Statins Fail to Improve Outcome in Experimental Cerebral Malaria and Potentiate Toll-Like Receptor-Mediated Cytokine Production by Murine Macrophages

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Abstract. Cerebral malaria is responsible for a large proportion of the estimated one million deaths caused by Plasmodium falciparum malaria annually. This disease is associated with excessive pro-inflammatory cytokine production resulting from dysregulated host responses to infection. On the basis of reports indicating potent activity against host-mediated inflammatory disorders such as sepsis, we examined the activity of statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) on malaria-associated inflammation in vivo and in vitro. Simvastatin failed to improve survival or alter parasitemia in C57BL/6 mice infected with Plasmodium berghei ANKA, an experimental model of cerebral malaria. In vitro statin treatment potentiated production of tumor necrosis factor and interleukin-6 by murine peritoneal macrophages in response to P. falciparum glycosylphosphatidyl inositol, a Toll-like receptor 2 (TLR2) ligand. Statin treatment also potentiated pro-inflammatory cytokine production stimulated by a panel of TLR2 and TLR4 ligands. Our results indicate that statins fail to confer protection in experimental cerebral malaria and potentiate TLR-mediated pro-inflammatory cytokine production by primary murine macrophages.

INTRODUCTION

Plasmodium falciparum malaria is the most important parasitic disease in the world, causing approximately 500 million human infections and greater than one million deaths annually, with a large proportion of this mortality caused by cerebral malaria. This disease represents one of the leading causes of non-bacterial sepsis in the developing world. 1,2 Similar to other causes of sepsis, cerebral malaria is characterized by an exaggerated host immune response to infection, including the upregulation of pro-inflammatory cytokines (e.g., interferon-γ [IFN-γ], tumor necrosis factor [TNF], and lymphotixin-α). 3,4 Dramatic increases in drug resistance in the malaria parasite and insecticide resistance in the mosquito vector have contributed to global resurgence in drug-resistant malaria over the past several decades, and effective new interventions are urgently needed. 5 One strategy to expedite drug discovery is the investigation of novel therapeutic indications of drugs previously approved for other clinical conditions.

Statins are one of the most widely prescribed classes of drugs in the developed world, with potent cholesterol-lowering activity and an excellent safety record. 6 They act upon the rate-limiting step in cholesterol biosynthesis by competitively inhibiting 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, thereby blocking the conversion of HMG-CoA into mevalonate. However, statins have also been shown to have pleiotropic effects independent of their impact on cholesterol levels. These effects have been attributed to disruption of small GTP-binding proteins in the Ras superfamily, which derive their important post-translational isoprenoid modifications from the cholesterol biosynthesis pathway. 6–9 Therapeutic statin-mediated immunomodulatory activity has been demonstrated in animal models of asthma and sepsis. 10–17 Specifically, statins have been reported to improve survival in the cecal-ligation and puncture (CLP) and lipopolysaccharide (LPS)–injection models of sepsis. 10–14 Based on clinical similarities between sepsis and cerebral malaria, the objective of this study was to examine the impact of statins on the innate immune response and clinical outcome in experimental cerebral malaria.

Sepsis and cerebral malaria are characterized by dysregulated inflammatory responses to infection. Innate inflammatory responses are mediated in part by Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns expressed by microbial pathogens. Upon ligand binding, TLRs activate NF-κB and activator protein-1 (AP-1) in cell-signaling cascades mediated by myeloid differentiation primary response protein 88 and mitogen-activated protein kinases, 18 leading to release of pro-inflammatory cytokines, which although necessary for immune activation, may contribute to immunopathologic changes and tissue injury. Parasite products, including P. falciparum glycosylphosphatidyl inositol (GPI), have been shown to induce the production of pro-inflammatory cytokines, such as TNF by activation of TLR2. 18 To test the hypothesis that statins would improve outcome in cerebral malaria, similar to their reported effects in murine models of sepsis, we examined the effects of statins in vivo in the Plasmodium berghei ANKA model of severe malaria. 19 Our study also tested the immunomodulatory action of statins upon TLR-mediated inflammatory responses in vitro to determine possible mechanisms for the effects of statins on disorders associated with generalized inflammation such as cerebral malaria and sepsis. We report that statin treatment failed to improve survival or alter parasitemia in vivo. Furthermore, contrary to our expectations, statins enhanced, rather than suppressed, TLR-induced pro-inflammatory cytokine production by primary murine macrophages in vitro.

MATERIALS AND METHODS

Reagents. Lipopolysaccharide (Escherichia coli 055:B5) was obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Lipopeptides FSL-1 and Pam3CSK4 were obtained from InvivoGen (San Diego, CA). Simvastatin and atorvastatin were obtained from Toronto Research Chemicals

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(Toronto, Ontario, Canada). For in vitro experiments, 21.4 mg of atorvastatin was dissolved in 1.5 mL of 100% ethanol to give a final concentration of 12.3 mM. The solution was heated in a shaker at 55°C until it was completely dissolved. The atorvastatin was then passed through a 0.2-μm filter, aliquoted, and stored at −20°C. Simvastatin was activated to its acid form as described by Matsumoto and others. Briefly, 20 mg of simvastatin was dissolved in 400 μL of 100% ethanol. A total of 2.4 mL of 0.1 M NaOH was added to this solution, and the solution was heated to 50°C for 2 hours. A total of 3.6 mL of solution A (81 mM Na₂HPO₄, 15 mM NaH₂PO₄) was then added to give a final concentration of 7.47 mM simvastatin, and the solution was then incubated at 40°C for 30 minutes. Activated simvastatin was passed through a 0.2-μm filter, aliquoted, and stored at −20°C. dl-mevalonic acid lactone was obtained from Sigma-Aldrich Canada Ltd. A total of 1 g was dissolved in 10 mL of 10 mM KH₂PO₄ to give a final concentration of 0.77 M. This solution was stored at −20°C and filtered (0.22 μm) prior to use.

For in vivo experiments, simvastatin was dissolved in 100% ethanol to give a concentration of 25 mg/mL (59.7 M). This solution was filtered (0.22 μm) and aliquoted for storage at −20°C. Simvastatin was added to sterile phosphate-buffered saline prior to injection, to give a final concentration of 0.1 mg/mL (0.2 M). Treatment was initiated one day prior to infection with P. berghei at a dose of 2 mg/kg. The vehicle-treated group received the same dose of ethanol dissolved in sterile phosphate-buffered saline, and all injections were given intraperitoneally once a day in a volume of 0.5 mL.

Mice. All animal experiments were performed after approval of the University of Toronto Animal Care Committee, in accordance with institutional guidelines. C57BL/6 wild-type mice were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada) and housed under pathogen-free conditions. All mice were generously provided by Dr. Thomas Hawn, University of Toronto Animal Care Committee, in accordance with institutional guidelines. C57BL/6 wild-type mice were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada) and housed under pathogen-free conditions with a 12-hour light cycle. Mice used in the P. berghei experiment were of the same age (7–9 weeks) and sex (male). Tlr2−/−C57BL/6 mice were generously provided by Dr. Thomas Hawn, University of Washington, Seattle, WA), and Tlr4−/−(C57BL/10ScNJ) mice were obtained from Jackson Laboratories (West Grove, PA). All mouse strains, including C57BL/6 mice, were bred and housed in the same facility.

Parasite cultures. Plasmodium falciparum ITG parasite cultures were maintained as previously described and treated with Mycoplasma removal agent (ICN Pharmaceuticals, Costa Mesa, CA). Polymerase chain reaction analysis and the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD) were used to ensure that cultures were mycoplasma free and endotoxin free, respectively. Culture supernatants containing P. falciparum GPI were collected and frozen in aliquots at −20°C for use in experiments. Protein-free P. falciparum GPI purified by high-performance liquid chromatography was obtained as previously described.

Infection with P. berghei. Plasmodium berghei parasites (MR4; American Type Culture Collection, Manassas, VA) were cultivated by passage through C57BL/6 mice as previously described. Experimental infections were introduced (day 0) by intraperitoneal injection of 5 × 10⁵ parasitized erythrocytes. Parasitemia was monitored using Giemsa-stained blood smears prepared on days 3, 5, and 6 post-infection. At least 1,000 cells were counted, and parasitemia was expressed as the percentage of parasitized erythrocytes in the erythrocytes counted.

Assay of in vivo cytokine production. Cardiac puncture was performed on 10 P. berghei-infected mice (5 simvastatin-treated and 5 vehicle-treated mice) on days 1, 3, 5, and 6 after infection. Blood was collected with a heparinized needle and centrifuged at 13,000 rpm for 10 minutes at 4°C. The plasma was then aliquoted and stored at −80°C. Tumor necrosis factor (TNF), IFN-γ, interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1) were measured using a cytometric bead array (BD Biosciences, Mississauga, Ontario, Canada).

Murine peritoneal macrophages. Murine macrophages were harvested from the peritoneum using ice-cold RPMI 1640 medium (Invitrogen, Burlington, Ontario, Canada) four days after intraperitoneal injection of 2 mL of sterile 3% thioglycollate. The cells were washed twice with cold RPMI 1640 medium and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (heat-inactivated), 1.5 mM l-glutamine, and gentamicin (25 mg/mL) (Invitrogen). Cells were seeded (150,000/well) in 96-well plates and incubated at 37°C in an atmosphere of 5% CO₂.

Measurement of cytokine levels. Murine macrophages were isolated as described above. Adherent cells were incubated overnight, then treated for 24 hours with statins (± mevalonate) or vehicle. This treatment was repeated with the addition of relevant ligands (P. falciparum GPI, LPS, Pam3CSK₄, or FSL-1), for another 24 hours, after which supernatants were assayed using commercially available enzyme-linked immunosorbent assay kits for TNF (eBioscience, San Diego, CA) or IL-6 (BD Biosciences). Supernatants were stored at −20°C.

Statistical analysis. A log-rank test was performed on the Kaplan-Meier survival curves, a Student’s t-test was carried out on the in vivo cytokine data, and a t-test with Welch’s correction was performed on the in vitro cytokine data. Tests were performed with Excel (Microsoft, Redmond, WA) and Prism version 4.0b (Macintosh version; GraphPad Software, Inc., La Jolla, CA). A general linear model using SPSS version 15.0 (SPSS, Inc., Chicago, IL) was used to test the overall effect of statin treatment on pro-inflammatory cytokine production from TLR agonist-stimulated macrophages.

RESULTS

Effect of simvastatin on protection of mice from cerebral malaria. To examine the effects of statin treatment on clinical outcome in experimental cerebral malaria, simvastatin was administered once a day beginning one day prior to inoculation with P. berghei at a dose (2 mg/kg/day) based on previous studies in which statins improved outcome in both the CLP and systemic LPS mouse models of experimental sepsis. Treatment with simvastatin was continued until mice died or recovered from cerebral malaria. Simvastatin failed to improve survival compared with vehicle-treated C57BL/6 mice (P=0.61), and no mice survived past day 7 post-infection (Figure 1A). Simvastatin-treated animals exhibited characteristic signs of cerebral malaria, including ruffled hair coat, rapid respiration, and lack of mobility. In addition, quantitative parasitemia was not affected by treatment with simvastatin (Figure 1B).

Effect of simvastatin on the cytokine profile of C57BL/6 mice during P. berghei infection. Several cytokines have been implicated in the pathogenesis of cerebral malaria in humans and in the P. berghei rodent model. On days 1, 3, 5, and 6 post-infection, cardiac puncture was performed to obtain plasma
for cytokine measurements by cytometric bead array analysis. Levels of IFN-γ were significantly decreased in the statin-treated group on day 5 ($P < 0.05$) (Figure 2A). Levels of IL-6 were increased on day 6 in the statin-treated group, although this difference did not reach statistical significance ($P = 0.15$). Similarly, levels of MCP-1 were lower on day 5 in the statin-treated group ($P = 0.16$) (Figure 2B and C). Levels of TNF were not statistically different in the two groups (Figure 2D).

**Effect of statins on TLR2- and TLR4-mediated TNF and IL-6 production in vitro.** Because of the failure of statins to improve clinical outcome in experimental cerebral malaria, we examined the ability of statins to modulate the production of the pro-inflammatory cytokines TNF and IL-6 in murine peritoneal macrophages after stimulation with TLR2 and TLR4 ligands. Macrophages were pre-treated with simvastatin for 24 hours before stimulation with *P. falciparum* GPI purified by high-performance liquid chromatography. Treatment with simvastatin significantly increased TNF and IL-6 production. This effect was dose dependent and was abrogated when statins were administered together with 200 μM mevalonate, a metabolite that reverses statin-mediated inhibition of the cholesterol synthesis pathway (Figures 3A and 4A). Similar results were observed with another member of the statin class of drugs, atorvastatin (see supplementary Figures 1A and 2A online at www.ajtmh.org).

In addition to *P. falciparum* GPI, the effects of other TLR2 ligands (FSL-1 [Figures 3B and 4B] and Pam3CSK4 [Figures 3C and 4C]) and the TLR4 ligand LPS (Figures 3D and 4D) were also examined after pretreatment with simvastatin. As with *P. falciparum* GPI, simvastatin augmented production of TNF and IL-6 in response to these TLR ligands. This augmentation of cytokine production was dose dependent and prevented by mevalonate. Although atorvastatin produced a similar effect, simvastatin appeared more potent in its ability to up-regulate cytokine production (see supplementary Figures 1 and 2 online at www.ajtmh.org).

Receptor controls were used to document the specificity of each ligand for its cognate TLR. *Plasmodium falciparum* GPI stimulated TNF production (Figure 5A) in wild-type and TLR4−/− macrophages. *Plasmodium falciparum* GPI-induced TNF production was markedly reduced or abolished in CD36−/− and TLR2−/− macrophages, respectively. The IL-6 response was similarly reduced in TLR2−/− macrophages (Figure 6A). As expected, LPS induced production of large amounts of TNF and IL-6 in wild-type, TLR2−/−, and CD36−/− macrophages but not in TLR4−/− macrophages (Figures 5B and 6B). Lipopeptides Pam3CSK4 (Figures 5C and 6C) and FSL-1 (Figures 5D and 6D) induced TNF and IL-6 production in all macrophages except for TLR2−/−. Lipopeptide FSL-1 was also dependent on CD36 for stimulation of TNF and IL-6 production (Figures 5D and 6D).

**DISCUSSION**

Based on previous reports of statin therapeutic efficacy in experimental sepsis, we investigated whether statin treatment would improve clinical outcome in experimental murine cerebral malaria, in which the host immune response contributes to immunopathologic changes and associated morbidity and mortality. However, contrary to our expectations, simvastatin failed to improve survival or affect parasitemia in experimental cerebral malaria (Figure 1).
study showed that treatment of experimental cerebral malaria. Therefore, the decrease in survival, which suggested a protective role for this cytokine during cerebral malaria. Further more, IL-6 levels were greater in statin-treated mice on day 6 (Figure 2B). Although the difference in IL-6 levels observed in simvastatin-treated versus control mice did not reach statistical significance, it should be noted that increased levels of this cytokine have been associated with poor outcomes in human infection and experimental cerebral malaria.

The reduction in MCP-1 observed on day 5 (Figure 2C) is characteristic of statin treatment. Given the reported association of increased TNF production with severity of neurologic illness in cerebral malaria in humans and mice, it is important to note that statin treatment failed to decrease TNF production in vivo (Figure 2D).

Two previous studies reported that statins inhibit P. falciparum growth in vitro. This anti-parasitic activity may not extend to P. berghei because our study failed to show any difference in parasitemia in vivo (Figure 1B). Our in vivo experiments underscore differences between murine models for sepsis, an acute challenge where survival is measured in hours, and cerebral malaria, where neurologic symptoms are generally not observed until at least day 6 post-infection. The lack of efficacy in experimental cerebral malaria versus sepsis may be caused by these temporal differences or to other features of cerebral malaria such as parasite burden, which statins did not affect in our study and in the study of Kobbe and others.

Our in vitro work characterized the effect of statins on the production of TNF and IL-6 by peritoneal murine macrophages after stimulation by TLR2 and TLR4 ligands. As mentioned previously, TNF and IL-6 are believed to play mechanistic roles in the reduction in MCP-1 observed on day 5 (Figure 2C). This cytokine was measured in response to the following TLR2 and TLR4 agonists: A, high-performance liquid chromatography–purified Plasmodium falciparum glycosylphosphatidyl inositol (100 nM); B, lipopeptide FSL-1 (20 ng/mL); C, lipopeptide PAM3CSK4 (50 ng/mL); and D, lipopolysaccharide (5 ng/mL). This effect was reversed by the addition of mevalonate. Cells were pre-treated (statins, mevalonate, or media) for 24 hours, and supernatants were collected 24 hours after administration of the TLR ligand. Data are the mean ± SD. All experiments were performed at least three times (each in triplicate) and statistical analysis using a general linear model confirmed that for each TLR ligand, statin treatment resulted in a dose-dependent elevation in cytokine production of TNF and IL-6 by peritoneal murine macrophages (TLR2/4)–induced interleukin-6 production by primary murine macrophages.
in the pathogenesis of cerebral malaria similar to their respective roles in other systemic inflammatory disorders. Although statin treatment failed to modify clinical outcome in experimental cerebral malaria, we were interested in determining the effect of statins on TLR-induced inflammatory responses in macrophages in vitro, especially given their reported therapeutic efficacy in experimental sepsis. We chose to focus our attention on macrophages because of their importance as cellular mediators of innate immune responses in cerebral malaria and sepsis. Previous studies have demonstrated that statins attenuate LPS-induced up-regulation of surface TLR2 and TLR4 in human monocytes, in addition to decreasing basal TLR4 expression and TLR-mediated down-stream signaling in CD14+ human monocytes. Statins have also been reported to reduce elevated TLR4 expression observed on blood monocytes in patients with chronic heart failure.

Challenging the hypothesis that statins function exclusively as anti-inflammatory agents, our study conclusively demonstrated that simvastatin or atorvastatin paradoxically sensitized murine macrophages to subsequent *P. falciparum* GPI stimulation, with increasing TNF and IL-6 production in a dose-dependent manner (Figures 3 and 4A and supplementary Figures 1 and 2A). Mevalonate was effective in reversing this augmentation of cytokines, supporting our conclusion that statins affect macrophages by disrupting the cholesterol biosynthesis pathway rather than interfering with a TLR:agonist interaction.

Furthermore, we examined whether this response was limited to the malarial GPI antigen or was a general TLR-related phenomenon by treating macrophages with Pam, CSK, or FSL-1 (TLR2 ligands) and with LPS (a TLR4 ligand). Statins potentiated TNF and IL-6 production by murine peritoneal macrophages stimulated by all of the TLR agonists examined (Figures 3 and 4B–D and supplementary Figures 1 and 2B–D). Thus, our study has identified a novel effect of statins on TLR-mediated responses in primary murine macrophages. These results suggest that the reported therapeutic efficacy of statins in experimental sepsis may be attributable to modulation of endothelial activation (as suggested by Merx and others), rather than inhibition of macrophage pro-inflammatory cytokine production per se. The discordance between the effect of statins on TNF production in vitro (enhancement) and in vivo (no significant change) in our study may reflect the complex biological responses and networks responsible for the pathogenesis of experimental cerebral malaria. Another contributing factor could be that statins exert differential effects on cell types other than macrophages (e.g., vascular endothelial cells).

Our findings in primary murine macrophages are in agreement with those of previous studies, which demonstrated statin-mediated up-regulation of LPS-induced TNF, IL-12, and IL-6 production by human dendritic cells and bone marrow–derived murine dendritic cells. Another study also reported increased LPS-induced pro-inflammatory cytokine production in human monocytes after treatment with statins.

In summary, the results of the current study indicate that statin treatment fails to improve outcome in experimental cerebral malaria and sensitizes, rather than inhibits, TLR-induced pro-inflammatory cytokine production by macrophages in vitro.

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Note: Supplementary Figure 1 (Atorvastatin increases TLR2/4-induced TNF production by primary murine macrophages in vitro) and Supplementary Figure 2 (Atorvastatin increases TLR2/4-induced IL-6 production by primary murine macrophages in vitro) appear online at www.ajtmh.org.

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