**Statins reduce spirochetal burden and modulate immune responses in the C3H/HeN
mouse model of Lyme disease**

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Abstract

Lyme disease (LD) is a systemic disorder caused by *Borrelia burgdorferi*. Lyme spirochetes encode for a functional 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGR EC 1.1.1.88) serving as a rate limiting enzyme of the mevalonate pathway that contribute to components critical for cell wall biogenesis. Statins have been shown to inhibit *B. burgdorferi in vitro*. Using a mouse model of Lyme disease, we found that statins contribute to reducing bacterial burden and altering the murine immune response to favor clearance of spirochetes.

1. Introduction

Lyme disease is a multiphasic systemic disorder caused by the spirochetal pathogen *Borrelia burgdorferi* [1]. This bacterium is transmitted to mammalian hosts from arthropod vectors, specifically *Ixodes* spp. ticks [2]. Lyme disease is the most prevalent arthropod borne disease in the United States with over 25,000 cases confirmed by the Centers for Disease Control and Prevention (CDC) in 2014. The risk of infection is highest in areas where tick vectors are found in close association with infected reservoir hosts and humans [3, 4].

The genome of *B. burgdorferi* is very limited [5], and *B. burgdorferi* scavenges many required compounds from its arthropod and vertebrate host environments. Therefore, any intact metabolic pathway in *B. burgdorferi* serves as a potential target for inhibition of the bacterium. Sequence analysis of the borrelial genome indicates the presence of homologs (*bb0683-bb0688*) of the mevalonate pathway (MP) leading to the synthesis of isopentenyl-5-pyrophosphate (IPP) [5]. IPP is an essential component of several isoprenoids and a precursor for peptidoglycan synthesis contributing to the

1 structural integrity of several organisms [6, 7]. Previous studies from our laboratory have
2 shown that *B. burgdorferi* possesses a functional MP [8]. The rate-limiting step of the
3 MP is the reduction of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) to
4 mevalonate by HMG-CoA reductase (HMGR; EC 1.1.1.88) (8). We also determined that
5 *B. burgdorferi* has a functional HMGR and that enzyme activity could be inhibited using
6 two commercially available HMGR inhibitors (statins) [8]. Though the mevalonate
7 pathway is found in many genera of bacteria known to cause human disease, including
8 *Staphylococcus*, *Streptococcus*, *Listeria*, and *Borrelia*, the potential antimicrobial use of
9 statins has not been fully explored [6, 5].

10 **2. Materials and Methods**

11 *2.1. Bacterial strains and growth conditions.* A clonal derivative of *B.*
12 *burgdorferi* sensu stricto strain B31, MSK5 [9], which contains all plasmids was used for
13 infectivity experiments. *B. burgdorferi* cultures were grown in 1% CO₂ at 32°C in
14 Barbour-Stoenner-Kelly II (BSK-II) liquid medium supplemented with 6% normal rabbit
15 serum (Pel-Freez, Biologicals, Rogers, AR).

16 *2.2. Statin inactivation.* Statins were activated as previously described [8].
17 Briefly, 25mg of Lovastatin or Simvastatin (Sigma-Aldrich, St. Louis, MO) was
18 dissolved in 500 µl of EtOH preheated to 55°C. 250 µl of 0.6 M NaOH and 5 ml of
19 ddH₂O were then added to the samples, which were then incubated at room temperature
20 for 30 minutes. Following incubation, the pH was brought to 8.0 with 1 M HCl at which
21 time ddH₂O was added to the samples to bring them to a final concentration of 4 mg/ml.
22 The statins were aliquoted and stored at -20°C for use.

2.3. *Infectivity studies.* All animal procedures were done in accordance with the approved animal use protocol from the Institutional Animal Care and Use Committee of The University of Texas at San Antonio. Groups ($n=5$) of mice were treated with activated simvastatin, lovastatin, or vehicle control at 5mg/kg every other day by oral gavage beginning at day 7 before infection. Groups ($n=5$) of 6-week-old female C3H/HeN mice (Charles River Laboratories, Wilmington, MA) were infected at a dose of 10^3 spirochetes per mouse intradermally. One group ($n=5$) was left uninfected. On day 14 postinfection, the spleen, left inguinal lymph node, heart, bladder, and a piece of abdominal skin were removed aseptically from infected mice and the tissues were processed to facilitate isolation of spirochetes in BSK-II growth medium [10]. All cultures were blind passed after 5 days into fresh BSK-II growth medium to minimize the toxicity associated with the degradation of host tissues and to facilitate growth of spirochetes. The cultures were scored for growth of *B. burgdorferi* after 2 to 3 weeks using dark-field microscopy [10].

2.4. *Quantitative real-time PCR analysis.* A portion of skin, spleen, right inguinal lymph node, and right tibiotarsal joint was collected aseptically, and total DNA was extracted using the High Pure PCR template preparation kit (Roche Applied Bioscience, Piscataway, NJ). The manufacturer's suggested protocol for extracting nucleic acids from the tail of the mouse was adapted to obtain total genomic DNA from different infected tissues. Briefly, the tissue samples were homogenized in 200 μ l of lysis buffer containing proteinase K (final concentration, 2 mg/ml) and collagenase (final concentration, 1 mg/ml; Sigma Chemicals, St. Louis, MO). After incubation at 56°C overnight in a water bath, total genomic DNA was extracted according the

1 manufacturer's suggested protocol. Known amounts of mouse or spirochete genomic
2 DNA were used as standards to determine the total numbers of spirochetes in different
3 mouse tissues. Total genomic DNA isolated from different infected mouse tissues was
4 subjected to quantitative real-time PCR using SYBR green PCR master mix with a final
5 concentration of 0.3 μ M of oligonucleotides using the ABI Prism 7300 system (Applied
6 Biosystems). Spirochetes were enumerated by real-time PCR analysis using primers
7 specific to a borrelial gene, *flaB* (F-5' TCTTTTCTCTGGTGAGGGAGCT; R-5'
8 TCCTTCCTGTTGAACACCCTCT), and normalized the total DNA extracted from
9 different tissues to the number of copies of a mouse *β -actin* (F-5'
10 CAAGTCATCACTATTGGCAACGA; R-5' CCAAGAAGGAAGGCTGGAAAA). The
11 spirochete burden was expressed as the number of borrelial *flaB* copies per 10⁶ mouse *β -*
12 *actin* copies.

13 2.5. *Enzyme-linked immunosorbent assays.* A clonal isolate of *B. burgdorferi*
14 strain B31, MSK5, was grown under conditions mimicking the fed-tick (pH 6.8/37°C) to
15 a density of 1 x 10⁸ spirochetes/ml. The cells were harvested by centrifugation and
16 washed four times with in HBSS. The final pellet was disrupted by sonication and the
17 cells were resuspended in ELISA coating buffer (50 mM sodium carbonate, pH 9.6) at a
18 final concentration of 100 μ g/ml following quantification using the BCA protein assay
19 (Pierce, Thermo Fisher Scientific, Rockford, IL). 96-well MaxiSorp ELISA plates
20 (Thermo Fisher Scientific, Rochester, NY) were coated with 100 μ l of total sonicate in
21 coating buffer and incubated overnight at 4°C. Following incubation, the coated plates
22 were washed three times in ELISA wash buffer (0.80 mM Na₂HPO₄, 137 mM NaCl, 0.27
23 mM KCl, 0.15 mM KH₂PO₄ containing 0.5% Tween-20) and blocked for two hours at

room temperature in ELISA wash buffer supplemented with 3% bovine serum albumin (BSA). After blocking, the wells were washed three times with ELISA wash buffer, then coated with serum derived from infected or control mice which was serially diluted in ELISA wash buffer supplemented with 1% BSA and the plates were incubated for 1 hour at room temperature. The plates were washed three times with ELISA wash buffer. The wells were coated with secondary antibody (α -IgG and α -IgM) diluted in ELISA wash buffer supplemented with 1% BSA and incubated at room temperature for 1 hour. The plates were washed three times with ELISA wash buffer and the wells were coated with OPD buffer (50 mM Na₂HPO₄, 50 mM citric acid, pH 5.0, OPD tablets (Thermo Fisher Scientific), H₂O₂). The plates were then incubated in the dark for 15 minutes at room temperature. The absorbance was measured using a Synergy HT Plate Reader (BioTek, Winooski, VT) at 450nm. 50% binding titers were calculated using non-linear regression curve fit analysis with SlideWrite 7.0 software [11].

2.6. Cytokine analysis. On day 14 postinfection, serum was collected from all mice. Cytokine levels in the serum of individual mice were analyzed using the Bio-Plex Protein Array System (Luminex-based technology) (Bio-Rad Laboratories, Hercules, CA) as described previously [12] for the presence of interferon (IFN)- γ , interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-17, tumor necrosis factor (TNF)- α , and granulocyte-colony stimulating factor (G-CSF) expression as well as chemokines (macrophage inflammatory protein [MIP]-1 α (CCL3), MIP-1 β (CCL4), macrophage chemoattractant protein [MCP]-1 (CCL2), KC (CXCL1), and regulated upon activation, normal T cell expressed and secreted [RANTES] (CCL5)). The data reported are the

1 averages of all mice per group for all cytokines that were significantly differentially
2 expressed.

3
4 2.7. *Statistical analysis.* The quantitative real-time PCR were analyzed using a
5 one-way analysis-of-variance test with Bonferroni's multiple-comparison test. The
6 unpaired Student's *t* test (two-tailed) was used to analyze cytokine/chemokine and
7 ELISA data. All statistical data were calculated using GraphPad Prism version 4.0 for
8 Macintosh (GraphPad Prism Software, San Diego, CA). Statistical significance was
9 accepted when *P* values were less than 0.05 [10].

10 3. Results

11 3.1. *Infectivity analysis of statin treated C3H/HeN mice* – C3H/HeN mice, with or
12 without statin treatment, infected with 10^3 spirochetes per mouse, exhibited dissemination
13 of spirochetes to all tissues with a few exceptions (Table 1). Though there was no
14 significant difference in bacterial dissemination to distal tissues between statin-treated
15 and untreated mice, we wanted to determine whether there were differences in the
16 numbers of bacteria migrating to specific tissues. To that end, total genomic DNA was
17 extracted from a portion of skin, spleen, right inguinal lymph node, and right tibiotarsal
18 joint and subjected to quantitative real-time PCR analysis using primers specific for a *B.*
19 *burgdorferi* gene (*flaB*) and a mouse gene (*β -actin*). As shown in Figure 1, there was a
20 significant decrease in the numbers of bacteria in each of the tissues tested with the
21 exception of the joints. There were higher levels of reduction seen in the lymph nodes
22 and spleens of mice treated with simvastatin when compared to the same tissues from

mice treated with lovastatin, while there was a higher level of reduction in the skin of lovastatin-treated mice, though these data were not significant.

3.2. *Statins affect antibody titers in infected C3H/HeN mice* - Enzyme-linked immunosorbent assays against *B. burgdorferi* whole cell sonicates were performed to determine the antibody profile present in the mice after 14 days of infection with *B. burgdorferi* and treatment with simvastatin or lovastatin. As shown in Figure 2A, there was a significant reduction in total IgG antibodies following treatment with simvastatin and lovastatin. IgM titers were also significantly reduced in statin-treated mice (Fig. 2A).

3.3. *Statins elicit a T_H2 type response in treated mice* – The typical cytokine profile of C3H/HeN mice infected with *B. burgdorferi* is a pro-inflammatory, T_H1/T_H17 response, with a downregulation in the T_H2 response [13-16]. Cytokine analysis of all serum samples was performed to determine if there was a differential cytokine profile in response to statin therapy. Statin therapy alone had little to no effect on the levels of various cytokines, however in infected mice, lovastatin treatment significantly upregulated IL-4, IL-5, IL-9, IL-10, and IL-13, cytokines involved in a T_H2 immune response (Fig. 2B). Lovastatin-treated mice also showed an upregulation in T_H1 cytokines IL-2 and IL-12p70. Simvastatin had no significant effect on cytokine levels, with the exception of IL-9.

4. Discussion

The inhibitory property of statins has several implications for interactions of *B. burgdorferi* with its hosts. As our *in vitro* data showed that statins were able to exert an inhibitory effect on *B. burgdorferi*, we wanted to test the effects of statins on a susceptible C3H/HeN model of Lyme disease. Though there was no defect in

1 dissemination, we did see a significant decrease in bacterial burden in all tissues tested,
2 with the exception of the joint (Fig. 1) in statin-treated mice.

3 Though we have previously found that statins are able to inhibit survival of *B.*
4 *burgdorferi* *in vitro* [8], the levels of statins in the blood of treated animals do not reach
5 the concentrations necessary for complete bactericidal effects *in vitro* [17]. However, the
6 numbers of bacteria reaching distal tissues are significantly lower in statin-treated mice,
7 providing evidence that even some bactericidal activity can have an impact on the course
8 of a borreliac infection.

9 Statins have also been previously shown to negatively affect bacterial growth and
10 survival, even in bacteria that do not encode an *hmgR* homolog due to their cholesterol
11 lowering properties [18]. Bacteria which require host cholesterol have defects in growth
12 when cholesterol levels are lowered. As *B. burgdorferi* absolutely require cholesterol for
13 their outer membranes [19], it is likely that the lowered levels of cholesterol would
14 provide less cholesterol for *B. burgdorferi* to sequester, thus preventing *B. burgdorferi*
15 from properly forming its membrane.

16 In the vertebrate host, there is a robust antibody response to *B. burgdorferi*
17 infection that plays a role in bacterial clearance. When infected mice were treated with
18 statins, we saw a significant decrease in the titers of whole IgG and IgM, two classes of
19 Abs known to be elevated during the response against *B. burgdorferi* [20, 21]. We saw
20 no difference in Ab titers in statin-treated uninfected mice (data not shown).

21 The typical cytokine profile of C3H/HeN mice infected with *B. burgdorferi* is a
22 pro-inflammatory, T_H1/T_H17 response, with a downregulation in the T_H2 response. This
23 has been shown to contribute to increased disease severity as measured by increased

arthritis [13]. In infected mice treated with lovastatin, there was significant upregulation of T_H2 cytokines, some of which have been shown to be upregulated during convalescence in humans and have roles in controlling inflammation [22]. Previously, it has been demonstrated that statins can drive a T_H2 response in other disease models [23, 24]. Lovastatin-treated mice also showed an upregulation in T_H1 cytokines IL-2 and IL-12p70, implicated in clearance of *B. burgdorferi*, and whose loss is shown to lead to increased Lyme arthritis [25].

We have previously shown that simvastatin and lovastatin inhibit borrelial growth under in vitro growth conditions [8]. It appears that statin treatment contributes to decreased bacterial burden in infected mice, which could be due to either direct interference with spirochetal growth or by limiting available cholesterol to the bacteria [19]. Moreover, it is also possible that alterations in the immune response to the spirochetes could lead to increased bacterial clearance. It is interesting to speculate that statins could potentially have a number of as yet uncharacterized primary or secondary anti-borrelial effects. These pharmacological properties can be further exploited to enhance bacterial clearance and/or reduce persistence in reservoir hosts to subsequently reduce the incidence of Lyme disease.

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Figure Legends

FIGURE 1. Statins lower bacterial burden in infected C3H/HeN mice. Quantitative real-time PCR analysis of the spirochetal burden in mice infected with *B. burgdorferi* and treated with statins. Groups ($n = 5$) of 6-week-old C3H/HeN female mice were infected intradermally with *B. burgdorferi* strain B31 isolate MSK5 at 10^3 spirochetes per mouse. Each group was treated with 5 mg/kg of lovastatin, simvastatin, or vehicle control, every other day by oral gavage. Total genomic DNA was isolated from tissues (skin, spleen, lymph node, and joint) using the High Pure PCR template preparation kit, and quantitative real-time PCR was performed. Numbers of borrelial *flaB* copies were normalized against total mouse *β -actin* copies. Data shown represent 2 independent experiments. All tissues showed a significant decrease in burden with the exception of the joints. The asterisks indicate levels of significance as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

FIGURE 2. Statins affect antibody titers in treated mice and lovastatin elicits a T_H2 type response in mice infected with *B. burgdorferi*. A) Enzyme-linked immunosorbent assays using individual sera from C3H/HeN mice against whole cell *B. burgdorferi* strain B31 clonal isolate MSK5 with a-IgG, a-IgG2a, a-IgG2b, and a-IgM secondary antibodies. Levels of IgG and IgM were significantly lower in statin-treated mice when compared to levels in untreated mice. There were no significant differences seen in titers of IgG2a and IgG2b (data not shown). Asterisks indicated antibody titers significantly different

1 between statin-treated and untreated mice. B) Bio-Plex cytokine/chemokine analysis of
2 individual sera from C3H/HeN mice treated with statins. Only cytokines and chemokines
3 showing significant differences are shown. The y-axis gives cytokine and chemokine
4 levels in pg/ml. IL-4, IL-5, IL-9, IL-10, and IL-13 are T_H2 cytokines whose levels are
5 significantly higher in lovastatin-treated mice. IL-2 and IL-12p70, important for
6 clearance of *B. burgdorferi*, are also upregulated in lovastatin-treated mice. All data
7 shown are average of 2 independent experiments. The asterisks indicate levels of
8 significance as follows: **, $P < 0.01$; *, $P < 0.05$.

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Tables

Table 1. Statins do not affect bacterial dissemination.

Treatment and Dose of MSK5	No. of cultures positive/No. tested						No. of mice infected/No. tested
	Skin	Spleen	Lymph Node	Heart	Bladder	All Sites	
Control							
0	0/10	0/10	0/10	0/10	0/10	0/50	0/10
10 ³	10/10	10/10	10/10	10/10	10/10	50/50	10/10
Simvastatin							
0	0/10	0/10	0/10	0/10	0/10	0/50	0/10
10 ³	10/10	10/10	10/10	10/10	9/10	49/50	10/10
Lovastatin							
0	0/10	0/10	0/10	0/10	0/10	0/50	0/10
10 ³	10/10	9/10	10/10	10/10	10/10	49/50	10/10

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Figure 2

FIGURE 2. Statins affect antibody titers in treated mice and lovastatin elicits a T_H2 type response in mice infected with *B. burgdorferi*. **A)** Enzyme-linked immunosorbent assays using individual sera from C3H/HeN mice against whole cell *B. burgdorferi* strain B31 clonal isolate MSK5 with α -IgG, α -IgG2a, α -IgG2b, and α -IgM secondary antibodies. Levels of IgG and IgM were significantly lower in statin-treated mice when compared to levels in untreated mice. There were no significant differences seen in titers of IgG2a and IgG2b (data not shown). Asterisks indicated antibody titers significantly different between statin-treated and untreated mice. **B)** Bio-Plex cytokine/chemokine analysis of individual sera from C3H/HeN mice treated with statins. Only cytokines and chemokines showing significant differences are shown. The y-axis gives cytokine and chemokine levels in pg/ml. IL-4, IL-5, IL-9, IL-10, and IL-13 are T_H2 cytokines whose levels are significantly higher in lovastatin-treated mice. IL-2 and IL-12p70, important for clearance of *B. burgdorferi*, are also upregulated in lovastatin-treated mice. All data shown are average of 2 independent experiments. The asterisks indicate levels of significance as follows: **, $P < 0.01$; *, $P < 0.05$.