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Short communication

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.

Keywords: Lyme disease; *Borrelia burgdorferi*; Cell wall biogenesis; Statins

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

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2. Materials and methods

2.1. Bacterial strains and growth conditions

A clonal derivative of *B. burgdorferi* sensu stricto strain B31, MSK5 [9], which contains all plasmids was used for infectivity experiments. *B. burgdorferi* cultures were grown in 1% CO_2 at 32 °C in Barbour-Stoenner-Kelly II (BSK-II) liquid medium supplemented with 6% normal rabbit serum (Pel-Freez, Biologicals, Rogers, AR).

2.2. Statin inactivation

Statins were activated as previously described [8]. Briefly, 25 mg of Lovastatin or Simvastatin (Sigma–Aldrich, St. Louis, MO) was dissolved in 500 µl of EtOH preheated to 55 °C. 250 µl of 0.6 M NaOH and 5 ml of ddH_2O were then added to the samples, which were then incubated at room temperature for 30 min. Following incubation, the pH was brought to 8.0 with 1 M HCl at which time ddH_2O was added to the samples to bring them to a final concentration of 4 mg/ml. 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 5 mg/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 2–3 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 µl 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 manufacturer's suggested protocol. Known amounts of mouse or spirochete genomic DNA were used as standards to determine the total numbers of spirochetes in different mouse tissues. Total genomic DNA isolated from different infected mouse tissues was subjected to quantitative real-time PCR using SYBR green PCR master mix with a final concentration of 0.3 µM of oligonucleotides using the ABI Prism 7300 system (Applied Biosystems). Spirochetes were enumerated by real-time PCR analysis using primers specific to a borrelial gene, *flaB* (F-5′ TCTTTTCTCTGGTGAGGGAGCT; R-5′ TCCTTCCTGTTGAACACCCTCT), and normalized the total DNA extracted from different tissues to the number of copies of a mouse *β-actin* (F-5′ CAAGTCATCACTATTGGCAACGA; R-5′ CCAAGAAGGAAGGCTGGAAAA). The spirochete burden was expressed as the number of borrelial *flaB* copies per 10^6 mouse β-*actin* copies.

2.5. Enzyme-linked immunosorbent assays

A clonal isolate of *B. burgdorferi* strain B31, MSK5, was grown under conditions mimicking the fed-tick (pH 6.8/37 °C) to a density of 1×10^8 spirochetes/ml. The cells were harvested by centrifugation and washed four times with in HBSS. The final pellet was disrupted by sonication and the cells were resuspended in ELISA coating buffer (50 mM sodium carbonate, pH 9.6) at a final concentration of 100 µg/ml following quantification using the BCA protein assay (Pierce, Thermo Fisher Scientific, Rockford, IL). 96-well MaxiSorp ELISA plates (Thermo Fisher Scientific, Rochester, NY) were coated with 100 µl of total sonicate in coating buffer and incubated overnight at 4 °C. Following incubation, the coated plates were washed three times in ELISA wash buffer (0.80 mM Na_2HPO_4, 137 mM NaCl, 0.27 mM KCl, 0.15 mM KH_2PO_4 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 h 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 h. The plates were washed three times with ELISA wash buffer and the wells were coated with OPD buffer (50 mM Na_2HPO_4, 50 mM citric acid, pH 5.0, OPD tablets (Thermo Fisher Scientific), H_2O_2). The plates were then incubated in the dark for 15 min at room temperature. The absorbance was measured using a Synergy HT Plate Reader (BioTek, Winooski, VT) at 450 nm. 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