Cell Host & Microbe
Article

Cholesterol Lipids of *Borrelia burgdorferi* Form Lipid Rafts and Are Required for the Bactericidal Activity of a Complement-Independent Antibody

Timothy J. LaRocca,[1] Jameson T. Crowley,[1] Brian J. Cusack,[1] Priyadarshini Pathak,[2] Jordi Benach,[3] Erwin London,[2] Juan C. Garcia-Monco,[1] and Jorge L. Benach[1,*]
[1]Department of Molecular Genetics and Microbiology, and Center for Infectious Diseases
[2]Department of Biochemistry and Cell Biology
Stony Brook University, Stony Brook, NY 11794, USA
[3]ALBA Synchrotron Light Source (CELLS) Cerdanyola del Vallès, 08290 Barcelona, Spain
*Correspondence: jbenach@notes.cc.sunysb.edu
DOI 10.1016/j.chom.2010.09.001

SUMMARY

Borrelia burgdorferi, **the agent of Lyme disease, is unusual as it contains free cholesterol and cholesterol glycolipids. It is also susceptible to complement-independent bactericidal antibodies, such as CB2, a monoclonal IgG1 against outer surface protein B (OspB). We find that the bactericidal action of CB2 requires the presence of cholesterol glycolipids and cholesterol. Ultrastructural, biochemical, and biophysical analysis revealed that the bacterial cholesterol glycolipids exist as lipid raft-like microdomains in the outer membrane of cultured and mouse-derived *B. burgdorferi* and in model membranes from *B. burgdorferi* lipids. The order and size of the microdomains are temperature sensitive and correlate with the bactericidal activity of CB2. This study demonstrates the existence of cholesterol-containing lipid raft-like microdomains in a prokaryote, and we suggest that the temperature dependence of *B. burgdorferi* lipid raft organization may have significant implications in the transmission cycle of the spirochetes which are exposed to a range of temperatures.**

INTRODUCTION

The *Borrelia* include the agents of Lyme disease (Benach et al., 1983; Burgdorfer et al., 1982) and relapsing fever (Johnson, 1977) and are extracellular spirochetes that are susceptible to antibodies (Connolly and Benach, 2005; LaRocca and Benach, 2008). The antibody response is the primary means of host defense against the *Borrelia* and is linked to the pathogenesis of these infections. Antibodies were first used to confirm the spirochetal etiology of Lyme disease, and serology is critical for diagnosis of the disease.

Lytic complement is not required for clearance of *Borrelia* infections (Bockenstedt et al., 1993; Connolly and Benach, 2001; Newman and Johnson, 1981), and resistance to complement is mediated by the binding of complement regulator inhibitors (Bykowski et al., 2008). There are complement-independent antibodies to *Borrelia* that are bactericidal in vitro and in vivo. The monoclonal antibodies CB2 (IgG1) and H6831 (IgG2a) against the outer surface lipoprotein B (OspB) of *B. burgdorferi* and CB515 (IgM) and H4825 (IgG2a) against variable major proteins (Vmps) of relapsing fever *Borrelia* exhibit this unique bactericidal activity (Coleman et al., 1992; Connolly et al., 2004; Sadziene et al., 1993). Fab fragments and the variable regions alone had the same effect (Coleman et al., 1992; Sadziene et al., 1993; LaRocca et al., 2008). Structural changes occur in OspB upon binding of CB2 or H6831 (Becker et al., 2005; Katona et al., 2000).

Complement-deficient mice generated IgM antibodies coincident with the clearance of relapsing fever spirochetemia identical to wild-type (WT) mice (Connolly and Benach, 2001; Connolly et al., 2004). B cell-deficient mice had a peak spirochetemia that was longer and higher than that of WT mice. These experiments demonstrated that complement-independent bactericidal antibodies function in vivo. CB2 creates pores that lead to osmotic lysis as part of its bactericidal mechanism (LaRocca et al., 2009). Exposure to CB2 resulted in the creation of outer membrane projections and openings. Additionally, CB2 bound to *E. coli* expressing OspB was not bactericidal, indicating that factors specific to the *Borrelia* outer membrane are required for the bactericidal mechanism (LaRocca et al., 2009).

The outer membrane of *Borrelia* contains phosphatidylcholine and phosphatidylglycerol, as well as numerous lipoproteins (Belisle et al., 1994; Brandt et al., 1990; Jones et al., 1995; Radolf et al., 1994, 1995). Incorporation of cholesteryl glucoside into the membrane of *B. hermsii* (Livermore et al., 1978) prompted the demonstration that *B. burgdorferi* also contained antigenic glycolipids (Wheeler et al., 1993). Antibodies to these glycolipids crossreact with gangliosides, and vice versa (García Moncó et al., 1993; Garcia-Monco et al., 1995).

B. burgdorferi has three glycolipids, two of which contain cholesterol. These glycolipids were identified as cholesteryl 6-*O*-acyl-β-D-galactopyranoside or cholesteryl 6-*O*-palmitoyl-β-D-galactopyranoside (ACGal/Bb-GL-I), cholesteryl-β-D-galacto-pyranoside (CGal), and mono-α-galactosyl-diacylglycerol (MGalD) and exist in other *Borrelia* species (Ben-Menachem

et al., 2003; Schröder et al., 2003; Stübs et al., 2009). Free cholesterol and cholesterol esters also exist in the outer membrane of *Borrelia* (Stübs et al., 2009). The presence of cholesterol and cholesterol glycolipids in prokaryotes is unusual, although there are some exceptions such as *Helicobacter*, *Mycoplasma*, *Ehrlichia*, *Anaplasma*, and *Brachyspira* (Haque et al., 1995; Hirai et al., 1995; Lin and Rikihisa, 2003; Smith, 1971; Trott et al., 2001). In eukaryotic cell membranes, sterols help form microdomains called lipid rafts (Brown and London, 2000; London, 2002). Lipid rafts are ordered, rigid, cholesterol-rich areas of the membrane that have lipid-anchored proteins, notably the glycosylphosphatidylinositol (GPI)-anchored proteins that have important roles in maintaining lateral heterogeneities in cell membranes and in biological sorting processes. Lipid rafts are important for receptor clustering and lateral sorting of proteins (Brown, 1998; Epand, 2008), as well as elasticity, endocytosis, exocytosis, and vesicle formation and budding (Chen and Rand, 1997; Nichols, 2003; Salaün et al., 2004; Wang et al., 2000). The presence of cholesterol in the outer membrane of *Borrelia* suggests that lipid raft microdomains, similar to those in eukaryotes, may exist in these bacteria. If this is the case, lipid rafts may be important for lateral sorting and coalescence of certain *Borrelia* antigens and could influence membrane effects in response to CB2.

We show here that cholesterol and cholesterol glycolipids are critical for the bactericidal mechanism of the complement-independent mAb, CB2. We also show evidence for the existence of lipid raft microdomains in *B. burgdorferi*.

RESULTS

CB2 Causes Removal of Antigens from the Surface of *B. burgdorferi* in Membrane Vesicles

CB2 exerts its bactericidal effects by creating membrane projections and blebs on the surface of *B. burgdorferi*, and we proposed that vesicle removal formed the lytic pores (LaRocca et al., 2009). To determine whether vesicle removal is important in the bactericidal mechanism of CB2, we measured release of vesicles in *Borrelia* supernatants with the fluorescent, lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH). DPH fluoresces in hydrophobic but not aqueous environments and shows a linear response with membrane bilayer concentration (London and Feligenson, 1978). Spirochetes were treated with CB2, CB10, or a control without antibody in the presence of dextran T500 to prevent lysis (LaRocca et al., 2009). Greater release of vesicles from CB2-treated *B. burgdorferi* was observed relative to the controls that had the normal, constitutive level of membrane vesicle release (Figure 1A). Vesicle release was correlated with an increased release of OspB and OspA but not of cytosolic DnaK (Figure 1B). Negative-stain transmission electron microscopy (TEM) demonstrated removal of membrane vesicles that contained OspB (Figure 1C). The membrane vesicles also contained OspA, concordant with our earlier observations with confocal microscopy where we observed release of vesicles containing OspA as a later event in the bactericidal mechanism after release of vesicles containing OspB (Escudero et al., 1997). The late release of vesicles containing OspA may be due to the lack of membrane stability induced by the binding of CB2 to OspB.

The release of vesicles caused by CB2 suggested that cholesterol and cholesterol glycolipids could be constituents of these structures, and they were quantified in supernatants after CB2 treatment. Polyclonal rabbit anti-asialo GM1 cross-reacts with *B. burgdorferi* cholesterol glycolipids and was used to detect the cholesterol glycolipids in the vesicles induced by treatment with CB2 (Figure 1D). When these supernatants were resolved by electrophoresis on native gels and transferred to nitrocellulose, immunoblot reactivity to OspB, OspA, and cholesterol glycolipids occurred at the same mobility, but not DnaK (Figure 1E), confirming the inclusion of the lipoproteins in the membrane vesicles and also the results in Figures 1B and 1D.

Cholesterol Is Required for the Bactericidal Mechanism of CB2

The possibility of a molecular interaction between OspB and cholesterol glycolipids led us to test whether the bactericidal mechanism of CB2 is dependent on cholesterol. Methyl-β-cyclodextrin (MβCD) has been used to deplete cholesterol in eukaryotic cells (Zidovetzki and Levitan, 2007) and bacteria (Lin and Rikihisa, 2003). Given the known toxicity of MβCD, we determined the experimental conditions for its use. There was no change in spirochete numbers after an MβCD exposure of 3 hr (Figure 2A), and spirochetes exposed to 10 mM MβCD for 30 min grew normally once returned to normal medium (Figure 2B). Protein levels did not change in the pellet or supernatant after MβCD treatment for 1 hr (Figure S1A available online). Furthermore, addition of cholesterol was not toxic as exposure to 10 and 20 μg/ml of excess cholesterol did not affect spirochete growth (Figure 2B). These experiments show that there are no obvious pleiotropic effects of MβCD or of excess cholesterol at the concentrations and incubation times used for the cholesterol-depletion experiments, and that the spirochetes remain viable (do not lyse). When *B. burgdorferi* were treated with 10 mM MβCD for 30 or 60 min, there was a 50% reduction in total cholesterol detected fluorometrically (Figure 2C). Lipid extracts from supernatants and pellets of MβCD-treated spirochetes were separated by thin-layer chromatography (TLC) and showed that MβCD removes free cholesterol, and the glycolipids ACGal/Bb-GL-I and CGal from *B. burgdorferi* while not affecting MGalD or phospholipids (Figure 2D). Probing MβCD-treated supernatants with antibody to asialo GM1 in immunoblots confirmed that cholesterol glycolipids were removed from *B. burgdorferi* (Figure 2D). Lipid extracts were also analyzed by mass spectrometry, which showed that MβCD removed the two cholesterol glycolipids and confirmed the TLC findings (Table S1).

To determine the role of cholesterol and cholesterol glycolipids in the bactericidal mechanism of CB2, we pretreated spirochetes with MβCD for 30 min and exposed them to the antibody. In contrast to the 50% decrease in organisms without MβCD treatment, after a 15 min incubation with CB2, there was no reduction (Figure 2E). Spirochetes depleted of cholesterol exhibited decreased vesicle release after CB2 treatment compared to controls (Figure 2F). The difference in vesicle release resulting from treatment with CB2 or CB10 may be due to the greater affinity of CB2 for OspB. The affinity of CB2 for recombinant OspB is three orders of magnitude greater (Kd = 1.1 nM) than the affinity of CB10 for recombinant OspA (Kd = 1.6 μM). Thus, membrane cholesterol enhances the bactericidal action of CB2.

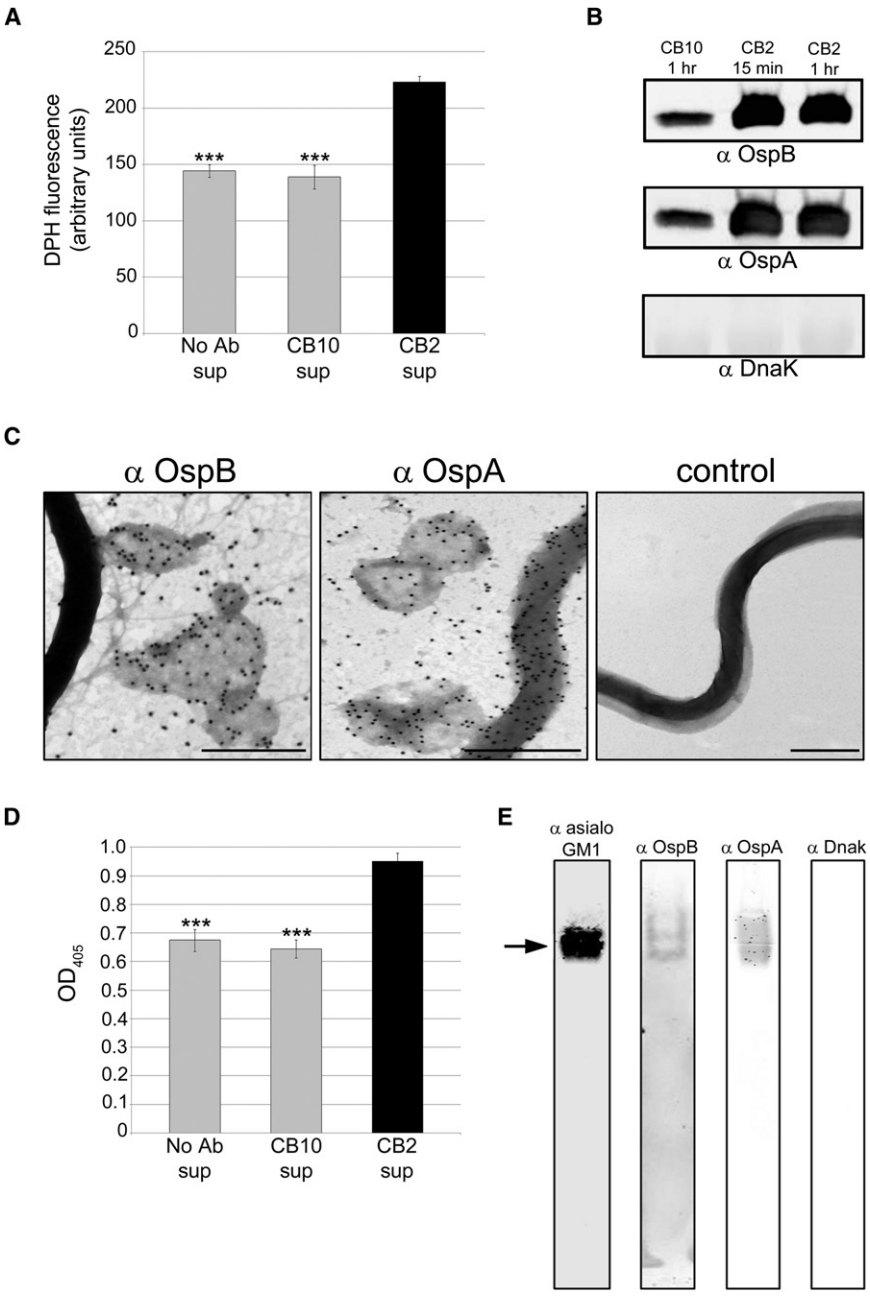

Figure 1. CB2 Removes Antigens from the Surface of *B. burgdorferi* in Membrane Vesicles

(A) CB2 treatment caused increased release of membrane vesicles from *B. burgdorferi* relative to treatment with CB10 (control IgG against OspA) or no antibody (Ab) as measured by DPH fluorescence in the cell-free supernatant (sup).

(B) Immunoblots showing that OspB and OspA are released into the supernatant upon CB2 treatment for 15 min or 1 hr while the cytosolic DnaK is not.

(C) Negative-stain TEM images showing that CB2 causes removal of OspB and OspA (18 nm colloidal gold) from the surface in large and small membrane vesicles (dark gray around colloidal gold) after 15 min of exposure. Controls (secondary colloidal gold conjugate only) did not show labeling. Scale bars represent 500 nm.

(D) ELISA showing greater release of cholesterol glycolipids into the supernatant (see A).

(E) Native PAGE immunoblot of supernatants from spirochetes treated with CB2 for 30 min. Bands representing OspB, OspA, and cholesterol glycolipids (anti-asialo GM1) had the same mobility (arrow), indicating colocalization of these molecules in CB2-induced vesicles; cytosolic DnaK was not present in the supernatant.

Results in (A), (B), (D), and (E) are from triplicate experiments. ANOVA, ***$p < 0.001$. Error bars represent the standard deviation.

To show that the decreased bactericidal effect of CB2 was specific for cholesterol depletion, we mixed MβCD with 20 μg/ml cholesterol prior to treatment of spirochetes, followed by CB2 treatment. Spirochete numbers decreased to levels similar to treatment with CB2 alone (Figure 3A). We next assessed the bactericidal effect of CB2 when cholesterol was replaced after depletion. Cholesterol replacement was confirmed by incorporation of the fluorescent NBD-cholesterol into *B. burgdorferi* after MβCD treatment (Figure 3B). Replacing cholesterol in spirochetes resulted in its incorporation into the cholesterol glycolipid fraction as detected with anti-asialo GM1 in slot blots (Figure 3B). Cholesterol replacement also restored the bactericidal effect of CB2 (Figure 3C). Furthermore, addition of 20 μg/ml exogenous cholesterol during CB2 treatment enhanced the bactericidal effect (Figure 3D). To determine whether cholesterol replacement or deletion have an effect on CB2 binding to OspB, we performed surface labeling of OspB by indirect immunofluorescence of *B. burgdorferi* and western blots quantitated by densitometry. Using both experimental approaches, we concluded that the binding of CB2 to OspB after cholesterol replacement or deletion does not change, indicating that the accessibility of OspB to the antibody remains the same (Figures S1B and S1C). These experiments show that membrane cholesterol is required for the bactericidal mechanism of CB2.

The Cholesterol Glycolipids of *B. burgdorferi* Are Constituents of the Membrane Vesicles Created by CB2

The localization of the cholesterol glycolipids in relation to OspB was characterized with transmission electron microscopy (TEM) after treatment of spirochetes with CB2. An anti-rabbit IgG conjugated to colloidal gold of 6 nm was used to detect anti-asialo GM1 bound to cholesterol glycolipids and an anti-mouse IgG conjugated to colloidal gold of 18 nm was used to detect CB2 bound to OspB (Figure 4). There was an abundance of cholesterol glycolipids in outer membrane projections created

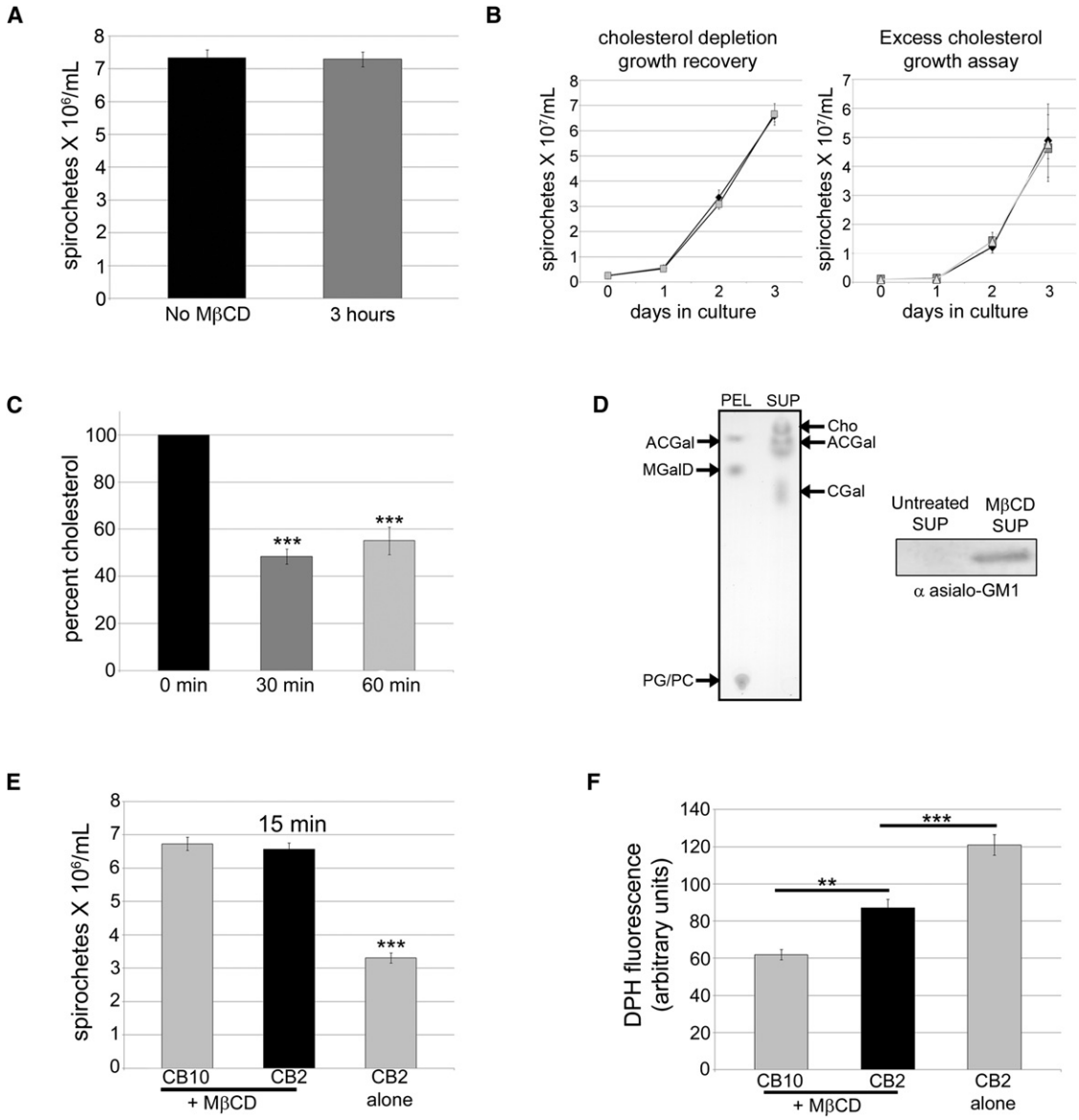

Figure 2. Cholesterol Depletion Affects the Bactericidal Mechanism of CB2 against *B. burgdorferi*

(A) Ten millimolar MβCD is not toxic to *B. burgdorferi* after a 3 hr exposure.

(B) Left: *B. burgdorferi* grow normally after cholesterol depletion with MβCD for 30 min (black diamonds) compared to untreated spirochetes (gray squares). Right: no difference in the growth of *B. burgdorferi* with 10 (dark gray squares), or 20 μg/ml (light gray squares) of excess cholesterol; controls (black diamonds).

(C) Ten millimolar MβCD depletes cholesterol in *B. burgdorferi* by 50% after 30 or 60 min of treatment.

(D) Left: chloroform-methanol (85:15) TLC showing that MβCD removes cholesterol (Cho) and cholesterol glycolipids (ACGal, CGal) into the supernatant (SUP), while the MβCD-treated *B. burgdorferi* pellet (PEL) contains ACGal, MGalD (cholesterol-free glycolipid), phosphatidyl choline (PC), and phosphatidyl glycerol (PG). Right: immunoblot of supernatants (sup) from control or MβCD-treated spirochetes probed with anti-asialo GM1 confirming that MβCD removes the cholesterol glycolipids.

(E) Spirochetes were exposed to MβCD for cholesterol depletion and then treated with CB2 or the control IgG CB10 for 15 min.

(F) DPH fluorescence vesicle assay of spirochete supernatants shows that depletion of cholesterol with MβCD reduces the number of vesicles released from *B. burgdorferi* upon CB2 exposure. Spirochete counts were done by direct dark-field microscopy enumeration.

Results for (A)–(C), (E), and (F) are from triplicate experiments. ANOVA, ***$p < 0.001$, **$p < 0.01$. Error bars represent the standard deviation. See also Table S1 and Figure S1.

by CB2 (Figures 4A–4C) and in released membrane vesicles containing OspB and bound CB2 (Figures 4A–4D), demonstrating the presence of these lipids in the released structures. The cholesterol glycolipids formed distinct clusters or microdomains surrounding OspB in membrane vesicles and projections.

The Cholesterol Glycolipids of *B. burgdorferi* Bind OspB

The clustering of cholesterol glycolipids around OspB in the outer membrane and in released vesicles (Figure 4) shows that these molecules associate and that OspB exists in microdomains in the outer membrane of *B. burgdorferi*. It is possible

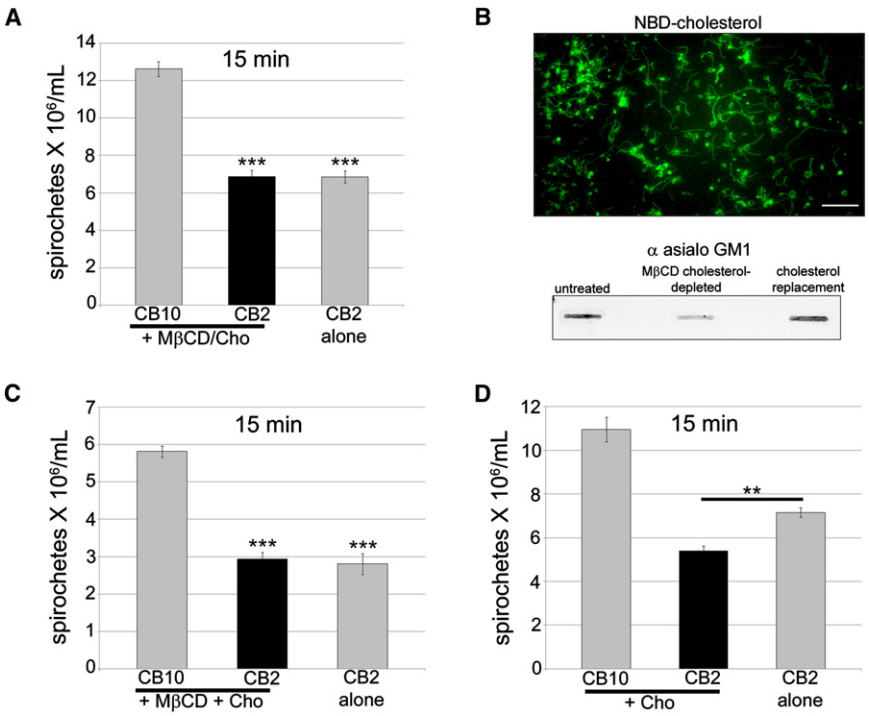

Figure 3. Cholesterol Is Required for the Bactericidal Mechanism of the Complement-Independent Antibody, CB2

(A) Inhibition of 10 mM MβCD with 20 μg/ml of soluble cholesterol (+ MβCD/Cho) restored the bactericidal effect of CB2.

(B) Top: spirochetes exposed to 10 mM MβCD and treated with the fluorescent cholesterol-NBD show that cholesterol has been reincorporated into their membrane. Bottom: slot blot shows that cholesterol replacement results in its incorporation in the cholesterol glycolipids as detected by anti-asialo GM1.

(C) CB2 regains its bactericidal effect against spirochetes that have had their cholesterol replaced after depletion (MβCD + Cho).

(D) Addition of excess cholesterol (20 μg/ml, + Cho) during CB2 treatment enhances the bactericidal effect of the antibody against spirochetes.

Results for (A), (C), and (D) are from triplicate experiments. ANOVA, ***$p < 0.001$, **$p < 0.01$. Error bars represent the standard deviation. See also Figure S1.

that OspB interacts with cholesterol glycolipid directly or via the interaction of its covalently linked palmitates with the tightly packed hydrophobic core of the ordered domains, as is the case for other lipid-anchored proteins that interact with lipid rafts. Crystal structures of both OspA and OspB share a putative binding cleft for which there is no proposed ligand (Becker et al., 2005; Li et al., 1997). At the bottom of this cleft are three charged amino acids (arg-162, glu-184, and arg-214) that may be functionally important as a result of their location and sequence conservation. It has been postulated that this cleft could bind small molecules, so we tested whether the cholesterol glycolipids bind to this cleft. Blind docking analyses with the program VINA were performed with the crystal structure of OspB and ACGal, Bb-GL-I, and CGal. The top-scored binding sites in OspB were those involving the cleft lined with the previously mentioned triad of arg-162, glu-184, and arg-214. The binding energy calculated in silico by VINA of the top ranking solution of each docked complex at 25°C was −6.5 kcal/mol (K_i ~17 μM) for ACGal, −7.3 kcal/mol (K_i ~4 μM) for CGal, and −5.4 kcal/mol for Bb-GL-I (K_i ~109 μM). However, the conformations adopted by these molecules, the weak hydrogen bond and steric interactions with OspB and the extended solvent exposure of these hydrophobic molecules, point to a weak interaction of these cholesterol glycolipids with the OspB cleft, consistent with the relatively low binding energies (Figure S2). This suggests that the cleft in OspB has low affinity for the cholesterol glycolipids of *B. burgdorferi*, although it could also be enhanced if OspB is held in the outer membrane by other interactions. Docking analyses and modeling show that the glycolipid binding site faces the membrane and that it would be relatively near to its surface (~30–40 Å). Furthermore, the N terminus of OspB is close to the cleft, and in all the binding models predicted by the docking analyses, the cleft faces the cellular membrane (Figure S2, arrow) which strengthens the possibility of an interaction.

Detergent Resistant Membranes Containing OspB and Cholesterol Glycolipids Can Be Isolated from *B. burgdorferi*

The clustering of cholesterol into distinct microdomains (Figure 4) is a characteristic of lipid rafts. To determine whether the cholesterol glycolipid-rich regions of *B. burgdorferi* exhibit other characteristics of lipid rafts, we performed solubilization experiments with *B. burgdorferi* with Triton X-100 (TX-100) at 4°C, followed by separation of the lysate on a discontinuous OptiPrep density gradient. Lipid rafts resist solubilization by TX-100 at 4°C, forming detergent-resistant membranes (DRMs) (Brown and Rose, 1992). DRMs float in the gradient and are found at the 25% density with some at 20% and 30%. Each density fraction was analyzed by slot blot for cholesterol glycolipids, as well as specific proteins that may associate with the DRMs. Cholesterol glycolipids were concentrated in the 25% and 30% fractions with some in the 20% fraction, demonstrating association of these glycolipids with DRMs (Figure 5A). The outer membrane proteins OspB, OspA, and P66 partitioned with cholesterol glycolipids in the DRMs in the 20%, 25%, and 30% fractions, suggesting that these proteins normally reside in lipid rafts (Figure 5A). The cytoplasmic Lon-1 protease (Coleman et al., 2009) was included as a control and was only found in the 35% fraction, typical for a soluble protein (Figure 5A). We also determined the lipid composition of the DRM and non-DRM (solubilized) fractions. We found that TX-100 treatment solubilized PC, PG, and MGalD but not ACGal/Bb-GL-I, CGal, or free cholesterol when lipids were analyzed by TLC (Figure S3). This result shows that the DRMs are composed of a distinct subset

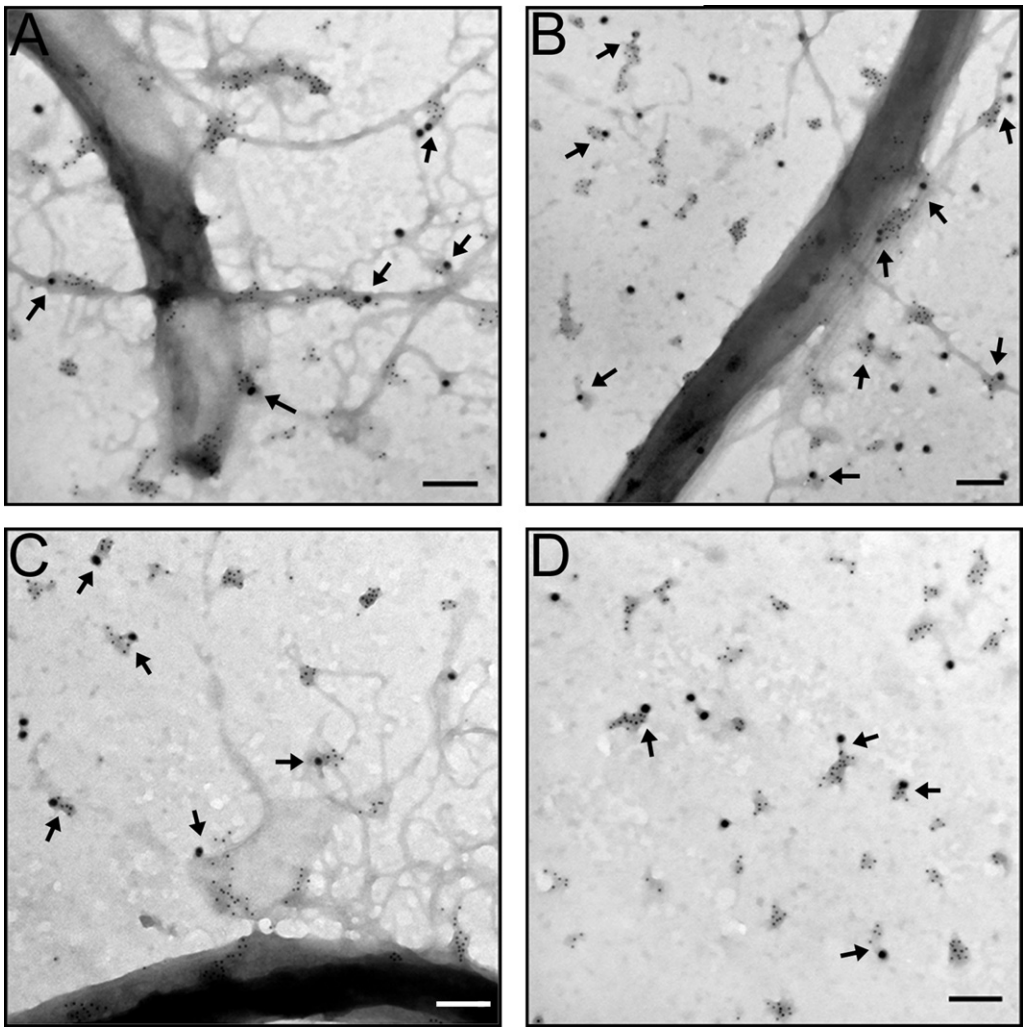

Figure 4. The Cholesterol Glycolipids of *B. burgdorferi* Are Constituents in the Projections and Vesicles Induced by CB2
Negative-stain TEM images of CB2-treated *B. burgdorferi* that were labeled with immunogold for CB2 bound to OspB (18 nm colloidal gold) and cholesterol glycolipids (6 nm colloidal gold). Cholesterol glycolipids are associated with projections (A–C) and vesicles (B–D) induced by CB2. (D) specifically focuses on released membrane vesicles. Projections and released vesicles contain cholesterol glycolipids clustered around OspB (arrows), suggesting the existence of cholesterol-rich microdomains. Controls included secondary gold conjugates alone and normal mouse and rabbit serum controls. Scale bars represent 100 nm. See also Figure S2.

of *B. burgdorferi* lipids. The observation that DRMs can be isolated from *B. burgdorferi* and that they are rich in cholesterol lipids is suggestive of the existence of lipid rafts. Indeed, after cholesterol depletion of spirochetes that disrupts lipid rafts, and thus the DRMs, the same lipoproteins solubilize and are found in the 35% fraction (Figure 5B).

Multilamellar Vesicles of *B. burgdorferi* Lipids Spontaneously Form Ordered Lipid Domains

To confirm that the insolubility of *B. burgdorferi* lipids in TX-100 was linked to their ability to form ordered domains (lipid rafts), we studied the properties of multilamellar vesicles (MLVs) composed of extracted *B. burgdorferi* lipids and compared them to those of vesicles forming a raft (liquid ordered, Lo) state or a nonraft (liquid disordered, Ld) state. Figure 5C shows that DRMs can be obtained by treating *B. burgdorferi* MLVs with TX-100 at room temperature (RT). The degree of insolubility in TX-100 is slightly less than that of sphingomyelin/cholesterol vesicles (SM/cho 2:1), which form the Lo state, and much more than for dioleoylphosphatidylcholine /cholesterol vesicles (DOPC/cho 2:1), which form the Ld state. DPH fluorescence anisotropy measurements (for membrane order) indicated that at RT the degree of order in the *B. burgdorferi* MLVs was intermediate between Lo state SM/cho and Ld state DOPC/cho (Figure 5D). This shows that the tendency of *B. burgdorferi* lipids to form an ordered state bilayer is not a detergent artifact. Measurement of temperature dependence of fluorescence anisotropy (Figure 5E) shows that the high degree of order in *Borrelia* lipids relative to that in nonraft (DOPC/cho) vesicles persists to high temperatures.

Although detergent-resistance and anisotropy experiments demonstrate a degree of lipid order, they do not distinguish

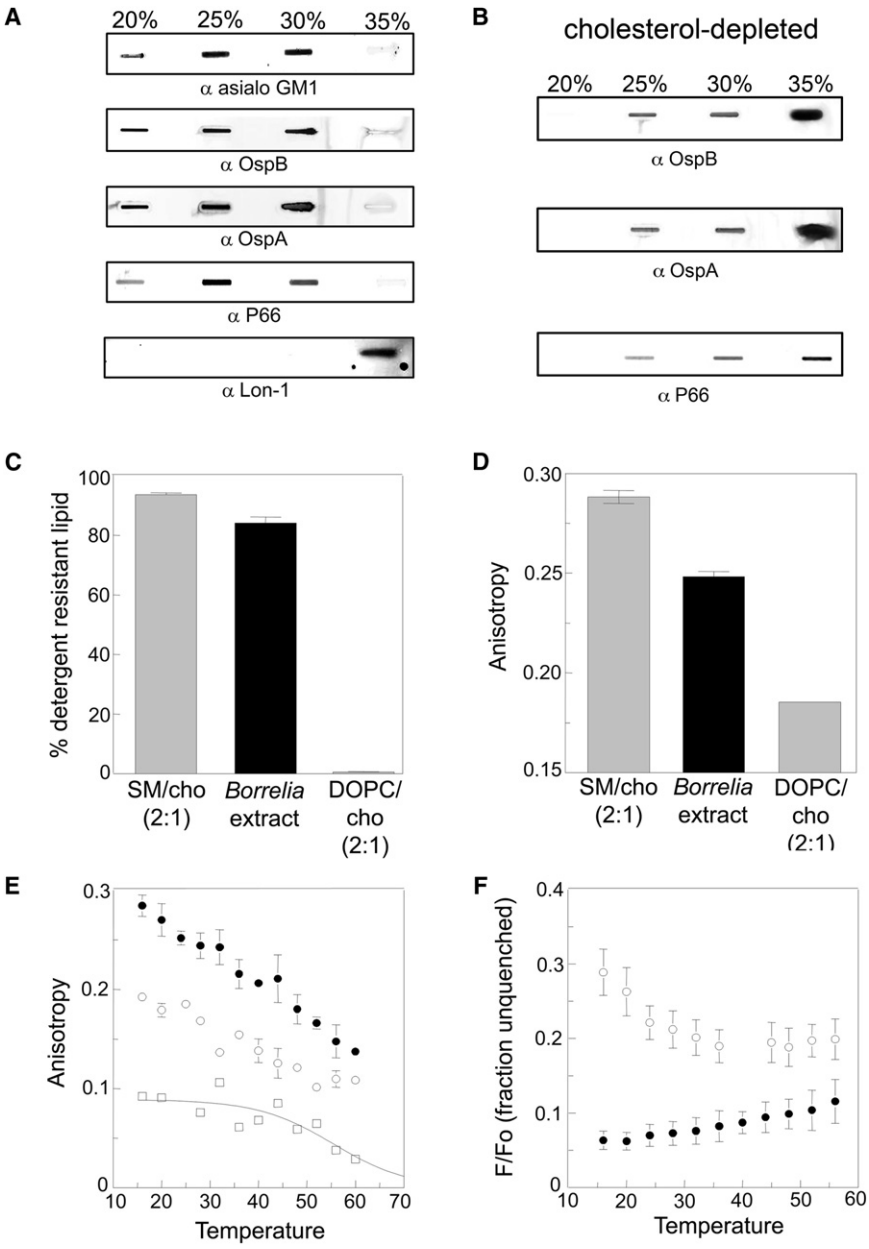

Figure 5. Lipid Rafts Exist in *B. burgdorferi*

(A) Whole spirochetes were subjected to TX-100 treatment at 4°C and Optiprep density gradient separation. Gradient fractions were slot blotted and probed with anti-asialo GM1, OspB, OspA, P66, or Lon-1 protease.

(B) Spirochetes were treated as in (A) but were exposed to 10 mM MβCD prior to solubilization in TX-100. Cholesterol depletion leads to solubilization of lipoproteins (35% fraction).

(C) *B. burgdorferi* lipids in the form of MLV model membranes display resistance to solubilization by TX-100. Lipid ratios are mol:mol. SM/cho (2:1) Lo, sphingomyelin/cholesterol vesicles; DOPC/cho (2:1) Ld, dioleoylphosphatidylcholine /cholesterol vesicles.

(D) Fluorescence anisotropy of DPH demonstrates a high degree of order among lipids in *B. burgdorferi* MLVs.

(E) DPH fluorescence anisotropy in *Borrelia* MLVs as a function of temperature. MLVs contained *B. burgdorferi* lipid extract (filled circles) or 2:1 DOPC/cholesterol (open circles). Open squares show the difference between the value for *Borrelia* lipids and that for the DOPC/cho fit to a sigmoidal curve that has a limiting value of zero at high temperature.

(F) FRET as a function of temperature for *Borrelia* lipids in model membranes demonstrates the coexistence of Lo (raft) and Ld domains (high F/Fo values). MLVs contained *Borrelia* lipid extract (open circles) or DOPC/cholesterol 2:1 (filled circles). F/Fo is the ratio of fluorescence in samples containing donor (NBD-DPPE) and acceptor (rhodamine-DOPE) to that in samples containing donor.

(A) and (B) are representative experiments, and (C)–(F) are from triplicate experiments. *$p < 0.05$, ***$p < 0.001$. Error bars represent the standard deviation. See also Figure S3.

between a homogeneous bilayer that has a uniformly high degree of order and one that contains coexisting Lo (raft) and Ld domains. To distinguish this, a Förster resonance energy transfer (FRET) assay was used. This evaluates the degree of segregation of NBD-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-7-nitro-2-1,3-benzoxadiazol-4-yl (NBD-DPPE), which has a moderate affinity for rafts, and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl (rhodamine-DOPE), which mainly localizes within disordered domains (Ayuyan and Cohen, 2008). When lipid raft and nonraft domains coexist, these probes segregate from one another, and FRET is weak. The temperature dependence of FRET in the *Borrelia* MLVs was compared to that of Ld state (nonraft) DOPC/cho vesicles. The *Borrelia* MLVs show weak FRET (high F/Fo values) at low temperature in contrast to DOPC/cho (Figure 5F). At high temperature, the FRET values in the *Borrelia* MLVs approach those in DOPC/cholesterol, showing that the segregation of the probes in the *Borrelia* lipid MLVs is lost at high temperature (Figure 5F). This is typical of the melting of ordered domains at high temperature, such that ordered domain formation, and segregation of molecules into different domains is lost (Bakht et al., 2007; Xu et al., 2001). Combined, these results demonstrate that the *B. burgdorferi* lipids have the ability to form ordered domains (lipid rafts) that segregate from Ld domains over a wide range of temperatures.

Temperature Affects Both the Bactericidal Action of CB2 and Cholesterol Glycolipid Organization

To determine whether the bactericidal effects of CB2 were temperature dependent, we held spirochetes at different temperatures between 4°C and 42°C and then treated with CB2. The bactericidal activity of CB2 increased with increasing temperature (Figure 6A). Additionally, the rate of CB2-induced lysis

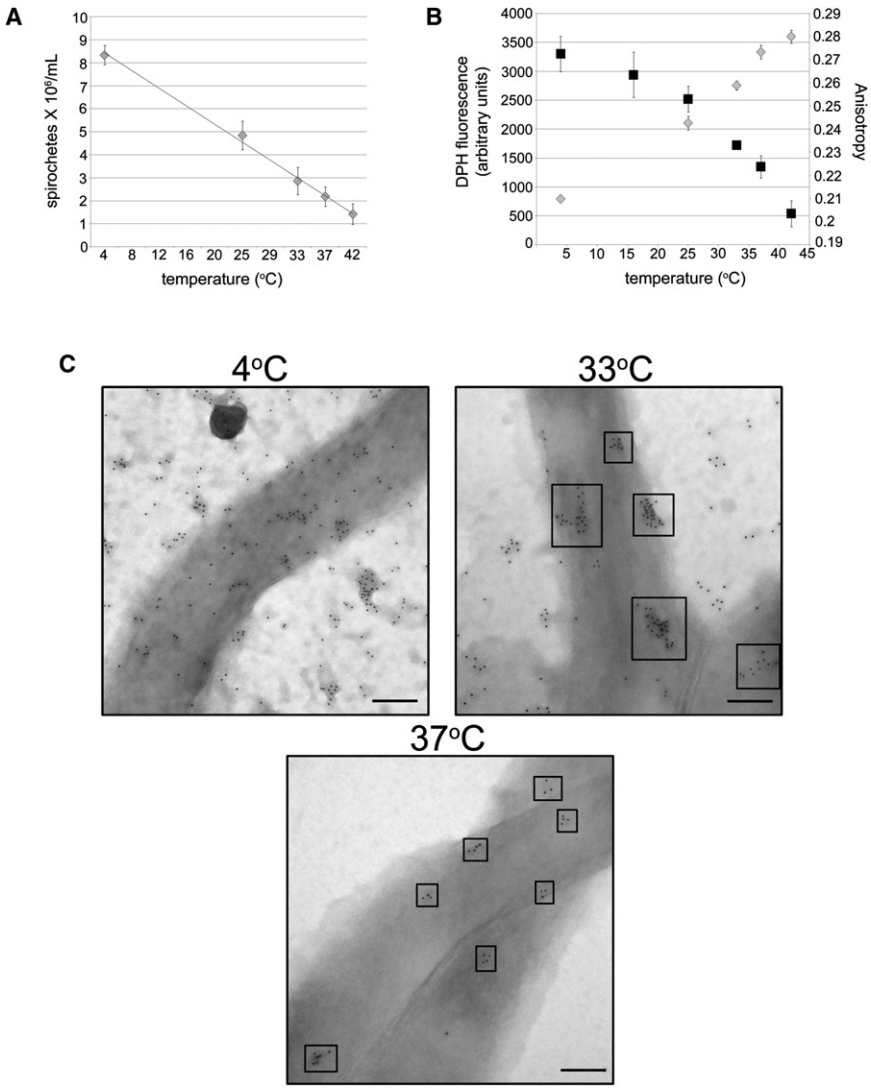

Figure 6. Modulation of Outer Membrane Order with Temperature Affects the Bactericidal Activity of CB2 and the Organization of the Cholesterol Glycolipids

(A) Killing assay showing that the bactericidal activity of CB2 is directly proportional to temperature.

(B) Permeability of the *B. burgdorferi* membrane is directly proportional to temperature, as measured by DPH incorporation (gray diamonds). Lipid order, as measured by anisotropy (black squares), is inversely proportional to temperature. Results are from triplicate experiments.

(C) *B. burgdorferi* were labeled for cholesterol glycolipids (6 nm colloidal gold) at 4°C, 33°C, and 37°C and analyzed to observe the native organization of microdomains and the effect of temperature on organization and size. At 4°C, the microdomains are greatly enlarged and cholesterol glycolipids and dispersed. At 33°C, the microdomains exist as distinct clusters of ~100 nm; at 37°C the microdomains form smaller (~40 nm) clusters. Scale bars represent 100 nm. Error bars represent the standard deviation. See also Figure S4.

increased as temperature increased (Figures S4A–S4D). A decrease in the degree of ordered microdomain formation as temperature increased was also detected by DPH fluorescence. The intensity of DPH fluorescence bound to *B. burgdorferi* increased with increasing temperature (Figure 6B, left y axis), suggesting that DPH inserts into the membrane of *B. burgdorferi* more readily at higher temperatures. In addition, the anisotropy of DPH fluorescence decreased as temperature increased as a result of greater membrane disorder (Figure 6B, right y axis). Anisotropy values of whole *B. burgdorferi* and their temperature dependence were concordant with those obtained in model membranes of *B. burgdorferi* lipids (Figure 5E). This is consistent with a decrease in order and a decrease in the raft formation or size at high temperatures (Fujita et al., 2007).

Indeed, temperature had a significant effect on the organization of lipid microdomains in the outer membrane of *B. burgdorferi* (Figure 6C). Cholesterol glycolipids organized into distinct clusters at 33°C (~100 nm) and at 37°C (~40 nm). At 4°C larger, more dispersed clusters were noted. Temperature might alter other membrane properties; however, it did not affect the levels of several membrane proteins or the level of CB2 binding to *B. burgdorferi* (Figure S4E). The visualization of distinct cholesterol microdomains (clusters) in the outer membrane of intact *B. burgdorferi* cells is highly indicative of lipid rafts. That the cholesterol microdomains can be seen even without antibody treatment indicates that this is the native organization of the cholesterol glycolipids in the outer membrane of *B. burgdorferi* and not the result of the CB2-induced damage seen in Figure 4. Furthermore, OspB colocalizes with the cholesterol glycolipids rafts at different temperatures (Figure 7A), suggesting that this association is maintained as the size of rafts changes with temperature. Lipid rafts are also present in spirochetes isolated from ten mouse bladders, without cultivation, demonstrating that these microdomains occur in vivo (Figure 7B). That the cholesterol glycolipid microdomains are present in vivo suggests that this is the normal organization of the cholesterol glycolipids when spirochetes are in the mammalian host. The in vivo organization of the cholesterol glycolipids is similar to the organization of cultured organisms at 37°C with tight clusters.

DISCUSSION

Three main conclusions are derived from these studies. First, we show that cholesterol glycolipids of *B. burgdorferi* are required for the bactericidal mechanism of CB2, the complement-independent IgG against OspB. Second, a cross-reaction with the cholesterol glycolipids that could have implications for the

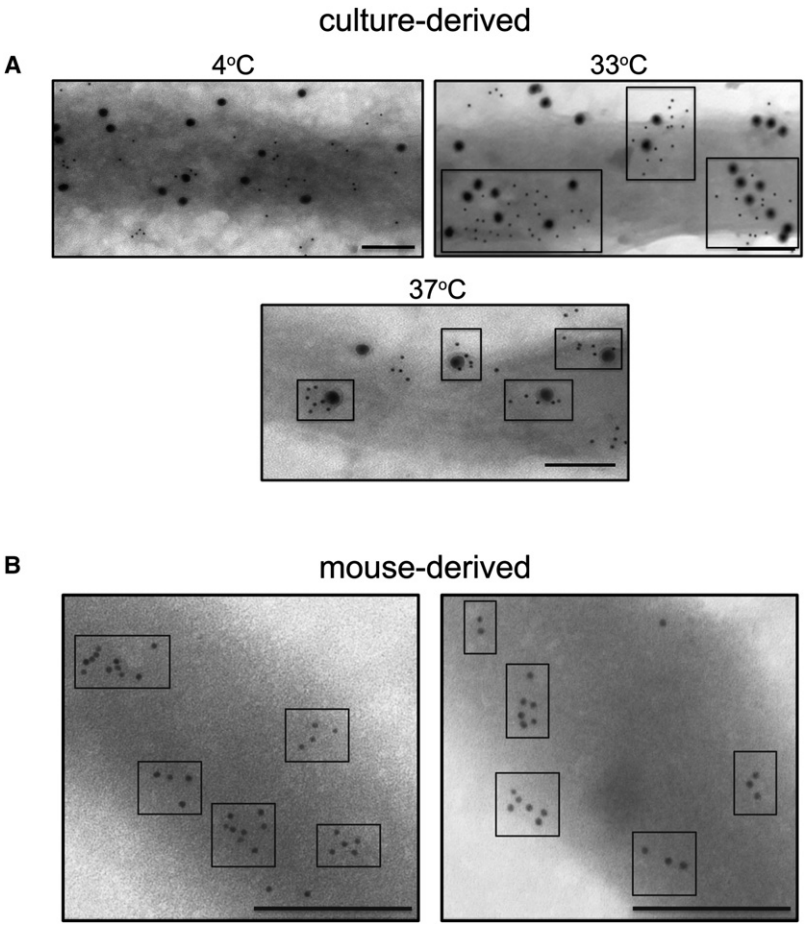

Figure 7. Cholesterol Glycolipid Microdomains Associate with OspB at Different Temperatures and Exist in *B. burgdorferi* Harvested Directly from Mice

(A) *B. burgdorferi* held at 4°C, 33°C, or 37°C were fixed and labeled with anti-asialo GM1 (6 nm colloidal gold) and CB2 (18 nm colloidal gold) followed by secondary colloidal gold conjugates. OspB in the glycolipid microdomains organize differently depending on the temperature (boxes).

(B) *B. burgdorferi* harvested directly (without cultivation) from the bladders of ten C3H/HeN mice 20 days after inoculation were labeled for cholesterol glycolipids (6 nm colloidal gold) to demonstrate the presence of clustering in vivo (boxes).

Scale bars represent 100 nm.

pathogenesis of Lyme disease has been documented. Third, we have also provided evidence for the existence of lipid rafts in *Borrelia* from cultures at various temperatures and directly isolated from experimentally infected mice. This has implications for other prokaryotes.

We demonstrated the requirement of cholesterol and cholesterol glycolipids for the bactericidal action of CB2. Depletion of cholesterol and cholesterol glycolipids decreased the bactericidal activity of CB2, whereas replacement of cholesterol restored it. Additional evidence for a role of cholesterol in CB2 action comes from the formation and composition of the vesicles and surface projections that increase upon CB2 exposure. Ultrastructural and biochemical approaches showed that released vesicles include the antigen OspB, with bound CB2, and cholesterol glycolipids in the form of microdomains. Lipid raft organization impacts CB2 function. Ultrastructural evidence suggests that the clustering of the cholesterol glycolipids into distinct microdomains is maintained in the membrane vesicles and projections observed after CB2 treatment.

The lipid binding properties of OspB would explain its association with these lipid rafts. The structural docking analyses showed that the cleft of OspB can bind the cholesterol glycolipids in a weak interaction. This could be enhanced by the interaction of OspB (and also very likely OspA) with the cholesterol glycolipids through its covalent palmitoylation at the amino terminal cysteine of the lipoprotein. Binding to cholesterol, a raft component, could induce raft association. This could be enhanced by palmitoylation, which is also believed to impart an affinity for lipid rafts in eukaryotes (Epand, 2008). The hemagglutinin and neuraminidase of influenza do not associate with rafts if their palmitic acids are removed (Shvartsman et al., 2003; Zhang et al., 2000).

The model for the bactericidal action based on the evidence presented here requires that the binding of CB2 to OspB induces an unstable boundary between microdomains and the surrounding bilayer resulting in formation of projections, membrane fission leading to shedding of vesicles rich in cholesterol glycolipids and OspB, and formation of pores that result in the lysis of the spirochete (LaRocca et al., 2009). This raises the question as to how the interaction of OspB with cholesterol glycolipids might influence membrane fission induced by CB2. One possibility is that upon CB2 binding, OspB undergoes a conformational change so that it expands the outer leaflet of the membrane bilayer. This could result from a penetration of OspB into the outer leaflet, a net movement of lipid into the outer leaflet from the inner leaflet, or a decrease in the packing of the outer leaflet lipids so that they take up more space per molecule. Whatever the mechanism for the expansion of the outer leaflet, it could be amplified by the high concentration of OspB within domains rich in cholesterol glycolipids. Once the outer leaflet expands, the bilayer would spontaneously bend to maintain contact with the inner leaflet, thus forming projections. If this bending is sufficiently severe, the boundary between the domains rich in OspB and the remainder of the membrane would be unstable, leading to vesiculation from the surface of *B. burgdorferi.* When OspB is more dispersed within the membrane, such as when cholesterol glycolipid is absent, CB2 binding may not cause the same changes in OspB conformation or membrane structure, thereby reducing membrane fission (vesiculation). An alternate model is that membrane disruption could arise from a change in the OspB-bound lipid upon CB2 binding. For example, the binding of CB2 could change OspB structure in such a manner that the

distance between palmitates and bound cholesterol glycolipid increases, resulting in outer leaflet expansion, which would have the consequences noted above.

The ultrastructural evidence for the organization of cholesterol glycolipids into clustered microdomains suggests that these are analogous to the lipid rafts that form in cholesterol-rich eukaryotic cells. The microdomains were ~100 nm in diameter at the optimal in vitro growth temperature for *B. burgdorferi* (33°C), consistent with the small size of eukaryotic lipid rafts (Pike, 2009). This evidence was strengthened by biochemical and biophysical experiments that documented the existence of a prokaryotic version of lipid raft microdomains. First, we found that DRMs could be isolated from *B. burgdorferi* and were rich in cholesterol glycolipids. OspB, OspA, and P66 were shown to reside in the DRMs, suggesting that the outer membrane proteins normally localize in these microdomains. In addition, the physical behavior of *B. burgdorferi* lipids in MLVs showed that they formed lipid raft-like ordered domains as determined by TX-100 detergent resistance, anisotropy, and FRET. Combined, these data indicate that microdomains form in the outer membrane of *B. burgdorferi* and can be considered close analogs of eukaryotic lipid rafts. Therefore, the discovery that *Borrelia* has lipid rafts could be a key to the understanding of lipoprotein function, to the role of the cholesterol glycolipids in both physiological and pathological conditions, and to the pathogenesis of Lyme disease and relapsing fever.

The lack of lipid rafts in the membranes of prokaryotes may be due to the absence of cholesterol in all but a few bacteria, namely *Helicobacter*, *Mycoplasma*, *Ehrlichia*, *Anaplasma*, *Brachyspira*, and, of course, *Borrelia*. We now show that lipid rafts exist in prokaryotes. These findings represent a substantial shift in the thinking about lipid rafts, expanding their biological relevance to prokaryotes.

Lastly, the evolution of the eukaryotic cell membrane with its well-defined, segregated cholesterol-sphingolipid domains, is not completely understood. It is possible that prokaryotes, such as *Borrelia*, that have ordered lipid domains represent transitional forms that in the distant past led to the evolution of the eukaryotic cell membrane. Thus, there is an evolutionary significance to the ordered cholesterol-glycolipid domains in the outer membrane of *Borrelia*.

Changes in the organization of lipid rafts in *B. burgdorferi* in response to temperature may also be of functional importance. Ultrastructural evidence showed that the microdomains decrease in size as temperature increases. In fact, spirochetes harvested directly from mice have lipid rafts that are organized in the same manner and size as those from spirochetes in culture at 37°C. Therefore, the in vivo and culture (at 37°C) lipid raft organization is concordant. In addition, biophysical data showing that membrane order and fluidity decreases as temperature increases (both in cells and MLVs) are in concordance with the ultrastructural observations. Furthermore, these changes were correlated to differences in CB2 bactericidal activity. Lower temperatures decreased CB2 bactericidal activity, while the opposite was true for higher temperatures. This is additional evidence of a link between CB2 action and lipid raft organization.

The temperature dependence of lipid raft organization in *B. burgdorferi* may have significant implications in the transmission cycle of the spirochetes. Due to their arthropod-to-mammal life cycle, *B. burgdorferi* are exposed to a wide range of temperatures, from ambient temperature in the tick vector to 37°C during mammalian infection. Thus, lipid raft organization in *Borrelia* may change in response to environmental cues and may play a significant role in their transmission, such as allowing the spirochetes to sense their location, within the vector or the mammalian host. The complex of outer membrane lipoproteins within lipid rafts could carry out a sensor function, and, importantly, it could have a signaling role in the differential expression of these lipoproteins as *B. burgdorferi* moves from the tick to the mammal.

The cross-reactivity of the antibody to asialo GM1 with lipid antigens of *Borrelia* has been known for some time, and antibodies generated against the lipid antigens of *B. burgdorferi* cross-react with ganglioside GM1 (Gal-β-1-3-GalNAc-β-1-4-Gal-β-1-4-Glc-β-1-1-ceramide). We suggest that this cross-reactivity is partially due to the shared β1-3 linkage of the galactose (Willison and Kennedy, 1993). That these immunogenic glycolipids share an epitope with a prominent host molecule raises the possibility of an antibody-mediated effect in the pathogenesis of Lyme disease. This idea is not a new one; however, sufficient evidence for the contribution of autoreactivity to the pathologic manifestations of Lyme disease has been lacking. The cross-reactivity among the cholesterol glycolipids with GM1 may prompt a reappraisal of antibody- and cell-mediated (Kinjo et al., 2006) damage as part of the pathogenesis of the spirochetoses.

In summary, using an antibody as a biological tool, we have documented the existence of membrane microdomains in the membrane of the prokaryote *B. burgdorferi*. These microdomains contain the cholesterol glycolipids of *Borrelia* and contribute to the bactericidal mechanism of CB2. Their lipid composition and physical properties indicates that they are similar to the lipid rafts of eukaryotic membranes. Several antigens of *B. burgdorferi* were shown to reside in lipid rafts, one of which, OspB, is the target of the complement-independent bactericidal IgG, CB2.

EXPERIMENTAL PROCEDURES

Bacteria, Cultures, Mice, and Antibodies

B. burgdorferi strain B31 were grown in microaerophillic conditions in BSK-H medium (Sigma) at 33°C. CB2 and CB10 hybridoma supernatants were obtained as described (Coleman et al., 1992). C3H/HeN mice were inoculated intradermally with 2×10^4 spirochetes; after 20 days, the bladders from ten mice were removed and dispersed in Hank's balanced salt solution (HBSS, GIBCO). The suspension was centrifuged at 400 × g, and the supernatant was examined for spirochetes.

CB2 Bactericidal Assays

So that the effect of cholesterol depletion on the bactericidal mechanism of CB2 could be tested, *B. burgdorferi* were treated with MβCD for 30 min in HBSS, centrifuged, washed, and treated with 2 μg/ml CB2 or CB10 for 15 min followed by dark-field enumeration. CB10 is an IgG1*k* complement-dependent antibody to OspA. So that the specificity of MβCD for cholesterol on CB2 bactericidal activity could be tested, 10 mM MβCD was mixed with 20 μg/ml cholesterol (Invitrogen) for 1 hr and then used to treat spirochetes for 30 min at 33°C . For cholesterol replacement bactericidal assays, spirochetes had their cholesterol depleted, incubated with 20 μg/ml cholesterol at 33°C for 1 hr, harvested by centrifugation, and treated with CB2 or CB10 for 15 min. To add excess cholesterol, spirochetes were incubated with 20 μg/ml cholesterol for 1 hr at 33°C followed by treatment with CB2 or

CB10 and dark-field enumeration. To test the temperature-dependence and kinetic assays of the bactericidal effect of CB2, spirochetes were held at 4°C, 25°C, 33°C, 37°C, or 42°C for 1 hr. For experiments to assess growth of spirochetes in the presence of excess cholesterol, 10 and 20 μg of cholesterol in 100% ethanol were added to the cultures; controls consisted of adding an equal volume of ethanol.

Detection Methods for Lipids and Vesicle Content

B. burgdorferi were depleted of cholesterol and supernatant and pellet were separated for lipid extraction with choloroform-methanol (1:2) (Bligh and Dyer, 1959). Extracts (30 μl) were resolved on an HPTLC silica plate (EM separations) with chloroform-methanol (85:15) and stained with iodine vapor. Standards were phosphatidylcholine, phosphatidylglycerol (Avanti Polar Lipids), and cholesterol (Sigma), as well as known R_f values from identical solvent systems (Schröder et al., 2003; Stübs et al., 2009). MβCD-treated and untreated supernatant extracts were also analyzed on western blots probed with rabbit polyclonal antibody to ganglioside asialo GM1 as above.

For detection of cholesterol glycolipids in supernatant vesicles by ELISA, spirochetes (2.5×10^8/ml in HBSS) with 6% dextran T500 (Pharmacia) were depleted of cholesterol and treated with 2 μg/ml CB2, CB10, or no antibody for 30 min at 33°C, followed by centrifugation. Supernatants were used to coat the wells of an ELISA plate, followed by probing with polyclonal rabbit IgG against asialo GM1 and an alkaline phosphatase conjugated goat anti-rabbit IgG (1:1000, Sigma) and resolved by the substrate p-nitrophenyl phosphate (pNPP, Sigma) at 405 nm in a SpectraMax M2 plate reader.

Supernatants were analyzed by western blot with anti-OspB (mouse IgG1), anti-OspA (mouse IgG1), and anti-DnaK (mouse IgG1) as a negative control and resolved with an anti-mouse IgG IR800 conjugate. Reactivity of the immunoblots was by scanning in an Odyssey infrared scanner. For all ELISA and immunoblots, controls included secondary antibody conjugates without the primary antibody.

Detergent-Resistance of Whole *B. burgdorferi* and Model Membrane Vesicles

DRMs were identified and isolated from whole *B. burgdorferi* by detergent insolubility and gradient separation using the caveolea/rafts isolation kit (Sigma) according to the manufacturer's instructions. For gradient separations that analyzed the effect of cholesterol depletion/raft disruption, spirochetes were treated with 10 mM MβCD for 30 min prior to TX-100 treatment. Gradient fractions were analyzed by slot blot with anti-asialo GM1, CB2, CB10, anti-P66 (rabbit polyclonal IgG), or 1:100 anti-Lon1 protease (rabbit polyclonal IgG). The secondary antibodies used were anti-rabbit IgG IR800 and anti-mouse IgG IR800 conjugates. All slot blots were read in an Odyssey infrared scanner.

MLVs were made from ethanolic solutions of lipids and fluorescent probes, mixed, and dried under nitrogen, redissolved in 20 μl chloroform, and then redried under nitrogen in high vacuum for 1 hr. The dried lipid film was then dispersed at 70°C in 1 ml PBS (pH 7.4), by vortexing, and cooled to RT. MLV samples containing 500 μM total lipid (assuming the average molecular weight of *B. burgdorferi* lipids was 760 g/mol) were prepared in PBS. Light scattering at 400 nm was measured at RT with a Beckman 650 spectrophotometer. Next, 46 μl 10% v/v Triton X-100/PBS was added, and the samples were incubated for 2.5 hr at RT. OD at 400 nm was remeasured. Estimated % detergent resistant lipid was calculated from the ratio of OD remaining after incubation with detergent to that before detergent addition.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at doi:10.1016/j.chom.2010.09.001.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants R37-AI-027044 and ROI-AR-040445 and Northeast Biodefense Center grant U54-AI-057158 (Lipkin) to J.L.B. and by grant R01-GM-048596 to E.L. T.J.L. was supported by T32-AI-007539 from the National Institute of Allergy and Infectious Diseases.

Received: April 20, 2010
Revised: July 20, 2010
Accepted: September 1, 2010
Published: October 20, 2010

REFERENCES

Ayuyan, A.G., and Cohen, F.S. (2008). Raft composition at physiological temperature and pH in the absence of detergents. Biophys. J. *94*, 2654–2666.

Bakht, O., Pathak, P., and London, E. (2007). Effect of the structure of lipids favoring disordered domain formation on the stability of cholesterol-containing ordered domains (lipid rafts): identification of multiple raft-stabilization mechanisms. Biophys. J. *93*, 4307–4318.

Becker, M., Bunikis, J., Lade, B.D., Dunn, J.J., Barbour, A.G., and Lawson, C.L. (2005). Structural investigation of *Borrelia burgdorferi* OspB, a bactericidal Fab target. J. Biol. Chem. *280*, 17363–17370.

Belisle, J.T., Brandt, M.E., Radolf, J.D., and Norgard, M.V. (1994). Fatty acids of *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins. J. Bacteriol. *176*, 2151–2157.

Ben-Menachem, G., Kubler-Kielb, J., Coxon, B., Yergey, A., and Schneerson, R. (2003). A newly discovered cholesteryl galactoside from *Borrelia burgdorferi*. Proc. Natl. Acad. Sci. USA *100*, 7913–7918.

Benach, J.L., Bosler, E.M., Hanrahan, J.P., Coleman, J.L., Habicht, G.S., Bast, T.F., Cameron, D.J., Ziegler, J.L., Barbour, A.G., Burgdorfer, W., et al. (1983). Spirochetes isolated from the blood of two patients with Lyme disease. N. Engl. J. Med. *308*, 740–742.

Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. *37*, 911–917.

Bockenstedt, L.K., Barthold, S., Deponte, K., Marcantonio, N., and Kantor, F.S. (1993). *Borrelia burgdorferi* infection and immunity in mice deficient in the fifth component of complement. Infect. Immun. *61*, 2104–2107.

Brandt, M.E., Riley, B.S., Radolf, J.D., and Norgard, M.V. (1990). Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. Infect. Immun. *58*, 983–991.

Brown, R.E. (1998). Sphingolipid organization in biomembranes: what physical studies of model membranes reveal. J. Cell Sci. *111*, 1–9.

Brown, D.A., and London, E. (2000). Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J. Biol. Chem. *275*, 17221–17224.

Brown, D.A., and Rose, J.K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell *68*, 533–544.

Burgdorfer, W., Barbour, A.G., Hayes, S.F., Benach, J.L., Grunwaldt, E., and Davis, J.P. (1982). Lyme disease-a tick-borne spirochetosis? Science *216*, 1317–1319.

Bykowski, T., Woodman, M.E., Cooley, A.E., Brissette, C.A., Wallich, R., Brade, V., Kraiczy, P., and Stevenson, B. (2008). *Borrelia burgdorferi* complement regulator-acquiring surface proteins (BbCRASPs): Expression patterns during the mammal-tick infection cycle. Int. J. Med. Microbiol. *298* (*Suppl 1*), 249–256.

Chen, Z., and Rand, R.P. (1997). The influence of cholesterol on phospholipid membrane curvature and bending elasticity. Biophys. J. *73*, 267–276.

Coleman, J.L., Rogers, R.C., and Benach, J.L. (1992). Selection of an escape variant of *Borrelia burgdorferi* by use of bactericidal monoclonal antibodies to OspB. Infect. Immun. *60*, 3098–3104.

Coleman, J.L., Katona, L.I., Kuhlow, C., Toledo, A., Okan, N.A., Tokarz, R., and Benach, J.L. (2009). Evidence that two ATP-dependent (Lon) proteases in *Borrelia burgdorferi* serve different functions. PLoS Pathog. *5*, e1000676.

Connolly, S.E., and Benach, J.L. (2001). Cutting edge: the spirochetemia of murine relapsing fever is cleared by complement-independent bactericidal antibodies. J. Immunol. *167*, 3029–3032.

Connolly, S.E., and Benach, J.L. (2005). The versatile roles of antibodies in *Borrelia* infections. Nat. Rev. Microbiol. *3*, 411–420.

Connolly, S.E., Thanassi, D.G., and Benach, J.L. (2004). Generation of a complement-independent bactericidal IgM against a relapsing fever *Borrelia*. J. Immunol. *172*, 1191–1197.

Epand, R.M. (2008). Proteins and cholesterol-rich domains. Biochim. Biophys. Acta *1778*, 1576–1582.

Escudero, R., Halluska, M.L., Backenson, P.B., Coleman, J.L., and Benach, J.L. (1997). Characterization of the physiological requirements for the bactericidal effects of a monoclonal antibody to OspB of *Borrelia burgdorferi* by confocal microscopy. Infect. Immun. *65*, 1908–1915.

Fujita, A., Cheng, J., Hirakawa, M., Furukawa, K., Kusunoki, S., and Fujimoto, T. (2007). Gangliosides GM1 and GM3 in the living cell membrane form clusters susceptible to cholesterol depletion and chilling. Mol. Biol. Cell *18*, 2112–2122.

García Moncó, J.C., Wheeler, C.M., Benach, J.L., Furie, R.A., Lukehart, S.A., Stanek, G., and Steere, A.C. (1993). Reactivity of neuroborreliosis patients (Lyme disease) to cardiolipin and gangliosides. J. Neurol. Sci. *117*, 206–214.

Garcia-Monco, J.C., Seidman, R.J., and Benach, J.L. (1995). Experimental immunization with *Borrelia burgdorferi* induces development of antibodies to gangliosides. Infect. Immun. *63*, 4130–4137.

Haque, M., Hirai, Y., Yokota, K., and Oguma, K. (1995). Steryl glycosides: a characteristic feature of the Helicobacter spp.? J. Bacteriol. *177*, 5334–5337.

Hirai, Y., Haque, M., Yoshida, T., Yokota, K., Yasuda, T., and Oguma, K. (1995). Unique cholesteryl glucosides in *Helicobacter pylori*: composition and structural analysis. J. Bacteriol. *177*, 5327–5333.

Johnson, R.C. (1977). The spirochetes. Annu. Rev. Microbiol. *31*, 89–106.

Jones, J.D., Bourell, K.W., Norgard, M.V., and Radolf, J.D. (1995). Membrane topology of *Borrelia burgdorferi* and *Treponema pallidum* lipoproteins. Infect. Immun. *63*, 2424–2434.

Katona, L.I., Ayalew, S., Coleman, J.L., and Benach, J.L. (2000). A bactericidal monoclonal antibody elicits a change in its antigen, OspB of *Borrelia burgdorferi*, that can be detected by limited proteolysis. J. Immunol. *164*, 1425–1431.

Kinjo, Y., Tupin, E., Wu, D., Fujio, M., Garcia-Navarro, R., Benhnia, M.R., Zajonc, D.M., Ben-Menachem, G., Ainge, G.D., Painter, G.F., et al. (2006). Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. Nat. Immunol. *7*, 978–986.

LaRocca, T.J., and Benach, J.L. (2008). The important and diverse roles of antibodies in the host response to *Borrelia* infections. Curr. Top. Microbiol. Immunol. *319*, 63–103.

LaRocca, T.J., Katona, L.I., Thanassi, D.G., and Benach, J.L. (2008). Bactericidal action of a complement-independent antibody against relapsing fever Borrelia resides in its variable region. J. Immunol. *180*, 6222–6228.

LaRocca, T.J., Holthausen, D.J., Hsieh, C., Renken, C., Mannella, C.A., and Benach, J.L. (2009). The bactericidal effect of a complement-independent antibody is osmolytic and specific to Borrelia. Proc. Natl. Acad. Sci. USA *106*, 10752–10757.

Li, H., Dunn, J.J., Luft, B.J., and Lawson, C.L. (1997). Crystal structure of Lyme disease antigen outer surface protein A complexed with an Fab. Proc. Natl. Acad. Sci. USA *94*, 3584–3589.

Lin, M., and Rikihisa, Y. (2003). *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. Infect. Immun. *71*, 5324–5331.

Livermore, B.P., Bey, R.F., and Johnson, R.C. (1978). Lipid metabolism of *Borrelia hermsi*. Infect. Immun. *20*, 215–220.

London, E. (2002). Insights into lipid raft structure and formation from experiments in model membranes. Curr. Opin. Struct. Biol. *12*, 480–486.

London, E., and Feligenson, G.W. (1978). A convenient and sensitive fluorescence assay for phospholipid vesicles using diphenylhexatriene. Anal. Biochem. *88*, 203–211.

Newman, K., Jr., and Johnson, R.C. (1981). *In vivo* evidence that an intact lytic complement pathway is not essential for successful removal of circulating *Borrelia turicatae* from mouse blood. Infect. Immun. *31*, 465–469.

Nichols, B. (2003). Caveosomes and endocytosis of lipid rafts. J. Cell Sci. *116*, 4707–4714.

Pike, L.J. (2009). The challenge of lipid rafts. J. Lipid Res. Suppl. *50*, S323–S328.

Radolf, J.D., Bourell, K.W., Akins, D.R., Brusca, J.S., and Norgard, M.V. (1994). Analysis of *Borrelia burgdorferi* membrane architecture by freeze-fracture electron microscopy. J. Bacteriol. *176*, 21–31.

Radolf, J.D., Goldberg, M.S., Bourell, K., Baker, S.I., Jones, J.D., and Norgard, M.V. (1995). Characterization of outer membranes isolated from *Borrelia burgdorferi,* the Lyme disease spirochete. Infect. Immun. *63*, 2154–2163.

Sadziene, A., Thompson, P.A., and Barbour, A.G. (1993). *In vitro* inhibition of *Borrelia burgdorferi* growth by antibodies. J. Infect. Dis. *167*, 165–172.

Salaün, C., James, D.J., and Chamberlain, L.H. (2004). Lipid rafts and the regulation of exocytosis. Traffic *5*, 255–264.

Schröder, N.W., Schombel, U., Heine, H., Göbel, U.B., Zähringer, U., and Schumann, R.R. (2003). Acylated cholesteryl galactoside as a novel immunogenic motif in *Borrelia burgdorferi* sensu stricto. J. Biol. Chem. *278*, 33645–33653.

Shvartsman, D.E., Kotler, M., Tall, R.D., Roth, M.G., and Henis, Y.I. (2003). Differently anchored influenza hemagglutinin mutants display distinct interaction dynamics with mutual rafts. J. Cell Biol. *163*, 879–888.

Smith, P.F. (1971). Biosynthesis of cholesteryl glucoside by *Mycoplasma gallinarum*. J. Bacteriol. *108*, 986–991.

Stübs, G., Fingerle, V., Wilske, B., Göbel, U.B., Zähringer, U., Schumann, R.R., and Schröder, N.W. (2009). Acylated cholesteryl galactosides are specific antigens of borrelia causing lyme disease and frequently induce antibodies in late stages of disease. J. Biol. Chem. *284*, 13326–13334.

Trott, D.J., Alt, D.P., Zuerner, R.L., Wannemuehler, M.J., and Stanton, T.B. (2001). The search for Brachyspira outer membrane proteins that interact with the host. Anim. Health Res. Rev. *2*, 19–30.

Wang, Y., Thiele, C., and Huttner, W.B. (2000). Cholesterol is required for the formation of regulated and constitutive secretory vesicles from the trans-Golgi network. Traffic *1*, 952–962.

Wheeler, C.M., Garcia Monco, J.C., Benach, J.L., Golightly, M.G., Habicht, G.S., and Steere, A.C. (1993). Nonprotein antigens of *Borrelia burgdorferi*. J. Infect. Dis. *167*, 665–674.

Willison, H.J., and Kennedy, P.G. (1993). The fine specificity of anti-GM1 antibodies. Q. J. Med. *86*, 216–217.

Xu, X., Bittman, R., Duportail, G., Heissler, D., Vilcheze, C., and London, E. (2001). Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide. J. Biol. Chem. *276*, 33540–33546.

Zhang, J., Pekosz, A., and Lamb, R.A. (2000). Influenza virus assembly and lipid raft microdomains: a role for the cytoplasmic tails of the spike glycoproteins. J. Virol. *74*, 4634–4644.

Zidovetzki, R., and Levitan, I. (2007). Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. Biochim. Biophys. Acta *1768*, 1311–1324.