Hypercholesterolemia and ApoE deficiency result in severe infection with Lyme disease and relapsing-fever *Borrelia*

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The Lyme disease (*Borrelia burgdorferi*) and relapsing-fever (*Borrelia hispanica*) agents have distinct infection courses, but both require cholesterol for growth. They acquire cholesterol from the environment and process it to form cholesterol glycolipids that are incorporated onto their membranes. To determine whether higher levels of serum cholesterol could enhance the organ burdens of *B. burgdorferi* and the spirochetemia of *B. hispanica* in laboratory mice, apolipoprotein E (apoE)-deficient and low-density lipoprotein receptor (LDLR)-deficient mice that produce large amounts of serum cholesterol were infected with both spirochetes. Both apoE- and LDLR-deficient mice infected with *B. burgdorferi* had an increased number of spirochetes in the joints and inflamed ankles compared with the infected wild-type (WT) mice, suggesting that mutations in cholesterol transport that result in high serum cholesterol levels can affect the pathogenicity of *B. burgdorferi*. In contrast, elevated serum cholesterol did not lead to an increase in the spirochetemia of *B. hispanica*. In the LDLR-deficient mice, the course of infection was indistinguishable from the WT mice. However, infection of apoE-deficient mice with *B. hispanica* resulted in a longer spirochetemia and increased mortality. Together, these results argue for the apoE deficiency, and not hypercholesterolemia, as the cause for the increased severity with *B. hispanica*. Serum hyperlipidemias are common human diseases that could be a risk factor for increased severity in Lyme disease.

cholesterol | Lyme disease | relapsing fever | *Borrelia* | tick-borne

Cholesterol is an essential structural component of the cell membrane of vertebrate animals, and it is required for membrane integrity and fluidity. In addition to being a component of the membrane, cholesterol is the precursor of steroid hormones and bile. In eukaryotic cells, cholesterol and sphingolipids are the main components of membrane microdomains known as lipid rafts. These microdomains are characterized as being more tightly packed than the surrounding bilayer and enriched with proteins involved in signaling (1–3).

In the bloodstream of humans and other vertebrates, cholesterol is transported in lipoprotein complexes. Apolipoprotein E (apoE) binds cholesterol for transport through the circulatory system as apoE-containing chylomicrons and very-low-density lipoprotein (VLDL) particles. These apoE–cholesterol particles are internalized through the interaction with the low-density lipoprotein receptors (LDLRs). LDLR is one of the cell-surface receptors in cells that binds to apoE to clear the lipoprotein particles from the blood (4). Both apoE-deficient ($apoE^{-}$) and LDLR-deficient ($LDLR^{-}$) mice show elevated serum cholesterol levels and develop atherosclerotic plaques (5, 6). These mice are the most used mouse models for hyperlipidemia and atherosclerosis research.

Lyme disease and relapsing-fever *Borrelia* have very distinct infection courses and niches in the host. In experimental mouse infections, relapsing-fever borreliae multiply in the blood, reaching high numbers (spirochetemia), until antibodies, mostly of the IgM class, clear the first peak, which is followed by several smaller peaks of antigenically variable organisms. Therefore, infection with *Borrelia hispanica*, an agent of relapsing fever, can be monitored by direct enumeration of the spirochetes from blood. Conversely, *Borrelia burgdorferi*, the agent of Lyme disease, does not have an overt spirochetemia, and it is difficult to detect in blood, but invades tissues including the skin, heart, and joints, where it can be quantified by molecular methods.

The outer membrane of *Borrelia* is composed of phospholipids, including phosphatidylcholine and phosphatidylglycerol (7). The borreliae also have cholesterol glycolipids: cholesteryl 6-*O*-acyl-β-D-galactopyranoside and cholesteryl-β-D-galacto-pyranoside in *B. burgdorferi*; and 6-*O*-acylated cholesteryl β-D-glucopyranoside and cholesteryl β-D-glucopyranoside in relapsing-fever *Borrelia*. They also have noncholesterol glycolipids, monogalactosyl-diacylglycerol in *B. burgdorferi* and monoglucosyl-diacylglycerol in relapsing-fever *Borrelia*, as well as many lipoproteins (7–15). The presence of cholesterol is not common among prokaryotes, but it is increasingly being reported in bacterial pathogens other than *Borrelia* spp., including species of *Helicobacter*, *Mycoplasma*, *Ehrlichia*, *Anaplasma*, and *Brachyspira* (16–20).

Recently, we demonstrated that *B. burgdorferi* acquires cholesterol from host cells (21). Cholesterol can remain free in the membrane or can be internalized and glycosylated by undetermined enzymes (22). Subsequently, cholesterol glycolipids are exported to the membrane, where they form lipid rafts (23, 24) that are cholesterol-rich domains with a selective presence of lipoproteins (25). The borreliae require cholesterol for growth and have to recruit it from the host because they cannot synthesize it. In this study, our goal was to determine whether serum hypercholesterolemia could lead to greater yields of bacteria in

Significance

Elevated levels of cholesterol and other lipid abnormalities are common diseases of adults. Cholesterol is an essential nutrient for *Borrelia*. To test whether increased levels of cholesterol could affect the infection with *Borrelia*, we used two types of mice with different deficiencies in cholesterol transport that result in increased cholesterol levels and infected them with two species of *Borrelia*. Infection with the agent of Lyme disease resulted in higher severity, increased number of bacteria in the joints, and ankle swelling. The higher mortality found in infections with relapsing-fever *Borrelia* was associated with an apolipoprotein E deficiency and was independent of cholesterol levels. Elevated serum lipids are common diseases that could be a risk factor for increased severity in Lyme disease.

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vivo by providing added cholesterol in a manner that would be accessible to the spirochetes. To this end, we infected apoE$^-$ and LDLR$^-$ mice that have increased levels of serum cholesterol with *B. burgdorferi* and *B. hispanica*. From these experiments, we concluded that infection of apoE$^-$ and LDLR$^-$ mice with *B. burgdorferi* resulted in greater severity of infection. In contrast, immune dysfunctions associated with deficiencies in the apoE$^-$ mouse model, and not high cholesterol levels, led to increased severity in infection with relapsing-fever *Borrelia.*

Results

Means (±SD) of serum cholesterol levels for all of the groups of mice used in this study are shown in Table 1.

The apoE and LDLR Deficiency Resulted in Greater Spirochetal Burdens and Inflammation in the Joints in Infection with *B. burgdorferi.* The apoE$^-$ and LDLR$^-$ mice used in this study had a C57BL/6 background that leads them to develop mild to moderate arthritis when infected with 2×10^4 *B. burgdorferi* (26). To measure the spirochetal burden in mice, we carried out a quantitative PCR in different organs and tissues. Quantitative PCR revealed a significant increase in spirochete numbers in the joints of apoE$^-$ and LDLR$^-$ mice compared with WT. Also, there were modest increases in spirochete numbers in the hearts, but not in the ears (Fig. 1*A*). Inflammation of the ankle joints was clearly visible in all of the apoE$^-$ and LDLR$^-$ mice, and not in the WT mice, and this finding was confirmed with measurements of ankle width (Fig. 1*B*).

The levels of total serum immunoglobulins of both IgG and IgM were significantly higher in the apoE$^-$ mice (Fig. 1*C*), as were class-specific antibodies, as detected by ELISA (Fig. 1*D*). Combined, these data indicate that the apoE deficiency can enhance the development of arthritis due to infection with *B. burgdorferi* and that this development appears to be independent of total and specific Ig production.

The LDLR$^-$ mice developed comparable levels of total serum IgG to those of the WT, but total serum IgM was significantly elevated (Fig. 1*E*), as were IgM antibodies (Fig. 1*F*). The increase in the numbers of *B. burgdorferi*, as well as the inflammation of the joints, was shared by both the apoE$^-$ and LDLR$^-$ mice, suggesting that the hypercholesterolemia is responsible for these differences.

Feeding the Atherogenic Diet to WT Mice Does Not Result in Greater Severity of Infection with *B. burgdorferi.* WT mice (C57BL/6J) were fed with the atherogenic diet for 3 wk before infection and thereafter for 21 d until the termination of the experiment. This experiment was performed to determine whether lower levels of cholesterol had the same effect as the higher levels in the ApoE- and LDLR-deficient mice. Diet-induced increases in serum cholesterol did not result in higher levels of organisms in joints, ears, and hearts compared with WT mice fed a regular diet as measured by quantitative PCR (Fig. S1). Likewise, mean ankle width of mice fed the atherogenic diet was not significantly higher than that of the controls. These data indicate that diet-induced increases in the levels of serum cholesterol (Table 1) in WT mice do not lead to greater severity of infection by Lyme disease *Borrelia*.

Table 1. Mean ± SD of the serum levels of cholesterol (mg/dL) at the time of infection

Mouse strain	Serum levels of cholesterol, mg/dL (± SD)	*n*
WT	79 ± 12	14
WT/diet*	226 ± 18	4
apoE$^-$	3,912 ± 839	18
LDLR$^-$	4,540 ± 777	14

*WT/diet, WT mice fed an atherogenic diet.

The LDLR Deficiency Does Not Result in Severe Infection with *B. hispanica.* Because the relapsing-fever *Borrelia* are in the bloodstream, measuring the impact of increased serum cholesterol is particularly important. There was an increase in the spirochetemia peak in the LDLR$^-$ mice that did not reach statistical significance, and the organisms were cleared on day 6 after inoculation, at the same time as the WT mice (Fig. 2*A*). Total serum immunoglobulins (IgG and IgM) (Fig. 2*B*) and specific antibodies of both Ig classes (Fig. 2*C*) were not significantly higher in the LDLR$^-$ compared with the WT mice.

Feeding the Atherogenic Diet to WT Mice Does Not Result in Greater Severity of Infection with *B. hispanica.* As with experiments with *B. burgdorferi*, the effect of diet-induced hypercholesterolemia was measured with *B. hispanica*. WT mice (C57BL/6J) were fed with an atherogenic diet for 3 wk before infection and thereafter for 15 d until the termination of the experiment. This diet did not result in higher levels or longer duration of spirochetemia (Fig. 3*A*), despite a doubling of the serum cholesterol levels (Table 1). These mice had significantly lower levels of both total IgG and specific IgG antibodies (Fig. 3 *B* and *C*). These data indicate that diet-induced increases in the levels of serum cholesterol in WT mice do not lead to greater severity of infection in relapsing-fever *Borrelia*.

The apoE Deficiency Results in Severe Infection with *B. hispanica.* The spirochetemia of *B. hispanica* in apoE$^-$ mice lasts approximately three times longer (72 h) than in the WT mice (<24 h) (Fig. 4*A*). In addition, there was a 50% mortality rate in the apoE$^-$ mice beginning on day 7 after infection, whereas none of the WT mice died (Fig. 4*B*). This result is the most striking finding because murine infection with *B. hispanica* is not fatal, even in immunodeficient mice (B-cell, complement components, and TLR-2 deficient), where the spirochetemia can reach levels of $\sim 10^8$ to 3×10^8 spirochetes per mL (27–29). To further document the severity of the infection, we measured the activity of the apoE$^-$ and WT mice for a week before infection and thereafter for the duration of the infection period. There were no differences in activity between apoE$^-$ and control mice during the preinfection week. After infection, the WT mice had a sharp decrease in activity coinciding with the spirochetemia peak on days 5 and 6 after infection, and this decrease was followed by a rapid recovery in activity beginning on day 7 (Fig. 4*C*). The apoE$^-$ mice had the same decrease in activity at the spirochetemia peak as the WT. However, apoE$^-$ mice did not show a recovery in activity because it kept decreasing throughout the course of the experiment. (Fig. 4*E*). The suppressed levels of locomotor activity are in agreement with the lengthier spirochetemia and high mortality in the apoE$^-$ mice and an overall severity of the infection.

IgM is a critical factor in the spirochetemia clearance in relapsing fever (27–29). To determine whether a defect in total Ig production accounted for the increased severity of infection in apoE$^-$ mice, we measured total serum IgM and IgG. Likewise, to determine whether the specificity of the immunoglobulins had been altered, we measured both IgM and IgG antibodies. The results showed that apoE$^-$ mice had significantly higher levels of IgM and IgG immunoglobulins, suggesting that a deficiency in the production of total serum antibodies is not the reason for the longer spirochetemia and the associated mortality in these mice (Fig. 4 *D* and *F*).

Because the determinations of total serum immunoglobulins as well as antibodies were performed initially at the end of the experiments—and therefore, only on the survivors at day 15 after infection (Fig. 4 *D* and *F*)—additional determinations were made on mice on day 6 after infection in separate experiments to address the possibility that dead mice did not develop a proper

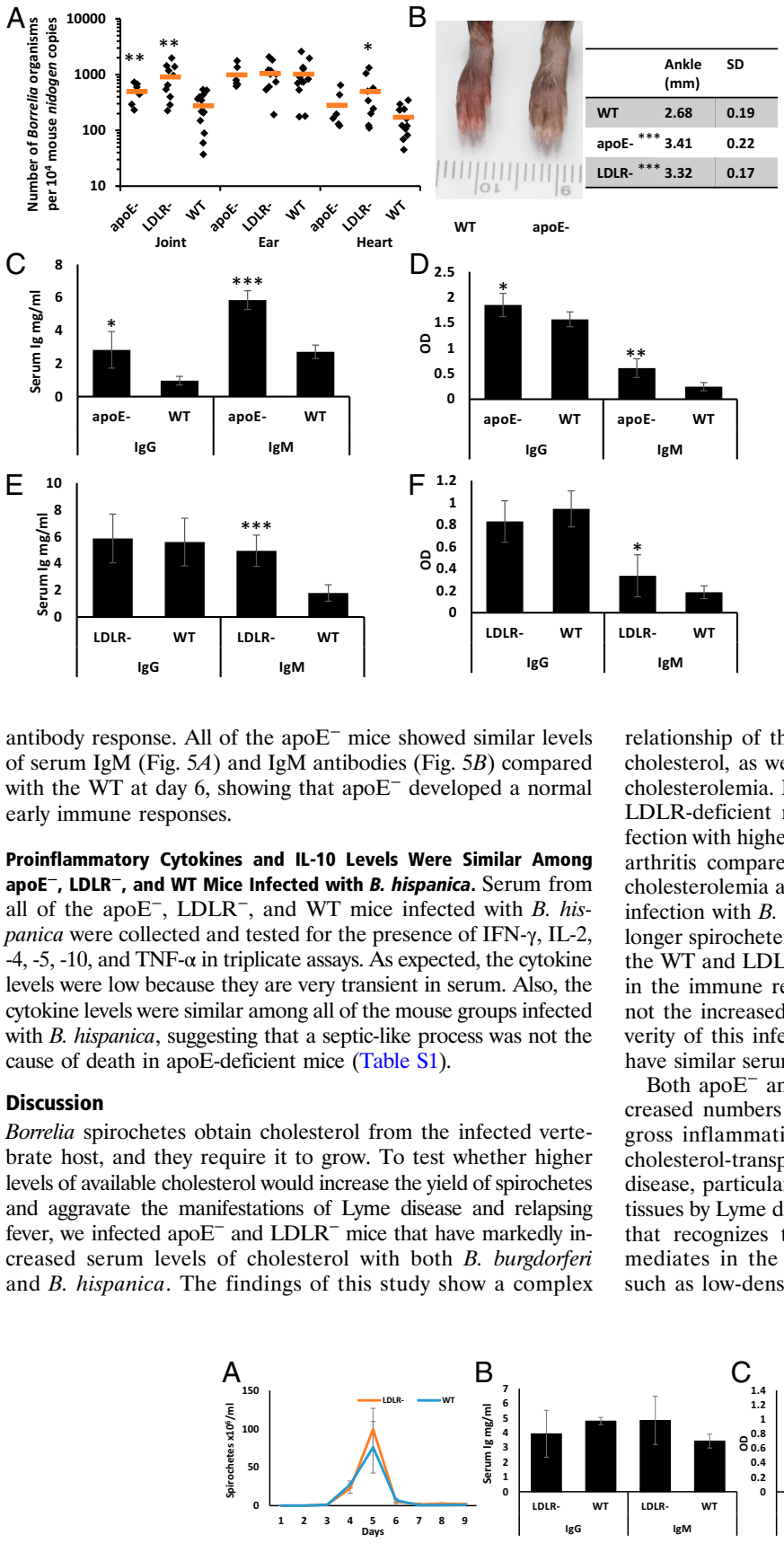

Fig. 1. The infection of apoE$^-$, LDLR$^-$, and WT mice with *B. burgdorferi*. (*A*) Quantitative real-time PCR analysis of spirochete burdens in joint, ear, and heart tissue. $^{**}P < 0.01$; $^{*}P < 0.05$ (compared with WT of the same tissue). (*B*) Representative photograph of a swollen ankle of apoE$^-$ (right) and WT (left*L*) mice. Ankles of LDLR$^-$ mice were similar to those of apoE$^-$ mice. Mean ± SD of ankle width of apoE$^-$, LDLR$^-$, and WT mice values are shown. $^{***}P < 0.001$ ($n = 8$). (*C*) Mean ± SD of total serum immunoglobulins (IgG and IgM) in apoE$^-$ and WT mice. $^{***}P < 0.001$; $^{*}P < 0.05$ ($n = 8$). (*D*) Mean ± SD of the OD of IgG and IgM antibodies in apoE$^-$ and WT mice. $^{**}P < 0.01$; $^{*}P < 0.05$ ($n = 8$). (*E*) Mean ± SD of total serum Ig (IgG and IgM) in LDLR$^-$ and WT mice. $^{***}P < 0.001$ ($n = 8$). (*F*) Mean ± SD of the OD of IgG and IgM antibodies in LDLR$^-$ and WT mice. $^{*}P < 0.05$ ($n = 8$).

antibody response. All of the apoE$^-$ mice showed similar levels of serum IgM (Fig. 5*A*) and IgM antibodies (Fig. 5*B*) compared with the WT at day 6, showing that apoE$^-$ developed a normal early immune responses.

Proinflammatory Cytokines and IL-10 Levels Were Similar Among apoE$^-$, LDLR$^-$, and WT Mice Infected with *B. hispanica*. Serum from all of the apoE$^-$, LDLR$^-$, and WT mice infected with *B. hispanica* were collected and tested for the presence of IFN-γ, IL-2, -4, -5, -10, and TNF-α in triplicate assays. As expected, the cytokine levels were low because they are very transient in serum. Also, the cytokine levels were similar among all of the mouse groups infected with *B. hispanica*, suggesting that a septic-like process was not the cause of death in apoE-deficient mice (Table S1).

Discussion

Borrelia spirochetes obtain cholesterol from the infected vertebrate host, and they require it to grow. To test whether higher levels of available cholesterol would increase the yield of spirochetes and aggravate the manifestations of Lyme disease and relapsing fever, we infected apoE$^-$ and LDLR$^-$ mice that have markedly increased serum levels of cholesterol with both *B. burgdorferi* and *B. hispanica*. The findings of this study show a complex relationship of these two *Borrelia* species with levels of serum cholesterol, as well as with the deficiencies that lead to hypercholesterolemia. Infection with *B. burgdorferi* in both apoE- and LDLR-deficient mice resulted in increased severity of the infection with higher numbers of spirochetes in the joints and gross arthritis compared with the WT mice, suggesting that hypercholesterolemia affects the severity of Lyme disease. In contrast, infection with *B. hispanica* in apoE-deficient mice resulted in a longer spirochetemia and a higher mortality rate compared with the WT and LDLR$^-$ mice. This finding indicates that anomalies in the immune response associated with apoE deficiency—and not the increased cholesterol level—are responsible for the severity of this infection, because both apoE$^-$ and LDLR$^-$ mice have similar serum cholesterol values.

Both apoE$^-$ and LDLR$^-$ infected with *B. burgdorferi* had increased numbers of spirochetes in the joints, accompanied by gross inflammation, suggesting that hypercholesterolemia in cholesterol-transport-deficient mice affects the severity of the disease, particularly in the joints, which are prominent targeted tissues by Lyme disease *Borrelia*. LDLR is a cell-surface receptor that recognizes the apolipoproteins apoB100 and apoE and mediates in the endocytosis of cholesterol-rich lipoproteins such as low-density lipoproteins (LDLs), intermediate-density

Fig. 2. The infection of LDLR$^-$ mice with *B. hispanica*. (*A*) Mean ± SD of the spirochetemia of LDLR$^-$ and WT mice ($n = 8$). (*B*) Mean ± SD of total serum Ig (IgG and IgM) of LDLR$^-$ and WT mice at day 8 after infection ($n = 8$). (*C*) Mean ± SD of the OD of the IgG and IgM antibodies produced by LDLR$^-$ and WT mice at day 8 after infection ($n = 8$).

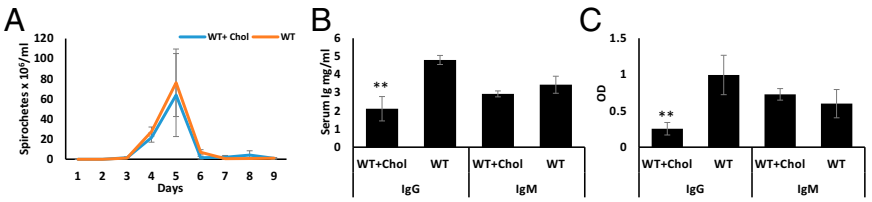

Fig. 3. The infection of WT mice fed the atherogenic diet with *B. hispanica*. (*A*) Mean ± SD of the spirochetemia of WT mice fed the atherogenic diet ($n = 4$). (*B*) Mean ± SD of total serum Ig (IgG and IgM). $^{**}P < 0.01$ ($n = 4$). (*C*) Mean ± SD of the OD of IgG and IgM antibodies at 15 d after infection. $^{**}P < 0.01$ ($n = 4$).

lipoproteins (IDLs), and chylomicrons (30). apoE is a class of lipoprotein found in chylomicrons, VLDLs, and IDLs (31). Nonetheless, apoE$^-$ and LDLR$^-$ mice have elevated LDL and VLDL and decreased HDL levels (32–34).

These findings raised the question as to whether normal mice fed with an atherogenic diet would also be affected in the same manner as the apoE$^-$ and LDLR$^-$ mice were. Cholesterol serum levels in WT mice fed with an atherogenic diet are modest compared with apoE$^-$ and LDLR$^-$ mice, but are significantly higher than WT fed with a regular diet. Moreover, the serum cholesterol levels of WT mice fed with normal or atherogenic diets probably better represent the physiological cholesterol levels that *B. burgdorferi* would encounter in the host during infection. The number of spirochetes in the joints and the severity of arthritis were similar between WT mice fed with the atherogenic diet and WT mice fed with a normal diet. These results indicate that a moderate increase of serum cholesterol does not have an impact in murine Lyme disease. Combined, these results also suggest that cholesterol transport dysfunctions that result in elevated serum cholesterol beyond normal physiological levels can increase the number of spirochetes in joints and exacerbate Lyme disease arthritis, whereas moderate increases do not seem to affect the severity of the infection.

There are several human single-gene disorders of cholesterol transport (for review, see ref. 35). The most common genetic disorder is the familial hypercholesterolemia (FH) that is inherited in an autosomal codominant pattern and affects the *LDLR* gene. As a result, persons affected with this genetic disorder have high cholesterol levels, specifically very high levels of LDL. The severity of this disorder depends on the alleles affected, the heterozygous being the more benign form of FH and also being more common, affecting 1:500 persons. The homozygous FH is more severe and much more rare, occurring in one in a million births. One of the characteristics of FH is the accumulation of cholesterol in tissues (xanthomas), including deposition in major joints such as elbows, knees, and ankles that coincidentally are targeted by Lyme disease *Borrelia* during infection. Interestingly, there are significant differences in the cholesterol balance between mouse and human (36) that result in a less dramatic phenotype when the LDLR is nonfunctional in mice compared with humans (35), suggesting that the exacerbation of Lyme disease observed in LDLR$^-$ mice could be even more prominent in humans. Taking these studies together with our results, it is possible

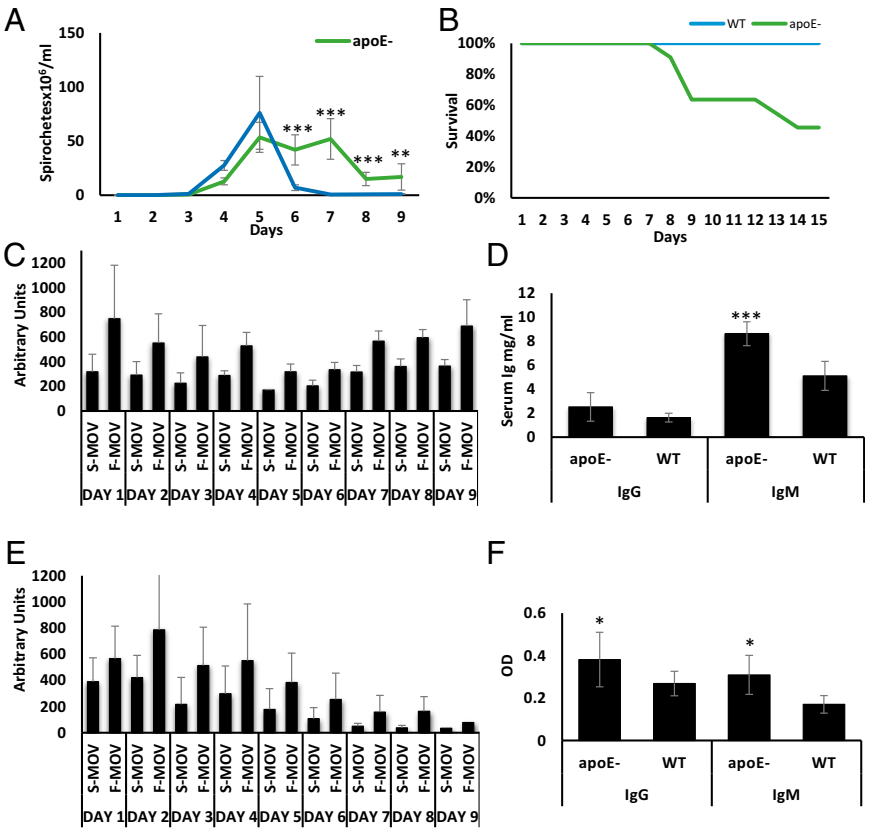

Fig. 4. The infection of apoE$^-$ mice with *B. hispanica*. (*A*) Mean ± SD of the spirochetemia in apoE$^-$ and WT mice. $^{***}P < 0.001$; $^{**}P < 0.01$ ($n = 14$). (*B*) Survival curve of apoE$^-$ and WT mice infected with *B. hispanica* ($n = 14$). (*C*) Activity plot of WT mice for 9 d of infection with *B. hispanica*. S-MOV, slow movements; F-MOV, fast movements ($n = 4$). (*D*) Mean ± SD of total serum immunoglobulins (IgG and IgM) of apoE$^-$ and WT mice at 15 d after infection. $^{***}P < 0.001$ ($n = 7$). (*E*) Activity plot of apoE$^-$ mice for 9 d of infection with *B. hispanica* ($n = 4$). (*F*) Mean ± SD of the OD of IgG and IgM antibodies produced by apoE$^-$ and WT mice at 15 d after infection. $^{*}P < 0.05$ ($n = 7$).

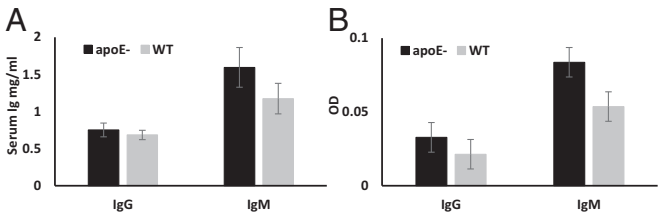

Fig. 5. (*A*) Mean ± SD of total serum Ig (IgG and IgM) of apoE$^-$ mice at day 8 after infection. (*B*) Mean ± SD of the OD of the IgG and IgM antibodies produced by apoE$^-$ mice at day 8 after infection ($n = 4$).

that Lyme disease could be aggravated in patients with genetic disorders in cholesterol transport such as FH.

Relapsing-fever spirochetes such as *B. hispanica* also recruit cholesterol from the host. As mentioned earlier, these spirochetes are prominently found in blood, whereas Lyme disease spirochetes are only transient in blood and are predominantly found in organs and tissues, including skin, joints, heart, and bladder. Therefore, both agents could be affected differently by increased levels of serum cholesterol. Infection of apoE$^-$ mice with *B. hispanica* resulted in a longer period of spirochetemia and a 50% mortality rate. In contrast, the courses of infection of LDLR$^-$ and WT mice with *B. hispanica* were similar, suggesting that the higher level of serum cholesterol in the LDLR$^-$ mice alone is not sufficient to affect the severity of relapsing fever. These observations suggest that the blood-borne infection with *B. hispanica* was not affected by the levels of serum cholesterol. Instead, the exacerbation of the infection in the apoE$^-$ mice was likely associated with a pleiotropic immune defect, because these mice had a longer spirochetemia, and, hence, a defect in clearance. In addition, and not surprisingly, moderate increases of cholesterol achieved by feeding WT mice with an atherogenic diet did not affect the severity of the relapsing-fever infection either.

Development of antibodies to both *B. burgdorferi* and *B. hispanica* is required for the initial host response. The production of antibodies of the IgM class is important to control the infection caused by both organisms, and it is particularly critical for the clearance of *B. hispanica* from the blood. Indeed, the clearance of the first spirochetemia peak takes place after the infected host starts producing specific IgM antibodies. However, the severity of the *B. hispanica* infection in apoE$^-$ does not seem to be related to the antibody response because the levels of serum immunoglobulins, as well as of specific *B. hispanica* antibodies, were elevated. This finding is not surprising because apoE$^-$ mice have increased production of antibodies related to an overall Th2 response (37, 38). In addition, when we tested early antibody production in infected mice, we found similar levels of immunoglobulins, so barring an undisclosed problem with antibody affinity, the humoral response does not appear to be implicated in the increased severity of relapsing fever in apoE$^-$ mice. There is a substantial body of literature on apoE deficiency in bacterial infections, but no consensus has emerged so far. For example, apoE deficiency resulted in increased mortality in infections with *Listeria monocytogenes,* and it was associated with increased serum concentrations of TNF-α, suggesting an impaired innate response to infection by this bacterium (39). A high-cholesterol diet increased infectivity of *Anaplasma phagocytophilum* in apoE$^-$ mice, and it was associated with the increased production of macrophage inflammatory protein MIP-2 and its receptor CXCR2 (40). In addition, the IFN-γ response appears to be delayed in apoE$^-$ mice, and this delay had an effect on the inability to control infection with *Mycobacterium tuberculosis* (41). From the above reports, it is clear that apoE is a major regulator of the immune response with protean functions spanning innate and acquired immunity to bacterial infection. However, these organisms are obligate or facultative intracellular bacteria, and therefore whatever immune defects are responsible for the increased severity of infection in apoE-deficient mice might differ from that of *B. hispanica.* For this reason, we also measured the levels of serum cytokines in consideration of the possibility that the death of apoE$^-$ mice infected with *B. hispanica* may have been due to a septic-like process. The serum levels for these cytokines were not significantly different among the various infections as well as controls, suggesting that the exacerbation of the disease in the apoE$^-$ mice was not due to differences in serum cytokine levels. Together, these results suggest that the immune deficiency in *B. hispanica*-infected apoE$^-$ mice is complex and not related to the humoral response or to serum levels of prominent cytokines.

We conclude that hyperlipidemias, which are common diseases, could be risk factors for increased severity in Lyme disease, particularly in patients with genetic disorders in cholesterol transport. In addition, immune dysfunctions associated with deficiencies in the apoE cholesterol transport system lead to increased severity in relapsing-fever infections.

Materials and Methods

Bacteria and Mice. *B. burgdorferi* B31-A3 and *B. hispanica* Sp1 (42) were used to infect 8-wk-old mice of three different strains: B6.129P2-Apoe^{tm1Unc}/J (apoE$^-$), B6.129S7-Ldlrtm1Her/J (LDLR$^-$), and C57BL/6J (WT), as controls, from Jackson Laboratory. In this study, we followed the mouse nomenclature of the Jackson Laboratory (apoE$^-$ and LDLR$^-$). *B. burgdorferi* were grown at 33 °C in BSK-II medium supplemented with 6% (vol/vol) rabbit serum (Sigma) and inoculated intradermally with 2×10^4 spirochetes. *B. hispanica* were harvested from infected mice, and 2×10^4 spirochetes were inoculated intradermally in blood diluted in BSK-II medium. apoE- and LDLR-deficient mice were fed with the atherogenic diet TD02028 (Harlan Laboratories) for 3 wk before infection and for the duration of the infection period with both organisms. For some experiments, WT mice were also fed with the atherogenic diet for 3 wk before infection and during the period of infection. Serum cholesterol levels were measured just before infection (see below). Mice infected with *B. burgdorferi* were euthanized at 21 d after inoculation, and skin, heart, bladder, and joints were collected. Ankle width of mice infected with *B. burgdorferi* was measured with a digital micrometer (Starret). The number of spirochetes in culture and the spirochetemia of mice infected with *B. hispanica* were determined by direct enumeration in a darkfield microscope. Mice infected with *B. hispanica* were euthanized at 15 d after inoculation. All animal experimentation was conducted under the protocol approved by the Institutional Animal Care and Use Committee of Stony Brook University.

Serology. Direct serum levels of total IgG and IgM were determined with the IgG and IgM kits (Abcam) following the instructions of the manufacturer and read with a VERSAmax spectrophotometer (Molecular Devices) at a wavelength of 545 nm. Measurements of Ig class-specific antibodies were done by ELISA (43). Briefly, flat- bottomed, 96-well polystyrene assay plates (Becton-Dickinson) were coated with sonicated *B. burgdorferi* antigen at a concentration of 5 mg/mL in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4 °C, and the plates were washed with PBS (pH 7.2) containing 0.05% Tween 20 (PBS-Tween). Mouse serum at the appropriate dilutions in PBS-Tween containing 0.5% BSA was added to the wells at 37 °C for 1 h, followed by washes and addition of 100 mL of alkaline phosphatase-conjugated antisera [either goat antisera to affinity-purified murine IgG (γ-chain specific) or IgM (μ-chain specific) (Sigma] for 3 h at 37 °C. The plates were again washed in PBS-Tween and incubated for 1 h at 37 °C with the substrate *p*-nitrophenyl phosphate (Sigma), and the reaction was stopped with 5 M NaOH. Optical density (OD) was read with a VERSAmax spectrophotometer (Molecular Devices) at a wavelength of 405 nm.

Serum cholesterol determinations were made using the cholesterol kit (Molecular Probes Life Technologies) following the recommendations of the manufacturer and read with a SPECTRAmax spectrophotometer (Molecular Devices) at a wavelength of 545 nm.

Serum cytokine levels in apoE$^-$, LDLR$^-$, and WT mice infected with *B. hispanica* were measured by using a mouse multiplex cytokine assay (Bio-Rad) for the detection of GM-CSF, IFN-γ, IL1-β, IL-2, -4, -5, -10, and TNF-α following the manufacturer's instructions.

Activity. The activity of mice was measured with the Panlab Infrared Actimeter (Panlab, S.L., Letica Scientific Instruments), which allows for the study of spontaneous locomotor activity. The system is composed of a 2D square

frame, a frame support, and a control unit that is operated directly through the SEDACOM computer software. Each frame has 16 × 16 infrared beams for subject detection. Activity was defined as the number of beam disruptions over a 24-h test session at 15-min intervals. The activity meter was set to measure fast (F-MOV; scratching, grooming, and twitching) and slow (S-MOV) locomotor horizontal activity.

Quantitative PCR of *B. burgdorferi* in Mouse Tissues. Total DNA was isolated from mouse joint, ear, and heart tissue by using the DNeasy kit (Qiagen), according to the manufacturer's protocol. Genomic DNA was further cleaned and concentrated by using a modified protocol developed in house with SpinSmart PCR Purification columns (Denville Scientific) (44). DNA concentrations were determined by using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific), and all samples were then diluted to a uniform concentration of 10 ng/μL. Published primers were used to amplify the *B. burgdorferi flaB* gene and the mouse *nidogen* gene (45, 46). Quantitative PCR was performed and analyzed on a 7500 Real-Time PCR System (Applied Biosystems). Each reaction included 50 ng of template DNA, 300 nM of each primer, 250 ng/μL BSA, and 1× SYBR Green master mix (Applied Biosystems) in a final volume of 25 μL. The thermal profile was 1 cycle at 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 s, 55 °C (*nidogen*) or 60 °C (*flaB*) for 30 s, and 72 °C for 1 min. All reactions were performed in duplicate within a 96-well plate, and each gene was amplified three times in separate plates, thus yielding six estimates of the number of gene copies. The average quantity of *Borrelia flaB* copies was normalized by the average quantity of mouse *nidogen* copies (45).

Statistics. One-tailed *t* tests were performed to test whether the spirochete burden of apoE$^-$ and LDLR$^-$ mice was higher than that of WT mice. Two-tailed *t* tests were performed to test whether the immunoglobulins levels were different in apoE$^-$ and LDLR$^-$ mice from that of WT. Data were analyzed with the GraphPad InStat statistical program (Version 3.10; GraphPad Software) or Minitab (Version 16; Minitab).

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1. Brown DA, London E (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J Biol Chem* 275(23):17221–17224.
2. Schroeder R, London E, Brown D (1994) Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. *Proc Natl Acad Sci USA* 91(25):12130–12134.
3. Brown DA, London E (1998) Structure and origin of ordered lipid domains in biological membranes. *J Membr Biol* 164(2):103–114.
4. Goldstein JL, Brown MS (2009) The LDL receptor. *Arterioscler Thromb Vasc Biol* 29(4): 431–438.
5. Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM, Maeda N (1992) Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc Natl Acad Sci USA* 89(10):4471–4475.
6. Ishibashi S, et al. (1993) Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest* 92(2):883–893.
7. Brandt ME, Riley BS, Radolf JD, Norgard MV (1990) Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. *Infect Immun* 58(4):983–991.
8. Belisle JT, Brandt ME, Radolf JD, Norgard MV (1994) Fatty acids of *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins. *J Bacteriol* 176(8):2151–2157.
9. Radolf JD, Bourell KW, Akins DR, Brusca JS, Norgard MV (1994) Analysis of *Borrelia burgdorferi* membrane architecture by freeze-fracture electron microscopy. *J Bacteriol* 176(1):21–31.
10. Radolf JD, et al. (1995) Characterization of outer membranes isolated from *Borrelia burgdorferi*, the Lyme disease spirochete. *Infect Immun* 63(6):2154–2163.
11. Jones JD, Bourell KW, Norgard MV, Radolf JD (1995) Membrane topology of *Borrelia burgdorferi* and Treponema pallidum lipoproteins. *Infect Immun* 63(7):2424–2434.
12. Ben-Menachem G, Kubler-Kielb J, Coxon B, Yergey A, Schneerson R (2003) A newly discovered cholesteryl galactoside from *Borrelia burgdorferi*. *Proc Natl Acad Sci USA* 100(13):7913–7918.
13. Schröder NW, et al. (2003) Acylated cholesteryl galactoside as a novel immunogenic motif in *Borrelia burgdorferi* sensu stricto. *J Biol Chem* 278(36):33645–33653.
14. Stübs G, et al. (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(20):13326–13334.
15. Livermore BP, Bey RF, Johnson RC (1978) Lipid metabolism of *Borrelia hermsi*. *Infect Immun* 20(1):215–220.
16. Lin M, Rikihisa Y (2003) *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. *Infect Immun* 71(9):5324–5331.
17. Haque M, Hirai Y, Yokota K, Oguma K (1995) Steryl glycosides: A characteristic feature of the *Helicobacter* spp.? *J Bacteriol* 177(18):5334–5337.
18. Hirai Y, et al. (1995) Unique cholesteryl glucosides in *Helicobacter pylori*: Composition and structural analysis. *J Bacteriol* 177(18):5327–5333.
19. Smith PF (1971) Biosynthesis of cholesteryl glucoside by *Mycoplasma gallinarum*. *J Bacteriol* 108(3):986–991.
20. Trott DJ, Alt DP, Zuerner RL, Wannemuehler MJ, Stanton TB (2001) The search for Brachyspira outer membrane proteins that interact with the host. *Anim Health Res Rev* 2(1):19–30.
21. Crowley JT, et al. (2013) Lipid exchange between *Borrelia burgdorferi* and host cells. *PLoS Pathog* 9(1):e1003109.
22. Ostberg Y, Berg S, Comstedt P, Wieslander A, Bergström S (2007) Functional analysis of a lipid galactosyltransferase synthesizing the major envelope lipid in the Lyme disease spirochete *Borrelia burgdorferi*. *FEMS Microbiol Lett* 272(1):22–29.
23. LaRocca TJ, et al. (2010) Cholesterol lipids of *Borrelia burgdorferi* form lipid rafts and are required for the bactericidal activity of a complement-independent antibody. *Cell Host Microbe* 8(4):331–342.
24. LaRocca TJ, et al. (2013) Proving lipid rafts exist: Membrane domains in the prokaryote *Borrelia burgdorferi* have the same properties as eukaryotic lipid rafts. *PLoS Pathog* 9(5):e1003353.
25. Toledo A, et al. (2014) Selective association of outer surface lipoproteins with the lipid rafts of *Borrelia burgdorferi*. *MBio* 5(2):e00899–e14.
26. Ma Y, et al. (1998) Distinct characteristics of resistance to *Borrelia burgdorferi*-induced arthritis in C57BL/6N mice. *Infect Immun* 66(1):161–168.
27. Connolly SE, Benach JL (2001) Cutting edge: The spirochetemia of murine relapsing fever is cleared by complement-independent bactericidal antibodies. *J Immunol* 167(6):3029–3032.
28. Alugupalli KR, et al. (2003) The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. *J Immunol* 170(7):3819–3827.
29. Connolly SE, Benach JL (2005) The versatile roles of antibodies in *Borrelia* infections. *Nat Rev Microbiol* 3(5):411–420.
30. Francke U, Brown MS, Goldstein JL (1984) Assignment of the human gene for the low density lipoprotein receptor to chromosome 19: Synteny of a receptor, a ligand, and a genetic disease. *Proc Natl Acad Sci USA* 81(9):2826–2830.
31. Brown MS, Goldstein JL (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science* 232(4746):34–47.
32. Aprahamian T, et al. (2004) Impaired clearance of apoptotic cells promotes synergy between atherogenesis and autoimmune disease. *J Exp Med* 199(8):1121–1131.
33. Mehta JL, et al. (2007) Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet. *Circ Res* 100(11):1634–1642.
34. Hasty AH, et al. (2001) Severe hypercholesterolemia, hypertriglyceridemia, and atherosclerosis in mice lacking both leptin and the low density lipoprotein receptor. *J Biol Chem* 276(40):37402–37408.
35. Ikonen E (2006) Mechanisms for cellular cholesterol transport: Defects and human disease. *Physiol Rev* 86(4):1237–1261.
36. Dietschy JM, Turley SD (2002) Control of cholesterol turnover in the mouse. *J Biol Chem* 277(6):3801–3804.
37. Nazzal D, et al. (2010) Apolipoprotein E-deficient mice develop an anti-*Chlamydophila pneumoniae* T helper 2 response and resist vascular infection. *J Infect Dis* 202(5): 782–790.
38. Zhou X, Paulsson G, Stemme S, Hansson GK (1998) Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J Clin Invest* 101(8):1717–1725.
39. Roselaar SE, Daugherty A (1998) Apolipoprotein E-deficient mice have impaired innate immune responses to *Listeria monocytogenes* in vivo. *J Lipid Res* 39(9):1740–1743.
40. Xiong Q, Wang X, Rikihisa Y (2007) High-cholesterol diet facilitates *Anaplasma phagocytophilum* infection and up-regulates macrophage inflammatory protein-2 and CXCR2 expression in apolipoprotein E-deficient mice. *J Infect Dis* 195(10):1497–1503.
41. Martens GW, et al. (2008) Hypercholesterolemia impairs immunity to tuberculosis. *Infect Immun* 76(8):3464–3472.
42. Toledo A, et al. (2010) Phylogenetic analysis of a virulent *Borrelia* species isolated from patients with relapsing fever. *J Clin Microbiol* 48(7):2484–2489.
43. Coleman JL, Benach JL (1987) Isolation of antigenic components from the Lyme disease spirochete: Their role in early diagnosis. *J Infect Dis* 155(4):756–765.
44. Monzón J, Kays R, Dykhuizen DE (2014) Assessment of coyote-wolf-dog admixture using ancestry-informative diagnostic SNPs. *Mol Ecol* 23(1):182–197.
45. Morrison TB, Ma Y, Weis JH, Weis JJ (1999) Rapid and sensitive quantification of *Borrelia burgdorferi*-infected mouse tissues by continuous fluorescent monitoring of PCR. *J Clin Microbiol* 37(4):987–992.
46. Toledo A, Coleman JL, Kuhlow CJ, Crowley JT, Benach JL (2012) The enolase of *Borrelia burgdorferi* is a plasminogen receptor released in outer membrane vesicles. *Infect Immun* 80(1):359–368.