Type I Interferon Counteracts Antiviral Effects of Statins in the Context of Gammaherpesvirus Infection

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ABSTRACT

The cholesterol synthesis pathway is a ubiquitous cellular biosynthetic pathway that is attenuated therapeutically by statins. Importantly, type I interferon (IFN), a major antiviral mediator, also depresses the cholesterol synthesis pathway. Here we demonstrate that attenuation of cholesterol synthesis decreases gammaherpesvirus replication in primary macrophages *in vitro* and reactivation from peritoneal exudate cells *in vivo*. Specifically, the reduced availability of the intermediates required for protein prenylation was responsible for decreased gammaherpesvirus replication in statin-treated primary macrophages. We also demonstrate that statin treatment of a chronically infected host attenuates gammaherpesvirus latency in a route-of-infection-specific manner. Unexpectedly, we found that the antiviral effects of statins are counteracted by type I IFN. Our studies suggest that type I IFN signaling counteracts the antiviral nature of the subdued cholesterol synthesis pathway and offer a novel insight into the utility of statins as antiviral agents.

IMPORTANCE

Statins are cholesterol synthesis inhibitors that are therapeutically administered to 12.5% of the U.S. population. Statins attenuate the replication of diverse viruses in culture; however, this attenuation is not always obvious in an intact animal model. Further, it is not clear whether statins alter parameters of highly prevalent chronic herpesvirus infections. We show that statin treatment attenuated gammaherpesvirus replication in primary immune cells and during chronic infection of an intact host. Further, we demonstrate that type I interferon signaling counteracts the antiviral effects of statins. Considering the fact that type I interferon decreases the activity of the cholesterol synthesis pathway, it is intriguing to speculate that gammaherpesviruses have evolved to usurp the type I interferon pathway to compensate for the decreased cholesterol synthesis activity.

Intracellular cholesterol is acquired via two major mechanisms: internalization of exogenous cholesterol via the low-density lipoprotein receptor (LDLR) and endogenous cholesterol synthesis. The latter mechanism (Fig. 1A) operates in all cell types and is positively regulated by sterol regulatory element-binding protein 2 (Srebp2). Srebp2, a transcription factor synthesized as an inactive membrane-associated precursor, is cleaved by Golgi apparatus-specific proteases under conditions of low cholesterol to release an active product (1). This active cleaved product induces expression of most enzymes involved in cholesterol synthesis, including the rate-limiting enzyme hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR) (2). Active Srebp2 also increases the expression of LDLR to promote the acquisition of exogenous cholesterol. Due to the critical role of cholesterol for cell viability, genetic disruption of Srebp2 leads to early embryonic lethality (3).

Elevated plasma cholesterol levels are a potent risk factor for atherosclerosis, and the targeting of the cholesterol synthesis pathway using HMGCR agents (statins) has proven to be one of the most successful medical therapies. Statins are prescribed to ~12.5% of the adult population in the United States. Interestingly, statins decrease the replication of several RNA and DNA viruses in tissue culture (4–16). In some cases, statin-mediated attenuation of endogenous cholesterol synthesis is thought to interfere with viral entry or decrease the abundance of intracellular membranes that serve as platforms for viral replication and assembly/maturation. Additionally, intermediates of the cholesterol

synthesis pathway serve as the substrates for protein prenylation, a process that involves a covalent attachment of either geranylgeranyl or farnesyl to select intracellular proteins (Fig. 1A). Protein prenylation regulates protein localization and function and, as such, is important for several cellular pathways, including those regulated by small GTPases (17). Not surprisingly, some of the *in vitro* antiviral effects of statins have been ascribed to the reduced prenylation of virus or host proteins (4, 9, 10).

Intriguingly, the *in vitro* and *in vivo* antiviral effects of statins do not uniformly correlate. For some viruses (i.e., respiratory syncytial virus and hepatitis C virus), statins decrease viral replication both *in vitro* and *in vivo* (5, 18, 19). In contrast, in spite of the antiviral effects of statins on influenza virus replication in tissue culture, treatment of influenza virus-infected mice with statins either has no effect on viral replication, clearance, and lung pa-

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FIG 1 Disruption of the cholesterol biosynthesis pathway attenuates MHV68 replication in primary macrophages. (A) Diagrammatic representation of the key intermediates within the cholesterol biosynthesis pathway. The rate-limiting enzyme, HMG-CoA reductase, is pharmacologically inhibited by statins. Prenylation of proteins is mediated by the prenyltransferases using the indicated intermediates. PP, pyrophosphate. (B, C) Bone marrow-derived macrophages from BL6 mice were infected at an MOI of 0.01 PFU/cell (B) or 5 PFU/cell (C) and treated with the dimethyl sulfoxide (DMSO) carrier or 10 μ M pravastatin following virus adsorption and for the duration of the experiment. The viral titers were determined in triplicate cultures at the indicated time points for each condition. (D) Bone marrow-derived macrophages from mice heterozygous (*Srebp2*^{hypo/wt}) or homozygous (*Srebp2*^{hypo/hypo}) for a hypomorphic *Srebp2* allele were analyzed for the baseline expression of *Srebp2*-dependent genes via qRT-PCR. *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase. (E, F) Bone marrow-derived macrophages from *Srebp2*^{hypo/wt} and *Srebp2*^{hypo/hypo} mice were infected at an MOI of 0.01 PFU/cell (E) or 5 PFU/cell (F). The viral titers were determined in triplicate cultures at the indicated time points for each condition. Data are representative of those from 2 to 4 independent experiments. Here and throughout the study, error bars represent the standard errors of the means. *, $P < 0.05$.

thology or actually decreases survival rates (20–22). Interestingly, statin treatment of older hospitalized patients suffering from seasonal influenza correlates with improved overall survival; however, the factors directing improved survival are not known (23).

Along with experiencing intermittent acute viral infections, most humans acquire lifelong herpesvirus infections that are associated with diverse clinical manifestations ranging from mucosal lesions to cancer. In spite of the widespread use of statins, the

effects of these inhibitors on chronic human herpesvirus infections are poorly understood. Gammaherpesviruses, the focus of this study, establish lifelong infection in >95% of adults worldwide and are associated with several types of cancer (24). While simvastatin stimulates apoptosis of Epstein-Barr virus (EBV)-positive lymphoblastoid cells (25), the effect of statins on chronic EBV infection and viral lymphomagenesis in humans remains unknown. Studies of the interaction between EBV and the cholesterol synthesis pathway are limited by the exquisite species specificity of human gammaherpesviruses. To overcome this limitation, this study utilizes mouse gammaherpesvirus 68 (MHV68). MHV68 is genetically and biologically related to human gammaherpesviruses, including EBV (26, 27), and MHV68-infected mice offer a powerful animal model of gammaherpesvirus infection. In this study, we show that the pharmacologic or genetic attenuation of the cholesterol synthesis pathway decreased MHV68 replication in primary macrophage cultures. Intriguingly, attenuation of chronic infection by statins was dependent upon the route of initial virus inoculation. Unexpectedly, we found that type I interferon (IFN) signaling counteracted the antiviral effects of statins, offering an insight into the potential of statins as antiviral therapy.

MATERIALS AND METHODS

Ethics statement. All experimental manipulations of mice were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin (AUA971). All animal experiments adhered to the recommendations of the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (28) and the American Veterinary Medical Association guidelines on euthanasia.

Animal infection and primary cell cultures. C57BL/6 (BL6) mice, mice with a hypomorphic *Srebp2* allele (*Srebp2^{hyp}*) (29), and *IFNAR1^{-/-}* mice (30) were housed and bred at the Medical College of Wisconsin in a specific-pathogen-free facility in accordance with all federal and institutional guidelines. At 6 to 7 weeks of age, the mice were intranasally inoculated with 500 PFU of MHV68 (WUMS strain) or 15 μ l of phosphate-buffered saline (PBS; the carrier) while they were under light anesthesia; for intraperitoneal infection, virus was diluted in a volume of 300 μ l. In some experiments the mice were treated with lovastatin (20 mg/kg of body weight; Cayman Chemical, Ann Arbor, MI). Immediately before each treatment stock lovastatin solution dissolved in ethanol or a corresponding amount of ethanol was mixed with sterile PBS and the mixture was injected intraperitoneally. Mice were euthanized by CO₂ inhalation from a compressed gas source in a nonovercrowded chamber, as mandated by the *Guide for the Care and Use of Laboratory Animals* (28) and the American Veterinary Medical Association Panel on Euthanasia. Limiting-dilution assays were performed on splenocytes and peritoneal exudate cells to determine the frequency of cells harboring a viral genome or supporting viral reactivation, as previously described (31). For studies of acute infection, lungs were homogenized and the virus titer was determined using NIH 3T12 cells. For *in vitro* studies, bone marrow was harvested from mice when they were between 3 and 10 weeks of age. Primary bone marrow-derived macrophages were generated as previously described (32).

qRT-PCR analysis. Total RNA was harvested, DNase treated, reverse transcribed, and analyzed by quantitative reverse transcription-PCR (qRT-PCR) (33). Analysis was performed using the following primers: IDI1 Forward (5'-GCC-AGC-AAC-AAC-CAG-AAT-TT-3'), IDI1 Reverse (5'-GTA-TGT-TTC-CTC-AGC-CCT-ACT-C-3'), *SREBP2* Forward (5'-CTC-ACT-CCT-ACC-TCC-CAT-AGA-A-3'), and *SREBP2* Reverse (5'-AAA-TGA-GAG-GCT-GGT-TGC-T-3').

Viral stock preparation and infections. MHV68 stocks were prepared and titers were determined on NIH 3T12 cells (32). Bone marrow-derived

macrophages were infected with MHV68 at the multiplicity of infection (MOI) indicated below for 1 h to allow adsorption and washed twice with PBS prior to medium replenishment. Supplementation experiments were performed by adding the indicated compound(s) to the replenishment medium following viral adsorption. Pravastatin, lovastatin, squalene, geranylgeranyl pyrophosphate, and prenyltransferase inhibitors were purchased from Cayman Chemical (Ann Arbor, MI). Mevalonolactone was from Sigma-Aldrich (St. Louis, MO). Recombinant mouse beta 1 IFN (IFN- β 1) was purchased from BioLegend (San Diego, CA).

Western blot analyses. Cell lysates were collected and analyzed as previously described (33). The antibodies used were anti- β -actin (1:20,000; Novus Biological, Littleton, CO), anti-Srebp2 (1:1,000; Abcam, Cambridge, MA), and a secondary goat anti-mouse or anti-rabbit immunoglobulin horseradish peroxidase-conjugated secondary antibody (1:25,000; Jackson ImmunoResearch, West Grove, PA).

Statistical analyses. Statistical analyses were performed using Student's *t* test (Prism; GraphPad Software).

RESULTS

The cholesterol synthesis pathway facilitates gammaherpesvirus replication in an MOI-dependent manner. To determine the extent to which statins affect MHV68 replication *in vitro*, primary macrophages, a physiologically relevant cell type (34), were infected at either a low or a high multiplicity of infection (MOI), and cultures were treated with pravastatin immediately following viral adsorption. The dose of pravastatin used was the lowest dose that induced maximum Srebp2 activation in primary macrophages; this dose was not associated with toxicity (data not shown). A significant decrease in MHV68 replication was found in statin-treated macrophages under conditions with a low but not a high MOI (Fig. 1B and C).

Next, a genetic system was used to define the effect of the cholesterol synthesis pathway on MHV68 replication. Because the genetic disruption of Srebp2 leads to early embryonic lethality (3), we utilized primary macrophages derived from mice homozygous for a hypomorphic *Srebp2* (*Srebp2^{hyp}*) allele (29). The *Srebp2^{hyp}* allele arose as a spontaneous mutation resulting in the R1038C substitution within the C-terminal regulatory domain of Srebp2. Homozygosity for the *Srebp2^{hyp}* allele caused decreased expression of Srebp2-regulated genes in primary macrophages (Fig. 1D). Interestingly, genetic attenuation of Srebp2 produced viral replication phenotypes that were similar to those observed in statin-treated macrophages: decreased MHV68 replication under conditions with a low but not a high MOI (Fig. 1E and F). Thus, pharmacologic or genetic attenuation of the cholesterol synthesis pathway in primary macrophages decreased MHV68 replication *in vitro* in an MOI-dependent manner.

Cholesterol pathway prenylation intermediates facilitate gammaherpesvirus replication. Diverse viruses utilize the cholesterol synthesis pathway to form membrane scaffolds for viral replication or as a source of the farnesyl and geranyl used for prenylation of viral and/or host proteins (4, 10, 18). To determine the step of the cholesterol synthesis pathway that facilitates MHV68 replication, statin-treated macrophages were supplemented with cholesterol synthesis intermediates downstream of HMG-CoA (Fig. 1A) and viral replication was examined. The attenuation of MHV68 replication observed in pravastatin-treated macrophages was rescued by mevalonate, a cholesterol synthesis intermediate downstream of HMGCR (Fig. 1A and 2A). This observation suggested that the statin-mediated attenuation of MHV68 replication is due to the inhibition of the cholesterol syn-

FIG 2 Cholesterol pathway intermediates facilitate gammaherpesvirus replication. Primary macrophages were derived from BL6 mice and infected at the indicated MOI (in numbers of PFU per cell). (A) Immediately following viral adsorption, macrophages were treated with DMSO (carrier), 20 μ M pravastatin, 20 μ M geranylgeranyl pyrophosphate (GG-PP), 200 μ M mevalonate, or a combination of a cholesterol synthesis intermediate and pravastatin. (B to E) Macrophages were treated (following viral adsorption) with either DMSO or the indicated prenyltransferase inhibitor (10 μ M). GGTI, geranylgeranyl transferase inhibitor; FTL, farnesyl transferase inhibitor. (F) Macrophages were treated with 20 μ M pravastatin (Prava), 100 μ M squalene, or a combination of both. The viral titers were determined in triplicate cultures at the indicated time points for each condition. Data are representative of those from 2 to 3 independent experiments.

thesis pathway and not off-target effects. Supplementation of pravastatin-treated macrophages with geranylgeranyl pyrophosphate, an intermediate used for protein geranylation, also rescued MHV68 replication (Fig. 2A), suggesting that steps in the cholesterol synthesis pathway at or downstream of prenylation facilitated viral replication (Fig. 1A).

To specifically address the extent to which protein prenylation facilitates MHV68 replication, infected primary macrophages were treated with the inhibitors of farnesyl transferase (FT) or geranylgeranyl transferase (GGT), two enzymes that mediate protein prenylation using intermediates of the cholesterol synthesis pathway. Treatment with the GGT inhibitor attenuated MHV68

replication at a low but not a high MOI (Fig. 2B and C). A similar outcome was observed when infected macrophages were treated with the FT inhibitor (Fig. 2D and E). Thus, inhibition of protein prenylation attenuated MHV68 replication in an MOI-dependent manner, similar to what was observed with statin treatment.

To determine the extent to which cholesterol synthesis intermediates downstream of prenylation substrates were important for MHV68 replication, statin-treated macrophages were supplemented with squalene. Squalene supplementation failed to rescue MHV68 replication in statin-treated macrophages (Fig. 2F). Thus, MHV68 replication was supported by intermediates of the cholesterol synthesis pathway utilized for protein prenylation.

Srebp2 activation is attenuated during early stages of MHV68 replication, in part through induction of type I IFN. The transcription factor Srebp2, a critical inducer of many cholesterol synthesis genes, is produced as an inactive endoplasmic reticulum-associated precursor that undergoes proteolytic cleavage and activation at the Golgi apparatus. The levels of active, cleaved Srebp2 are decreased in the presence of type I IFN (35). Correspondingly, type I IFN treatment decreases the level of expression of enzymes involved in the cholesterol synthesis pathway (35). Importantly, type I IFN is robustly induced in primary macrophage cultures infected with MHV68 at a high MOI (36), suggesting the possibility that type I IFN may regulate Srebp2 activity during MHV68 infection. To determine the extent to which type I IFN decreased Srebp2 activation during MHV68 infection, primary macrophages were derived from BL6 or IFNAR1^{-/-} mice, with macrophages from the latter lacking the ability to respond to type I IFN. Compared to the levels of active Srebp2 in mock-infected BL6 mouse macrophages, the levels of active Srebp2 were significantly decreased in BL6 mouse macrophages at 7 h postinfection, an early stage in viral replication characterized by low levels of expression of immediate early and early genes (33) (Fig. 3A). The levels of active Srebp2 in infected BL6 mouse macrophages increased as viral replication progressed. In contrast, the decrease in the amount of active Srebp2 was not as pronounced in IFNAR1^{-/-} mouse macrophages at 7 h postinfection (Fig. 3A). Correspondingly, expression of Srebp2-dependent genes was modestly decreased at 7 h postinfection in BL6 but not IFNAR1^{-/-} mouse macrophages (Fig. 3B). In contrast, by 24 h postinfection, the levels of IDI1 and Srebp2 mRNA showed a modest increase in infected BL6 and IFNAR1^{-/-} mouse macrophages compared to the levels in their respective mock-infected samples (Fig. 3C). Thus, type I IFN contributed to the decreased Srebp2 activity in MHV68-infected primary macrophages.

Inhibition of the cholesterol synthesis pathway does not suppress acute MHV68 replication *in vivo*. Considering the somewhat disparate results between the *in vitro* and *in vivo* effects of statins on viral replication in other systems, acute MHV68 infection was evaluated in statin- or carrier-treated BL6 mice. BL6 mice were treated with lovastatin or carrier (ethanol) every 2 days beginning at 4 days prior to infection, and lung virus titers were measured at 7 days postinfection (Fig. 4A). Statin treatment of mice prior to infection ensured the establishment of the systemic biological activity of statins throughout the course of acute infection (data not shown). Surprisingly, lovastatin-treated mice displayed slightly higher peak viral titers than the carrier-treated group, although the difference was not

FIG 3 SREBP2 activation is attenuated early in MHV68 infection. Bone marrow-derived macrophages from BL6 and IFNAR^{-/-} mice were infected with MHV68 at an MOI of 5 PFU/cell. Cells were collected and lysed at the indicated time points. Lysates were analyzed for active SREBP2 by Western blotting (A) or analyzed for SREBP2 and IDI1 expression via qRT-PCR (B, C). Data are normalized to those for wild-type mock-infected macrophages, and data from 2 to 3 independent experiments were pooled.

statistically significant (Fig. 4B). Thus, statin treatment did not decrease peak lung MHV68 titers during acute infection of BL6 mice.

Inhibition of the cholesterol synthesis pathway suppresses chronic gammaherpesvirus infection following inoculation by the intraperitoneal but not the intranasal route. Having determined that statin treatment does not attenuate peak MHV68 titers during acute infection, we next determined the effect of statins on latent MHV68 infection. Because a majority of humans are expected to already harbor herpesvirus infections prior to statin treatment, BL6 mice were first infected with MHV68 and subsequently treated with statin or the carrier starting at 10 days postinfection (Fig. 4G), a time when most infectious virus is cleared and latent infection is being established. At 21 days postinfection (11 days of treatment), latency parameters were examined in the spleen and peritoneum, two sites that support latent MHV68 infection. Because the effects of the cholesterol synthesis pathway were initially observed in macrophages and macrophages are thought to be the primary reservoir of the virus in the peritoneum

Acute infection

Latent infection, intraperitoneal inoculation

FIG 4 Statin treatment suppresses chronic MHV68 infection following inoculation by the intraperitoneal (I.P.) route. (A) Experimental design of acute infection studies. BL6 mice were treated with 20 mg/kg lovastatin or the ethanol carrier via intraperitoneal injection beginning at 4 days prior to infection. Treatment was continued every 2 days throughout the remainder of the experiment. On day 0 the mice were infected intranasally with 500 PFU of wild-type MHV68. (B) Lung MHV68 titers at 7 days postinfection. Each symbol represents the result for an individual animal. (C to H) BL6 mice were infected with 100 to 1,000 PFU of MHV68 via intraperitoneal injection and treated every other day with 20 mg/kg lovastatin beginning at 10 days postinfection (panel G shows the experimental design). At 21 days postinfection, the frequency of MHV68 reactivation (C, E) or MHV68 DNA-positive cells (D, F) in splenocytes (C, D) or peritoneal exudate cells (PEC) (E, F) was determined. (H) The absolute number of latently infected peritoneal cells in the indicated groups. Prior to the analyses, splenocytes and peritoneal cells from 4 to 5 mice/group in each experiment were pooled. Data from 2 to 3 independent experiments were pooled.

(34), parameters of viral latency were first examined following infection by the intraperitoneal route.

Low but similar levels of MHV68 reactivation from splenocytes were observed in both experimental groups (Fig. 4C). Further, the frequency of MHV68 DNA-positive splenocytes was also similar in carrier- and lovastatin-treated mice (Fig. 4D). In contrast, the frequency of MHV68 reactivation and MHV68 DNA-positive cells and the absolute number of virus-infected cells were significantly decreased in peritoneal cells from lovastatin-treated mice (at least 30-fold; Fig. 4E, F, and H). No persistent MHV68 replication was observed at either site (data not shown). Thus, statin treatment of intraperitoneally inoculated chronically infected mice led to a significant attenuation of MHV68 latency in the peritoneum but not the spleen.

As the parameters of MHV68 chronic infection are modified by the initial route of inoculation (37), the effect of statin treatment was examined in intranasally inoculated mice treated with statins following the resolution of the acute infection (Fig. 5E). As observed following intraperitoneal inoculation, lovastatin treatment had no effect on the parameters of splenic MHV68 infection (Fig. 5A and B). Intranasal inoculation resulted in minimal virus reactivation from peritoneal cells at 24 days postinfection in both experimental groups (Fig. 5C). However, in contrast to what was seen following intraperitoneal infection (Fig. 4F), statin treatment of intranasally infected mice led to much smaller decreases in the frequency and the absolute number of MHV68 DNA-positive peritoneal cells (~ 4 -fold, $P = 0.06$; Fig. 5D and F for frequency and absolute numbers, respectively). No persistent replication was observed at any of the examined sites (data not shown). Thus, statin treatment of latently infected mice had anatomic site- and route-of-inoculation-specific effects on the attenuation of MHV68 latency.

Type I IFN signaling counteracts the antiviral effects of statins *in vivo*. Type I IFN is an effective host response to gammaherpesvirus infection and has multiple antiviral effects that span the acute and chronic phases of MHV68 infection (38, 39). Interestingly, type I IFN signaling attenuates Srebp2 activation (35), including in MHV68-infected primary macrophages (Fig. 3). To define the potential interplay between cholesterol synthesis and type I IFN *in vivo*, BL6 or IFNAR1^{-/-} mice were treated with statin or the carrier starting at 4 days prior to infection and every 2 days thereafter for the duration of the experiment (Fig. 6A). The lung titers of MHV68 were analyzed at 10 days postinfection, a time when type I IFN is critical for the control of acute MHV68 replication (40). As expected, most carrier-treated BL6 mice cleared infectious MHV68 by 10 days postinfection (Fig. 6B). Interestingly, measurable lung MHV68 titers were observed in a majority of BL6 mice treated with lovastatin (a 27-fold increase in titer versus that for the carrier-treated group; Fig. 6B), suggesting that ongoing lovastatin treatment may actually delay infectious MHV68 clearance in wild-type mice.

As expected, lung MHV68 titers were significantly increased in carrier-treated IFNAR1^{-/-} mice compared to those in carrier-treated BL6 mice ($\sim 65,000$ -fold; Fig. 6B). Unexpectedly, treatment of IFNAR1^{-/-} mice with lovastatin reduced the lung MHV68 titers to the level observed in carrier-treated BL6 mice (Fig. 6B), suggesting that type I IFN counteracts the antiviral effects of lovastatin during acute MHV68 infection.

To determine the extent to which lovastatin could exert its antiviral effects in already infected animals, IFNAR1^{-/-} mice

were intranasally infected with MHV68 and treated with the carrier or lovastatin starting at 8 days postinfection (Fig. 6G). Lovastatin treatment did not alter either the frequency of MHV68 reactivation or the frequency of MHV68 DNA-positive cells in IFNAR1^{-/-} mouse spleens (Fig. 6C and D). In contrast to what was observed in intranasally inoculated BL6 mice (Fig. 5), lovastatin treatment significantly decreased the frequency of reactivation and MHV68 DNA-positive cells in the peritoneal cells of intranasally infected IFNAR1^{-/-} mice (>48 -fold and 23-fold, respectively; Fig. 6E and F). As in BL6 mice, no persistent replication was observed at 21 days postinfection in IFNAR1^{-/-} mice (data not shown). In summary, the antiviral effects of lovastatin against MHV68 were revealed by type I IFN deficiency in the context of both acute and chronic infections.

Type I interferon signaling counteracts the antiviral effects of statins *in vitro*. Having observed a significant antiviral effect of lovastatin in IFNAR1^{-/-} mice, we next determined the extent to which this antiviral effect is intrinsic to infected cells. In contrast to the rapid and significant type I IFN responses induced by MHV68 at a high MOI (36), MHV68 infection of primary macrophages at a low MOI was associated with very low type I IFN signaling for at least the first 3 to 4 days of infection (data not shown). Incidentally, statin treatment attenuated MHV68 replication *in vitro* under low-MOI but not high-MOI conditions (Fig. 1). To determine whether type I IFN treatment could rescue MHV68 replication in statin-treated cells, primary macrophages were infected at a low MOI and treated with pravastatin, type I IFN, or a combination of both. As expected, pravastatin treatment of primary macrophages attenuated viral replication, whereas low levels of type I IFN did not lead to a significant attenuation of MHV68 replication in statin-free macrophages at 48 h postinfection (Fig. 7A). However, when combined with pravastatin treatment, a low level of type I IFN was able to partially rescue MHV68 replication (Fig. 7A).

Similar results were obtained using genetic attenuation of the cholesterol synthesis pathway. Specifically, while low levels of type I IFN had a modest antiviral effect at 6 days postinfection of wild-type macrophages, the attenuated MHV68 replication observed in Srebp2^{hypo/hypo} mouse macrophage cultures was increased by type I IFN treatment (Fig. 7B). Thus, type I IFN counteracted the antiviral effects of the attenuated cholesterol synthesis pathway.

Finally, the effects of statins on MHV68 replication were examined under high-MOI conditions in IFN response-deficient macrophages. As previously demonstrated (36), high-MOI conditions resulted in significantly higher levels of MHV68 replication in carrier-treated macrophages from IFNAR1^{-/-} mice than in carrier-treated macrophages from BL6 mice (Fig. 7C). In spite of this greatly increased level of viral replication, treatment of IFNAR1^{-/-} mouse macrophages with pravastatin achieved a modest, statistically significant decrease in MHV68 yield (Fig. 7C). In contrast, pravastatin-treated MHV68-infected BL6 mouse macrophages produced virus titers similar to those produced by the carrier-treated controls. Thus, in agreement with our observations *in vivo*, type I IFN counteracted the antiviral effects of statins in primary macrophage cultures during *in vitro* infection. These findings suggest that MHV68 not only tolerates low-level type I IFN signaling but may actually usurp this antiviral pathway to facilitate viral replication in the context of attenuated host cholesterol synthesis.

Latent infection, intranasal inoculation

FIG 5 Statin treatment has a minimal effect on chronic MHV68 infection in intranasally (I.N.) inoculated BL6 mice. BL6 mice were intranasally infected with 500 PFU of wild-type MHV68 and treated every other day with 20 mg/kg lovastatin or ethanol carrier beginning at 10 days postinfection (E). The frequency of viral reactivation (A, C) or MHV68 DNA-positive cells (B, D) in splenocytes (A, B) or peritoneal cells (C, D) harvested at 24 days postinfection was determined. (F) The absolute number of latently infected peritoneal cells in the indicated groups. Prior to the analyses, splenocytes and peritoneal exudate cells from 4 to 5 mice/group in each experiment were pooled. Data from 2 to 3 independent experiments were pooled.

DISCUSSION

Working model. In this study, we demonstrated that MHV68 uses the host cholesterol synthesis pathway to facilitate viral replication and reactivation, likely through the use of cholesterol synthesis intermediates for protein prenylation. Our study has also uncovered an unexpected interaction between MHV68 and the cholesterol synthesis pathway that is modified by type I IFN signaling. Based on the insights provided by this study, we propose

the following working model (Fig. 7D). MHV68 infection induces type I IFN expression and signaling. This type I IFN signaling attenuates the activity of the cholesterol synthesis pathway by decreasing the levels of active Srebp2. MHV68, however, is able to usurp low-level type I IFN signaling to overcome the decreased activity of the cholesterol synthesis pathway. While several mechanisms may underlie this phenomenon, it is intriguing to speculate that type I IFN modifies protein prenylation processes in

FIG 6 Type I interferon counteracts the antiviral effects of statins. (A, B) BL6 and IFNAR1^{-/-} mice were treated with 20 mg/kg lovastatin or the ethanol carrier via intraperitoneal injection beginning 4 days prior to MHV68 infection. Treatment was continued every 2 days throughout the remainder of the experiment. On day 0 the mice were infected intranasally with 500 PFU of MHV68. Lung MHV68 titers were determined at 10 days postinfection; each symbol represents the result for an individual animal. (C to G) IFNAR1^{-/-} mice were intranasally infected with 100 to 500 PFU of MHV68 and treated every other day with 20 mg/kg lovastatin or the ethanol carrier beginning at 8 days postinfection (G). At 24 days postinfection, parameters of viral latency were determined as described in the legend to Fig. 3. Prior to the analyses, splenocytes and peritoneal exudate cells from 4 to 5 mice/group in each experiment were pooled. Data from 2 to 3 independent experiments were pooled.

FIG 7 Type I interferon signaling counteracts the antiviral effects of statins *in vitro*. (A and B) Primary bone marrow-derived wt or Srebp2^{hypo/hypo} macrophages were infected with MHV68 at an MOI of 0.01 PFU/cell. Following viral adsorption, macrophages were replenished with medium containing pravastatin (20 μ M), IFN- β (0.1 U/ml), or a combination of pravastatin and IFN- β (A) or with IFN- β (0.1 U/ml) (B). Viral titers were determined at 48 h (A) or 144 h (B) postinfection. Data are representative of those from at least 2 independent experiments. *, $P < 0.05$. (C) Primary bone marrow-derived macrophages of the indicated genotypes were infected at an MOI of 5 PFU/cell. Following viral adsorption, the macrophage cultures were replenished with medium containing pravastatin or the carrier. Viral titers were determined at 72 h postinfection. (D) Working model. Infection with MHV68 leads to the induction of type I IFN, which, in turn, downregulates the cholesterol synthesis pathway in a Srebp2-dependent manner. The virus usurps type I IFN signaling to counteract the antiviral effect of cholesterol synthesis downregulation, presumably by modifying protein prenylation. When the cholesterol synthesis pathway is inhibited in the absence of sufficient type I IFN signaling, the virus is unable to compensate, resulting in decreased viral replication.

fecting macrophages to benefit viral replication. Accordingly, artificial attenuation of the cholesterol synthesis pathway by statins leads to viral demise under circumstances when type I IFN signaling is insufficient (i.e., replication in cultured macrophages under low-MOI conditions) or absent (in IFNAR1^{-/-} mice).

Statins and gammaherpesvirus infection. Our study offers the first comprehensive analysis of the effect of statins on chronic gammaherpesvirus infection, providing a potential insight into the modification of chronic herpesvirus infections in a significant number of individuals treated with statins. In spite of the prevalent use of statins, the status of herpesvirus activity in statin-treated individuals has not been assessed, with the exception of a single study that found a small increase in the incidence of zoster in statin users (41). Importantly, markers of inflammation/immune activity were not taken into account in this study. We found that, in contrast to *in vitro* findings (Fig. 1), treatment of BL6 mice with statins prior to and during acute MHV68 infection did not attenuate MHV68 replication but, instead, produced a mild delay in the clearance of infectious virus. These results are in agreement with similar findings in the influenza virus system, where the clearly delineated antiviral effects of statins *in vitro* failed to man-

ifest in influenza virus-infected wild-type mice (20, 21). In contrast, treatment of IFNAR1^{-/-} mice with statins dramatically reduced acute MHV68 replication in the lungs, supporting the model where MHV68 usurps type I IFN signaling to overcome the antiviral effects of statins. Interestingly, in contrast to the findings of the animal studies, statin treatment of older patients hospitalized with severe influenza improved overall survival (23). Interestingly, dendritic cells from older donors display impaired type I IFN signaling in response to West Nile virus (42), suggesting an intriguing possibility that attenuated type I IFN signaling in elderly patients may facilitate the antiviral effects of statins against influenza virus. Accordingly, the high levels of type I IFN expression in wild-type mice infected with influenza virus may have counteracted the antiviral effects of statins *in vivo*.

Intriguingly, in the context of chronic infection, the antiviral effects of statins were modified by both the route and the anatomic site of infection. The antiviral effects of statins were most prominent following intraperitoneal inoculation compared with their effects following intranasal inoculation, in spite of the fact that virus inoculation and statin treatment were separated by 10 days. Further, the antiviral effects of statins were limited to the peritoneal cells and were not found in splenocytes. One possible expla-

nation for the observed phenotypes is the differential tropism of MHV68 in the spleen and peritoneum. In the spleen, a majority of latent virus is hosted by B cells, with an intricate B cell differentiation program defining viral reactivation (43–45). In contrast, macrophages support MHV68 latency in the peritoneum (34). Considering this differential tropism, the antiviral effects of lovastatin on peritoneal MHV68 latency are consistent with the antiviral activity of statins that we observed in the context of *in vitro*-infected primary macrophages.

However, even within the peritoneal cells, the extent of lovastatin antiviral activity was further modified by the route of infection. While it is possible that the route of viral infection modifies MHV68 tropism in the peritoneum, another possibility is that the route of infection shapes the extent of the host's type I IFN response. Introduction of MHV68 via the respiratory mucosa is expected to induce a robust type I IFN response. It is not clear whether the same robust type I IFN response is induced following intraperitoneal inoculation. The peritoneal cavity may offer a more immunosuppressive environment than the respiratory mucosa, in part due to the constitutive expression of interleukin-10 by peritoneal B cells (46, 47). It is also not clear whether type II IFN can substitute for type I IFN in the rescue of MHV68 replication. In our study, the greatest antiviral activity of statins *in vivo* was observed in IFNAR1^{−/−} mice, which express IFN-γ at levels similar to or higher than those detected in BL6 mice (data not shown), suggesting that the role of type I IFN may be unique.

Statins and the mechanism of antiviral activity. The cholesterol synthesis pathway facilitates the replication of diverse RNA and DNA viruses *in vitro* (4–16), similar to what was observed for MHV68 (Fig. 1 and 2). MHV68 replication benefited from the cholesterol synthesis intermediates used for protein prenylation (Fig. 2). While the protein(s) that assists with MHV68 replication upon prenylation remains unknown, one set of candidates is the cellular GTPases. These small signaling molecules are engaged during EBV latency or have been implicated in MHV68 spread (48–50). Although it is possible that a viral protein may be the target of prenylation, our bioinformatics screens failed to identify an annotated MHV68 protein with a conserved CAAX motif recognized by farnesyl or geranyl transferases (data not shown).

An important question to be solved in future studies is how type I IFN rescues MHV68 replication during statin exposure. One possibility is that type I IFN modifies the host prenylome and/or the specific activity of prenyltransferases to assist with viral replication under conditions when the cholesterol synthesis pathway is subdued. Under this scenario, unbiased analysis of the prenylome in MHV68-infected cells should offer insight into the specific pathways that may be targeted by interferon to compensate for the decreased prenylation. An argument for the positive effect of type I IFN on selective protein prenylation is based on the fact that mouse GBP-2, a GTPase highly induced by type I IFN, is readily prenylated, and this prenylation regulates its localization and function (51). Finally, it is intriguing to propose that MHV68 may further usurp and/or redirect the prenylation mechanisms modified by type I IFN to facilitate viral replication.

In summary, our studies reveal a complex interaction between chronic virus infection, the cholesterol synthesis pathway, and type I IFN signaling. Given the ever-increasing num-

ber of pharmaceutical agents that target the cholesterol synthesis pathway and the high prevalence of chronic virus infections in humans (52), it is prudent to consider the effects of these pharmaceutical agents on the pathogenesis of viral disease.

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