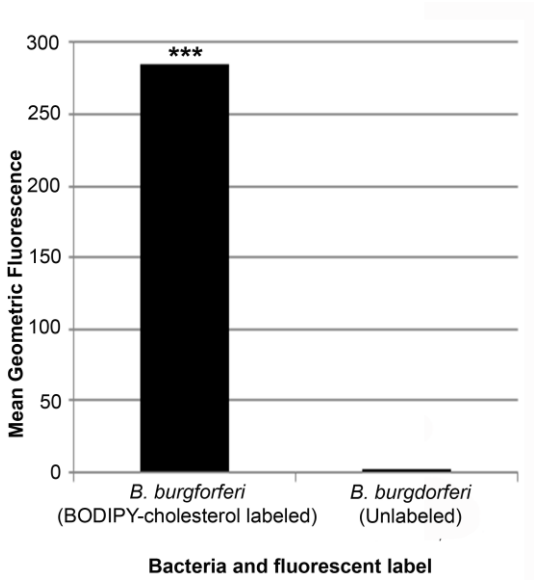

300
250
200
150
100
50
0
***
Mean Geometric Fluorescence
B. burgforferi
(BODIPY-cholesterol labeled)
B. burgdorferi
(Unlabeled)
Bacteria and fluorescent label

Figure 3.5. ***B. burgdorferi*** **incorporates BODIPY-cholesterol into their outer membrane as a component of the cholesterol-glycolipids.** *B. burgdorferi* were incubated with 4.0 mg/L of BODIPY-cholesterol for 4 hrs. *B. burgdorferi* pellets were extracted by Bligh and Dyer lipid extraction and analyzed using a chloroform-methanol (85/15) HPTLC. The glass HPTLC plate was developed using iodine and exposure to UV light. At the 0 min incubation time, the fluorescent cholesterol probe has the same migration profile as cholesterol and is not incorporated into the cholesterol-glycolipids.

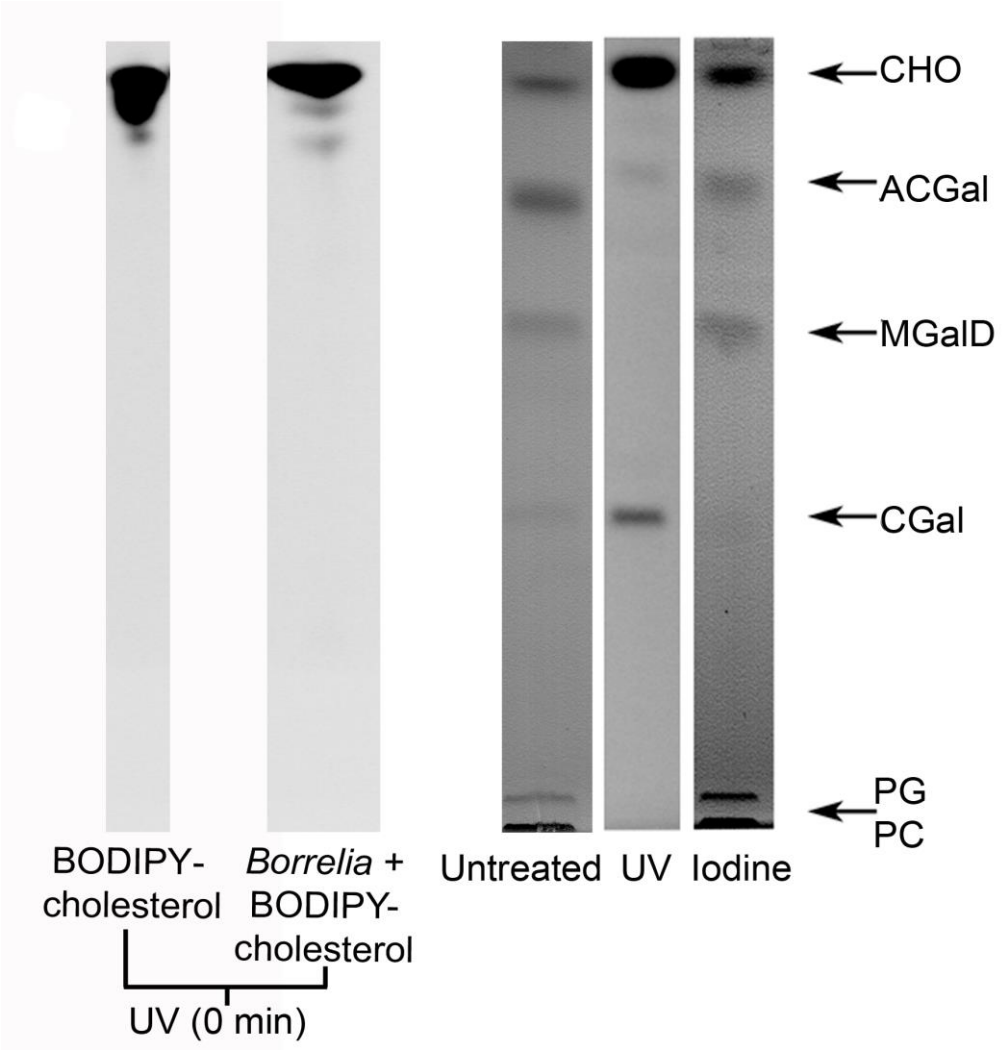
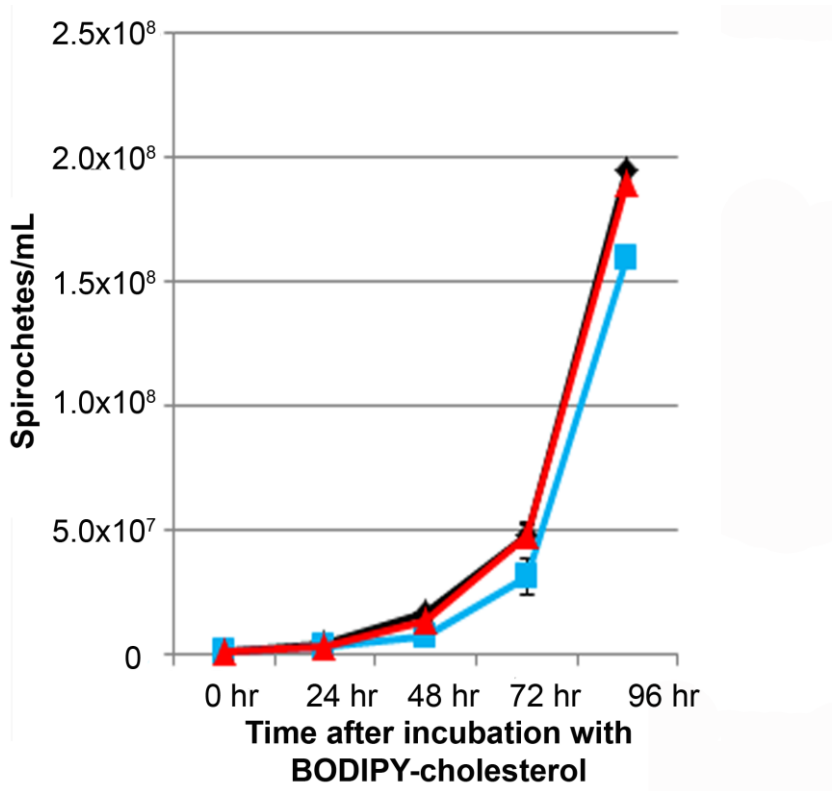

CHO
ACGal
MGalD
CGal
PG
PC
BODIPY-cholesterol
Borrelia + BODIPY-cholesterol
UV (0 min)
Untreated
UV
Iodine

Figure 3.6. ***B. burgdorferi*** **replicate after addition of BODIPY-cholesterol into their outer membrane, but serum is required for growth. A.** Following incubation of *B. burgdorferi* with 4.0 mg/L BODIPY-cholesterol for 4 hrs, the spirochetes were grown in BSK-II for up to 96 hrs, and there were no significant differences in growth compared to controls. Untreated control (black diamond); labeled with BODIPY-cholesterol (blue square); incubated with 0.25% DMSO (red triangle). **B.** *B. burgdorferi* were grown in BSK-II medium lacking free cholesterol with BODIPY-cholesterol or cholesterol as the primary source of sterol in the media to assess long term growth and cytotoxicity in the presence of exogenously added sterol. BSK-II (green); 4.0 mg/L BODIPY-cholesterol in cholesterol free BSK-II without serum (blue); 4.0 mg/L cholesterol in cholesterol free BSK-II without serum (black). Mean ± standard error of the mean from three separate experiments.

A


2.5x10^8
2.0x10^8
1.5x10^8
1.0x10^8
5.0x10^7
0
Spirochetes/mL
0 hr
24 hr
48 hr
72 hr
96 hr
Time after incubation with BODIPY-cholesterol

B

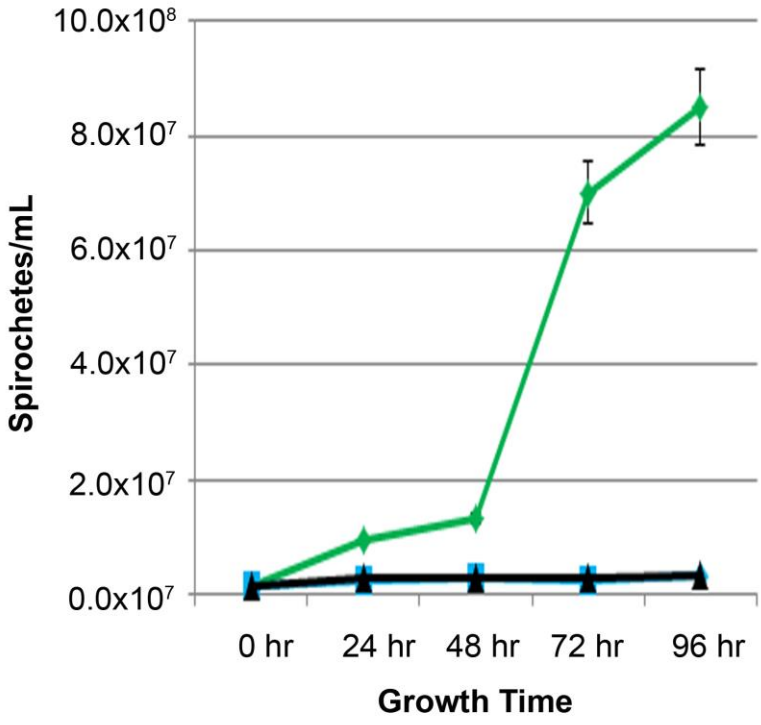

10.0x10^8
8.0x10^7
6.0x10^7
4.0x10^7
2.0x10^7
0.0x10^7
Spirochetes/mL
0 hr
24 hr
48 hr
72 hr
96 hr
Growth Time

Figure 3.7. ***B. burgdorferi*** **transfer BODIPY-cholesterol to HeLa cells.**

B. burgdorferi adhere to HeLa cells and transfer the *Borrelia*-derived fluorescent cholesterol analog BODIPY-cholesterol to the eukaryotic cells. *B. burgdorferi* labeled with BODIPY-cholesterol (green) were incubated with HeLa cells for 2 hrs. *B. burgdorferi* were washed and fixed, and spirochetes that remained attached were detected by labeling OspB (red). Spirochetes having both BODIPY-cholesterol (green) and OspB (red) and appear yellow in the merged confocal micrographs. Images were taken at multiple depths on the Z-axis.

A-D: 3.0 μm.

E-H: 5.0 μm.

I-L: 7.0 μm.

M-P: 9.0 μm.

Q-T: 11.0 μm.

Scale bar = 20 μm.

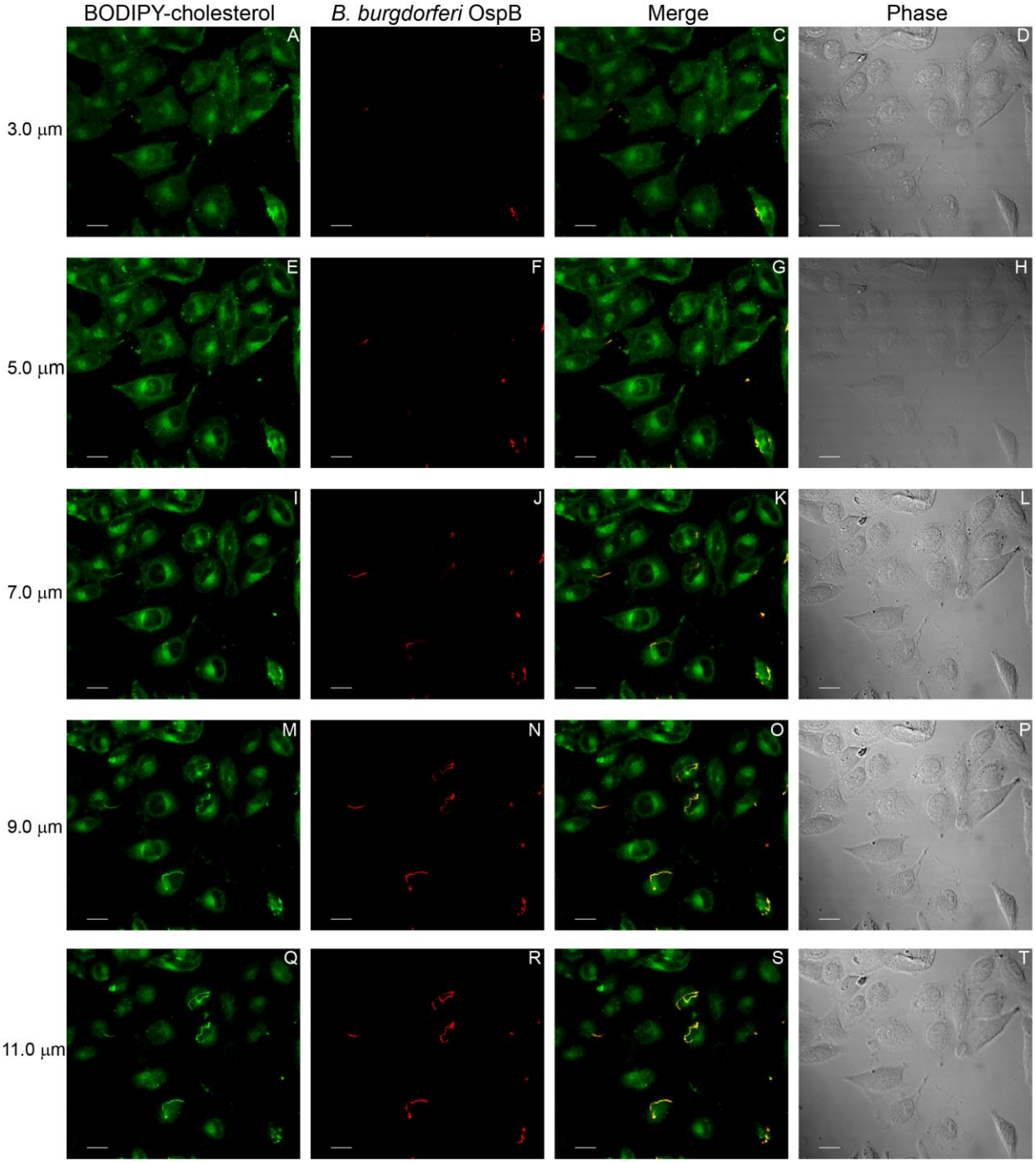

BODIPY-cholesterol
B. burgdorferi OspB
Merge
Phase
3.0 μm
5.0 μm
7.0 μm
9.0 μm
11.0 μm
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

Figure 3.8. *B. burgdorferi*-derived cholesterol is processed by the HeLa cell and localizes to the Golgi complex. A-C. Confocal microscopy demonstrated that the cis-Golgi marker GM130 (red) co-localizes (yellow) with the BODIPY-cholesterol fluorescence (green). **D.** In the control image where no *Borrelia* were added, the perinuclear localization (DAPI nuclear stain, blue) of GM130 is consistent with the other images. Scale bar = 20 μm.

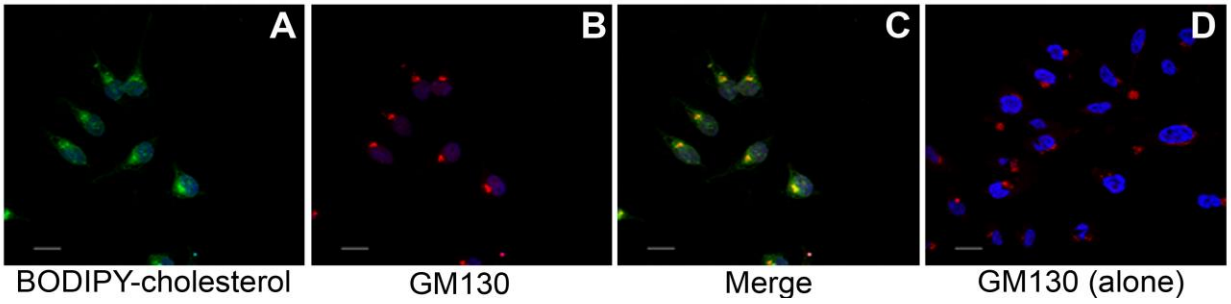
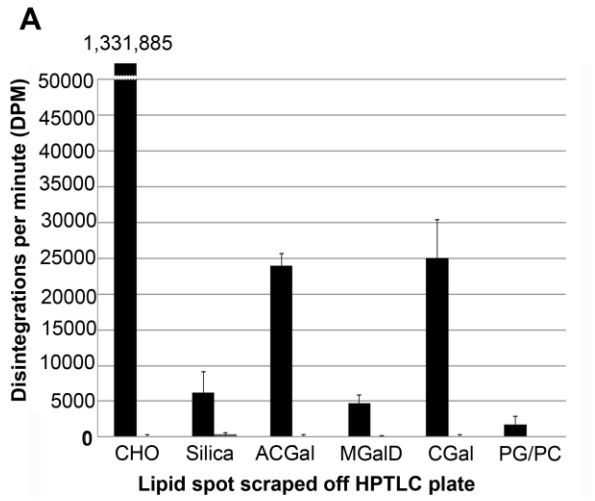
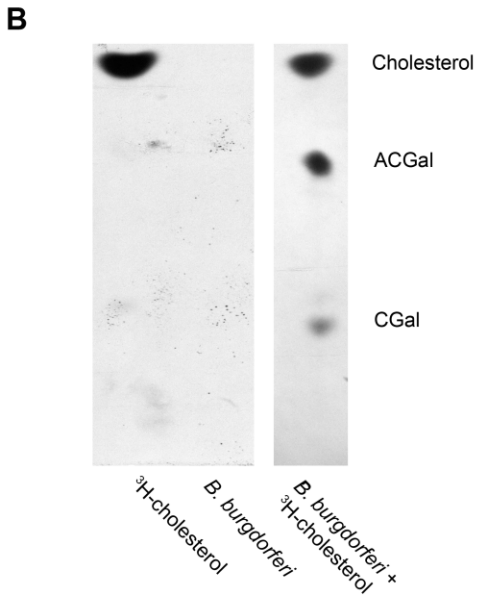
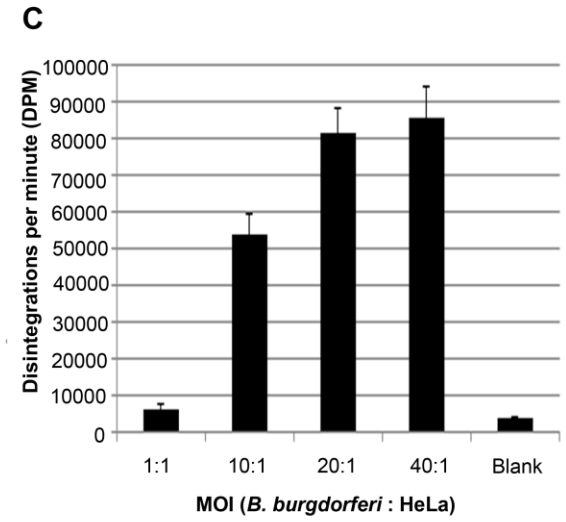

A
B
C
D
BODIPY-cholesterol
GM130
Merge
GM130 (alone)

Figure 3.9. *B. burgdorferi* transfer ^{3}H-cholesterol to HeLa cells. A. *B. burgdorferi* incubated with ^{3}H-cholesterol (black bars) utilized ^{3}H-cholesterol as a substrate for cholesterol-glycolipid synthesis and incorporated the radiolabeled sterol into their lipid fraction when compared to unlabeled *B. burgdorferi* (gray bars). DPMs are derived from silica scraped off the HPTLC plate. **B.** Exposure of a HPTLC plate to Kodak BioMax MR film without scraping demonstrates that the ^{3}H-cholesterol was incorporated into the cholesterol-glycolipids. Radioactive cholesterol was not observed in *B. burgdorferi* incubated with unlabeled cholesterol (middle lane). This data supports the results from HPTLC exposed to UV light from Figure 1E. **C.** Spirochetes labeled with 10.0 µCi ^{3}H-cholesterol transfer membrane lipids to HeLa cells in a dose dependent manner. Spirochetes were incubated at an MOI of 1:1, 10:1, 20:1, and 40:1 (bacteria: eukaryotic cells) with HeLa cells for 2 hours at 37˚C in BSK-II. HeLa cells were washed, lifted, and analyzed for transfer by liquid scintillation counting. **D.** *B. burgdorferi* transfer ^{3}H-cholesterol-labeled glycolipids to HeLa cells. Radiolabeled *B. burgdorferi* were incubated with HeLa cells at an MOI of 40:1 for 2 hrs. The bacteria were removed and the HeLa cell lipid extracts were analyzed by liquid scintillation. Experiments **A, C,** and **D:** mean ± standard error of the mean from three separate experiments.


A
1,331,885
Disintegrations per minute (DPM)
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
CHO
Silica
ACGal
MGalD
CGal
PG/PC
Lipid spot scraped off HPTLC plate


B
Cholesterol
ACGal
CGal
3H-cholesterol
B. burgdorferi
B. burgdorferi + 3H-cholesterol


C
Disintegrations per minute (DPM)
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
1:1
10:1
20:1
40:1
Blank
MOI (B. burgdorferi : HeLa)

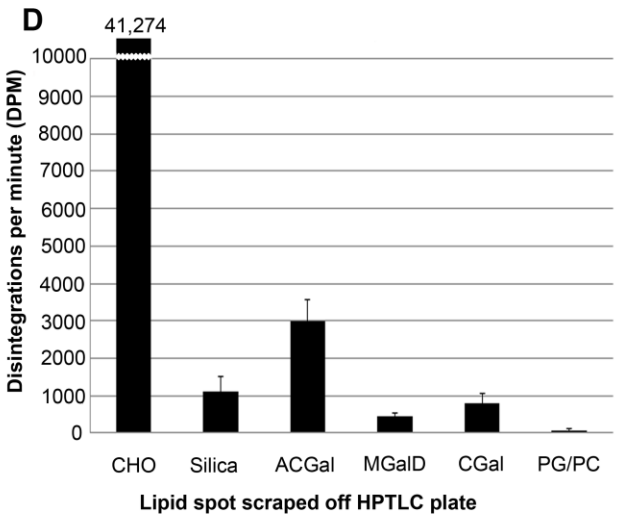

D
41,274
Disintegrations per minute (DPM)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
CHO
Silica
ACGal
MGalD
CGal
PG/PC
Lipid spot scraped off HPTLC plate

Figure 3.10. Exchange of lipids from *B. burgdorferi* to HeLa cells is specific and is also accomplished from conditioned media collected from BODIPY-labeled *Borrelia*. A-D. Spirochetes labeled with BODIPY-cholesterol were incubated for 2 hours at 37˚C in BSK-II to collect any released fluorescent material. **A**. The fluorescence of BODIPY-cholesterol (green) from the cell free supernatant was transferred to the HeLa cells after 2 hrs of incubation. **B, C.** No whole *B. burgdorferi* (red) are present in the preparations. **D.** Phase contrast. **E-H.** BODIPY-*Borrelia* cell free wash supernatant. No observable fluorescence indicates that the transfer of fluorescent lipids is specific and not derived from the medium, washes, or lysed bacteria. Scale bar = 20 μm.

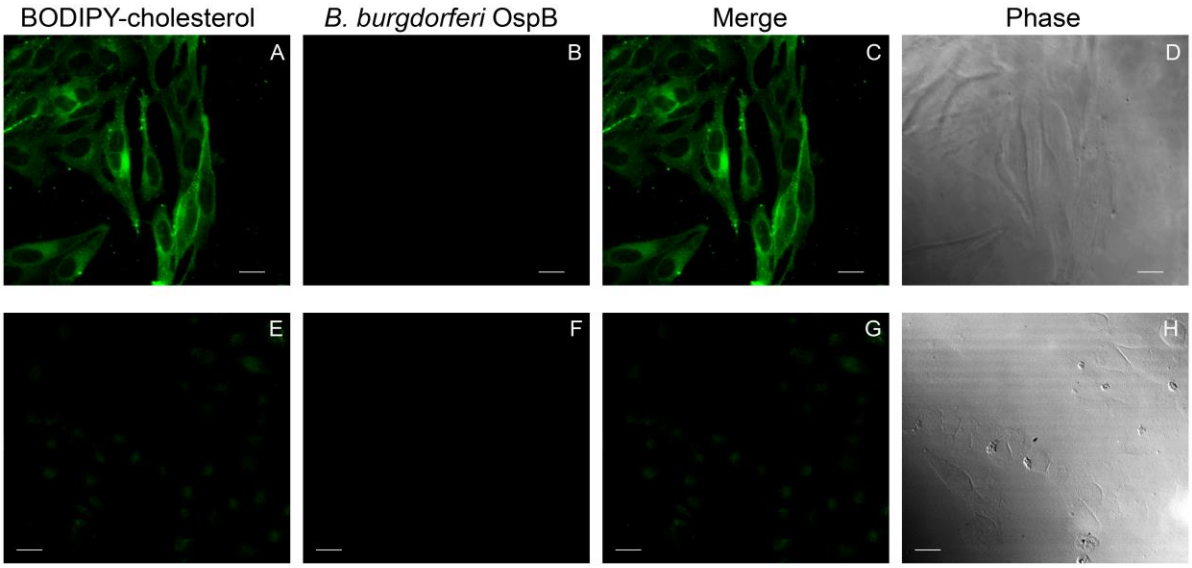
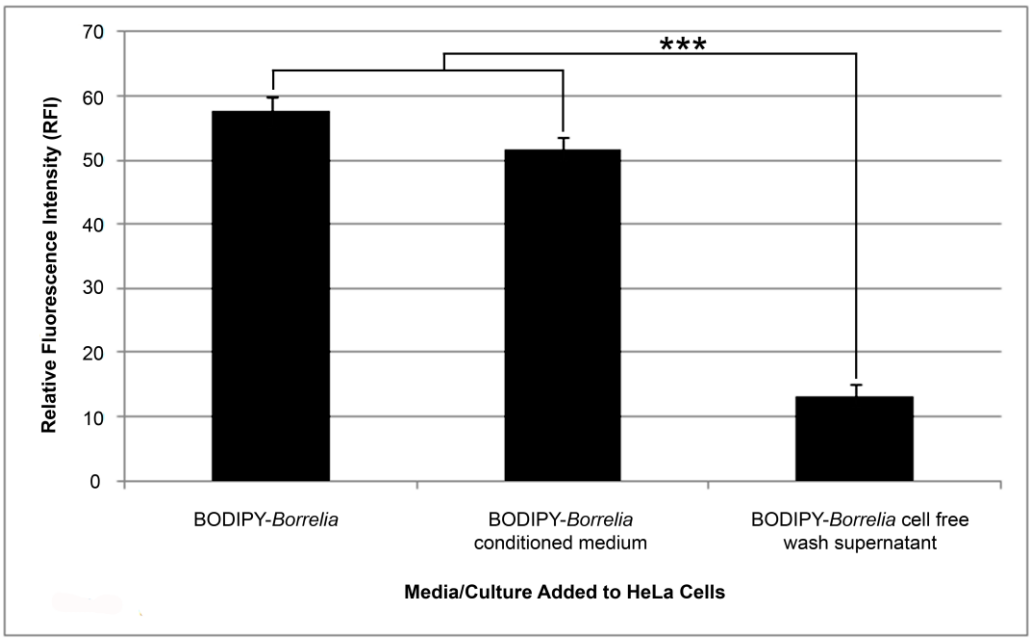

BODIPY-cholesterol
B. burgdorferi OspB
Merge
Phase
A
B
C
D
E
F
G
H

Figure 3.11. Transfer of *Borrelia*-derived cholesterol occurs through direct contact and cell-free conditioned medium.

A. The mean RFI +/- standard error of the mean of HeLa cells from 10 microscope fields were calculated from the BODIPY-*Borrelia*, BODIPY-*Borrelia* conditioned medium, and the BODIPY-*Borrelia* cell-free wash supernatant. ANOVA ***$p<0.001$,

B. Mean geometric fluorescence +/- standard error of the mean from three separate flow cytometry analyses of HeLa cells from the BODIPY-*Borrelia*, BODIPY-*Borrelia* conditioned medium, and the BODIPY-*Borrelia* cell-free wash supernatant. ANOVA***$p<0.001$.

A


***
70
60
50
40
30
20
10
0
Relative Fluorescence Intensity (RFI)
BODIPY-Borrelia
BODIPY-Borrelia conditioned medium
BODIPY-Borrelia cell free wash supernatant
Media/Culture Added to HeLa Cells

B

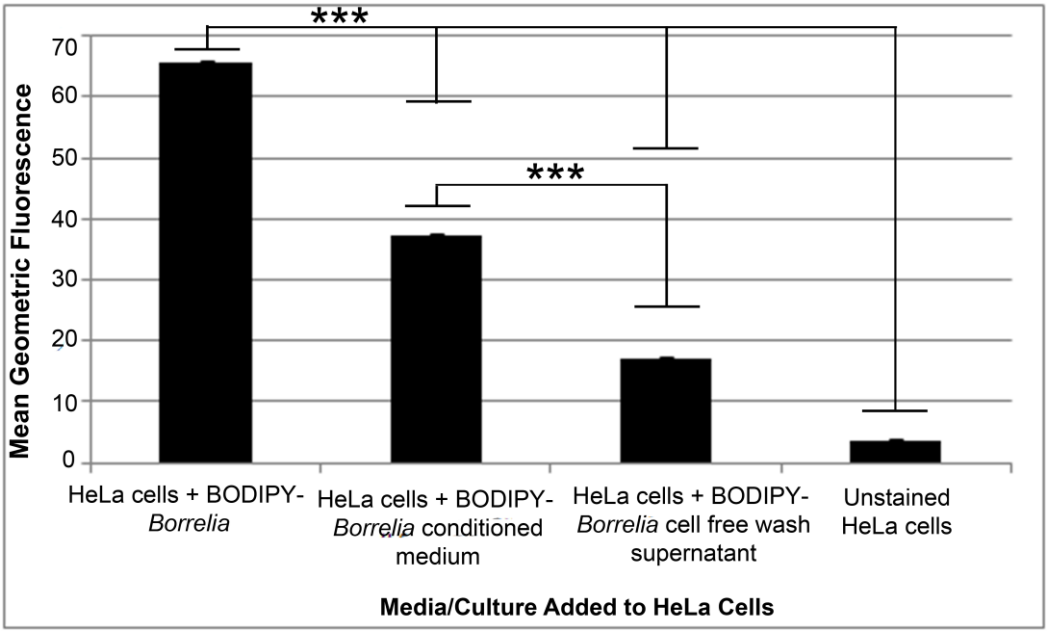

***
***
70
60
50
40
30
20
10
0
Mean Geometric Fluorescence
HeLa cells + BODIPY-Borrelia
HeLa cells + BODIPY-Borrelia conditioned medium
HeLa cells + BODIPY-Borrelia cell free wash supernatant
Unstained HeLa cells
Media/Culture Added to HeLa Cells

Figure 3.12. Transfer of BODIPY-cholesterol from *B. burgdorferi* to HeLa cells is time-dependent. *B. burgdorferi* labeled with BODIPY-cholesterol, and BODIPY- *Borrelia* conditioned medium, and cell-free wash supernatant were incubated with HeLa cells for 15 min, 30 min, 1 hr, and 2 hr.

A-F: HeLa cells exposed to *B. burgdorferi* labeled with BODIPY-cholesterol, BODIPY-*Borrelia* conditioned medium, or the cell-free wash supernatant for 15 min.

G-L: HeLa cells exposed to *B. burgdorferi* labeled with BODIPY-cholesterol, BODIPY-*Borrelia* conditioned medium, or the cell-free wash supernatant for 30 min.

M-R: HeLa cells exposed to *B. burgdorferi* labeled with BODIPY-cholesterol, BODIPY-*Borrelia* conditioned medium, or the cell-free wash supernatant for 1 hr.

S-X: HeLa cells exposed to *B. burgdorferi* labeled with BODIPY-cholesterol, BODIPY-*Borrelia* conditioned medium, or the cell-free wash supernatant for 2 hrs.

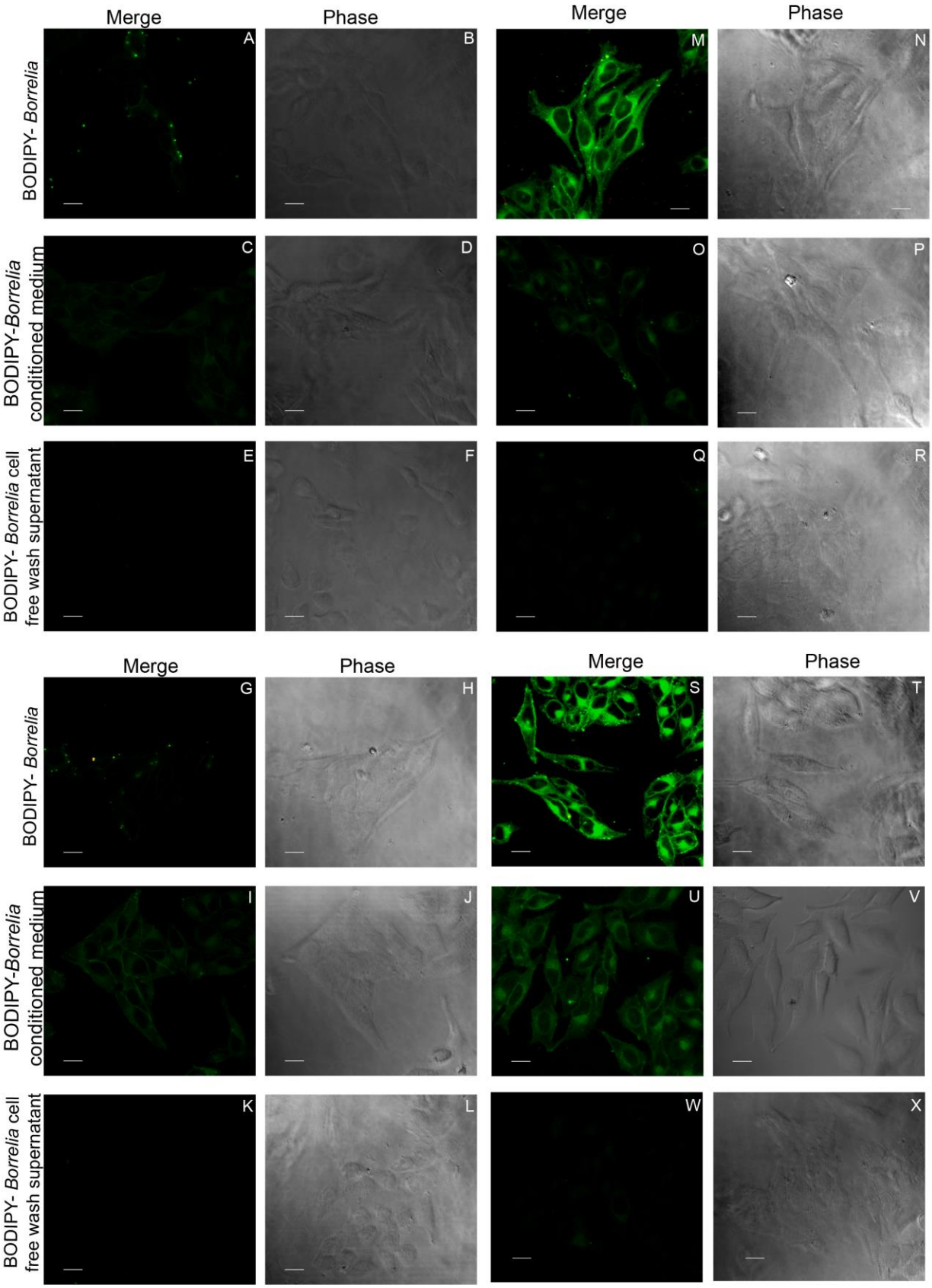
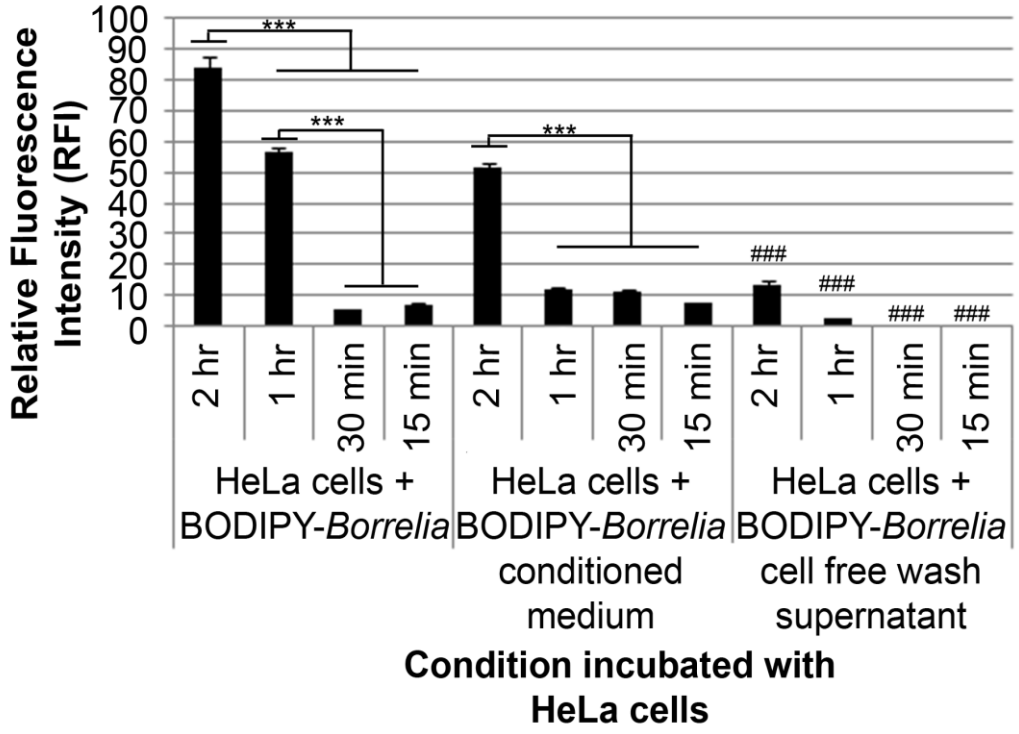

Merge
Phase
Merge
Phase
BODIPY- Borrelia
BODIPY-Borrelia conditioned medium
BODIPY- Borrelia cell free wash supernatant
A
B
M
N
C
D
O
P
E
F
Q
R
G
H
S
T
I
J
U
V
K
L
W
X

Figure 3.13. Transfer of *Borrelia*-derived cholesterol is time-dependent.

A. The mean relative fluorescence intensity (RFI) +/- standard error of the mean of HeLa cells from 10 microscope fields were calculated from the BODIPY-*Borrelia,* BODIPY-*Borrelia* conditioned medium, and the BODIPY-*Borrelia* cell-free wash supernatant. ANOVA ***p<0.001, ###p<0.001 (negative control is significantly less than comparable experimental condition).

B. Mean geometric fluorescence +/- standard error of the mean from three separate flow cytometry analyses of HeLa cells incubated following the experimental conditions. ANOVA *p<0.05, *p<0.01 ***p<0.001, ##p<0.01, ###p<0.001 (negative control is significantly less than associated experimental condition).

A


Relative Fluorescence Intensity (RFI)
100
90
80
70
60
50
40
30
20
10
0
***
***
***
###
###
###
###
2 hr
1 hr
30 min
15 min
2 hr
1 hr
30 min
15 min
2 hr
1 hr
30 min
15 min
HeLa cells + BODIPY-Borrelia
HeLa cells + BODIPY-Borrelia conditioned medium
HeLa cells + BODIPY-Borrelia cell free wash supernatant
Condition incubated with HeLa cells

B

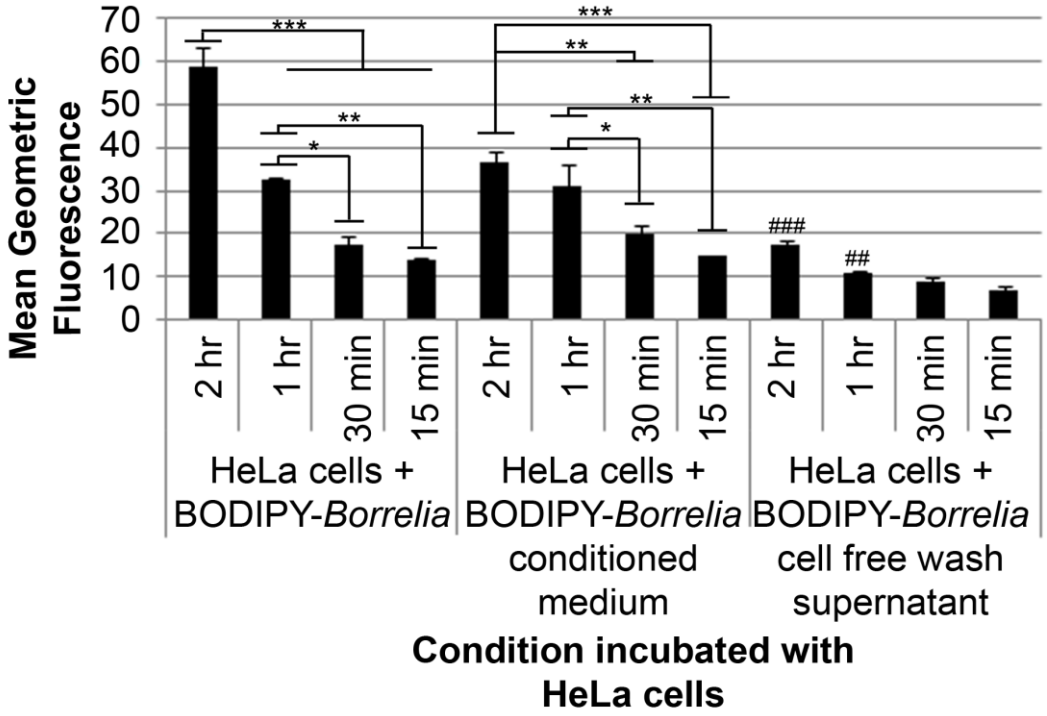

Mean Geometric Fluorescence
70
60
50
40
30
20
10
0
***
***
**
**
**
*
*
###
##
2 hr
1 hr
30 min
15 min
2 hr
1 hr
30 min
15 min
2 hr
1 hr
30 min
15 min
HeLa cells + BODIPY-Borrelia
HeLa cells + BODIPY-Borrelia conditioned medium
HeLa cells + BODIPY-Borrelia cell free wash supernatant
Condition incubated with HeLa cells

Figure 3.14. Transfer of BODIPY-cholesterol from *B. burgdorferi* to HeLa cells is more efficient at higher temperatures. *B. burgdorferi* labeled with BODIPY-cholesterol, BODIPY-*Borrelia* conditioned medium, and cell-free wash supernatant were incubated with HeLa cells for 2 hrs at 4˚C, 25˚C, and 37˚C. The preparations were probed for OspB by using the antibody CB2. Green fluorescence is the BODIPY-cholesterol and yellow is the colocalization of the CB2 (red) and BODIPY (green).

A-F: HeLa cells exposed to *B. burgdorferi* labeled with BODIPY-cholesterol, BODIPY-*Borrelia* conditioned medium, or cell-free wash supernatants for 2 hrs at 4˚C.

G-L: HeLa cells exposed to *B. burgdorferi* labeled with BODIPY-cholesterol, BODIPY-*Borrelia* conditioned medium, or cell-free wash supernatants for 2 hrs at 25˚C.

M-R: HeLa cells exposed to *B. burgdorferi* labeled with BODIPY-cholesterol, BODIPY-*Borrelia* conditioned medium, or cell-free wash supernatants for 2 hrs at 37˚C.

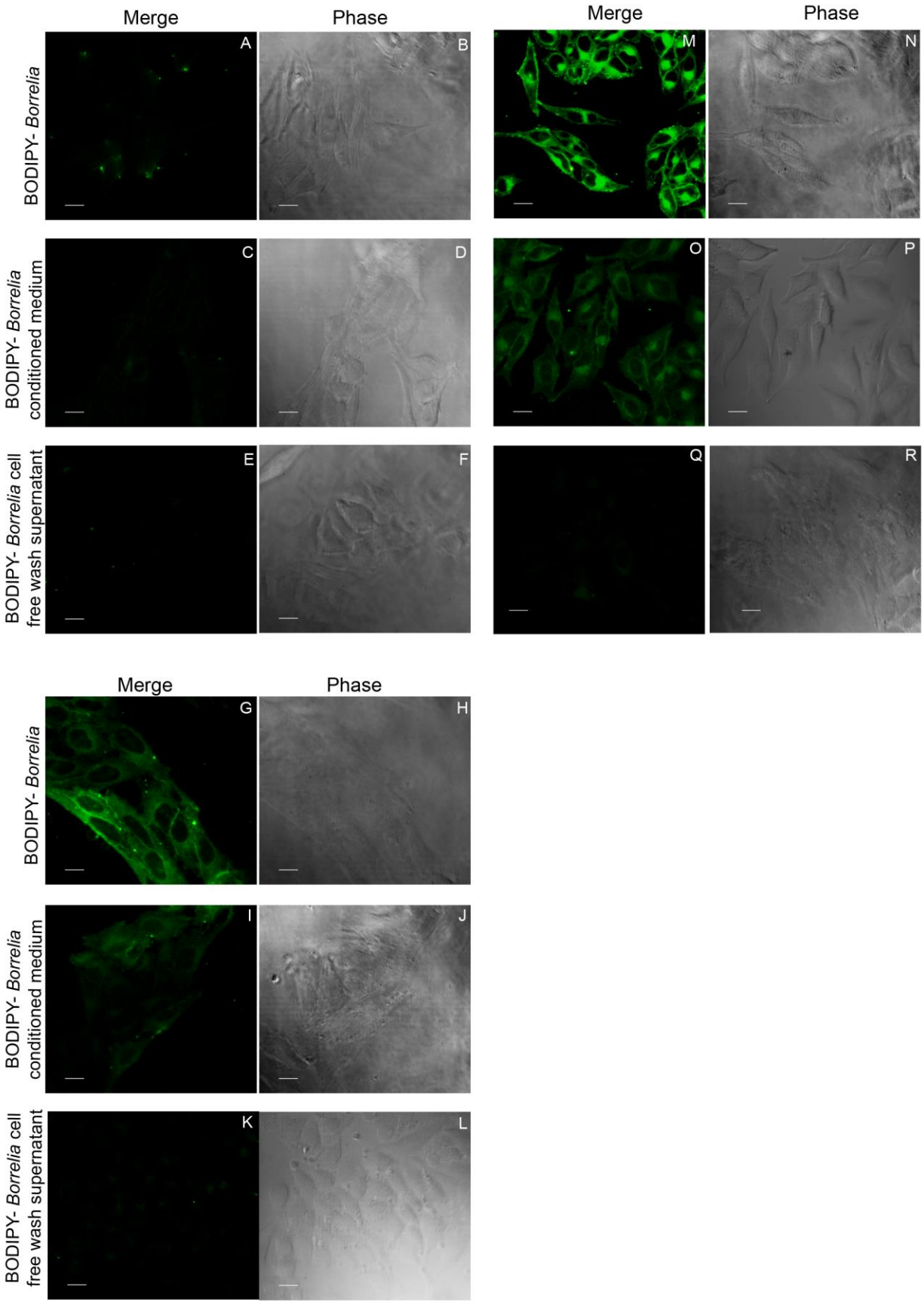
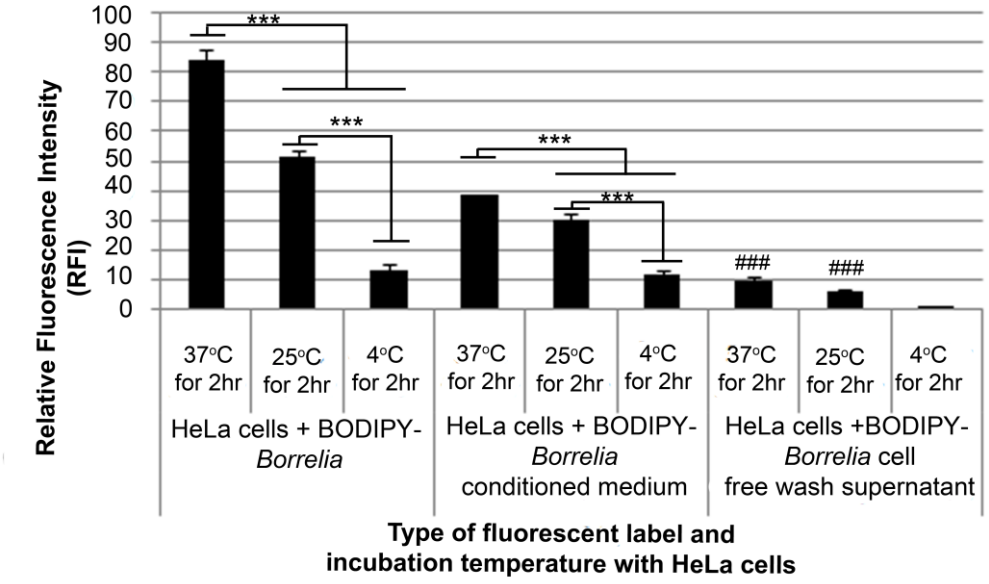

Merge
Phase
Merge
Phase
BODIPY- Borrelia
BODIPY- Borrelia conditioned medium
BODIPY- Borrelia cell free wash supernatant
A
B
C
D
E
F
M
N
O
P
Q
R
Merge
Phase
BODIPY- Borrelia
BODIPY- Borrelia conditioned medium
BODIPY- Borrelia cell free wash supernatant
G
H
I
J
K
L

Figure 3.15. Transfer of *Borrelia* derived cholesterol is temperature-dependent.

A. The mean relative fluorescence intensity (RFI) +/- standard error of the mean of HeLa cells from 10 microscope fields were calculated from the *B. burgdorferi* labeled with BODIPY-cholesterol, BODIPY-*Borrelia* conditioned medium, and cell-free wash supernatant. ANOVA ***p<0.001, ###p<0.001 (negative control is significantly less than associated experimental condition).

B. Mean geometric fluorescence +/- standard error of the mean from three separate flow cytometry analysis of HeLa cells incubated following the experimental conditions. ANOVA *p<0.05, *p<0.01 ***p<0.001, ###p<0.001 (negative control is significantly less than associated experimental condition).

A


Relative Fluorescence Intensity (RFI)
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
###
###
37°C for 2hr
25°C for 2hr
4°C for 2hr
37°C for 2hr
25°C for 2hr
4°C for 2hr
37°C for 2hr
25°C for 2hr
4°C for 2hr
HeLa cells + BODIPY-Borrelia
HeLa cells + BODIPY-Borrelia conditioned medium
HeLa cells +BODIPY-Borrelia cell free wash supernatant
Type of fluorescent label and incubation temperature with HeLa cells

B

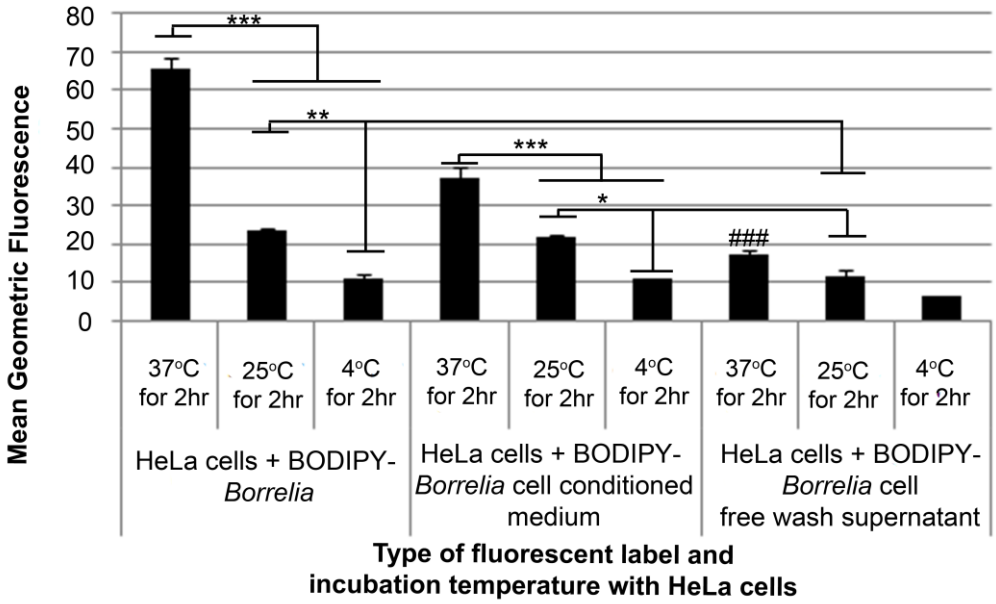

Mean Geometric Fluorescence
80
70
60
50
40
30
20
10
0
***
**
***
*
###
37°C for 2hr
25°C for 2hr
4°C for 2hr
37°C for 2hr
25°C for 2hr
4°C for 2hr
37°C for 2hr
25°C for 2hr
4°C for 2hr
HeLa cells + BODIPY-Borrelia
HeLa cells + BODIPY-Borrelia cell conditioned medium
HeLa cells + BODIPY-Borrelia cell free wash supernatant
Type of fluorescent label and incubation temperature with HeLa cells

Figure 3.16. Exchange of lipids from *B. burgdorferi* to HeLa cells is not dependent on actin polymerization. HeLa cells were treated with cytochalasin D to inhibit actin polymerization. The pretreatments were for 1 hour at 50 μg/mL, 10 μg/mL, and 1μg/mL. Confocal micrographs indicate that at multiple concentrations of cytochalasin D, the treatment of HeLa cells does not inhibit the transfer of BODIPY-cholesterol.

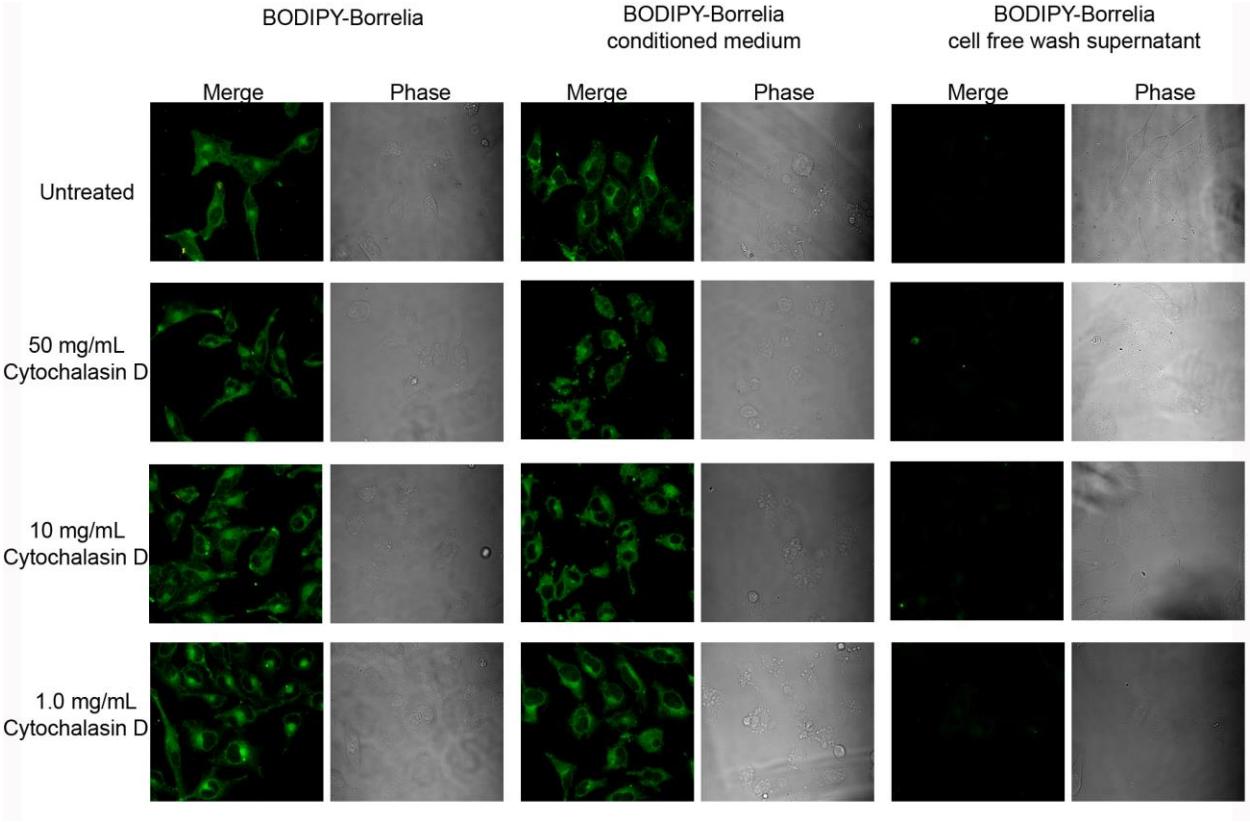

BODIPY-Borrelia
BODIPY-Borrelia conditioned medium
BODIPY-Borrelia cell free wash supernatant
Merge
Phase
Merge
Phase
Merge
Phase
Untreated
50 mg/mL Cytochalasin D
10 mg/mL Cytochalasin D
1.0 mg/mL Cytochalasin D

Figure 3.17. Cytochalasin D does not inhibit the transfer of *B. burgdorferi*-derived BODIPY-cholesterol. The mean RFI +/- standard error of the mean of HeLa cells from 10 microscope fields were calculated from the *B. burgdorferi* labeled with BODIPY-cholesterol, BODIPY-*Borrelia* conditioned medium, and cell-free wash supernatant. Each condition was treated for 1 hour at 50 μg/mL, 10 μg/mL, and 1μg/mL. RFI analysis indicates that there is no inhibition of transfer of *Borrelia* derived lipids when HeLa cells were treated with cytochalasin D. Results are reported as a mean +/- standard error of the mean.

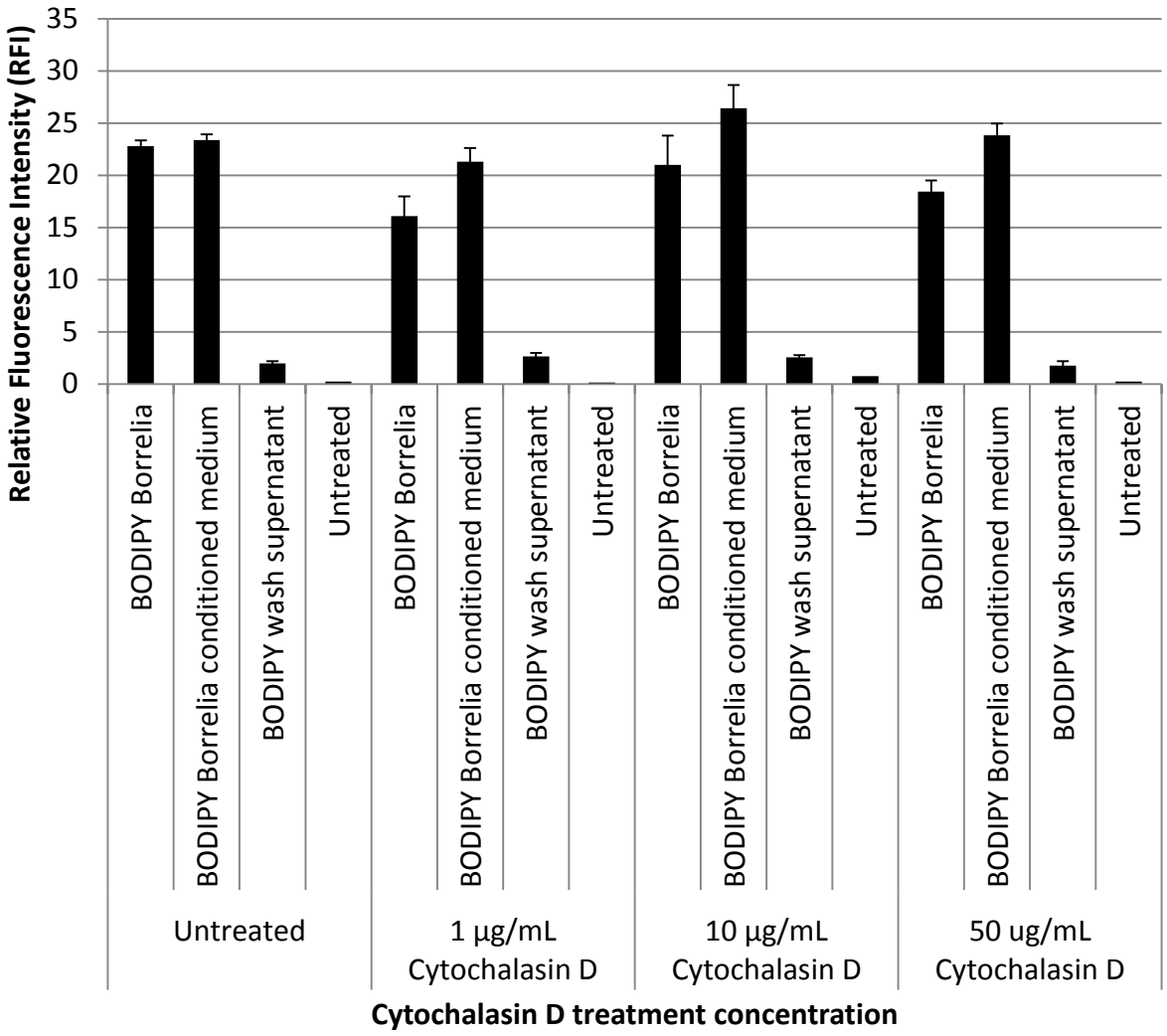

Relative Fluorescence Intensity (RFI)
35
30
25
20
15
10
5
0
BODIPY Borrelia
BODIPY Borrelia conditioned medium
BODIPY wash supernatant
Untreated
BODIPY Borrelia
BODIPY Borrelia conditioned medium
BODIPY wash supernatant
Untreated
BODIPY Borrelia
BODIPY Borrelia conditioned medium
BODIPY wash supernatant
Untreated
BODIPY Borrelia
BODIPY Borrelia conditioned medium
BODIPY wash supernatant
Untreated
Untreated
1 µg/mL Cytochalasin D
10 µg/mL Cytochalasin D
50 ug/mL Cytochalasin D
Cytochalasin D treatment concentration

Figure 3.18. Transfer of *B. burgdorferi*-derived lipids or treatment with cytochalasin D is not cytotoxic to HeLa cells over the course of the experiments. LDH release of HeLa cells was measured after cytochalasin D treatment and incubation with BODIPY-*Borrelia*, BODIPY-*Borrelia* conditioned medium, or BODIPY-*Borrelia* wash supernatant for 2 hrs. Cytochalasin D does not appear to cause significant levels of cytotoxicity. Results are reported as a mean +/- standard error of the mean.

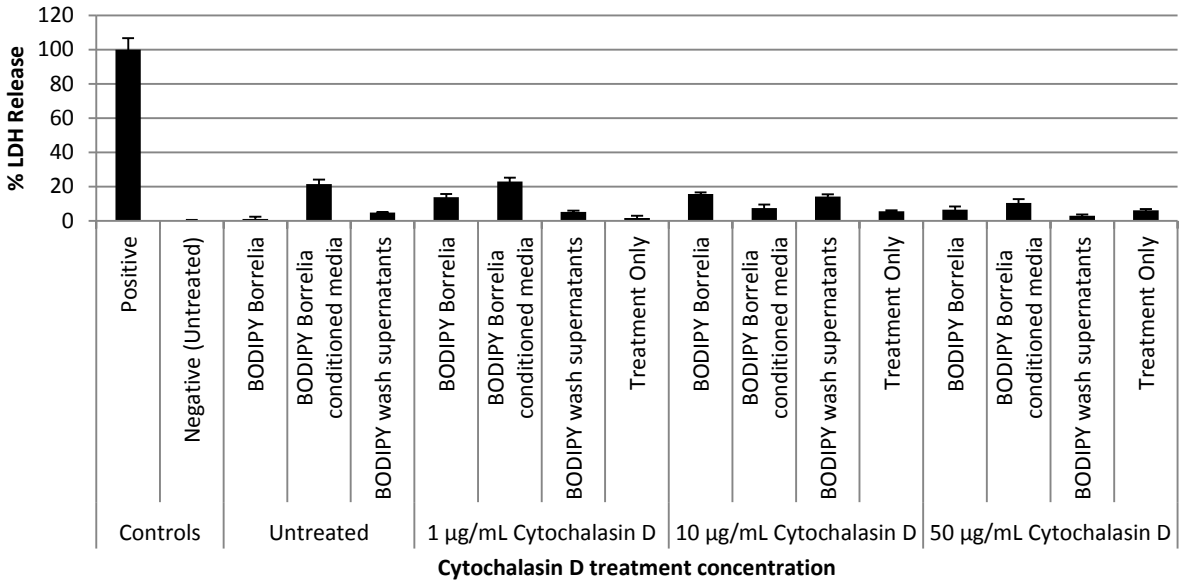
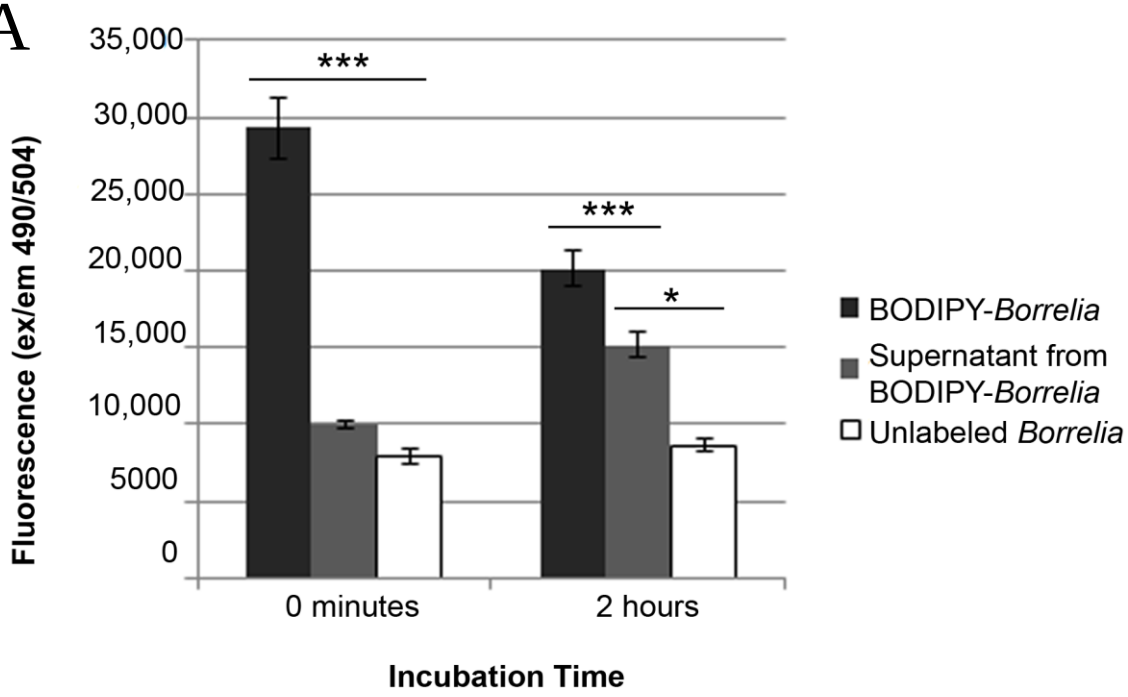

% LDH Release
120
100
80
60
40
20
0
Positive
Negative (Untreated)
BODIPY Borrelia
BODIPY Borrelia conditioned media
BODIPY wash supernatants
BODIPY Borrelia
BODIPY Borrelia conditioned media
BODIPY wash supernatants
Treatment Only
BODIPY Borrelia
BODIPY Borrelia conditioned media
BODIPY wash supernatants
Treatment Only
BODIPY Borrelia
BODIPY Borrelia conditioned media
BODIPY wash supernatants
Treatment Only
Controls
Untreated
1 µg/mL Cytochalasin D
10 µg/mL Cytochalasin D
50 µg/mL Cytochalasin D
Cytochalasin D treatment concentration

Figure 3.19. ***B. burgdorferi*** **labeled with BODIPY-cholesterol release the fluorescent cholesterol probe over time.** **A.** *B. burgdorferi* were labeled with 4.0 mg/L BODIPY-cholesterol and incubated for 2 hrs. After the centrifugation, the bacterial pellets and supernatants were measured for BODIPY-cholesterol fluorescence using the SpectraMaxM2. The fluorescence of the whole bacteria decreased while the supernatant fluorescence increased. ANOVA ***p< 0.001, *p<0.05. **B.** Labeled and unlabeled *B. burgdorferi* release similar amounts of membrane material. DPH was added to the supernatants to measure released OMV. Using the SpectraMaxM2, the DPH fluorescence was calculated for each sample at the specific time points. Experiments **A** and **B** represent the mean ± standard error of the mean from three separate experiments.

A


35,000
30,000
25,000
20,000
15,000
10,000
5000
0
Fluorescence (ex/em 490/504)
***
***
*
0 minutes
2 hours
Incubation Time
BODIPY-Borrelia
Supernatant from BODIPY-Borrelia
Unlabeled Borrelia

B

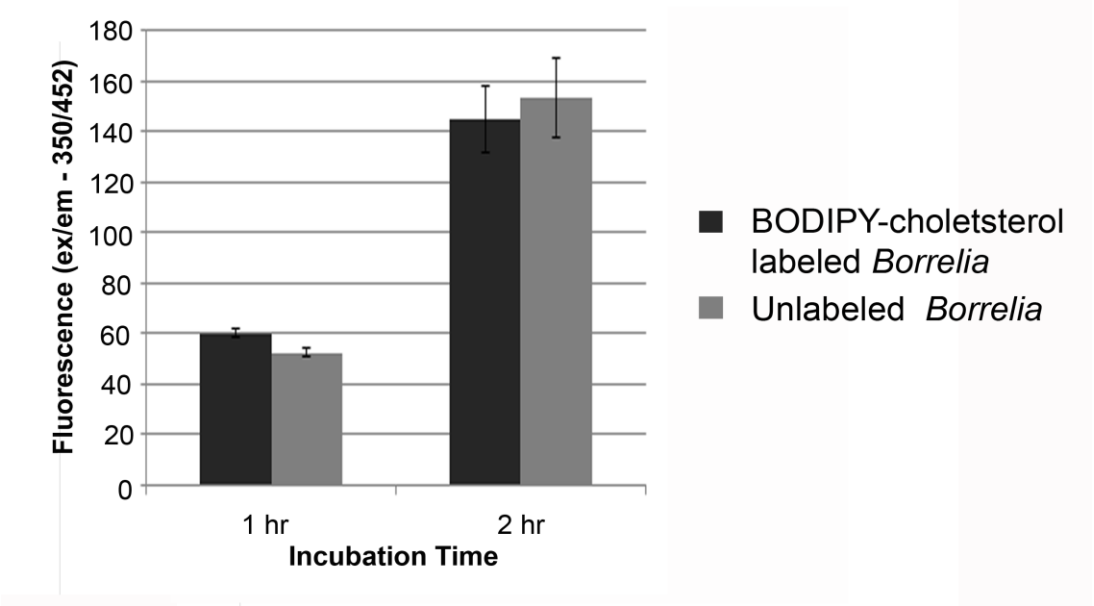
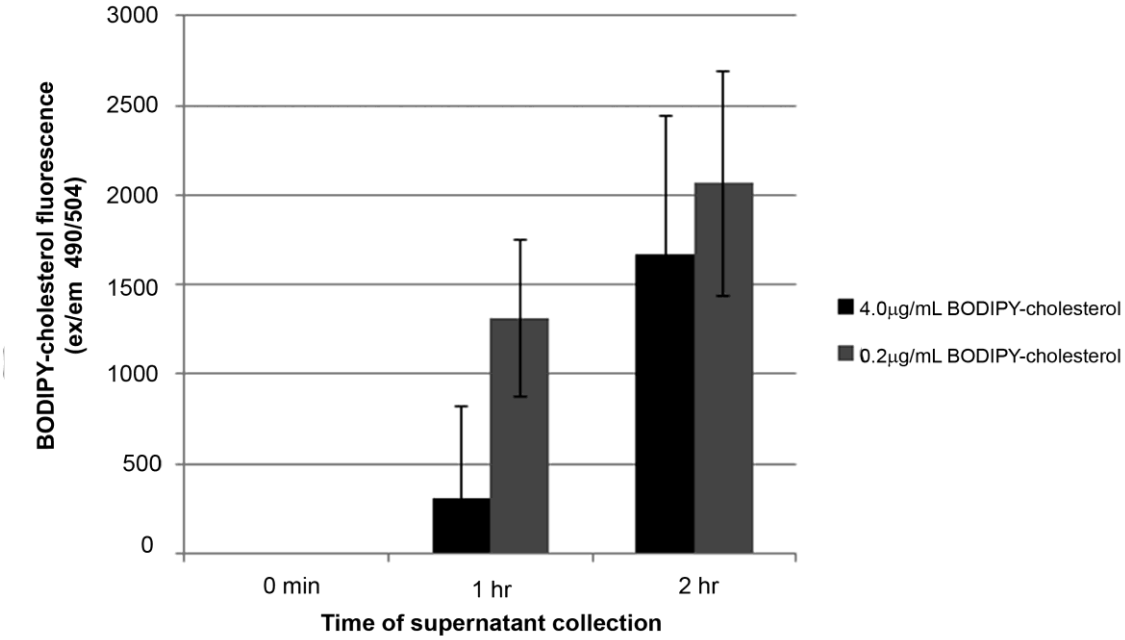

180
160
140
120
100
80
60
40
20
0
Fluorescence (ex/em - 350/452)
1 hr
2 hr
Incubation Time
BODIPY-choletsterol labeled Borrelia
Unlabeled Borrelia

Figure 3.20. The amount of BODIPY-cholesterol given to *B. burgdorferi* does not influence the amounts of cholesterol released by the spirochetes. *B. burgdorferi* were given 0.2 μg mL cholesterol (1X concentration of cholesterol found in cholesterol free BSK-II) and 4.0 μg/mL (20X concentration of cholesterol found in cholesterol BSK-II). **A.** *Borrelia* given BODIPY-cholesterol at concentrations of 0.2 μg/mL or 4.0 μg/mL released similar amounts of fluorescence. **B.** Total cholesterol measurements of the supernatants was measured using the Amplex Red Cholesterol quantification kit. The total cholesterol bar graph is a representative experiment that included 3 individual samples per group, while the BODIPY-cholesterol fluorescence data is a combination of 3 replicates. For both **A** and **B**, there were no significant differences in the fluorescence or cholesterol released between the *Borrelia* incubated with 0.2 μg/mL or 4.0 μg/mL of BODIPY cholesterol. Experiment **A** represents the mean ± standard error of the mean from three separate experiments.

A


3000
2500
2000
1500
1000
500
0
BODIPY-cholesterol fluorescence (ex/em 490/504)
0 min
1 hr
2 hr
Time of supernatant collection
4.0μg/mL BODIPY-cholesterol
0.2μg/mL BODIPY-cholesterol

B

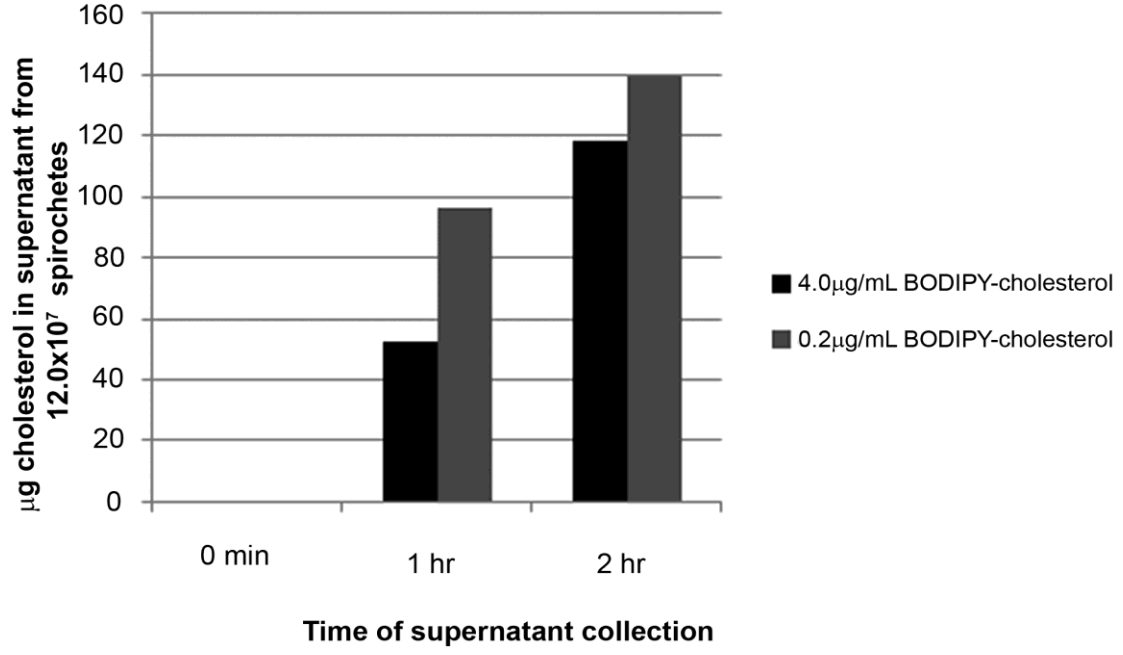
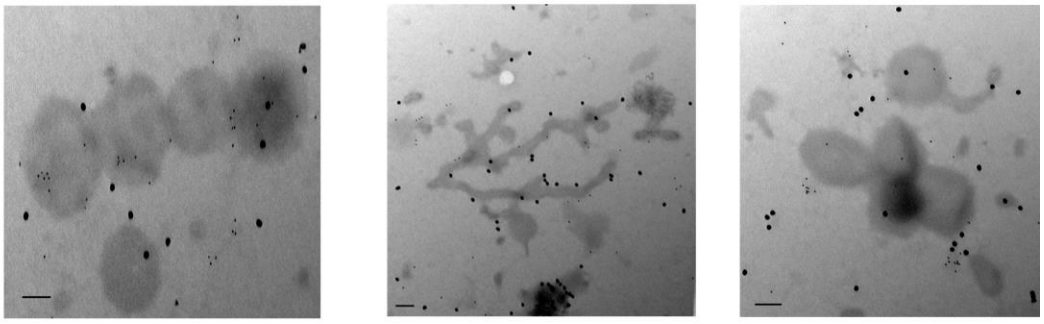
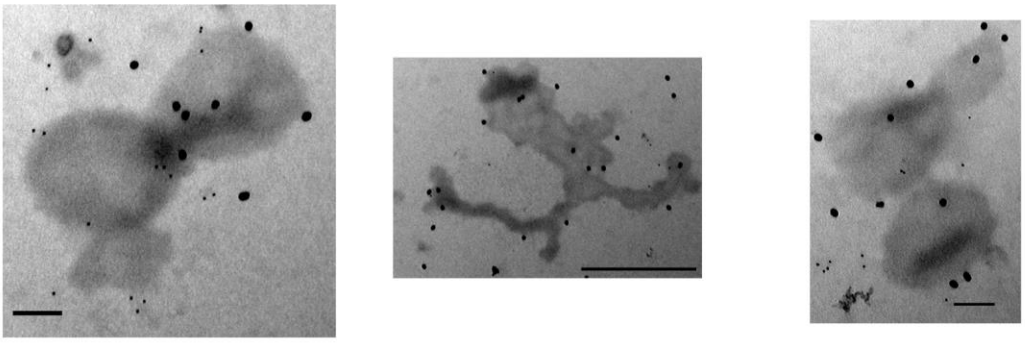

160
140
120
100
80
60
40
20
0
μg cholesterol in supernatant from 12.0x10⁷ spirochetes
0 min
1 hr
2 hr
Time of supernatant collection
4.0μg/mL BODIPY-cholesterol
0.2μg/mL BODIPY-cholesterol

Figure 3.21. BODIPY-cholesterol-labeled *B. burgdorferi* release OMV. Transmission electron micrographs showing isolated vesicles from both labeled and unlabeled *B. burgdorferi* OMV. The OMV are of similar size and shape. Immunogold labeling of OspB (18 nm) and *B. burgdorferi* glycolipids (6 nm) is seen throughout the vesicles. Scale bar = 100nm.

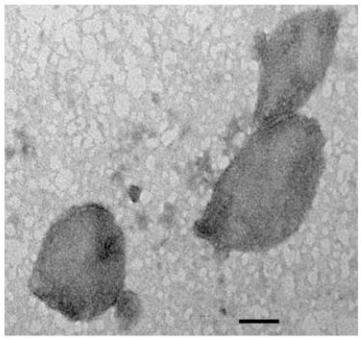
BODIPY-cholesterol labeled vesicles



Unlabeled vesicles



Isotype Control



<u>Figure 3.22.</u> The distribution of overall proteins across the Optiprep gradient is similar between BODIPY-cholesterol-labeled vesicles and unlabeled vesicles. A. Coomassie-stained SDS-PAGE of the 11 fractions collected from Optiprep gradient used to purify vesicles from the supernatants of labeled *B. burgdorferi*. **B.** Coomassie stained SDS-PAGE of the 11 fractions collected from Optiprep gradient used to purify vesicles from the supernatants of unlabeled *B. burgdorferi*. For panels **A** and **B** the most prominent outer membrane proteins (OspA and OspB) are highlighted to the left of the images.

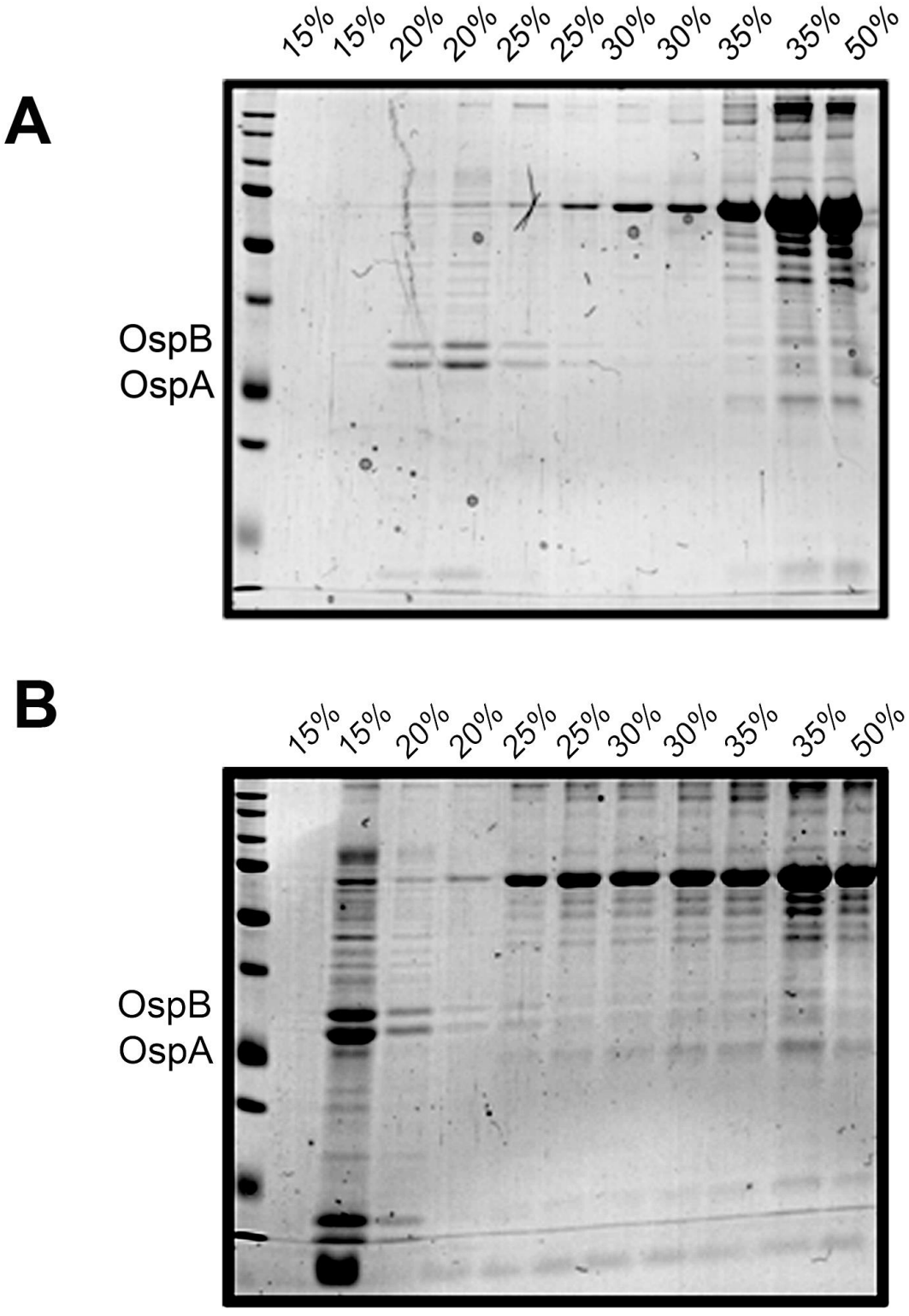

A
15%
15%
20%
20%
25%
25%
30%
30%
35%
35%
50%
OspB
OspA
B
15%
15%
20%
20%
25%
25%
30%
30%
35%
35%
50%
OspB
OspA

Figure 3.23. Both BODIPY-cholesterol-labeled and unlabeled purified OMV from *B. burgdorferi* contain outer membrane proteins. OMV from labeled and unlabeled *B. burgdorferi* were purified using an Optiprep density gradient. Each percentage (15%-35%) of the discontinuous gradient was collected in two fractions (1 mL each). **A.** SDS-PAGE western blot of the 11 fractions isolated from the supernatants of BODIPY-cholesterol labeled *B. burgdorferi.* Vesicles isolated from fractions 2-5 contain the outer membrane proteins OspB, OspA, and lp6.6. **B.** SDS-PAGE western blot of the 11 fractions isolated from the supernatants of unlabeled *B. burgdorferi.* Vesicles in fractions 2- 4 contain the outer membrane proteins OspB, OspA, and lp6.6.

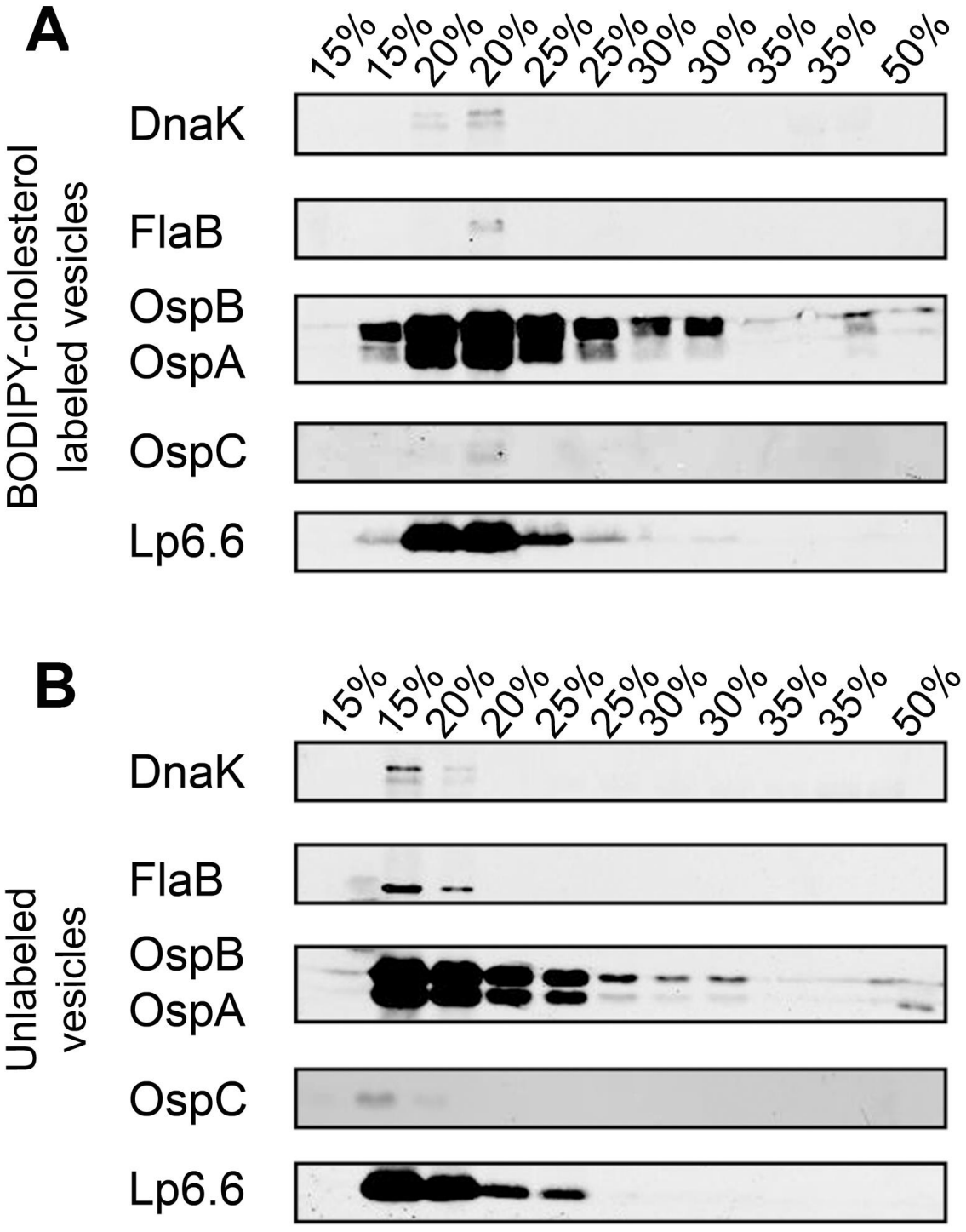

A
15%
15%
20%
20%
25%
25%
30%
30%
35%
35%
50%
DnaK
FlaB
OspB
OspA
OspC
Lp6.6
BODIPY-cholesterol labeled vesicles
B
15%
15%
20%
20%
25%
25%
30%
30%
35%
35%
50%
DnaK
FlaB
OspB
OspA
OspC
Lp6.6
Unlabeled vesicles

Figure 3.24. Both BODIPY-cholesterol-labeled and unlabeled purified OMV from *B. burgdorferi* contain the cholesterol-glycolipids. **Left:** Chloroform-methanol (85/15) HPTLC of isolated *B. burgdorferi* OMV. Fractions 15-25% of both labeled and unlabeled OMV were pooled and analyzed for glycolipid content by HPTLC resolution with iodine staining. Labeled and unlabeled OMV have both phospholipids (PG and PC), free cholesterol, ACGal, and CGal. Vesicles contain the cholesterol-glycolipids and phospholipids, but no MGalD. **Right:** UV exposed chloroform-methanol (85/15) HPTLC of isolated *B. burgdorferi* OMV. Vesicles derived from BODIPY-cholesterol labeled *B. burgdorferi* have the fluorescent cholesterol analog BODIPY-cholesterol.

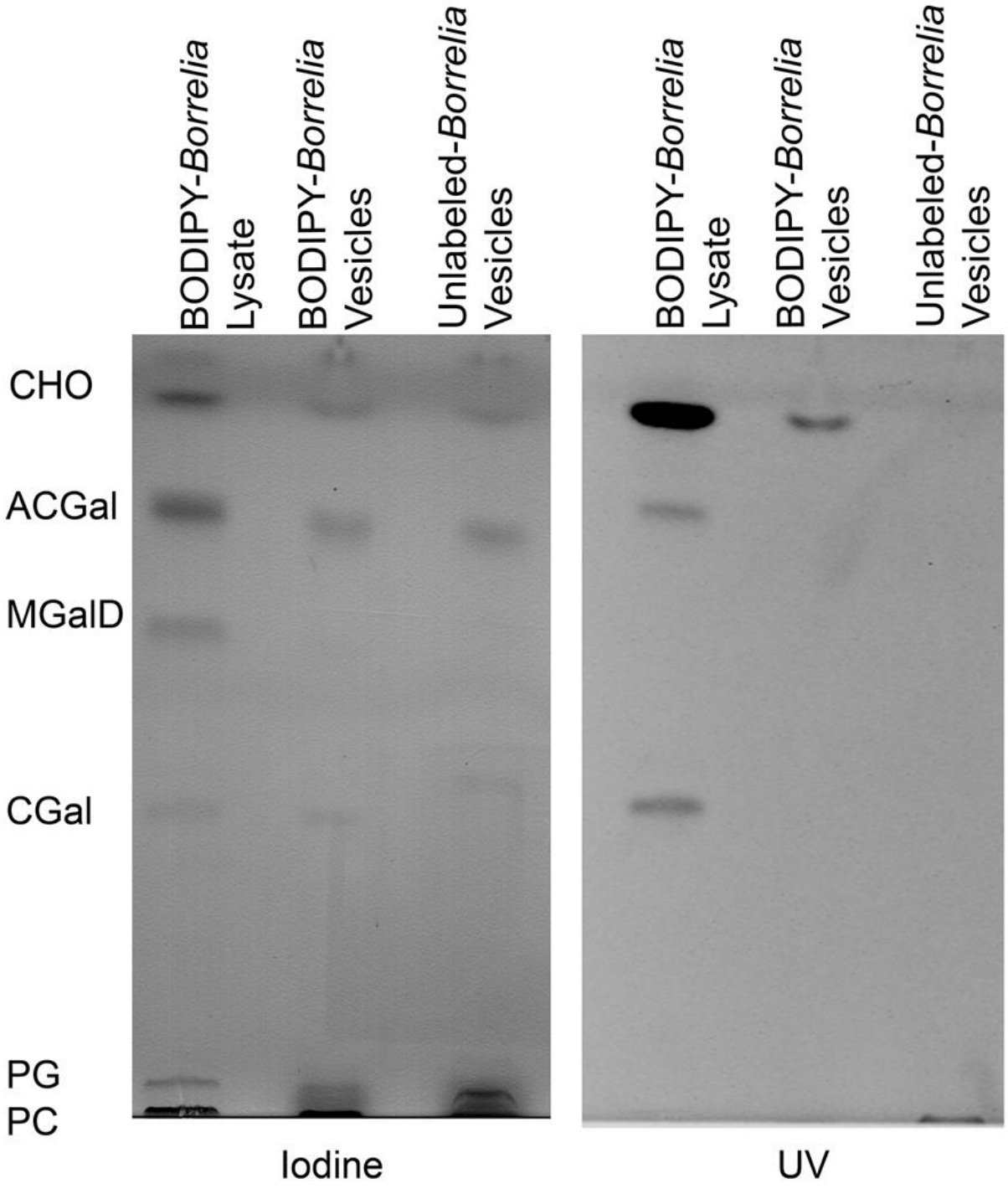

BODIPY-Borrelia Lysate
BODIPY-Borrelia Vesicles
Unlabeled-Borrelia Vesicles
BODIPY-Borrelia Lysate
BODIPY-Borrelia Vesicles
Unlabeled-Borrelia Vesicles
CHO
ACGal
MGalD
CGal
PG
PC
Iodine
UV

Figure 3.25. BODIPY-cholesterol-labeled vesicles fluoresce green. Vesicles from *B. burgdorferi* labeled with BODIPY-cholesterol and unlabeled *B. burgdorferi* were isolated by differential centrifugation and analyzed for fluorescence using the SpectraMaxM2 spectrophotometer. Some background interference caused by the vesicles in suspension is observed even in the unlabeled vesicles, but the most significant amount of fluorescence is observed at the interface of the 20% and 25% fractions. ANOVA ***$p<0.001$. The experiment represents the mean ± standard error of the mean from three separate measurements.

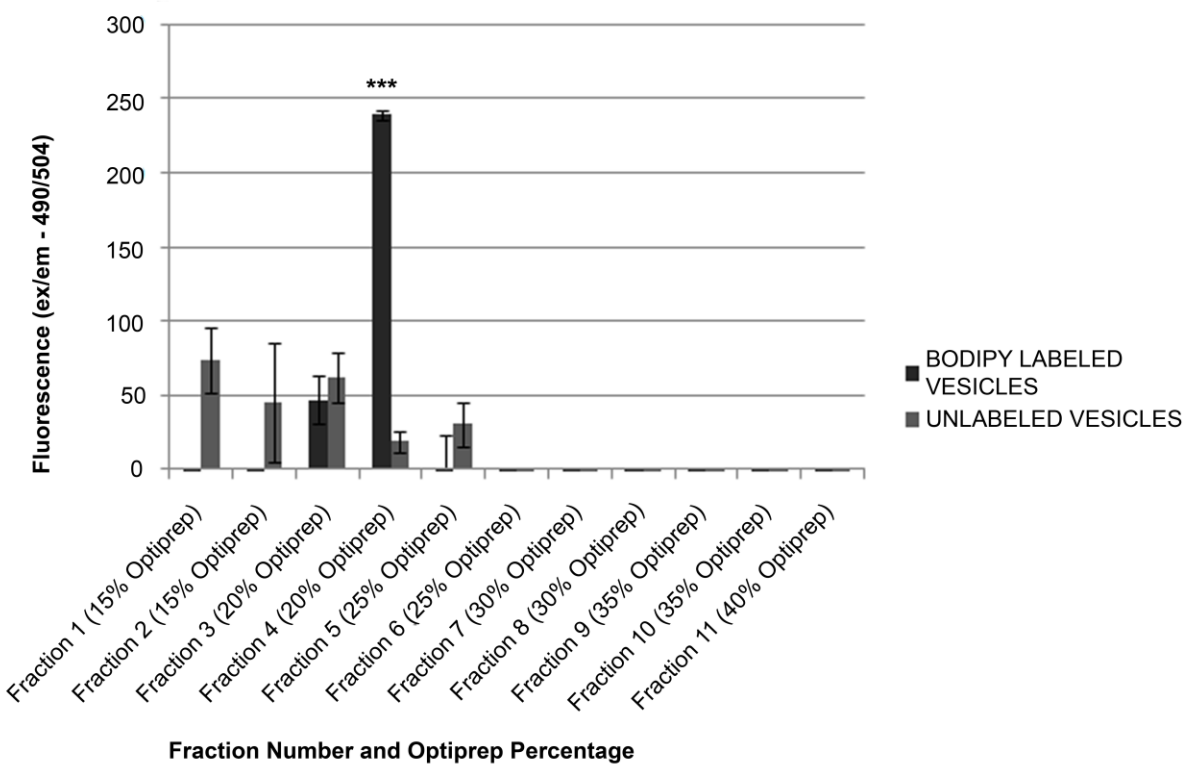

300
250
200
150
100
50
0
***
Fluorescence (ex/em - 490/504)
BODIPY LABELED VESICLES
UNLABELED VESICLES
Fraction 1 (15% Optiprep)
Fraction 2 (15% Optiprep)
Fraction 3 (20% Optiprep)
Fraction 4 (20% Optiprep)
Fraction 5 (25% Optiprep)
Fraction 6 (25% Optiprep)
Fraction 7 (30% Optiprep)
Fraction 8 (30% Optiprep)
Fraction 9 (35% Optiprep)
Fraction 10 (35% Optiprep)
Fraction 11 (40% Optiprep)
Fraction Number and Optiprep Percentage

Figure 3.26. OMV from *B. burgdorferi* contribute to the transfer of bacterial lipids to HeLa cells.

Vesicles were purified from *B. burgdorferi* labeled with BODIPY-cholesterol. The OMV were incubated with adherent HeLa cells for 2 hrs to observe transfer of bacterial lipids. **A.** Vybrant Cell Labeling Solution DiI was used to label the plasma membrane of HeLa cells. **B.** Single cell image of OMV labeled with BODIPY-cholesterol and Vybrant Cell Label Solution DiI. Labeled OMV (green) colocalize (yellow) at the plasma membrane (red). **C.** Merged three dimensional confocal micrograph demonstrates Vybrant Cell Labeling Solution DiI (red) is located at the plasma membrane of the HeLa cell. There is colocalization (yellow) of labeled OMV (green) on the outer edges or the surface of the HeLa cells. Scale bar = 20 μm

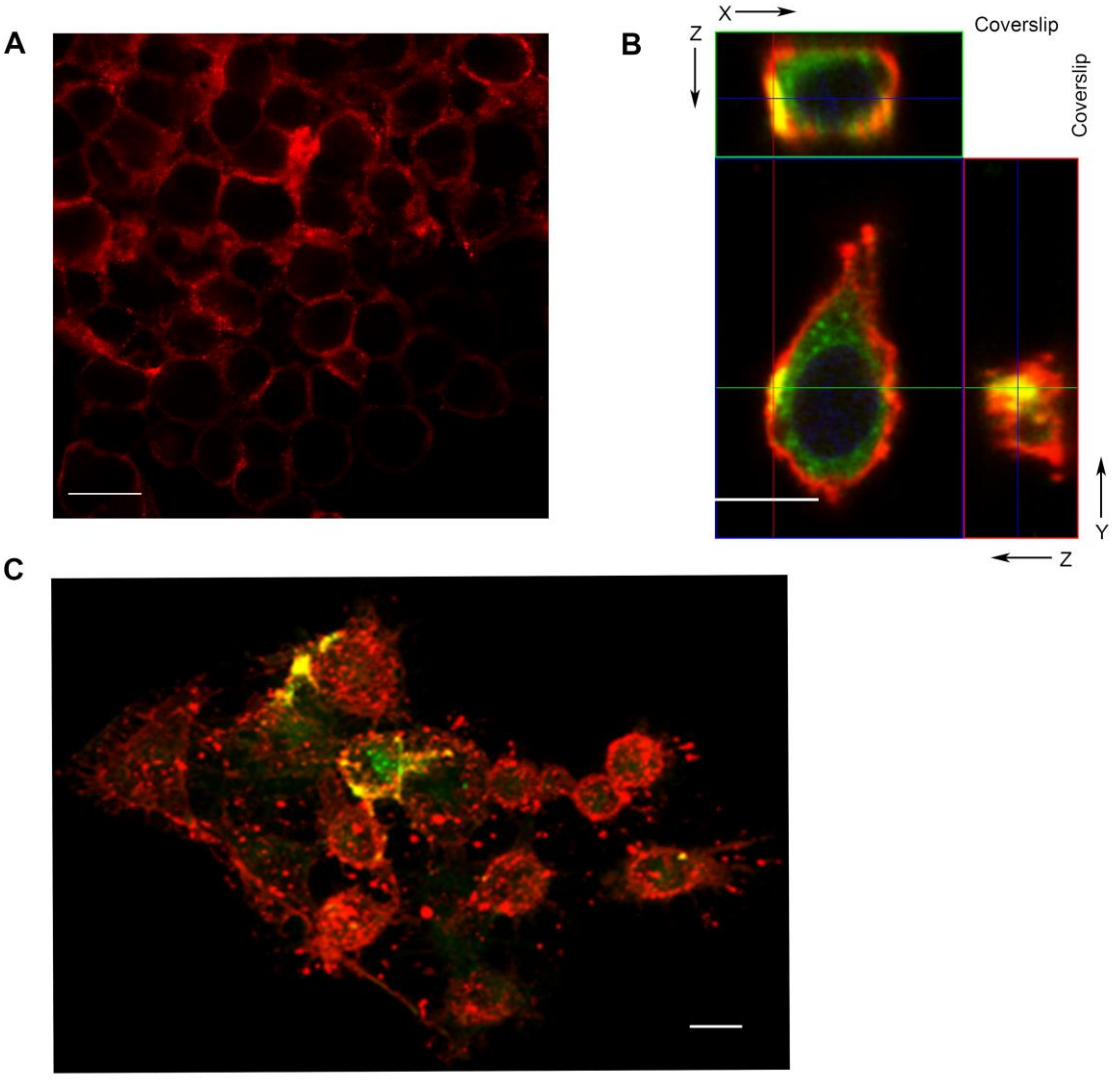

A
B
X
Z
Coverslip
Coverslip
Y
Z
C

Figure 3.27. ***B. burgdorferi*** **attach to HeLa cells and acquire cholesterol from the epithelial cell membranes.** HeLa cells incubated with BODIPY-cholesterol (green) and washed with MβCD were incubated with *B. burgdorferi* (red) for 1 hr at an MOI of 40:1. The cells were fixed, stained with CB2 to label the *B. burgdorferi* (red), and examined by confocal fluorescence microscopy. Colocalization (yellow) on the single confocal micrographs indicates the *B. burgdorferi* acquired the fluorescent cholesterol and is further indicated by arrows. Scale bars = 10 μm.

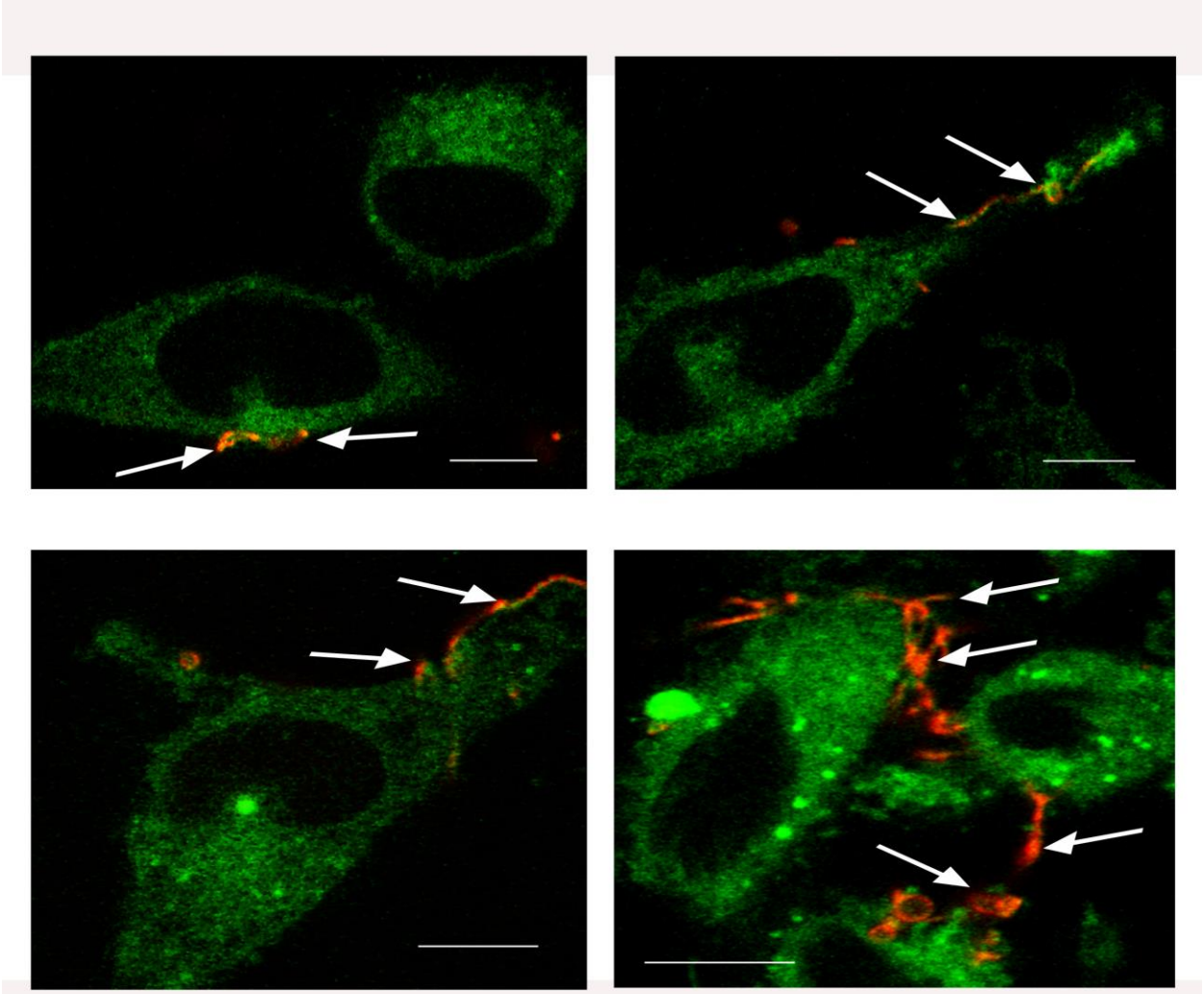
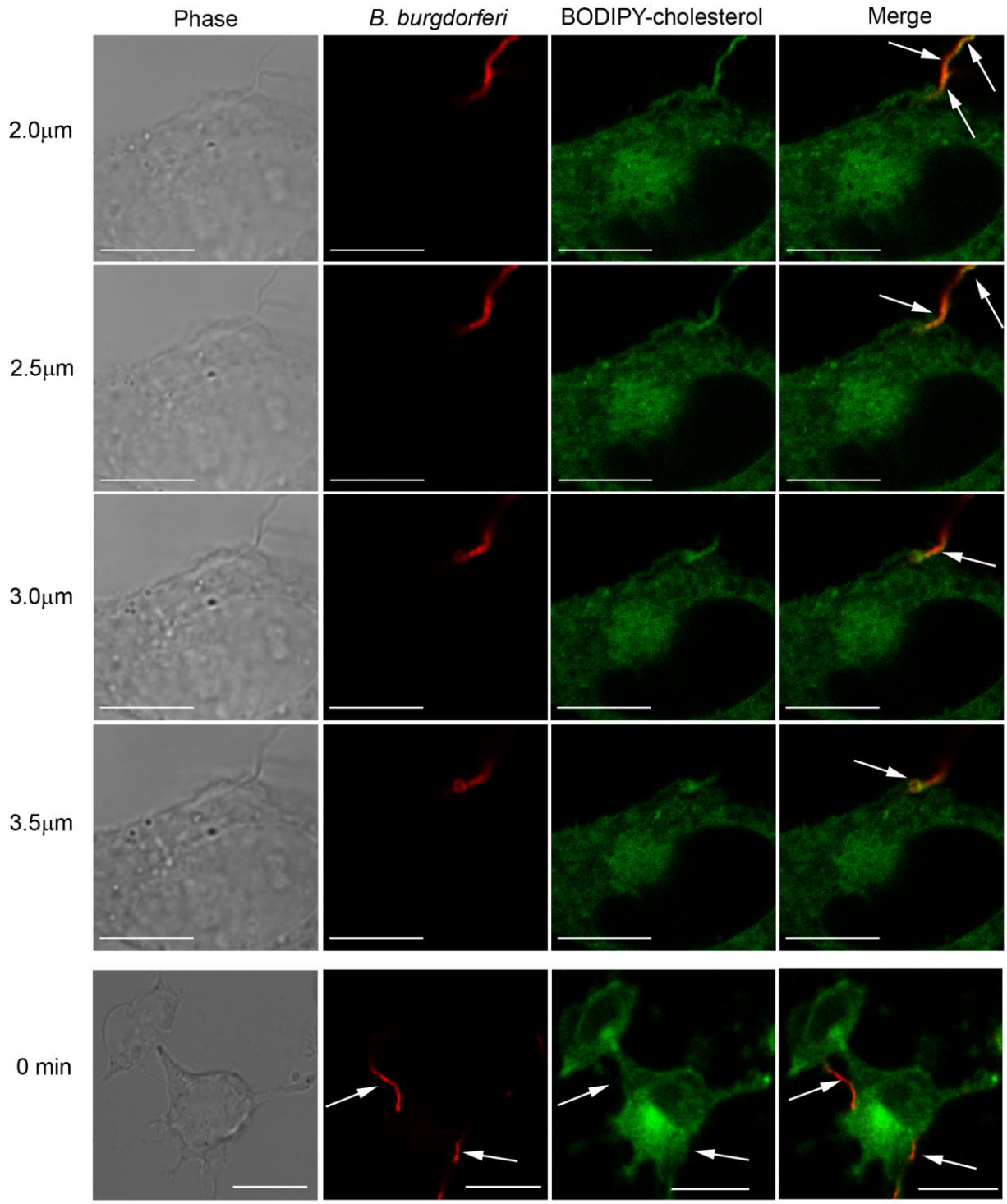


Figure 3.28. Cholesterol from epithelial cell membranes is acquired by attached *B. burgdorferi* and found throughout the spirochete.

HeLa cells incubated with BODIPY-cholesterol (green) and washed with MβCD were incubated with *B. burgdorferi* (red) for 1 hr at an MOI of 40:1. The cells were fixed, stained with CB2 (red) to label the spirochetes, and examined by confocal fluorescence microscopy. Four individual optical sections are shown at 2.0 µm, 2.5 µm, 3.0 µm and 3.5 µm. Confocal micrographs show colocalization (yellow, arrows) of BODIPY-cholesterol (green) and OspB (red) on the spirochete at the point of attachment. Scale bars = 10 µm. Confocal micrographs taken at the start of the experiment, 0 min panels (bottom), show that acquisition of BODIPY-cholesterol (green) has not occurred on the spirochetes (red) because there is no colocalization (yellow). The lack of colocalization is highlighted in the individual panels (arrows). Scale bars = 20 µm.


Phase
B. burgdorferi
BODIPY-cholesterol
Merge
2.0μm
2.5μm
3.0μm
3.5μm
0 min

CHAPTER 4 – GENERATION OF ANTI-*B. BURGDORFERI* GLYCOLIPID ANTIBODY AND ASSESSMENT OF ITS CROSS-REACTIVITY

4.1 CHAPTER SUMMARY

Infection with *B. burgdorferi* can result in the formation of cross-reactive antibodies. These antibodies have been shown to react against both gangliosides and non-protein antigens isolated from the lipid fraction of *B. burgdorferi*. Given the fact that *B. burgdorferi* has cholesterol and cholesterol glycolipids as a major component of their lipid profile and that cross-reactive antibodies are elicited during Lyme disease, we sought to study these antibodies alone and in the context of lipid exchange previously discussed in this dissertation. To study the antibodies generated against the *B. burgdorferi* cholesterol-glycolipids, we decided to experimentally generate a polyclonal antibody by injecting a rabbit and mice with the lipids. Prior to inoculation, extracted lipids were subjected to examination for contaminants. We treated the lipids with proteinase K to degrade contaminating peptides. Subsequent analysis of the lipid extract revealed that most of the contaminating protein was eliminated. We successfully made a polyclonal rabbit anti-*B. burgdorferi* cholesterol-glycolipids antibody. Examination of the serum collected from the rabbit indicated that the antibodies recognized the inoculum. We provide evidence using ELISAs that the polyclonal antibody recognizes the antigenic glycolipids of *B. burgdorferi* in vesicular or three-dimensional form and not the singular lipids in solution. The antibodies also reacted with whole *B. burgdorferi* and spirochete lysates. The polyclonal rabbit anti-*B. burgdorferi* cholesterol-glycolipids antibody was examined for its cross-reactivity with eukaryotic cells, and it was determined that the antibody cross-reacted with HeLa cells.

Furthermore, using a ganglioside-free cell line, GM95, we also show that the polyclonal antibody appears to cross-react with other epitopes on the surface of eukaryotic cells.

4.2 RESULTS

B. burgdorferi lipid extracts contain contaminating protein.

B. burgdorferi infection results in the formation of cross-reactive antibodies against gangliosides and non-protein antigens isolated from the lipid fraction of *B. burgdorferi*. To make a polyclonal antibody specific for the *Borrelia* glycolipids, it was necessary to initially test the extract to ensure that the sample was free of protein. These extracts were run on SDS-PAGE and stained with Coomassie blue to determine if there were any proteins in the sample. The lipids and a peptide contaminant were observed in the extracts (Figure 4.1). Mass spectrometry analysis determined that BSA the major protein in BSK-II medium.

Removal of contaminating protein from the *B. burgdorferi* lipid extract by protease digestion

Before a specific antibody was made to the non-protein antigens of *B. burgdorferi*, it was necessary to remove the protein contaminants. Removal of the protein contaminants was accomplished through protease treatment of the lipid extract. The first method utilized to remove BSA was treatment with trypsin that was immobilized on agarose beads. To optimize the digestion conditions, the trypsin alone was first added to 200 μg, 100 μg, 50 μg, or 25 μg of BSA. The reactions were run on SDS-PAGE and stained with Coomassie to determine the level of protein digestion. Trypsin alone appeared to be unable to digest the BSA (Figure 4.2, lanes 2-

5). With lower amounts of BSA, some digestion was observed (Figure 4.2), but the digestion did not result in complete elimination of the BSA.

To test whether the inability of trypsin to completely digest BSA was caused by the presence of 17 disulfide bonds in the protein, a similar experiment to the one conducted in Figure 4.2 was performed. However, the reducing agent DTT was included in the digestion conditions. The SDS-PAGE of the protein digestion indicated that addition of DTT to the BSA would enable efficient proteolysis (Figure 4.3). In all concentrations of BSA tested, the protein was efficiently digested when DTT was included in the reaction (Figure 4.3).

Since trypsin would require an additional chemical to for efficient proteolysis, we analyzed the ability of another protease to eliminate the BSA. Proteinase K was examined for its effectiveness in digesting BSA with and without the reducing agent DTT. The BSA digestions were repeated, but instead, proteinase K replaced trypsin as the protease. Using a similar experimental design, proteinase K did not need the addition of the reducing agent DTT to digest BSA (Figure 4.4A and 4.4B). However, unlike the trypsin, the proteinase K was not immobilized to agarose beads, and a band at ~MW of 28.5 kDa corresponding to the protease was present (Figure 4.4A). Therefore, an extra purification step, re-extraction of the lipids, was necessary to remove the protease. Together, the data from Figure 4.3 and Figure 4.4 suggested that proteinase K more efficiently digested BSA because DTT is not required for efficient digestion.

To further confirm that proteinase K was the more efficient protease at digesting contaminating proteins in the *B. burgdorferi* lipid extracts, protein amounts in the extracts were quantified by the BCA protein assay and the Coomassie Plus Assay. Negative controls included in the assay indicated that the lipids do not interfere with these quantification methods. The lipids

from *B. burgdorferi* were extracted using the Bligh and Dyer lipid extraction method and dried under constant nitrogen stream and resuspended in PBS. The different proteases (proteinase K or trypsin + DTT) were added to the reactions. After the digestion, the samples were analyzed by the two protein quantification assays. It was determined that both trypsin and proteinase K were unable to remove all the protein from the extracts. However, for both proteases measurements after the proteinase K-treated preparations were re-extracted, it was determined that the amount of detectable protein was decreased by ~2.0 - 3.0 fold when compared to untreated samples. It was possible that hydrophobic peptides were not completely removed even after a second Bligh and Dyer extraction or that the lipids themselves accounted for some level of interference in the assay. However, Western blot analysis for the presence of fully intact BSA indicated that the BSA had been digested because there were no bands present on the nitrocellulose membrane. Furthermore, data from the SDS-PAGE analysis and the protein quantification assays indicated that both proteases were capable of digesting similar amounts of protein when the experimental conditions were optimized. Given these conditions, however, proteinase K was chosen as the protease to degrade contaminating proteins in the lipid extracts, because it did not require the addition of DTT into the lipid mixture. This would ensure that the reaction mixture was as simple as possible, because it would eventually serve as the inoculum.

Because proteinase K is a promiscuous protease, it was necessary to assess if the protease significantly altered the lipids. After proteinase K treatment, the lipids of *Borrelia* were not significantly altered (Figure 4.5). We concluded this because the migration pattern of the protease-treated lipids on a TLC plate using the chloroform/methanol (85/15) mobile phase showed no differences when compared to an untreated control (Figure 4.5).

Generation and quality control of a polyclonal antibody specific for the non-protein antigens of *B. burgdorferi.*

Prior to injection, we determined the composition of the inoculum, the weight of the dried lipids, and the amount of protein that was calculated from the two protein quantification assays. The final lipid extract that would serve as the inoculum had a concentration of glycolipids in PBS of 7.0 mg/ml, while the protein concentration was 202.5, µg/mL. Despite the presence of measurable protein in the sample, the ratio of lipid to protein by mass was 35:1. Therefore, it was decided to inoculate animals, because the inoculum contained significantly more lipid than protein.

The animals (10 mice and 1 rabbit) were inoculated weekly for 7 weeks with the lipids resuspended in PBS (rabbit – 100 µg/mL [200 µL] and mice 50 µg/mL [100 µL]). The levels of antibody to the *B. burgdorferi* non-protein antigens were examined every week starting at the third week. The antibody reactivity against *Borrelia* lysates and the lipid extracts was determined using western blot. Samples were resolved on a 20% acrylamide SDS-PAGE gel. The samples were allowed to run approximately 2/3 down the gel, because the lipids run slightly ahead of the bromophenol blue front. It is important to note that the lipids do not resolve as a tight band, but instead appear as a large diffuse band (Figure 4.6). This is often noted in the electrophoretic profiles of lipids. The western blots revealed that the collected serum from the rabbit at week 3, week 4 and week 5 was reactive against the *B. burgdorferi* lysate and the lipid extract (Figure 4.6). The secondary antibody also showed some cross-reactivity against the lysates in all blots (Figure 4.6, lane 2 in all immunoblots). Sera from mice were nonreactive.

Evidence indicates that measuring the reactivity of an antibody against lipids by Western blotting is inconsistent, and a positive band is not very distinct. Due to the inconsistency and

inherent difficulty of measuring glycolipids by traditional SDS-PAGE and Western blot, we used an ELISA to measure the sensitivity of the glycolipid antibodies. For the ELISA, we used four experimental conditions: (i) lipid extracts in the form of small unilamellar vesicles (SUV), (ii) lipid extracts in a solvent, (iii) *Borrelia* lysates, and (iv) whole intact *Borrelia.* It is important to note that SUVs were used as an additional experimental condition to mimic the original inoculum (lipids dissolved in an aqueous buffer and not a solvent). Furthermore, this allowed us to compare the reactivity of the generated antibodies and examine if they were recognizing any conformational epitopes that could be maintained in a three-dimensional structure like a vesicle.

Data from the ELISAs suggested that the rabbit antibodies did not have high reactivity against the dried pure lipid extracts (Figure 4.7). However, antibody reactivity was observed to be higher when the SUVs were the target antigen in the ELISA (Figure 4.7). The pre-inoculation sera did not have reactivity against any of the antigens. This could indicate that a majority of the antibodies are recognizing conformational epitopes on the vesicles when they are suspended in an aqueous buffer. In addition to the reactivity against the SUVs, the generated polyclonal rabbit antibodies to the *B. burgdorferi* lipids showed high reactivity against the *Borrelia* lysate and the *Borrelia* whole bacteria (Figure 4.8). However, high baseline activity against the lysate and whole bacteria was also observed in the pre-inoculum serum. This high baseline reactivity could be caused by the fact that rabbit serum has elevated reactivity against *B. burgdorferi.* Therefore, taking both ELISAs into account, we concluded that the polyclonal antibody made to the *B. burgdorferi* lipid extract was most likely specific to the lipids when they were in an aqueous buffer. One trend that was observed across the experimental conditions was that the reactivity of the collected serum decreased over time (Figure 4.7 and 4.8). This most likely occurred because the animal's immune response was being habituated to the injections.

A polyclonal antibody made against the lipid extract of *B. burgdorferi* cross-reacts with eukaryotic cells.

To investigate if the rabbit polyclonal anti-*B. burgdorferi* lipid antibody recognized eukaryotic cells, *in vitro* fluorescence microscopy experiments were conducted. Because HeLa cells were used in previous experiments, we initially conducted these measurements with these cells. Initially the antibody reactivity was examined in the conditions of the transfer assay. The rabbit polyclonal anti-*Borrelia* lipid antibody had significant reactivity to HeLa cells when the eukaryotic cells were incubated with *B. burgdorferi* (Figure 4.9). Furthermore, the experimental antibody also was capable of detecting *B. burgdorferi* both from standard culture and derived from the coincubation experiment with HeLa cells (Figure 4.10). To determine whether the cross-reactivity of the polyclonal rabbit anti-*B. burgdorferi* glycolipid antibody to HeLa cells was influenced by the presence of *B. burgdorferi*, the reactivity of the antibody to HeLa cells alone was measured by fluorescence microscopy (Figure 4.11A). The distribution of labeling and level of reactivity appeared to be similar to the coincubation condition. This indicated that the observed cross-reactivity was not caused by the presence of *B. burgdorferi* or by anything the spirochetes deposited onto the surface of the HeLa cells. A positive control using anti-asialo-GM1 (Figure 4.11B) and the negative controls of the pre-inoculation sera and the secondary antibody alone (Figure 4.11C) were used in the experiment. The anti-asialo-GM1 control was used as documented for the earlier conditions (Figure 4.9 and Figure 4.11A and 4.11B). The results indicated that antibodies specific for the lipids of *Borrelia* could be recognizing similar epitopes on the HeLa cells. Furthermore, because the negative controls showed little to no

reactivity, we determined that the majority of the reactivity of the antibodies observed in Figures 4.9-4.11 was specific to the inoculation of the animals with the *Borrelia* lipid preparation.

We provided evidence that when using HeLa cells, both the anti-asialo GM1 antibody and the rabbit polyclonal anti-*Borrelia* glycolipid antibody reacted with the surface of eukaryotic cells. However, to examine if lipid transfer defined in Chapter 3 can increase the reactivity of antibodies to the non-protein antigens of *B. burgdorferi* for eukaryotic cells, it was necessary to study two different cell lines, a ganglioside knockout cell line, GM95, and the parent myeloma MEB4 cell line. Using these cell lines would allow us to detect small differences *in vitro*. The deficiency in gangliosides and glycolipids in the GM95 cells is attributed to the absence of a ceramide glucosyltransferase responsible for catalyzing the first glucosylation step of a ceramide molecule. [312-314].

The fluorescent microscopy experiments designed to look for cross-reactivity were repeated using the GM95 andMEB4 cells. The polyclonal antibody to asialo-GM1 and the rabbit polyclonal anti-*Borrelia* lipid antibody should react with theMEB4 cells and not the GM95 cells. For initial immunofluorescence experiments, we conducted a titration of the primary antibodies on the cells. The cells were seeded onto coverslips. The cells were washed with PBS and fixed with paraformaldehyde. The two experimental antibodies were added to the cells for 1 hr. After the 1 hr incubation, coverslips were washed, and the goat anti-rabbit secondary antibody conjugated to FITC was added. The initial titration of the primary antibodies indicated that both polyclonal antibodies, the rabbit polyclonal anti-*Borrelia* lipid antibody and the anti-asialo GM1 antibody, reacted with MEB4 cells and the GM95 cell line (Figure 4.12). Despite these latter cells having no gangliosides on the surface, there was significant fluorescence in these images (Figure 4.12). From this data there appears to be some level of cross-reactivity because the anti-

asialo GM1 antibody should not react with the GM95 cells as there are no gangliosides present in these cells. This reactivity was not due to the secondary antibody (Figure 4.12).

Because we observed significant cross-reactivity in the titration, we designed experimental conditions to evaluate different blocking agents. To examine if different blocking agents could limit the cross-reactivity, we used 5% BSA, 5% human serum, and no blocking agent in the fluorescent microscopy experiments (Figure 4.13). The cross-reactivity that was observed in the initial titration did not decrease when blocking agents were added (BSA or human serum) (Figure 4.13). It appears theMEB4 and GM95 cells will not serve as useful tools in the initial experiments designed to analyze antibody-mediated cytotoxicity. Nevertheless, it is also possible that even though there are no gangliosides on the GM95 cells, these polyclonal antibodies by nature have significant promiscuous cross-reactivity with many different epitopes on the surface of eukaryotic cells (lipids, sugars, or other structures) and could play a role in pathogenesis.

4.3 DISCUSSION

The exact mechanism that leads to chronic infections with *B. burgdorferi* is unknown. Autoimmunity is believed to play a role in the chronic nature of Lyme disease. However, clear evidence identifying a factor responsible for eliciting the autoreactive response has not occurred. In previous publications, cross-reactive antibodies to the non-protein antigens of *B. burgdorferi* were shown to be cross-reactive with gangliosides that are ubiquitous throughout the mammalian host [60,165,172,173]. Since these studies, the chemical composition of these non-protein antigens was solved [3-5,24], cholesterol was revealed to be a major component of the spirochetes' lipid content [3], and the cholesterol-glycolipids were shown to elicit strong

antibody responses during late stages of infection response [3-5,58,60,289]. Furthermore, in a previous publication, we showed that the cholesterol and cholesterol-glycolipids contribute to the formation of eukaryotic-like lipid rafts and used the anti-asialo-GM1 antibody to detect the cholesterol-glycolipids on spirochetes [6]. Given the findings that lipid antigens in *B. burgdorferi* contain cholesterol that can be derived from the mammalian host [127], and that there is similar lipid-raft domain formation shared by the spirochetes and eukaryotic host cells, there was new interest in studying the possible autoreactive nature of the antibodies to the cholesterol-glycolipids of *B. burgdorferi.*

It was of interest to make a polyclonal antibody specific for the *Borrelia* glycolipids that could be used to study the cross-reactive characteristics of antibodies against the cholesterol-glycolipids of *B. burgdorferi*. Based on other studies in the laboratory and previous publications [6,127], we believed that the Bligh and Dyer lipid extraction method isolated only the lipids and did not contain protein. However, preliminary data indicates that there is a protein contaminant in the lipid extracts. Mass spectrometry analysis of the peptide determined that the contaminating protein was BSA. BSA is a significant component of BSK-II [302]. With contaminating BSA s in the lipid extract, we could not inoculate animals and produce specific, effective antibody to the glycolipids of *B. burgdorferi.*

To remove the contaminating BSA in the *B. burgdorferi* lipid extract, we treated the lipids with proteases to degrade the protein. Both proteases tested, trypsin and proteinase K, could degrade BSA, but trypsin treatment required the addition of a reducing agent, DTT, to degrade all the BSA effectively. Proteinase K was more efficient in degrading BSA, and proteolysis did not require DTT. Despite treating the lipid extract with protease, BCA Protein Assay and the Coomassie Plus Assay indicated an inability to remove all of the contaminating

peptides. It is possible that small hydrophobic peptides were not completely removed or that the lipids themselves accounted for some level of interference in the assay. However, the final ratio of lipid to protein by mass was 35:1. Using this preparation, the majority of antibodies made to the inoculum would be specific for the lipids and that contributions of contaminating protein to the antibody response would likely be minor.

Inoculations of animals using a proteinase K treated lipid extract resulted in development of a polyclonal antibody to the glycolipids of *B. burgdorferi.* To determine the reactivity of the polyclonal antibody we initially tested the collected serum using a 20% acrylamide SDS-PAGE gel and western blot. We observed an antibody response to the lipids of *B. burgdorferi* in the rabbit throughout all the samples tested, but the mice were not reactive. Recognition of lipids by SDS-PAGE can be difficult because the lipids resolve as a diffuse lower band molecular weight band when subjected to electrophoresis. The positive control (anti-asialo GM1) did not robustly recognize the lipids of *B. burgdorferi.* Given the inconsistent recognition of the lipids by electrophoresis, it was necessary to use another method to confirm these findings. We did observed secondary antibody cross-reactivity across all samples including the positive control. These bands do not appear to be full length BSA. Separate analysis of the lipid extracts used for inoculation and subsequent quality control analyses with a monoclonal antibody to BSA indicated that the lipid extracts did not have BSA as a contaminant. This indicates that these bands are likely not specific to the inoculation of the animals.

Utilization of ELISAs allowed us to better assess the reactivity of the antibodies specific for the lipids of *B. burgdorferi.* We used the ELISA to examine the reactivity of the polyclonal antibody against pure lipid extracts as well as in the context of other *Borrelia* proteins (lysates and whole intact spirochetes). We showed that the rabbit antibodies do not have high reactivity

against the pure lipid extracts in a solvent. However, we observed high reactivity against the SUVs, *Borrelia* lysates, and the whole intact *Borrelia.* To explain this discrepancy we believe that the antibody is recognizing conformational epitopes on the lipids. When in a solvent (the pure lipid extracts in a solvent condition), the cholesterol-glycolipids are free to follow any conformation, the epitope is perhaps not maintained, and reactivity is lost. However, this epitope is more likely to be maintained when the lipids are in a three dimensional structure, such as a vesicle, that would be formed in an aqueous environment (SUVs, *Borrelia* lysates, and the whole intact *Borrelia*). In an aqueous environment, it is likely that the hydrophobic cholesterol and acyl chains are buried in vesicles or membrane bilayers away from the aqueous environment. Therefore, the likely antigen recognized by the antibodies is comprised of parts of the acyl chain, galactose, and cholesterol that are exposed to the aqueous environment. These findings are in line with previous publications. The first, from our laboratory, used anti-asialo-GM1 to detect the cholesterol-glycolipids on the spirochete [6]. The source of the cross-reactivity is believed to be the β1-3 linkage between the cholesterol and the galactose [6,79]. This region of the molecule is likely to be exposed in aqueous buffer and recognized by antibodies. The other publications provided evidence that indicated that host antibodies elicited against the cholesterol-glycolipids of *B. burgdorferi* do not react with the single components that make up the glycolipids [290]. Instead, antibodies reacted against the entire molecule that included the cholesterol, galactose, and the acyl chain (minimum of 4 carbons) [290]. However, given the elevated baseline activity against the lysate and whole bacteria in the pre-inoculum sera and the highly cross-reactive nature of these antibodies we cannot rule out the possibility that the polyclonal antibody is recognizing non-lipid antigens. Nonetheless, the ELISA data Together these observations indicate that a majority of the antibodies against the lipid extract of *B. burgdorferi* are likely

recognizing conformational epitopes on the glycolipids. It is important to note that the antibody response is specifically reactive to the pure lipid extracts because no cross-reactivity was observed with the controls.

After making the rabbit polyclonal anti-*B. burgdorferi* glycolipid antibody, it was of interest to investigate the cross-reactive nature of the antibody in the context of eukaryotic cells using *in vitro* fluorescence microscopy experiments. Using the same conditions as the transfer assay from **Chapter 3** to mimic an active infection (incubation of epithelial cells with whole *B. burgdorferi*), the rabbit polyclonal anti-*Borrelia* lipid antibody had significant reactivity to HeLa cells when the eukaryotic cells were incubated with the spirochetes. This reactivity was observed throughout the cells and on the surface where the majority of cross-reactivity is expected to occur. We also demonstrated using fluorescence microscopy that the antibody recognized spirochetes in the coincubation experiment with HeLa cells and whole *B. burgdorferi* from culture. This is an important control because the antibody was supposed to be specific for the lipids of *B. burgdorferi*. This is also consistent with the ELISA data presented in Figure 4.7 that indicates the antibody recognizes whole intact spirochetes. To determine whether that the recognition of the HeLa cells by the rabbit polyclonal anti-*B. burgdorferi* glycolipid antibody was dependent on the presence of *B. burgdorferi* or a factor released from the spirochetes, we also examined HeLa cells alone. The rabbit polyclonal anti-*B. burgdorferi* glycolipid antibody had similar levels of reactivity and distribution on the HeLa cells when compared to the coincubation conditions, but the coincubation conditions appeared to possibly have less cytoplasmic labeling. Furthermore, both of these conditions using the experimental antibody exhibited similar distribution and labeling intensity as the positive control anti-asialo-GM1. This indicates that the experimental antibody is cross-reacting with molecules on the surface and in

the epithelial cells. This reactivity was specific because the negative controls showed no reactivity.

From the experiments using the HeLa cells to test the reactivity of the experimental antibody, it was clear that there are high levels of cross-reactivity. To examine if transferred antigenic *Borrelia* cholesterol-glycolipids can cause eukaryotic cells to become targets of antibody-mediated immune response, it is necessary for us to use a clean background to examine small differences *in vitro*. To measure small differences in cross-reactivity, we obtained two cell lines, a ganglioside knockout cell line, GM95, and the parent myelomaMEB4 cell line. The polyclonal antibody to asialo-GM1 antibody and the rabbit polyclonal anti-*B. burgdorferi* glycolipid should react with the MEB4 cells and not the GM95 cells. Without gangliosides we believed that a majority of the cross-reactivity would be eliminated and could possibly detect transferred lipids on the surface of the eukaryotic cells. This would provide the basis to design future experiments to examine antibody-mediated autoimmunity.

Initial titration experiments of the primary experimental antibodies (polyclonal GM1 antibody and the rabbit polyclonal anti-*B. burgdorferi* glycolipid) indicated that both of the polyclonal antibodies reacted with MEB4 and GM95 cell lines. Despite these GM95 cells having no gangliosides, there was still significant fluorescence detected. Even the anti-asialo-GM1 antibody, which should not react with GM95 cells, did exhibit fluorescence. It is possible that these antibodies are both reacting with the GM95 cells because they are polyclonal antibodies. Despite being raised against specific antigens, the anti-asialo-GM1 antibody and the anti-*Borrelia* glycolipid antibody could be recognizing other common epitopes on the cell surface. Controls indicated that this reactivity was not due to the secondary antibody cross-reacting with these cells. To explore whether that the cross-reactivity was due to nonspecific

binding of the antibodies to the cells, we examined multiple blocking agents. We tested different blocking agents for cross-reactivity. BSA was used because it is a major component of the *Borrelia* media and the contaminating protein. In addition to BSA, we also used human serum and no blocking agent in the experimental conditions. From these experiments to test blocking agents, we show that the original cross-reactivity seen in both MEB4 and GM95 cells is not a result of the addition of a blocking agent (BSA or human serum) because there is not a significant difference when the three experimental conditions are compared.

Together, these results indicate that the antibodies for the cholesterol-glycolipids of *B. burgdorferi* react with numerous epitopes. Because we believe that these cross-reactive antibodies are recognizing a conformational motif, possibly the β1-3 linkage, it is conceivable that elimination of the gangliosides in the GM95 cells is not enough to create a clean background. There could be other molecules on the cell surface with similar conformations, linkages, or modifications that contribute to the cross-reactivity. Nevertheless, it appears that to some degree these polyclonal antibodies against the glycolipids of *B. burgdorferi*, by nature, could have significant promiscuous cross-reactivity with many different epitopes on the surface of eukaryotic cells (lipids, sugars, proteins, or other structures) and could play a role in pathogenesis.

The role of autoimmunity or cross-reactivity in Lyme disease is complex and not well understood. It has been shown in the literature and in this dissertation that *B. burgdorferi* can transfer antigens to cells [127]. These cells might then become the target of the host immune response – both antibodies and immune cells. Specifically, cross-reactive antibodies recognize both protein and lipid antigens in multiple types of host cells. Our results showed that this

occurs without *B. burgdorferi* or transferred antigens being present. We presented data in Chapter 4 that indicates that the experimentally generated rabbit polyclonal antibody to the lipids of *B. burgdorferi* is cross-reactive with multiple types of eukaryotic cells. Given the cross-reactivity, antibodies similar to the experimental rabbit polyclonal antibody could to be important for pathogenesis. Productions of these cross-reactive antibodies could be significant in determining the host response to infection by influencing the severity of the symptoms of Lyme disease. In some individuals, infection with *B. burgdorferi* could stimulate higher production of these antibodies and lead to increased disease manifestations like arthritis. Increased production may well result in enhanced arthritis or other auto-reactive manifestations observed in Post-Treatment Lyme Disease Syndrome. Understanding how *B. burgdorferi* infection influences production of these unique cross-reactive antibodies and how these antibodies could contribute to proposed autoimmunity could be important to improving treatment for chronic Lyme disease.

4.4 FIGURES

Figure 4.1. Peptide contaminants are present in *B. burgdorferi* lipid extract after Bligh and Dyer lipid isolation. Lipids from *B. burgdorferi* were isolated using Bligh and Dyer lipid extraction protocol. A Coomassie-stained SDS-PAGE demonstrated contaminating protein in the lipid extract.

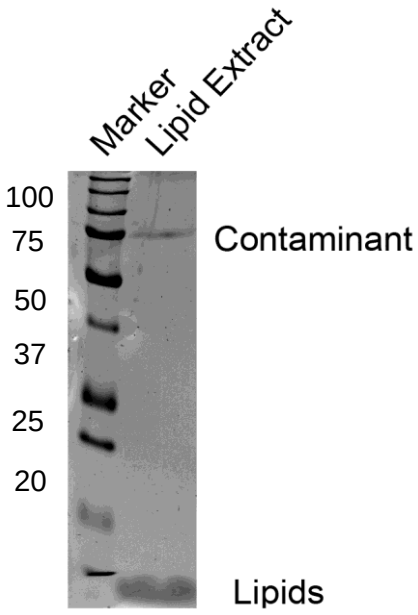
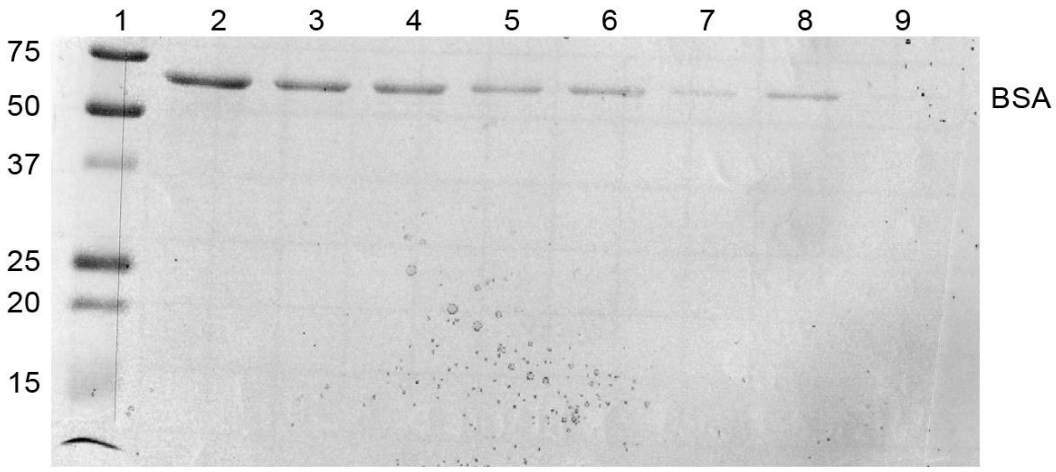
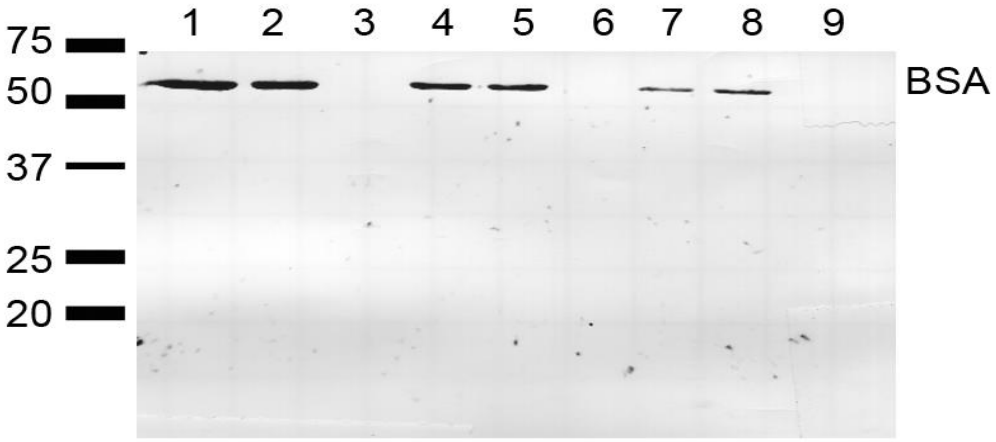

Marker
Lipid Extract
100
75
50
37
25
20
Contaminant
Lipids

Figure 4.2. Trypsin can digest small amounts of BSA. Trypsin was added to different amounts of BSA and was incubated overnight. The immobilized trypsin was removed from the reaction by centrifugation, and the samples were analyzed by SDS-PAGE and Coomassie staining to determine the level of digestion. Trypsin alone was unable to digest the higher amounts of BSA (lanes 2-5). Some digestion of BSA was observed in the last two conditions, 50 μg and 25 μg of BSA (lanes 6-9).



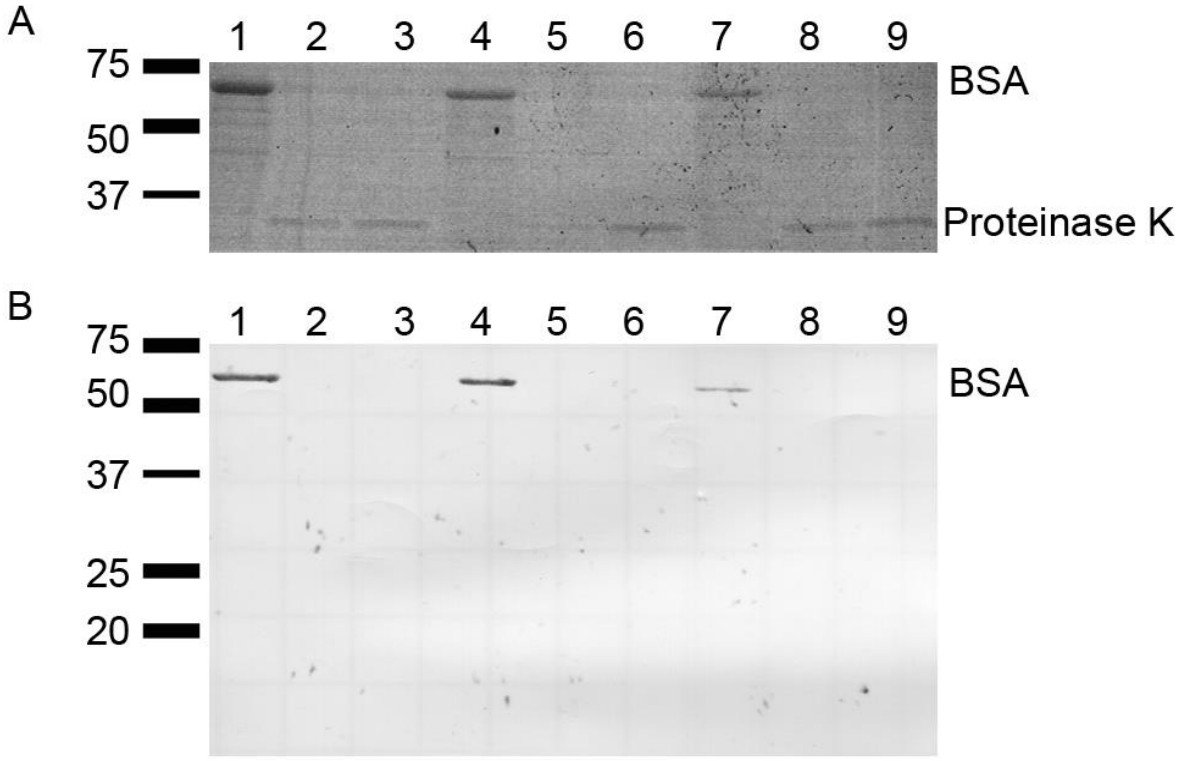
Lane	Condition
1	MW Marker
2	200 µg BSA
3	200 µg BSA + Trypsin
4	100 µg BSA
5	100 µg BSA + Trypsin
6	50 µg BSA
7	50 µg BSA + Trypsin
8	25 µg BSA
9	25 µg BSA + Trypsin

Figure 4.3. Addition of the reducing agent DTT allows for efficient degradation of BSA by trypsin. DTT was included in the overnight reaction between the protease trypsin and the BSA. The immobilized trypsin was removed from the reaction by centrifugation, and the samples were analyzed for digestion of BSA by SDS-PAGE and western blot. BSA was detected using a murine monoclonal antibody to BSA. Trypsin was able to efficiently digest BSA with the addition of DTT in all the conditions examined (lanes 3, 6, 9).



Lane	Condition
1	200 µg/mL BSA
2	200 µg/mL BSA + Trypsin
3	200 µg/mL BSA + Trypsin + DTT
4	100 µg/mL BSA
5	100 µg/mL BSA + Trypsin
6	100 µg/mL BSA + Trypsin + DTT
7	50 µg/mL BSA
8	50 µg BSA/mL + Trypsin
9	50 µg BSA/mL + Trypsin + DTT

Figure 4.4. Addition of the reducing agent DTT is not necessary for efficient degradation of BSA by Proteinase K. Proteainase K was added to different amounts of BSA with and without the reducing agent DTT. The samples were analyzed for digestion of BSA by western blot and Coomassie staining. **A.** Coomassie-stained SDS-PAGE of BSA treated with proteinase K. BSA is digested by the proteinase K. The protease is also observed in the reactions at the lower molecular weights. **B.** Western blot of BSA treated with Proteinase K. BSA is degraded by proteinase K. BSA was detected using a murine monoclonal antibody to BSA.



Lane	Condition
1	200 µg/mL BSA
2	200 µg/mL BSA + ProK
3	200 µg/mL BSA + ProK + DTT
4	100 µg/mL BSA
5	100 µg/mL BSA + ProK
6	100 µg/ug BSA + ProK + DTT
7	50 µg/mL BSA
8	50 µg/mL BSA + ProK
9	50 µg/mL BSA + ProK + DTT

<u>Figure 4.5.</u> Proteinase K does not alter or degrade the lipids of *B. burgdorferi.* The lipid extracts of *B. burgdorferi* were treated with proteinase K to examine if the protease alters the lipids. After a proteinase K treatment, it was necessary to re-extract the lipids by conducting another Bligh and Dyer lipid isolation. The lipids treated with proteinase K were analyzed by HPTLC, resolved using a choloroform:methanol mobile phase (85:15), and stained using iodine.

Left: Lipid extract prior to proteinase K.

Right: Lipid extract after proteinase K treatment and second Bligh and Dyer extraction.

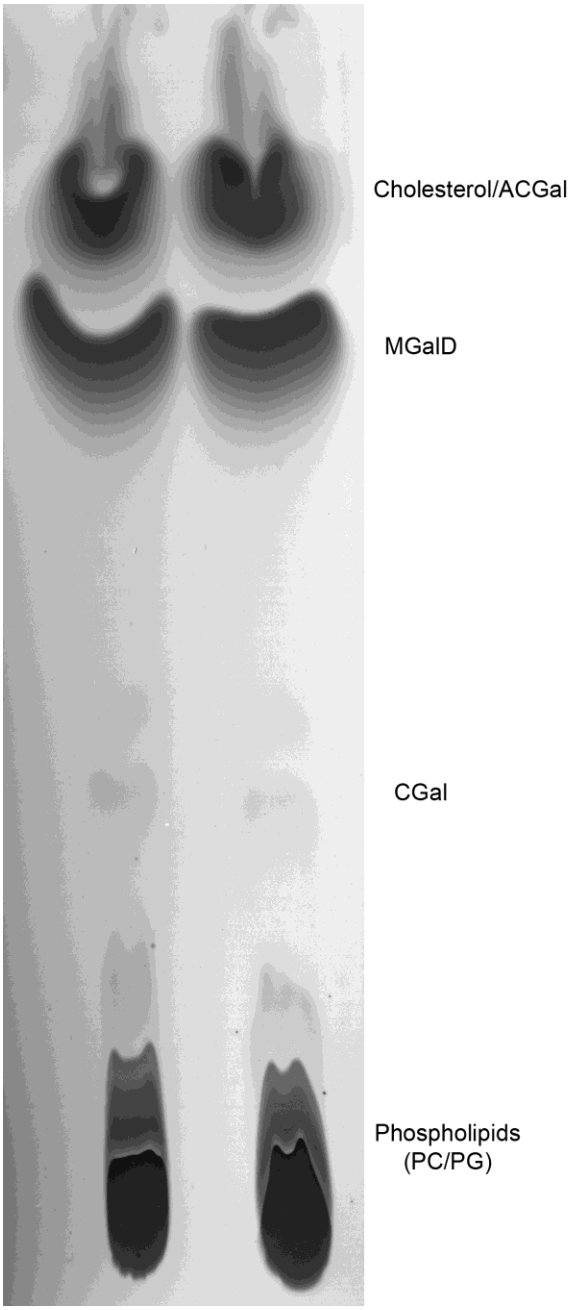

Cholesterol/ACGal
MGalD
CGal
Phospholipids
(PC/PG)

Figure 4.6. The rabbit inoculated with the *B. burgdorferi* lipid extract demonstrates increasing reactivity to the lipids over time. The antibody reactivity against *Borrelia* lysates and the lipid extracts was determined using Western blot. Samples were run on a 20% acrylamide SDS-PAGE gel approximately 2/3 down the gel. The lipids are observed as a large hazy band. The absorbed secondary antibody does show some cross-reactivity against the lysates (lane 2, ~50 kDa) in all blots. The αGM1 antibody was used as an internal positive control.

1: MW Standard

2: *Borrelia* lysate

3: Lipid extract

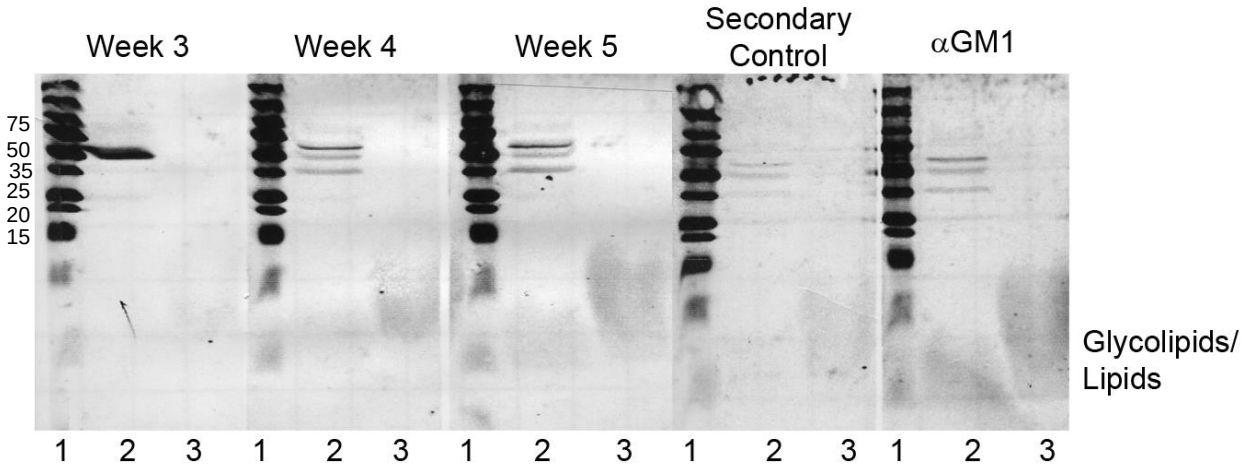

Week 3
Week 4
Week 5
Secondary Control
αGM1
75
50
35
25
20
15
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
Glycolipids/ Lipids

Figure 4.7. The polyclonal rabbit anti-*Borrelia* glycolipid antibody has high reactivity against small unilamellar vesicles (SUV), but not against dried lipid extracts. SUVs of the *Borrelia* lipid extracts were made prior to the ELISA experiments. ELISAs using the inoculated rabbit serum were conducted against lipid extracts and SLVs. The polyclonal rabbit anti-*Borrelia* lipid antibody appears to have higher reactivity with the SUVs than the dried lipid extracts. Other control antibodies used in the ELISA were α-GM1, pre-inoculation serum, and the secondary antibody alone. Results represent the mean +/- standard error of the mean of three individual experiments.

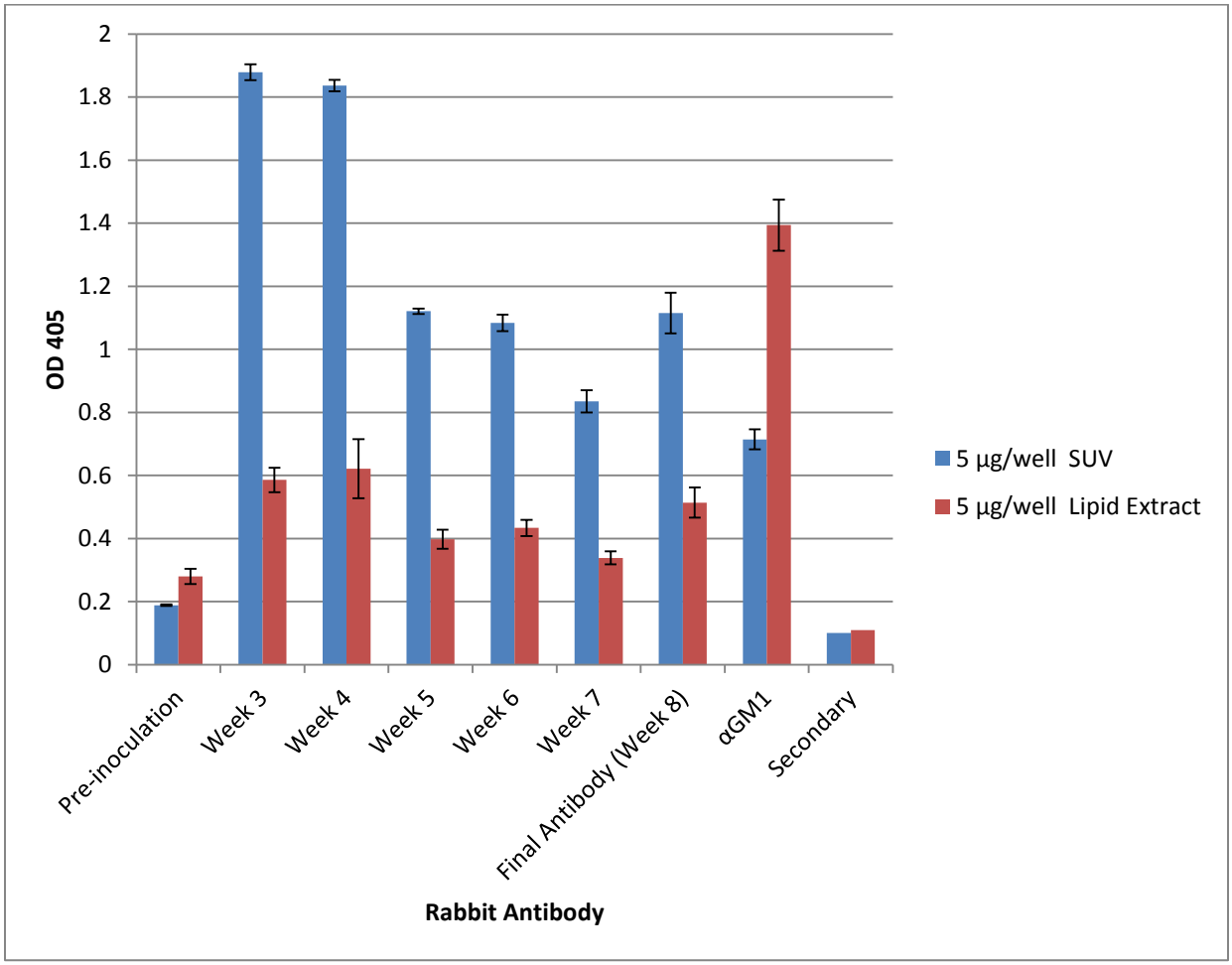

2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD 405
Pre-inoculation
Week 3
Week 4
Week 5
Week 6
Week 7
Final Antibody (Week 8)
αGM1
Secondary
Rabbit Antibody
5 µg/well SUV
5 µg/well Lipid Extract

Figure 4.8. The polyclonal rabbit anti-*Borrelia* glycolipid antibody has reactivity against *B. burgdorferi* lysates and whole spirochetes. ELISAs using the inoculated rabbit serum were conducted against *B. burgdorferi* lysates and whole spirochetes. The polyclonal rabbit anti-*Borrelia* glycolipid antibody does react with the lysates and the bacteria. However, the ELISAs also indicate that the pre-inoculation serum has some baseline reactivity against the lysate and the whole bacteria. Other control antibodies used in the ELISA were α-GM1and the secondary antibody alone. Results represent the mean +/- standard error of the mean of three individual experiments.

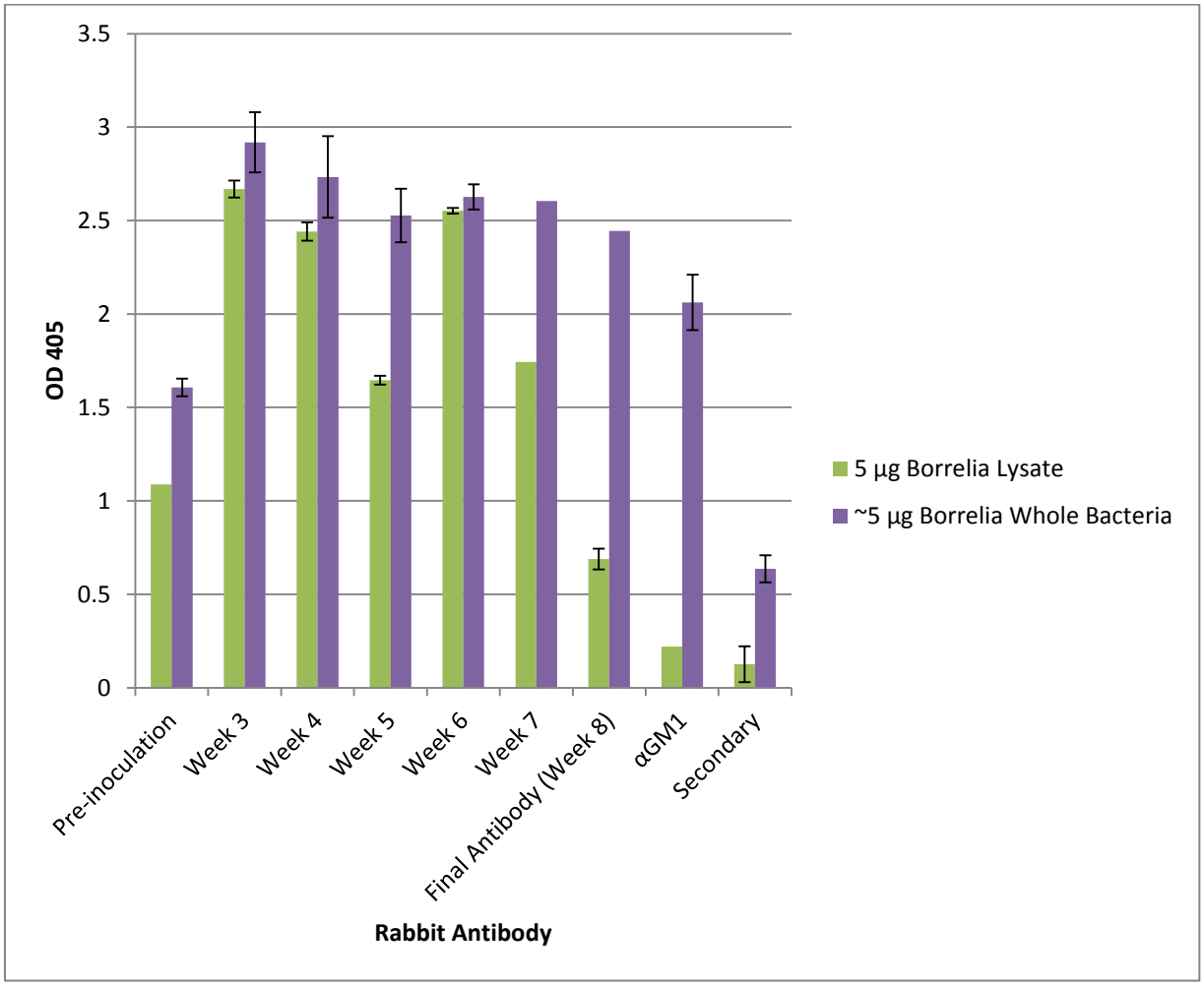
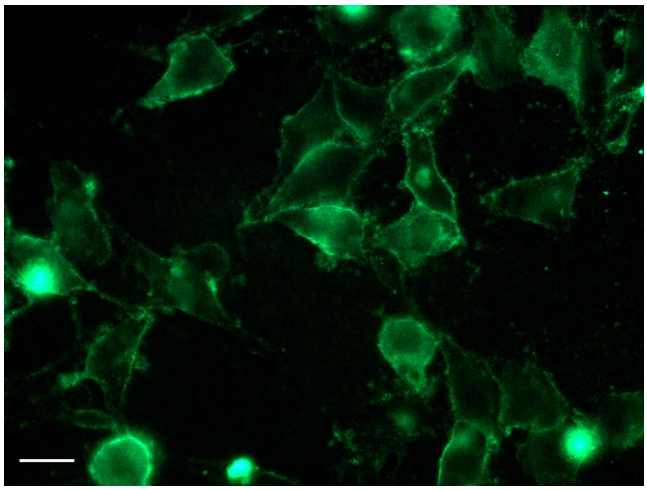
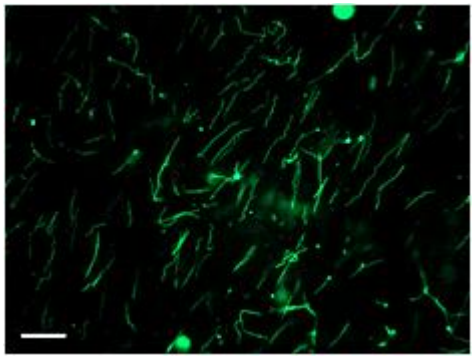

3.5
3
2.5
2
1.5
1
0.5
0
OD 405
Pre-inoculation
Week 3
Week 4
Week 5
Week 6
Week 7
Final Antibody (Week 8)
αGM1
Secondary
Rabbit Antibody
5 µg Borrelia Lysate
~5 µg Borrelia Whole Bacteria

Figure 4.9. The rabbit polyclonal anti-*Borrelia* lipid antibody reacts with HeLa cells that have been incubated with *B. burgdorferi*. The transfer assay was repeated by incubating unlabeled *B. burgdorferi* with HeLa cells for 2 hrs at a MOI of 20:1. Under these coincubation conditions, the rabbit polyclonal anti-*Borrelia* lipid antibody recognizes the surface of the HeLa cells. Size bar = 20 μm.



<u>Figure 4.10.</u> The rabbit polyclonal anti-*Borrelia* lipid antibody reacts with *B. burgdorferi*. **A.** *B. burgdorferi* from a BSK-II culture are recognized by the rabbit polyclonal anti-*Borrelia* lipid antibody. **B.** *B. burgdorferi* from a coincubation of HeLa cells and spirochetes are recognized by the rabbit polyclonal anti-*Borrelia* lipid antibody. Size bar = 20 μm.

A



B

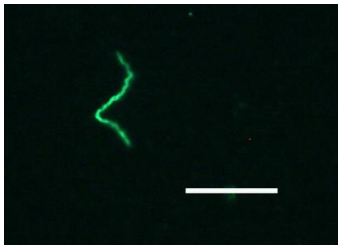
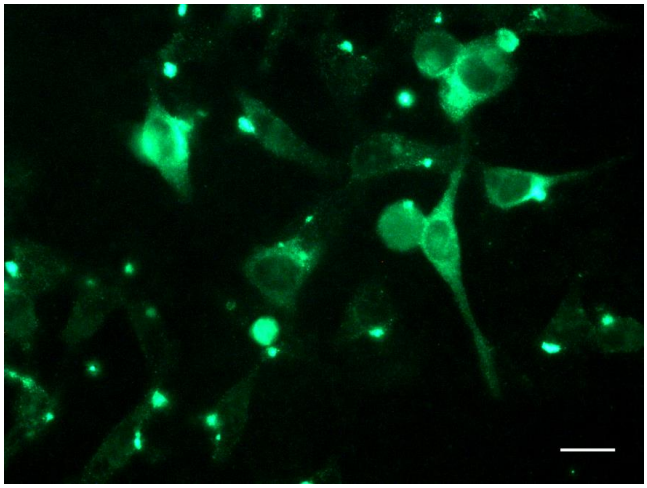
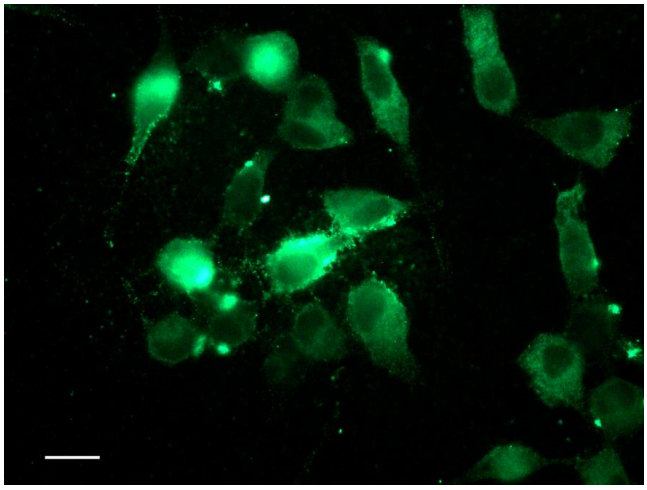


Figure 4.11. The rabbit polyclonal anti-*Borrelia* lipid antibody reacts with HeLa cells alone and the recognition is similar to an α-GM1 antibody. **A.** The rabbit polyclonal anti-*Borrelia* lipid antibody was used to detect the surface of HeLa cells without the presence of *B. burgdorferi*. **B.** The anti-asialo-GM1 antibody detects the surface of HeLa cells. **C.** Pre-inoculation serum and secondary antibody do not react with HeLa cells. Size bar = 20 μm.

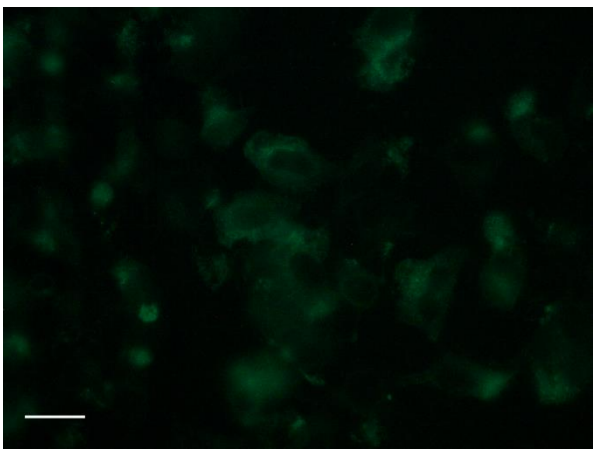
A



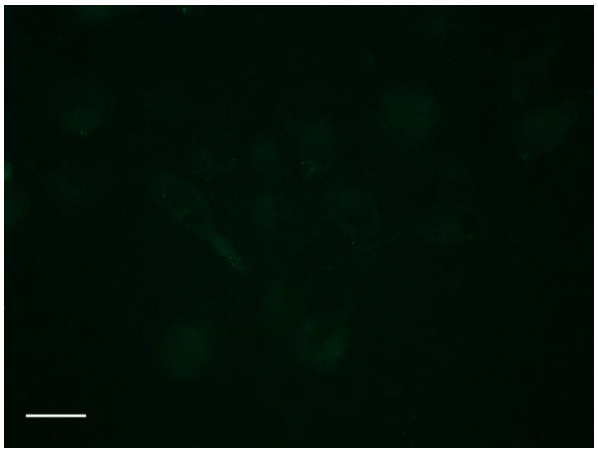
B



C



Pre-inoculation



Secondary Control

<u>Figure 4.12.</u> The rabbit polyclonal anti-*Borrelia* lipid antibody reacts with GM95 cells. A titration of primary the primary antibodies was completed. The antibodies studied were the rabbit polyclonal anti-*Borrelia* lipid antibody and antibody to GM1 and were diluted 1:100, 1:250, 1:500, and 1:1000. Subsequent analysis by immunofluorescence indicates both antibodies react with MEB4 and GM95 cells. These antibodies should not react with the GM95 because these cells lack gangliosides. The secondary antibody alone was used as a control.

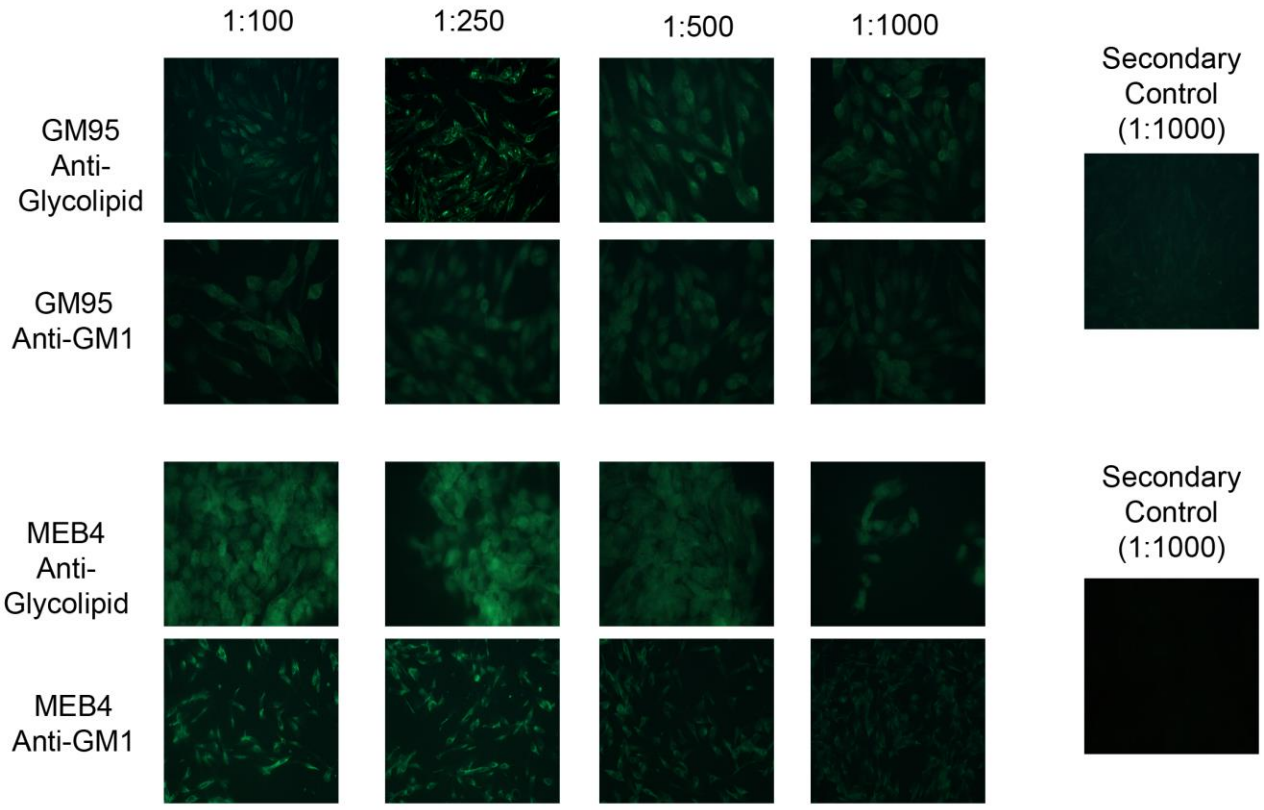
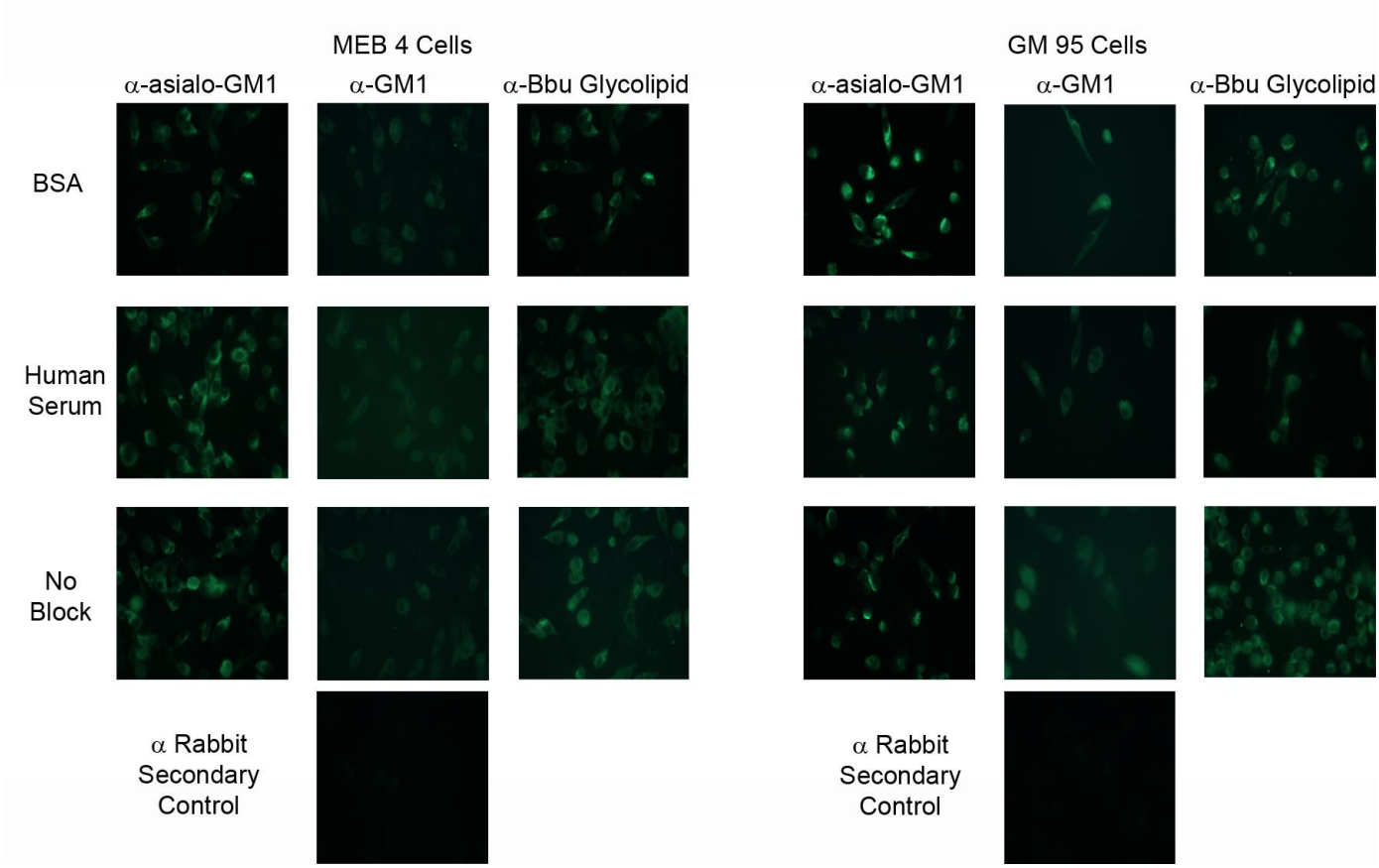

1:100
1:250
1:500
1:1000
Secondary Control (1:1000)
GM95 Anti-Glycolipid
GM95 Anti-GM1
MEB4 Anti-Glycolipid
MEB4 Anti-GM1
Secondary Control (1:1000)

Figure 4.13. The blocking agents BSA and human serum do not appear to contribute to the cross-reactivity of the rabbit polyclonal anti-*Borrelia* lipid antibody. BSA and human serum were examined for their ability to block GM95 andMEB4 cells in immunofluorescence experiments. BSA and human serum did not decrease the cross-reactivity of three antibodies, polyclonal rabbit α-asialo GM1, polyclonal rabbitα-GM1, and rabbit polyclonal α-*Borrelia* lipid antibody (α-Bbu Glycolipid), when compared to an unblocked sample. The secondary antibody alone was used as a negative control.


MEB 4 Cells
GM 95 Cells
α-asialo-GM1
α-GM1
α-Bbu Glycolipid
α-asialo-GM1
α-GM1
α-Bbu Glycolipid
BSA
Human Serum
No Block
α Rabbit Secondary Control
α Rabbit Secondary Control

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