



Statins Suppress Ebola Virus Infectivity by Interfering with Glycoprotein Processing

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ABSTRACT Ebola virus (EBOV) infection is a major public health concern due to high fatality rates and limited effective treatments. Statins, widely used cholesterol-lowering drugs, have pleiotropic mechanisms of action and were suggested as potential adjunct therapy for Ebola virus disease (EVD) during the 2013–2016 outbreak in West Africa. Here, we evaluated the antiviral effects of statin (lovastatin) on EBOV infection *in vitro*. Statin treatment decreased infectious EBOV production in primary human monocyte-derived macrophages and in the hepatic cell line Huh7. Statin treatment did not interfere with viral entry, but the viral particles released from treated cells showed reduced infectivity due to inhibition of viral glycoprotein processing, as evidenced by decreased ratios of the mature glycoprotein form to precursor form. Statin-induced inhibition of infectious virus production and glycoprotein processing was reversed by exogenous mevalonate, the rate-limiting product of the cholesterol biosynthesis pathway, but not by low-density lipoprotein. Finally, statin-treated cells produced EBOV particles devoid of the surface glycoproteins required for virus infectivity. Our findings demonstrate that statin treatment inhibits EBOV infection and suggest that the efficacy of statin treatment should be evaluated in appropriate animal models of EVD.

IMPORTANCE Treatments targeting Ebola virus disease (EVD) are experimental, expensive, and scarce. Statins are inexpensive generic drugs that have been used for many years for the treatment of hypercholesterolemia and have a favorable safety profile. Here, we show the antiviral effects of statins on infectious Ebola virus (EBOV) production. Our study reveals a novel molecular mechanism in which statin regulates EBOV particle infectivity by preventing glycoprotein processing and incorporation into virus particles. Additionally, statins have anti-inflammatory and immunomodulatory effects. Since inflammation and dysregulation of the immune system are characteristic features of EVD, statins could be explored as part of EVD therapeutics.

KEYWORDS Ebola virus, *Filoviridae*, antiviral, hemorrhagic fever

Ebola virus (EBOV) poses a threat to people throughout Africa, and, as the 2013–2016 outbreak demonstrated, to the rest of the world (1). The 2013–2016 outbreak was unprecedented in the history of the virus, with over 28,000 cases and more than 11,000 deaths (1). Despite the devastating consequences of EBOV infection, treatment options remain limited and experimental (2). Ebola virus disease (EVD) is associated with systemic inflammation, endothelial dysfunction, coagulopathy, vascular leakage, shock, and organ failure (3, 4). Statins, well-known cholesterol-lowering drugs, have potential beneficial effects beyond their ability to reduce cholesterol levels, including anti-inflammatory and immunomodulatory functions and the ability to reverse endothelial

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abnormalities (5, 6). For example, statins have been implicated in improving survival in sepsis patients (7–9); like EVD, sepsis is characterized by inflammation, endothelial dysfunction, and coagulopathy (6). Statins are already FDA approved for reducing high cholesterol, have a favorable safety profile, and are inexpensive. Thus, they were suggested as a possible adjunct therapy for EVD patients during the 2013–2016 outbreak (10).

Statins block 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme that catalyzes the conversion of HMG-CoA to mevalonate, a key intermediate for synthesis of cholesterol and isoprenoids (11). Since cholesterol plays important roles in membrane fluidity, organization, and signaling (12, 13), they serve as important platforms for viruses to enter cells (14, 15). Statins have been widely reported to block infection of many enveloped viruses by inhibiting the cholesterol/isoprenoid pathway (16–22). Cholesterol likewise contributes to the EBOV life cycle, including viral entry, fusion, and budding (23–29); EBOV has been reported to utilize cholesterol-enriched rafts as a platform for cell entry, as well as for assembly and budding from cells (25, 30–32). In addition, cholesterol-dependent interactions between EBOV glycoproteins (GPs) are essential for virus assembly (15). This further suggests that drugs lowering cholesterol levels, like statins, could be useful therapeutics for EVD patients.

EBOV virions project glycoprotein (GP) spikes that are synthesized and inserted into the host cell-derived envelope during budding (33). EBOV GP is synthesized in several forms. The most abundant form of GP is a secreted protein (sGP) translated from an unedited mRNA, whereas the structural GP is a product of the edited mRNA. The monomeric EBOV GP, a type I transmembrane glycoprotein, is processed by a complex series of events (34–36). An N-glycosylated, endoplasmic reticulum (ER)-resident form of GP precursor (preGP) undergoes N,O-glycosylation maturation in the Golgi apparatus to become GP₀ (36). GP₀ is then transported further into the trans-Golgi network, where the proprotein convertase furin or a furin-like protease cleaves GP₀ at a multibasic motif that is conserved in all EBOV strains (36). Cleavage results in the mature N,O-glycosylated GP₁ and in the GP₂ subunit linked by disulfide bond (34–38). These subunits interact to form GP_{1,2}, present on virions as trimeric spikes; GP₁ mediates receptor binding while GP₂ is critical for fusion of the EBOV envelope with the endosomal/lysosomal membrane (39, 40). However, unlike other viruses, cleavage of GP₀ by furin is not required for fusion (41, 42) or glycoprotein incorporation into virions (43–46).

Here, we report that a statin (lovastatin) suppresses infectious EBOV production in a human hepatoma cell line (Huh7) and in primary monocyte-derived macrophages, cell types that are *in vivo* targets for EBOV replication. Statin treatment inhibited processing of preGP into GP₁ in EBOV-infected cells or cells transfected with plasmids encoding GP_{1,2}; the effect was reversed by adding mevalonate. EBOV particles produced in statin-treated cells were depleted of the essential glycoprotein subunit GP₁ required for virus entry, suggesting that statins reduce EBOV infectivity by inhibiting glycoprotein maturation and incorporation into virions. In addition, we have tested the effect of 5 other types of statins, fluvastatin, simvastatin, atorvastatin, rosuvastatin, and pitavastatin, on EBOV replication. Of all the statins, simvastatin and pitavastatin were the most potent in reducing EBOV infectivity. Our results suggest that statins selectively inhibit preGP maturation and should be further investigated in *in vivo* models for EBOV infection.

RESULTS

Statin treatment inhibits EBOV infection. To test if statins affect EBOV replication, Huh7 cells were infected with the EBOV variant Mayinga (Ebola virus/H. sapiens-tc/COD/1976/Yambuku-Mayinga) at a multiplicity of infection (MOI) of 0.05. After 1 h of virus adsorption, the cells were treated with dimethyl sulfoxide (DMSO) (vehicle control) or with 20 μ M or 50 μ M lovastatin (referred to as “statin” here unless stated otherwise), the first clinically approved statin, in medium supplemented with lipoprotein-deficient serum (LPDS). LPDS eliminates the possible uptake of cholesterol from the

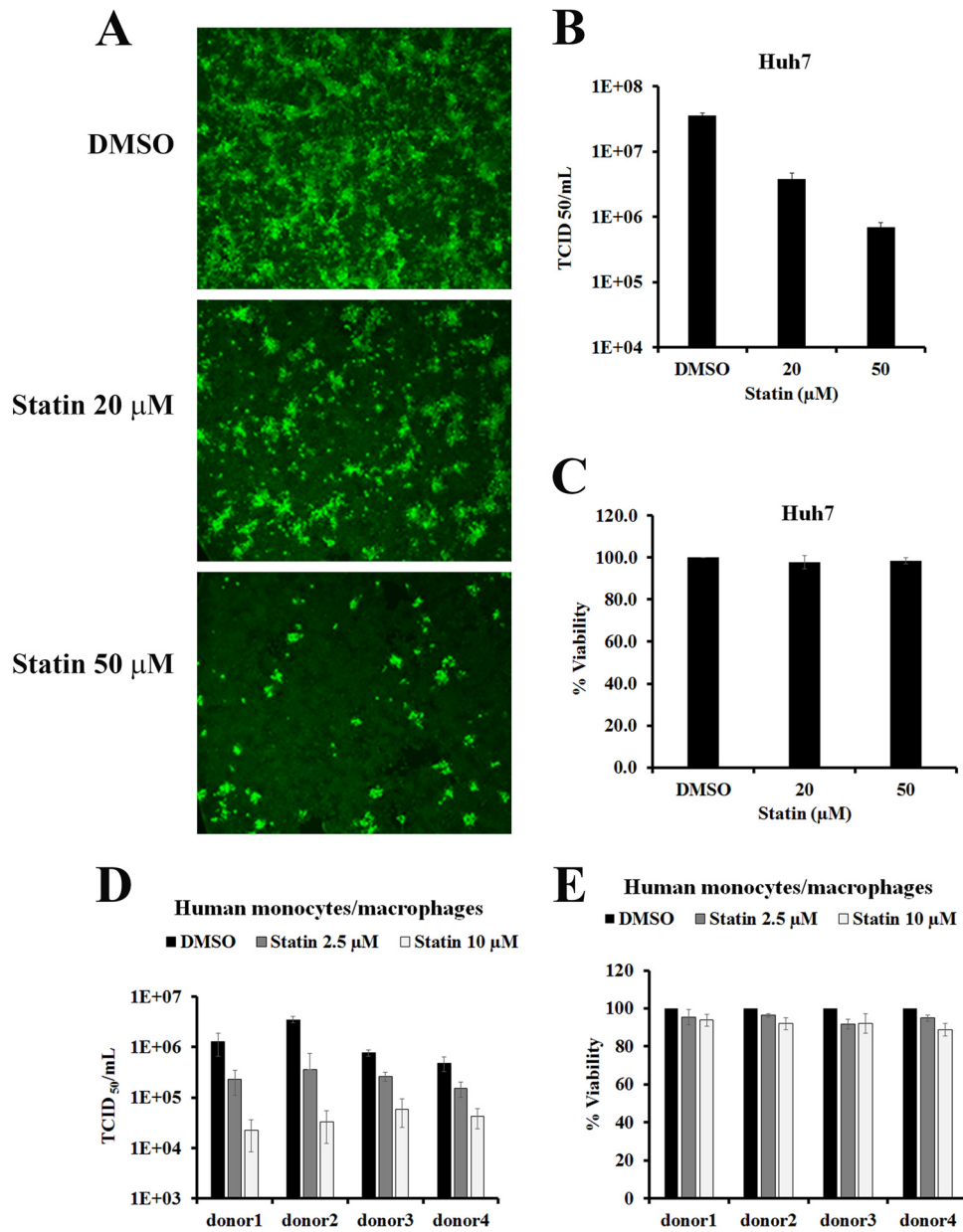


FIG 1 Statin inhibits Ebola virus infection. (A) Huh7 cells were infected with Ebola virus (EBOV) at an MOI of 0.05. After infection, cells were washed and then treated with various concentrations of statin or with DMSO (control). At 72 hpi, the cells were fixed, permeabilized, and stained with anti-EBOV rabbit polyclonal antibody. (B) Culture supernatants of Huh7 cells infected with EBOV and treated with statin or DMSO as in panel A were harvested 72 hpi, and viral titers were quantified by 50% tissue culture infective dose (TCID₅₀) determination. (C) Viability (percent) of statin-treated Huh7 cells was determined after 72 h of treatment. Values were normalized to DMSO-treated controls. (D) Human monocyte-derived macrophages from 4 separate donors were infected with EBOV at an MOI of 0.05, and cells were washed and then treated with various concentrations of statin or DMSO. Cell supernatants were harvested 72 hpi, and viral titers were quantified by TCID₅₀ determination. The results shown are means \pm standard deviations from triplicate wells and representative of two independent experiments. (E) Viability (percent) of statin-treated and mock-infected human monocytes/macrophages was determined after 72 h of treatment. Values were normalized to DMSO controls.

medium (47). After 72 h postinfection (hpi), cells were fixed and viral antigen expression was evaluated by immunofluorescence assays using polyclonal anti-EBOV serum. As shown in Fig. 1A, EBOV antigen-positive staining was seen throughout infected Huh7 cells treated with DMSO only. However, EBOV-positive staining was reduced compared to controls in cells treated with statin at either concentration. To ensure that statin-mediated reduction in EBOV-positive staining was not due to cytotoxicity, cell viability

was assayed after 72 h of treatment. Cell viability was unaffected by either concentration of statin (Fig. 1C). These results suggest that statin reduced EBOV infection.

To determine if statin treatment can inhibit infectious EBOV production, we examined viral titers in supernatants of infected cells. High titers of infectious virus (1.5×10^7 /ml) were detected at 72 hpi in vehicle control-treated cell culture supernatants supplemented with LPDS. Treatment with statin under the same cell culture conditions reduced EBOV titers; 20 μ M statin decreased the production of infectious EBOV titers by >1.1 log, and 50 μ M decreased EBOV titers by 1.5 log (Fig. 1B). In contrast, statin treatment under similar conditions did not affect titers of adenovirus type 5, a nonenveloped virus (see Fig. S1 in the supplemental material).

The antiviral potency of statin treatment was also evaluated in primary human monocyte-derived macrophages, since these cells represent a major target of EBOV infection. To account for donor variations, cells from 4 different donors were tested. Cell viability of macrophages treated with 10 μ M statin was >80% for all the donors (Fig. 1E). Untreated cells yielded infectious titers ranging between 5×10^5 and 4×10^6 (Fig. 1D). Statin treatment efficiently reduced EBOV titers in macrophages from each donor; 2.5 μ M statin reduced infectious EBOV titers by 0.5 to 1.0 log, and 10 μ M statin reduced EBOV titers by 1 to 2 log (Fig. 1D).

Statin inhibition of EBOV infection is reversed by exogenous mevalonate but not by LDL. Statin blocks mevalonate generation and subsequent cholesterol biosynthesis by competitively inhibiting HMG-CoA reductase (11). To investigate whether the anti-EBOV effect of statin was dependent on its ability to specifically inhibit mevalonate production, we added mevalonate during statin treatment. Since inhibition of cholesterol synthesis can be compensated for by import of low-density lipoprotein (LDL)-derived cholesterol from outside the cells, we also looked at the effects of LDL supplementation during statin treatment. Huh7 cells were infected with EBOV as described above and then treated with statin with or without the indicated concentrations of LDL or mevalonate. As shown in Fig. 2A, addition of mevalonate reversed statin-mediated reduction in EBOV titers in a dose-dependent manner, while mevalonate alone had no effect on viral titers. Expression of viral glycoprotein GP₁/preGP and nucleoprotein (NP) was also restored when mevalonate was added during statin treatment (Fig. 2B). In contrast to mevalonate, adding LDL did not reverse the effects of statin treatment on viral titers (Fig. 2C). Altogether, we showed that inhibition of EBOV infection by statin treatment was reversed by mevalonate, the immediate downstream product of the reaction catalyzed by HMG-CoA reductase. These findings are consistent with statin reducing EBOV infectivity by inhibiting HMG-CoA reductase and not via off-target effects.

Statin treatment does not affect EBOV entry. To assess whether reduced production of infectious virus was due to the inhibition of EBOV entry into cells, we measured the levels of cell-associated EBOV NP RNA at 3 hpi. Huh7 cells pretreated with statin were infected with EBOV at an MOI of 3 to ensure synchronous infection. Levels of viral NP RNA in the lysed cells were measured by quantitative reverse transcription PCR (qRT-PCR) and normalized to cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. As shown in Fig. 3A, NP RNA levels did not significantly differ among the samples, indicating that statin did not affect the levels of internalized EBOV genome. On the other hand, treatment with the positive control U18666A, a Niemann-Pick C1 protein (NPC1) inhibitor previously shown to prevent EBOV glycoprotein-dependent entry (25), reduced NP RNA copy numbers in a dose-dependent manner. These results are consistent with statin not affecting EBOV entry.

Statin treatment impairs EBOV infectivity. To further examine the mechanism by which statin reduces the levels of EBOV released from infected cells, the specific infectivity of virions made in the presence of statin was determined by comparing 50% tissue culture infective doses (TCID₅₀) with copy numbers of viral RNA released into culture supernatants of infected cells at 48 hpi. To maximize the initial number of infected cells, Huh7 cells were infected with EBOV at an MOI of 2.0; MOIs higher than

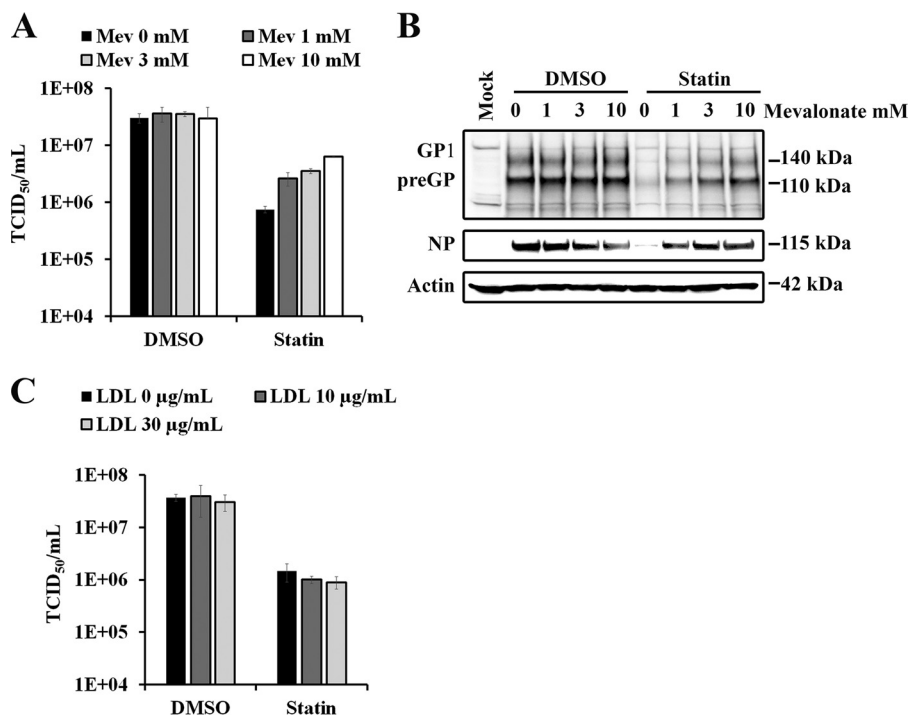


FIG 2 Mevalonate, but not low-density lipoproteins, restores the antiviral effect of statin. (A) Huh7 cells infected with EBOV as in Fig. 1A were treated with DMSO or 50 μ M statin in the presence of indicated concentrations of mevalonate (Mev). Culture supernatants of infected cells were harvested 72 hpi, and viral titers were quantified by determining TCID₅₀. (B) Glycoprotein (GP), nucleoprotein (NP), and actin expression was analyzed by Western blotting in lysates of Huh7 cells infected with EBOV and treated with DMSO or 50 μ M statin in the presence of indicated concentrations of Mev. (C) Huh7 cells infected with EBOV as in Fig. 1A were treated with DMSO or statin (50 μ M) in the presence of various concentrations of low-density lipoprotein (LDL). Culture supernatants of infected cells were harvested 72 hpi, and viral titers were quantified by TCID₅₀ determination.

2.0 caused a significant drop in cell viability at 48 hpi (data not shown). Infected cells were then treated either with DMSO (vehicle control) or with 20 μ M or 50 μ M statin. After 48 h, no differences were noted in extracellular EBOV RNA copy numbers released from cells treated with statin or with vehicle control (Fig. 3B). In contrast, yield of infectious virus (measured as TCID₅₀ per milliliter) in supernatants from statin-treated cells was approximately ~4 to 9 times lower than that in DMSO-treated samples (Fig. 3B). The ratio of TCID₅₀ per milliliter to viral RNA copy numbers (Fig. 3C) in statin-treated cells was reduced to ~5 to 10% of controls. Taken together, these data indicate that postinfection statin treatment decreased infectivity of newly synthesized EBOV particles.

Statin treatment inhibits EBOV glycoprotein maturation. To explore the mechanism responsible for reduced particle infectivity in statin-treated cells, we examined the impact of statins on the viral proteins involved in virus assembly and budding: the matrix protein VP40 and the envelope glycoprotein GP_{1,2}. We first determined whether statin treatment affected VP40 expression. Huh7 cells were transfected with plasmid expressing VP40 and then treated with statin or vehicle control; cell lysates were analyzed by Western blotting. As shown in Fig. S2, VP40 expression levels were similar in statin-treated and vehicle-treated cells. We then examined GP₁ expression in cells transfected with plasmid expressing EBOV GP_{1,2} and treated with statin. Two forms of GP were detectable: a 110-kDa form sensitive to both endoglycosidase H (endo H) and peptide-N-glycosidase F (PNGase F) digestion that had previously been identified as the N-glycosylated precursor present in the endoplasmic reticulum (designated preGP) and the 140-kDa form that was sensitive only to PNGase F digestion and was identified as the mature GP₁ (Fig. 4B), consistent with the presence of complex N- and O-glycans

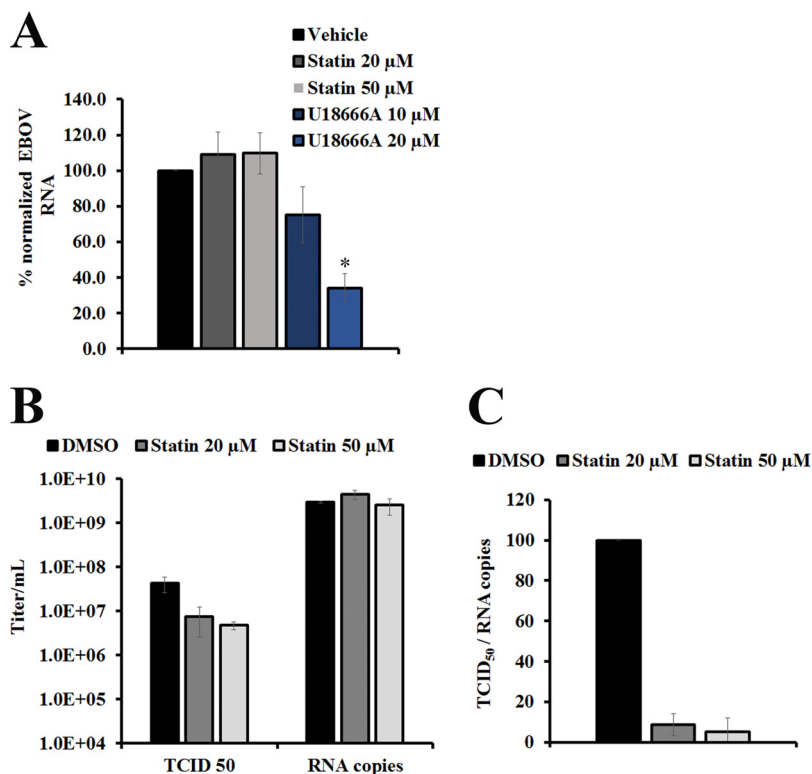


FIG 3 Statin inhibits specific infectivity of EBOV but does not affect entry. (A) Huh7 cells pretreated with the indicated concentrations of statin or DMSO for 48 h were infected with EBOV (MOI = 3.0). For a positive control, cells were pretreated for 1 h with various concentrations of U18666A before infection with EBOV. After 1 h, infected cells were washed with serum-free medium; fresh medium with or without statin or U18666A was then added back to the cells. After 3 h of incubation at 37°C, NP gene RNA copy numbers were determined by qRT-PCR and normalized to GAPDH mRNA. Results represent mean percent normalized NP RNA levels, with error bars indicating standard deviations calculated from 3 independent experiments. *, $P < 0.005$. (B) Culture supernatants of cells infected with EBOV (MOI = 2.0) and treated with DMSO or statin were harvested 48 hpi, and viral titers were quantified by TCID₅₀ determination. RNA was extracted from supernatants of cells, and absolute quantification of viral RNA copy numbers was done using a standard curve with known viral titers. (C) Ratios were calculated using TCID₅₀-per-milliliter values from panel B divided by the extracellular viral RNA copy numbers from panel A. Mean specific infectivity was calculated as a percentage of DMSO-treated samples. Mean values from triplicate wells are shown, and error bars indicate standard deviations. The graph shown is representative of 3 independent experiments.

(36). Most of the GP synthesized in statin-treated cells was the immature precursor glycoprotein (preGP) containing high-mannose sugar chains sensitive to endo H treatment (compare Fig. 4A with B). In contrast, the expression of mature N,O-glycosylated glycoprotein (GP₁), which was resistant to endo H treatment (compare Fig. 4A with B), decreased upon statin treatment. Statin did not similarly affect the glycosylation pattern of NPC1 (Fig. S3), suggesting that the observed effect on GP₁ was specific.

We next evaluated the effect of mevalonate on EBOV preGP maturation. Cells were treated with increasing concentrations of mevalonate, and GP₁ and preGP expression levels were determined. As shown in Fig. 4C, increasing mevalonate concentrations at least partially reversed statin-mediated inhibition of mature GP₁ expression. In parallel, a decrease in immature preGP expression was observed. Densitometry analysis indicated an increase in the GP₁/(GP₁ + preGP) ratio, indicating restoration of preGP maturation to GP₁. This ratio did not change in cells treated only with mevalonate. Partial rescue of preGP processing efficiency and viral titers (Fig. 2A) by mevalonate in statin-treated cells is consistent with statin reducing viral titers by a mechanism impeding preGP maturation.

Statin treatment inhibits preGP glycan maturation. EBOV GP_{1,2} is cleaved into subunits GP₁ and GP₂ by the proprotein convertase furin at the RRTRR₅₀₁ site. To

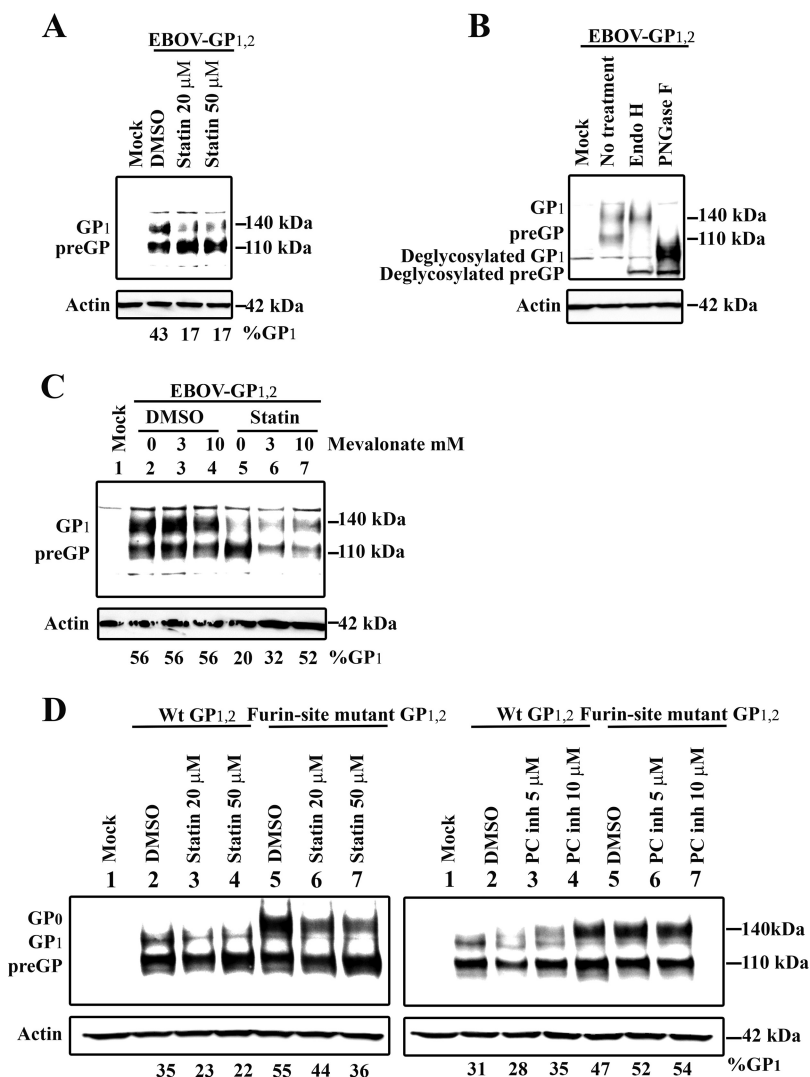


FIG 4 Statin inhibits EBOV GP processing. (A) Huh7 cells were transfected with a plasmid expressing EBOV GP_{1,2} and treated with statin or DMSO. GP and actin expression in cell lysates was then analyzed by Western blotting. To determine the extent of GP₁ cleavage, blot images were subjected to densitometry analysis. The percentage of GP₁ was determined by dividing the signal of GP₁ over the total amount of glycoprotein recognized by GP₁ MAb [GP₁/(GP₁ + preGP)]. (B) Cell lysates of EBOV GP_{1,2}-transfected cells either were left untreated or were digested with endo H or PNGase F. GP and actin expression was then visualized by Western blotting. (C) Huh7 cells transfected with a plasmid expressing EBOV GP_{1,2} were treated with 50 μM statin or DMSO in the presence of various concentrations of mevalonate. GP and actin expression in cell lysates was analyzed by Western blotting. The percentage of GP₁ was determined as in panel A. (D) Huh7 cells were transfected with a plasmid expressing either wild-type EBOV GP_{1,2} or GP_{1,2} in which the furin cleavage motif (furin-site mutant GP_{1,2}) had been mutated and were treated with statin (left panel), proprotein convertase inhibitor (PC inh, right panel), or DMSO control. GP and actin expression in cell lysates was then analyzed by Western blotting. To determine the extent of GP₀ cleavage, blot images were subjected to densitometry analysis. Percentage of GP₁ or GP₀ was determined by dividing the signal of GP₁ or GP₀ over the total amount of glycoprotein recognized by GP_{1,0} MAb [GP_{1,0}/(GP_{1,0} + preGP)].

investigate whether statin inhibits maturation of preGP glycan, we investigated the effect of statin on mutant EBOV GP_{1,2} resistant to furin cleavage. To generate this mutant, we replaced the RRTRR cleavage site with AGTAA, as described previously (44, 45). Huh7 cells were transfected with plasmids encoding wild-type or furin-resistant mutant EBOV GP_{1,2} and treated with either statin or furin-like protease inhibitor (proprotein convertase inhibitor). GP_{1,2} levels were determined in cell lysates by Western blotting. Unlike wild-type EBOV GP_{1,2}, which was processed into GP₁, mutant GP_{1,2} was observed as a higher-molecular-weight form of preGP consistent with the

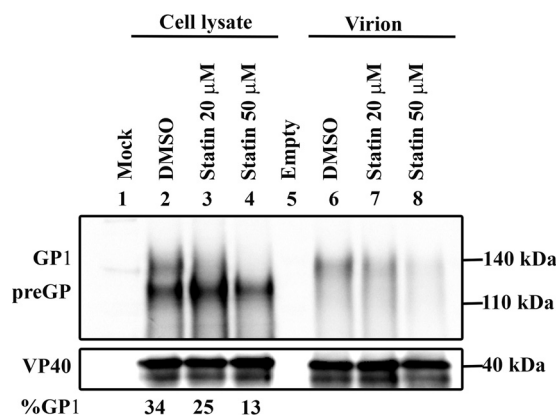


FIG 5 Statin inhibits GP processing and incorporation of GP₁ into EBOV particles. Huh7 cells infected with EBOV at an MOI of 2.0 were treated with DMSO or statin. Supernatants and cell lysates were collected, and EBOV particles were purified from supernatants by ultracentrifugation through a sucrose cushion. Purified EBOV particles were resuspended in 2× sample lysis buffer, and levels of GP and VP40 were analyzed by Western blotting. Percentage of GP₁ was determined by dividing the signal of GP₁ over the total amount of glycoprotein recognized by GP₁ MAbs [GP₁/(GP₁ + preGP)].

molecular weight of GP₀ (36) (Fig. 4D). Treating mutant EBOV GP_{1,2}-transfected cells with statin resulted in a dose-dependent decrease in GP₀ level compared to vehicle control, similar to samples with wild-type GP_{1,2}. No changes in preGP levels were detected in cells expressing either wild-type or furin-cleavage-resistant EBOV GP_{1,2}. In addition, the GP₀/(GP₀ + preGP) ratio in cells expressing the mutant EBOV GP_{1,2} was similar to that in cells expressing wild-type GP_{1,2}, indicating that statin treatment affected GP₁ glycan maturation independently of GP₀ cleavage by furin.

Statin treatment results in GP₁-deficient virions. To confirm the effect of statin treatment on EBOV GP maturation, we infected Huh7 cells with EBOV, treated the cells with DMSO or statin, and partially purified the viral particles released from infected cells. GP_{1,2} and VP40 expression in cell lysates and corresponding viral particles from statin-treated cells was analyzed by Western blotting. As shown in Fig. 5, blotting cell lysate samples from EBOV-infected, vehicle-treated cells showed bands of preGP (~110 kDa) and GP₁ (~140 kDa), as expected. Treatment with statin resulted in a selective decrease in GP₁ levels compared to preGP and VP40. These results are consistent with statin inhibiting preGP maturation of GP₁, as was observed in GP_{1,2}-transfected cells.

Analysis of viral particles from supernatants showed GP₁ migrating at ~140 kDa in the pelleted virions, and no uncleaved preGP or GP₀ was detected (Fig. 5). In cells treated with statin, GP₁ levels decreased in a dose-dependent manner while VP40 levels were unchanged. These results are consistent with the concept that statin treatment resulted in production of VP40-containing EBOV particles deficient in GP₁. Taken together, these results indicate that the lowered expression of GP₁ in statin-treated, EBOV-infected cells results in reduced incorporation of GP₁ into EBOV particles, leading to lower infectivity.

Multiple statins show antiviral activity against EBOV. To investigate the efficacy of other commonly prescribed statins, we compared the antiviral activities of lovastatin, fluvastatin, simvastatin, atorvastatin, rosuvastatin, and pitavastatin in Huh7 cells. As shown in Fig. 6A, at all doses tested, simvastatin and pitavastatin reduced EBOV infectious particle production most potently: 50 μM simvastatin or pitavastatin reduced viral titers by 2.5 log. Rosuvastatin inhibited EBOV production least effectively, reducing viral titers by ~1 log, while lovastatin, atorvastatin, and fluvastatin were moderately effective, reducing titers by 1.7, 1.5, and 1.4 log, respectively. The viability of untreated cells was similar to that of cells treated with each statin (Fig. 6B).

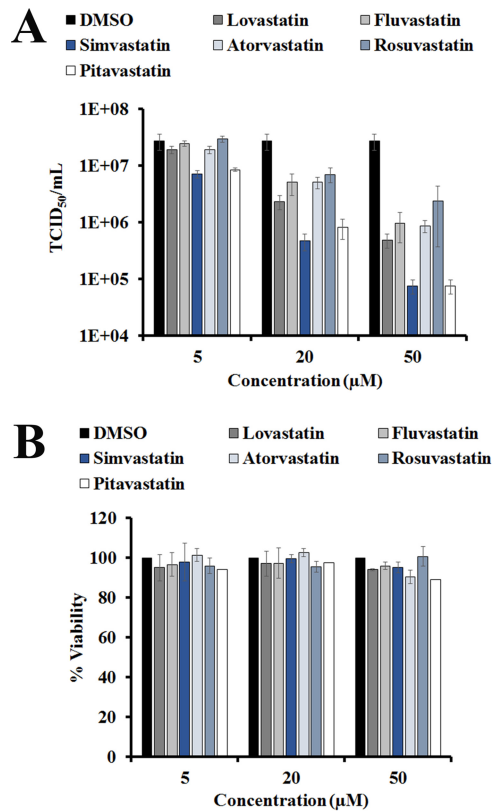


FIG 6 Antiviral activity of other statins against EBOV. (A) Huh7 cells were infected with Ebola virus (EBOV) at an MOI of 0.05. After infection, cells were washed and then treated with various concentrations of lovastatin, fluvastatin, simvastatin, atorvastatin, rosuvastatin, and pitavastatin or DMSO (control). Culture supernatants were harvested 72 hpi, and viral titers were quantified by 50% tissue culture infective dose (TCID₅₀) determination. (B) Viability (percentage) of Huh7 cells treated with lovastatin, fluvastatin, simvastatin, atorvastatin, rosuvastatin, and pitavastatin or DMSO (control) was determined after 72 h of treatment. Values were normalized to DMSO-treated controls.

DISCUSSION

Statins, well-known cholesterol-lowering drugs, have been proposed as therapeutic agents against certain viruses (16–18, 21, 22, 48–59). Statins are known for their anti-inflammatory and immunomodulatory effects as well as for preserving endothelial integrity; inflammation, immune system dysregulation, and endothelial dysfunction are major contributors of EVD pathogenesis (3, 60–62). Statin use was suggested as an adjunct therapy for EVD during the 2013–2016 outbreak (63). A clinical trial evaluating atorvastatin for use in EVD was registered with [clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02380625) (NCT02380625), but the trial was never initiated, presumably because the outbreak was waning prior to the scheduled study start date. Here, we provide evidence of the antiviral effects of statin treatment in a human liver cell line and in primary human macrophages, both major target cells of EBOV. The antiviral activity of statin in Huh7 cells was due to loss in particle infectivity rather than inhibition of viral entry (Fig. 3). Statin reduced the levels of GP₁, the envelope glycoprotein responsible for receptor binding and entry into cells, in GP_{1,2}-transfected (Fig. 4) and EBOV-infected (Fig. 5) cells. Finally, we found that virus particles produced in statin-treated cells had lower levels of GP₁ relative to VP40 matrix protein than did control cells. Thus, statin’s antiviral activity was due to its interference in GP₁ maturation, leading to production of EBOV particles with impaired infectivity.

While GP₁ levels were reduced in infected cells and in released EBOV particles, VP40 levels were not (see Fig. S2 in the supplemental material). Similarly, qRT-PCR analysis showed no changes in the extracellular levels of EBOV RNA after statin treatment, while TCID₅₀ values were reduced upon statin treatment (Fig. 3). Interaction of VP40 with minigenome RNA has been reported to be sufficient for packaging RNA into virus-like

particles (64). Our observation that the levels of extracellular VP40 and genomic RNA did not change even in the presence of little GP₁ is consistent with this report (64) and supports the idea that an interaction between the ribonucleoprotein components and VP40 is a critical step for the budding and release of viral particles (65).

Despite the abundant preGP present in cells transfected with furin-cleavage-resistant EBOV GP_{1,2}, statin treatment resulted in decreased GP₀ levels similar to those seen in cells transfected with wild-type EBOV GP_{1,2} (Fig. 4D). This indicates that statin affects a step prior to GP₀ cleavage, possibly by blocking transport of preGP out of the ER (36, 38). The observed decrease in the GP₁ steady-state levels could be due to degradation of GP₁ (via ER-associated or proteasomal degradation) resulting from its prolonged ER residency because of improper or insufficient maturation of preGP. Since cholesterol levels are lowest in the ER in the secretory pathway (66), ER-resident events involving transmembrane proteins might be particularly sensitive to very small deviations in cholesterol levels from a critical threshold. While most of statins' effect is associated with lowering cellular cholesterol levels, statins also blunt the nonsterol branch of the mevalonate pathway, decreasing formation of isoprenoids and altering protein prenylation, an often critical event in posttranslational modulation of proteins (67). Inhibitors of isoprenoid intermediates, such as geranylgeranyltransferase inhibitor (GGTI), which inhibits prenylation of Rho proteins, or farnesyltransferase inhibitor (FTI), which inhibits the prenylation of the Ras proteins geranyltransferase and farnesyltransferase, have been effective against certain viruses (19, 51). Whether statin's effects on GP₁ processing are mediated through the isoprenoid pathway is currently unclear and needs further investigation.

All statins tested in our study reduced EBOV titers, although with variable efficacy. Simvastatin and pitavastatin inhibited EBOV production most potently (0.5- to 2.5-log reduction at 5 to 50 μ M concentrations). Differences in the antiviral effects of individual statins may be due to many factors, such as chemical structures of each compound affecting pharmacokinetics and pharmacodynamics (68). One limitation of our study is that higher concentrations of statins were required to inhibit EBOV replication *in vitro* than are achievable in humans using current dosing regimens, as plasma levels of statins are usually low (maximum concentration of drug in serum [C_{max}], 0.019 to 0.031 μ M for simvastatin and 0.005 μ M for lovastatin, based on 40-mg oral dose [69]). Although statin concentrations are likely to be much higher in the liver (69), a major site of EBOV replication, comprehensive *in vivo* studies in appropriate animal models are required. Unfortunately, statins do not reliably decrease circulating cholesterol concentrations in rodents (70–72), and thus, such studies would require nonhuman primate models that recapitulate human EVD signs (73).

In summary, we provide evidence that statin treatment decreases production of infectious EBOV virions in a human liver cell line (Huh7) and primary human macrophages, both of which are primary target cells for EBOV infection. Statin reduced production of infectious EBOV particles in Huh7 cells by interfering with GP processing and reducing the amount of GP₁ incorporated into virus particles. The results of this study clearly show that statin inhibits EBOV infection. Our results, combined with statins' known role in suppressing inflammation (74) and preserving endothelial integrity (75), pathways that are impaired in EVD, argue for a potential benefit of using statins as adjunctive therapy in patients with EVD. Ideally, the use of an antiviral that exhibits additional effects in combination with a statin has the potential both to block virus replication and to decrease the deleterious effects of inflammation on the host. Clearly, the next step for evaluating statins for use in EVD would require testing in a nonhuman primate model of disease to ensure both safety and potential efficacy.

MATERIALS AND METHODS

Biosafety. All work with infectious virus was conducted in a biosafety level 4 laboratory at the Centers for Disease Control and Prevention (CDC; Atlanta, GA) according to the guidelines of CDC standard operating procedures.

Cells, virus, plasmids, reagents, and antibodies. Huh7 cells were from Apath, LLC (Brooklyn, NY), and were propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) fetal calf serum

(FCS; HyClone, Thermo Fisher Scientific, Waltham, MA) and $1\times$ nonessential amino acids (Life Technologies, Grand Island, NY, USA). For statin treatment, sterol-depleted medium (LPDS; Sigma-Aldrich, St. Louis, MO, USA; not heat inactivated) was used instead of medium with FCS to eliminate the possible uptake of cholesterol from the medium, as previously reported (47). Human monocyte-derived macrophages were isolated as described previously (76). Vero-E6 cells were obtained from the CDC core facility and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) FCS and 1% penicillin-streptomycin (Life Technologies). Wild-type EBOV variant Mayinga (Ebola virus/H. sapiens-tc/COD/1976/Yambuku-Mayinga) was from the CDC Viral Special Pathogens Branch reference collection. Lovastatin was from Calbiochem (Billerica, MA). Human LDL was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mevalonate was synthesized according to the method of Goldstein et al. (77).

The plasmid encoding EBOV GP_{1,2} was designed to express a human codon-optimized synthetic gene (GPco) corresponding to that of the EBOV Mayinga isolate. Briefly, the GPco (without RNA editing site) gene was purchased from GenScript (Piscataway, NJ) and cloned into the polymerase II (PolII) expression vector pCAGGS (78). EBOV GP resistant to furin cleavage (RRTRR₅₀₁ cleavage site replaced with AGTAA) was purchased from GenScript as described previously (44, 45). The plasmid encoding Flag-tagged VP40 has been described previously (79).

The following antibodies were used in this study: rabbit polyclonal antibody against EBOV NP (IBT Bioservices, Rockville, MD), rabbit polyclonal antibody against EBOV GP (IBT Bioservices), and rabbit polyclonal antibody against EBOV for immunofluorescence (in-house reagent 70331; Viral Special Pathogens Branch, CDC, Atlanta, GA). The anti-Flag antibody and mouse monoclonal anti-actin antibody were from Sigma (Sigma-Aldrich, St. Louis, USA). NPC1 antibody was from Abcam (Cambridge, MA).

Transfection and infection. To determine the effects of statin on GP processing or VP40 expression, 2.0×10^5 Huh7 cells plated in 12-well plates were transiently transfected with plasmids expressing either EBOV GP or Flag-tagged VP40; transfections were done using LT-1 reagent according to the manufacturer's instructions (Mirus, Madison, WI). After 24 h, cells were treated with statin in LPDS-containing DMEM; cells were harvested 48 h posttransfection. For EBOV infection, Huh7 cells were plated at 2×10^5 cells per well in 12-well plates. The next day, cells were infected with EBOV at the indicated MOI for 1 h. For control experiments, Huh7 cells were infected with adenovirus type 5 (ATCC, Manassas, VA) at an MOI of 0.05 for 1 h. Virus inoculum was removed, and cells were washed with serum-free medium. Fresh medium containing 10% LPDS and with or without statin was then added. Culture supernatants and cell lysates were harvested and analyzed as indicated.

TCID₅₀ and cell viability determination. Supernatants from EBOV-infected Huh7 cells and monocyte-derived macrophages were harvested 72 hpi, and virus titrations were performed in Vero-E6 cells. Three days postinfection, the cells were fixed, permeabilized, and stained to visualize viral proteins. For adenovirus type 5 titer, Vero-E6 cells were treated with 8 serial 10-fold dilutions of supernatants of infected Huh7 cells. After 10 days, the wells with cytopathic effects were counted for each dilution after crystal violet staining. Endpoint viral titers were determined, and TCID₅₀ was calculated as described previously (80). Results represent mean titers, with error bars indicating standard deviations calculated from 3 independent experiments. For human monocyte-derived macrophages, results represent mean titers with standard deviations from 3 replicate wells, representative of 2 independent experiments.

Cell viability was determined on statin-treated and mock-infected cells, using CellTiter-Glo (Promega) according to the manufacturer's instructions.

qRT-PCR. Huh7 cells were infected with EBOV for the indicated times, and then RNA was isolated from cells or from supernatants of infected cells using the MagMAX-96 total RNA isolation kit (Thermo Fisher Scientific). To determine viral RNA copy numbers, RNA was extracted from supernatants of infected cells. Absolute quantification of viral RNA copy numbers was done by measuring EBOV NP copy numbers using a standard curve with known viral titers serially diluted 5-fold. qRT-PCR was performed with the EBOV NP assay (81). To measure cell-associated EBOV NP gene levels, Huh7 cells pretreated with statin for 48 h or with U18666A (positive control) for 1 h were infected with EBOV at an MOI of 3. Cells were harvested after 3 h, and RNA was isolated from the cells. NP RNA levels were measured by qRT-PCR as described above and normalized to GAPDH mRNA. Results represent mean percent normalized NP RNA levels, with error bars indicating standard deviations calculated from 3 independent experiments.

Western blotting. At indicated times after transfection or infection, cell lysates were harvested by adding lysis buffer containing 50 mM NaCl, 5 mM EDTA, 1% NP-40, 1.0% SDS, and 0.5% sodium deoxycholate supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Lysates from infected cells were gamma irradiated at 2×10^6 rads using a high-energy ⁶⁰Co source to ensure complete virus inactivation, allowing work at biosafety level 2. Proteins were electrophoretically separated on either 3 to 8% Tris-acetate or 4 to 12% NuPAGE gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with buffer containing Tris-buffered saline, 0.1% Tween 20, and 5% nonfat dry milk and then probed overnight at 4°C with primary antibodies. Membranes were developed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. After detection of primary antibodies, the membranes were stripped and reprobed with antiactin antibody as a loading control. Results shown are representative of 3 independent experiments.

Immunofluorescence. At 72 hpi, the cells were washed twice with phosphate-buffered saline (PBS) and fixed with 10% formalin at room temperature for 20 min. After formalin fixation, the cells were washed three times with PBS and permeabilized with 0.1% Triton X-100 for intracellular staining. The primary antibodies were added at a 1:1,000 dilution in 1% bovine serum albumin in PBS for 1 h. The cells were then washed 3 times with PBS and incubated for 30 min with the secondary antibodies diluted 1:1,000 in 1% bovine serum albumin in PBS. Multiple final washes were done, and the images were taken using a Nikon Eclipse Ti-S.

Virion purification. EBOV virions were partially purified similarly to the procedure reported for Lassa virus (82). Briefly, supernatants from DMSO- or statin-treated and EBOV-infected cells were clarified by centrifugation at $1,500 \times g$ for 30 min. Clarified supernatants were subjected to ultracentrifugation ($100,000 \times g$ for 90 min at 4°C) through a 20% sucrose cushion to collect EBOV virions. Virions were suspended in $2\times$ Western lysis buffer, gamma irradiated at 5×10^6 rads using a high-energy ^{60}Co source, and analyzed by Western blotting to detect GP and VP40.

Endo H and PNGase F treatment. In order to investigate the modifications of EBOV glycoproteins, cell lysates were digested with endo H or PNGase F (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. The digested proteins were resolved by SDS-PAGE under reducing conditions and were analyzed by Western blotting.

Statistical analysis. Error bars in graphs represent standard deviations of the means from comparing Student's *t* tests for paired samples. Differences were considered significant for *P* values of <0.005 .

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.00660-18>.

FIG S1, TIF file, 22.6 MB.

FIG S2, TIF file, 22.7 MB.

FIG S3, TIF file, 22.9 MB.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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