LXR Alpha Restricts Gammaherpesvirus Reactivation from Latently Infected Peritoneal Cells

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ABSTRACT Gammaherpesviruses are ubiquitous viruses that establish lifelong infections. Importantly, these viruses are associated with numerous cancers and lymphoproliferative diseases. While risk factors for developing gammaherpesvirus-driven cancers are poorly understood, it is clear that elevated viral reactivation from latency often precedes oncogenesis. Here, we demonstrate that the liver X receptor alpha isoform (LXR α) restricts gammaherpesvirus reactivation in an anatomic-site-specific manner. We have previously demonstrated that deficiency of both LXR isoforms (α and β) leads to an increase in fatty acid and cholesterol synthesis in primary macrophage cultures, with a corresponding increase in gammaherpesvirus replication. Interestingly, expression of fatty acid synthesis genes was not derepressed in LXR α -deficient hosts, indicating that the antiviral effects of LXR α are independent of lipogenesis. Additionally, the critical host defenses against gammaherpesvirus reactivation, virus-specific CD8⁺ T cells and interferon (IFN) signaling, remained intact in the absence of LXR α . Remarkably, using a murine gammaherpesvirus 68 (MHV68) reporter virus, we discovered that LXR α expression dictates the cellular tropism of MHV68 in the peritoneal cavity. Specifically, LXR $\alpha^{-/-}$ mice exhibit reduced latency within the peritoneal B cell compartment and elevated latency within F4/80⁺ cells. Thus, LXR α restricts gammaherpesvirus reactivation through a novel mechanism that is independent of the known CD8⁺ T cell-based antiviral responses or changes in lipid synthesis and likely involves changes in the tropism of MHV68 in the peritoneal cavity.

IMPORTANCE Liver X receptors (LXRs) are nuclear receptors that mediate cholesterol and fatty acid homeostasis. Importantly, as ligand-activated transcription factors, LXRs represent potential targets for the treatment of hypercholesterolemia and atherosclerosis. Here, we demonstrate that LXR α , one of the two LXR isoforms, restricts reactivation of latent gammaherpesvirus from peritoneal cells. As gammaherpesviruses are ubiquitous oncogenic agents, LXRs may represent a targetable host factor for the treatment of poorly controlled gammaherpesvirus infection and associated lymphomagenesis.

KEYWORDS chronic infection, gammaherpesvirus, liver X receptor alpha, peritoneal B cells

Gammaherpesviruses are ubiquitous pathogens that establish lifelong infections in ~95% of the adult human population. There are two known human gammaherpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). Importantly, EBV and KSHV are associated with numerous lymphomas and other malignancies, including the body cavity-restricted primary effusion lymphoma (1). Furthermore, while risk factors for the development of gammaherpesvirus-driven cancers remain poorly understood, elevated viral reactivation often precedes oncogenesis (2–5). Thus, prevention of gammaherpesvirus-associated cancers requires a more thor-

Citation Lange PT, Jondle CN, Darrah EJ, Johnson KE, Tarakanova VL. 2019. LXR alpha restricts gammaherpesvirus reactivation from latently infected peritoneal cells. *J Virol* 93:e02071-18. <https://doi.org/10.1128/JVI.02071-18>.

Editor Jae U. Jung, University of Southern California

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Received 19 November 2018

Accepted 19 December 2018

Accepted manuscript posted online 2 January 2019

Published 5 March 2019

ough understanding of the virus-host interactions that dictate control of viral latency and reactivation. Due to the exquisite species specificity of human gammaherpesviruses, which significantly hinders identification of mechanisms that regulate viral reactivation *in vivo*, this study takes advantage of the murine gammaherpesvirus 68 (MHV68) model, a natural rodent pathogen that is closely related to EBV and KSHV (6–8) and represents a powerful animal model to define molecular and cellular mechanisms of the host that control chronic gammaherpesvirus infection.

Liver X receptors (LXRs) are transcription factors of the nuclear receptor superfamily. There are two isoforms of LXRs, LXR α and LXR β , which are transcribed from independent genes. While LXR α and LXR β share approximately 77% amino acid sequence identity, the expression patterns of the two isoforms differ greatly. LXR α is expressed in highly metabolic tissues, such as adipose tissue, liver, and intestine (9). Interestingly, LXR α is also expressed in some immune tissues and cell types, such as the spleen and thymus and macrophages (10). LXR β , on the other hand, is expressed ubiquitously (10). When activated by endogenous oxysterol ligands, LXRs induce the transcription of target genes, many of which encode proteins involved in fatty acid synthesis and cholesterol efflux. Thus, activation of LXRs, through synthetic or endogenous ligands, typically results in elevated fatty acid synthesis and cholesterol efflux (11–14). In addition to regulation of lipid metabolism, LXRs play important roles in the immune responses to pathogens. As such, LXRs are protective against *in vivo* infection with the intracellular pathogens *Listeria monocytogenes* (15) and *Mycobacterium tuberculosis* (16). Interestingly, while many reports describe anti-inflammatory activity of LXRs, along with negative regulation of T cell proliferation and CD4⁺ T cell differentiation (17, 18), these effects are likely context dependent, as LXRs facilitate T_H1 and T_H17 CD4⁺ T cell differentiation during *M. tuberculosis* infection (16).

Much of what is known about LXR biology in the context of viral infection has been defined using *in vitro* studies of RNA viruses and artificial synthetic LXR agonists. Activation of LXRs with artificial agonists was found to inhibit replication of hepatitis C virus (19, 20), HIV (21–23), and Newcastle disease virus (24) in cell culture, likely through perturbations of cellular cholesterol content. Only a few *in vivo* studies have defined the effects of synthetic LXR agonists during virus infection. Interestingly, while synthetic LXR agonists increased viral titers and the severity of pathology in a coxsackievirus infection model of myocarditis (25), treatment of humanized mice with synthetic LXR agonists suppressed HIV-1 replication (23, 26). Importantly, it is not known whether individual LXR isoforms and endogenous LXR ligands affect viral infection of an intact host.

We recently used a genetic approach to demonstrate that expression of both LXR isoforms attenuated replication of MHV68 in primary macrophages (27). Consistent with this antiviral activity, the expression of LXRs was elevated following viral infection in a type I interferon (IFN)-dependent manner. In contrast to the known ability of LXRs to activate their target genes in hepatocytes, we discovered that macrophages utilize LXRs to repress expression of enzymes involved in lipid synthesis, resulting in decreased activity of lipogenic pathways that support gammaherpesvirus replication (27). While these data demonstrate an antiviral role for LXRs during *in vitro* infection of primary macrophages, the role of LXRs during chronic gammaherpesvirus infection of an intact host remains unknown.

This study utilized genetic approaches to define the extent to which expression of LXR α , one of the two known LXR isoforms, affects chronic gammaherpesvirus infection. Expression of LXR α restricted reactivation of MHV68 from peritoneal cells during long-term infection. Interestingly, deficiency of LXR α alone did not result in derepression of LXR-dependent genes involved in lipid regulation as we had observed in LXR α ^{−/−} LXR β ^{−/−} macrophages *in vitro*. Furthermore, despite the elevated viral reactivation, LXR α ^{−/−} mice displayed normal virus-specific CD8⁺ T cell generation, recruitment, and antiviral cytokine production. Further, LXR α did not affect the ability of bone marrow-derived macrophages and latently infected peritoneal cells to respond to exogenous IFN treatment. Importantly, using a β -lactamase reporter virus (28), we

identified a dramatic change in the peritoneal tropism of MHV68 in mice lacking expression of LXR α . Thus, LXR α -mediated restriction of viral reactivation likely operates through a novel antiviral mechanism that dictates the peritoneal tropism of MHV68.

RESULTS

LXR α restricts MHV68 reactivation from peritoneal cells, but not splenocytes, in chronically infected animals. To determine the extent to which expression of LXR α affects chronic gammaherpesvirus infection of an intact host, C57BL/6J (BL6) and LXR $\alpha^{-/-}$ mice were intraperitoneally infected with 500 PFU of MHV68. At 16, 28, and 42 days postinfection (dpi), the frequencies of peritoneal exudate cells (PECs) and splenocytes that were viral DNA positive or supported viral reactivation *ex vivo* were determined using limiting-dilution assays.

MHV68 latency is established upon the clearance of acute infection, and the number of latently infected cells and viral reactivation peak at 16 days postinfection. Expression of LXR α did not alter viral reactivation (Fig. 1A) or the viral latent reservoir (Fig. 1B) in the spleen or the peritoneum at 16 days postinfection, indicating that LXR α does not affect the establishment of latent gammaherpesvirus infection. Intriguingly, following the establishment of latent infection, LXR $\alpha^{-/-}$ mice began to lose control of MHV68 reactivation from peritoneal cells, but not splenocytes (Fig. 1C) (~6.7-fold; 28 days postinfection). Despite increased reactivation, there was no significant change in the frequency of latently infected cells in LXR $\alpha^{-/-}$ mice (Fig. 1D), suggesting that LXR α specifically suppressed gammaherpesvirus reactivation. The ability of LXR α to suppress MHV68 reactivation from peritoneal cells was also observed in long-term-infected animals (Fig. 1E and F) (42 days postinfection).

To calculate the efficiency of viral reactivation, the percentage of peritoneal cells positive for MHV68 DNA or supporting *ex vivo* reactivation was determined for each time point, using average frequencies presented in Fig. 1A to F. The ratio of percent reactivating to percent viral-DNA-positive cells demonstrated comparable efficiencies of MHV68 reactivation in BL6 and LXR $\alpha^{-/-}$ mice at 16 days postinfection (Fig. 1G). In contrast, the efficiency of MHV68 reactivation from peritoneal cells was 2-fold and 16-fold higher in LXR $\alpha^{-/-}$ mice at 28 and 42 days postinfection, respectively (Fig. 1G). Of note, persistent MHV68 replication was not observed at any of the time points that were examined. Taken together, the data showed that LXR α decreased the efficiency of gammaherpesvirus reactivation from peritoneal cells, but not splenocytes, during long-term infection.

LXR α deficiency does not result in increased expression of proviral lipogenic genes during chronic MHV68 infection. We have previously demonstrated that expression of both LXR isoforms is stimulated by IFN signaling in cultured primary macrophages. Further, LXRs repressed the expression of cholesterol and fatty acid synthesis genes and activities of these proviral lipogenic pathways in the macrophage cultures (27). Having observed increased reactivation of MHV68 in peritoneal cells of chronically infected LXR $\alpha^{-/-}$ mice, we next sought to determine whether LXR α expression is necessary to limit lipogenic gene expression in infected animals.

At 28 days postinfection of BL6 mice, we detected a modest increase in LXR α expression and a modest decrease in LXR β expression compared to naive mice; however, these changes were not statistically significant (Fig. 2A and B). Interestingly, peritoneal cells from uninfected LXR $\alpha^{-/-}$ mice displayed reduced LXR β expression, suggesting that one LXR isoform may mediate homeostatic expression of the other. Importantly, the LXR target genes encoding fatty acid synthesis enzymes (FADS2, SCD2, and ACC) were not derepressed in the absence of LXR α (Fig. 2C to E). Instead, peritoneal cells from infected BL6 and LXR $\alpha^{-/-}$ mice showed comparable expression of each of the genes. Interestingly, expression of FADS2 and ACC was reduced in the peritoneum of infected BL6 and LXR $\alpha^{-/-}$ mice compared to naive controls (Fig. 2C and E), unveiling an interesting LXR α -independent virus-host interaction that limits lipogenic activity of the host at the site of long-term viral latency. In contrast, the expression of SCD2 did not differ between uninfected and infected mice (Fig. 2D). Taken together, these data

FIG 1 LXRα restricts MHV68 reactivation from peritoneal cells, but not splenocytes, in chronically infected animals. Mice of the indicated genotypes were intraperitoneally infected with 500 PFU wild-type (Continued on next page)

FIG 2 LXR α deficiency does not result in increased expression of proviral lipogenic genes during chronic MHV68 infection. Mice of the indicated genotypes were intraperitoneally infected with 500 PFU wild-type MHV68 for 28 days or mock infected. Peritoneal cells were isolated and subjected to qRT-PCR analysis to assess expression of the indicated genes. Gapdh, glyceraldehyde-3-phosphate dehydrogenase. Data are pooled from two independent experiments. Each symbol represents an individual animal, means and standard errors of the mean are shown. *, $P < 0.05$; **, $P < 0.01$; ****, $P < .0001$.

indicate that LXR α is not required to restrict lipogenic gene expression in peritoneal cells and that the observed hyperreactivation of MHV68 is likely not a result of increased fatty acid synthesis in the absence of LXR α .

MHV68-specific T cell responses remain intact in LXR $\alpha^{-/-}$ mice. Peritoneal reactivation of MHV68 is largely controlled by virus-specific CD8 $^{+}$ T cells (29, 30). Having observed a consistent increase in MHV68 reactivation from the peritoneal cells of LXR $\alpha^{-/-}$ mice, we tested the hypothesis that LXR α is important for the generation and recruitment of MHV68-specific CD8 $^{+}$ T cells during long-term infection. MHV68-specific CD8 $^{+}$ T cells were detected using major histocompatibility complex (MHC) class I tetramers that incorporated either an MHV68 orf6 or orf61 immunodominant epitope (Fig. 3A) (31). At 28 days postinfection, BL6 and LXR $\alpha^{-/-}$ mice exhibited similar

FIG 1 Legend (Continued)

MHV68. Mice were euthanized at 16 (A and B), 28 (C and D), or 42 (E and F) days postinfection, and splenocytes (right) or peritoneal exudate (left) cells were processed into single-cell suspensions. The cells were serially diluted and cocultured with MEFs to assess the frequency of cells reactivating latent virus (A, C, and E) or subjected to nested-PCR analysis to assess the frequency of cells harboring viral genomes (B, D, and F). The data are pooled from 2 to 4 independent experiments. The error bars represent standard errors of the mean. CPE, cytopathic effect. (G) The percentages of peritoneal cells positive for MHV68 DNA or supporting *ex vivo* reactivation were calculated for each time point using average frequencies of pooled data presented in panels A to F. Reactivation efficiency was defined as a ratio of percent reactivating to percent viral DNA-positive cells for each time point.

FIG 3 MHV68-specific T cell responses remain intact in $LXR\alpha^{-/-}$ mice. Mice of the indicated genotypes were intraperitoneally infected with 500 PFU wild-type MHV68 or mock infected. At 28 days postinfection, the mice were euthanized and splenocytes and peritoneal cells were processed into single-cell suspensions. Cells were stained with α -CD3, α -CD8, and α -CD44 antibodies, and MHC class I tetramers were loaded with the immunodominant peptides from the viral ORF6 (B, C, F, and G) or ORF61 (D, E, H, and I) proteins. (A) Representative flow plots from stained peritoneal cells. (B to I) Frequencies and total numbers of virus-specific CD8⁺ T cells for peritoneal cells (B to E) and splenocytes (F to I). The data are pooled from two independent experiments. Each symbol represents an individual animal; means and standard errors of the mean are shown.

FIG 4 $LXR\alpha$ does not regulate expression of IFN- γ by MHV68-specific $CD8^{+}$ T cells. Mice of the indicated genotypes were intraperitoneally infected with 500 PFU wild-type MHV68 or mock infected. At 28 days postinfection, the mice were euthanized, and peritoneal cells were isolated. The peritoneal cells were stimulated with 10 μ g/ml ORF6_{487–495} and ORF61_{524–531} immunodominant peptides in the presence of 10 μ g/ml brefeldin A for 6 h at 37°C. Cells were stained for flow cytometric analysis with α -CD3 and α -CD8 and intracellularly with α -IFN- γ antibodies. (A) Representative flow plots illustrating IFN- γ production. (B to E) Summarized frequencies and total numbers of IFN- γ -producing $CD8^{+}$ T cells from the peritoneal cavity (B and C) and spleen (D and E). (F) MFI of IFN- γ signal from IFN- γ^{+} peritoneal $CD8^{+}$ T cells. The data are pooled from at least two independent experiments. Each symbol represents an individual animal; means and standard errors of the mean are shown.

frequencies and total numbers of tetramer-positive, MHV68-specific $CD8^{+}$ cells in the peritoneum (Fig. 3B to E), indicating that the recruitment of MHV68-specific $CD8^{+}$ T cells was not regulated by $LXR\alpha$. Similar populations of MHV68-specific $CD8^{+}$ T cells were also observed in the spleens of infected BL6 and $LXR\alpha^{-/-}$ mice (Fig. 3F to I), indicating that the generation of MHV68-specific $CD8^{+}$ T cells was $LXR\alpha$ independent. Thus, the generation and recruitment of MHV68-specific $CD8^{+}$ T cells remained intact in the absence of $LXR\alpha$.

$LXR\alpha$ does not regulate expression of IFN- γ by MHV68-specific T cells. IFN- γ is a critical factor that controls MHV68 reactivation during chronic infection (30, 32–35), and both $CD8^{+}$ and $CD4^{+}$ MHV68-specific effector T cells can express IFN- γ following recognition of viral peptides in the context of MHC class I or class II, respectively. Importantly, treatment of primary murine macrophage cultures or mice with artificial LXR agonists has been reported to induce IFN- γ expression, suggesting that IFN- γ may be an LXR target gene (36). Therefore, having observed comparable numbers of MHV68-specific $CD8^{+}$ T cells in the peritoneum in BL6 and $LXR\alpha^{-/-}$ mice, along with poorly controlled viral reactivation, we next tested the hypothesis that $LXR\alpha$ deficiency compromises the ability of antiviral T cells to express IFN- γ , a critical antiviral cytokine.

Ex vivo restimulation of peritoneal cells (Fig. 4A to C) and splenocytes (Fig. 4D and

E) with the combination of immunodominant class I MHV68 peptides (ORF6_{487–495} and ORF61_{524–531}) generated similar frequencies and numbers of IFN- γ -expressing CD8⁺ T cells, regardless of LXR α expression. Further, the mean fluorescent intensities (MFI) of IFN- γ staining were similar in BL6 and LXR $\alpha^{-/-}$ mouse peritoneal CD8⁺ T cells (Fig. 4F), indicating that the amount of IFN- γ protein expressed on a per-cell basis was not affected by LXR α deficiency of MHV68-specific CD8⁺ T cells.

In addition to CD8⁺ T cells, CD4⁺ T cells are important for the control of chronic MHV68 infection (37–39) and are capable of producing IFN- γ in response to the MHC class II peptide gp150_{67–83} as long as 8 months after infection (40). Stimulation with gp150_{67–83} produced a measurable IFN- γ response from splenic CD4⁺ T cells (see Fig. S1A and B in the supplemental material), however, expression of IFN- γ from stimulated peritoneal CD4⁺ T cells was minimal (see Fig. S1C and D). Importantly, there was no difference in IFN- γ expression in response to gp150_{67–83} stimulation between BL6 and LXR $\alpha^{-/-}$ T cells. In summary, LXR α deficiency had no effect on the ability of MHV68-specific CD4 and CD8 T cells to express IFN- γ following exposure to immunodominant viral epitopes.

LXR α expression promotes neither signaling nor antiviral activity of IFN- γ . LXR α had no effect on generation, recruitment, or expression of antiviral IFN- γ by MHV68-specific T cells (Fig. 3 and 4). However, because T cells are not infected by MHV68, IFN- γ must engage receptors on infected cells in order to exert its antiviral activity. We and others have shown that peritoneal macrophages host a significant proportion of latent MHV68 following intraperitoneal inoculation of the virus (41–43). Furthermore, LXR α is robustly expressed in macrophages (13). Thus, we aimed to determine whether LXR α was necessary for optimal IFN signaling in primary macrophages.

Treatment of BL6 or LXR $\alpha^{-/-}$ bone marrow-derived macrophages with type I or type II IFN induced similar levels of STAT1 phosphorylation in both genotypes at 30 min postexposure (Fig. 5A). IFN signaling culminates in expression of interferon-stimulated genes (ISGs) that function as antiviral effectors. Expression of MND4, an ISG that attenuates MHV68 replication (44), was also induced to similar levels following 7 h of treatment of primary macrophages with either IFN- β or IFN- γ (Fig. 5B), indicating that LXR α expression did not promote IFN signaling in a primary cell type relevant for MHV68 infection.

Because primary macrophages derived from naive animals may not fully recapitulate the biology of peritoneal macrophages in long-term-infected mice, the antiviral activity of exogenous IFN- γ was assessed in peritoneal cells isolated from mice at 28 days post-MHV68 infection. Peritoneal cells were treated with exogenous IFN- γ immediately following explantation and for the duration of *ex vivo* reactivation. Consistent with previous reports (45, 46), exogenous IFN- γ efficiently suppressed MHV68 reactivation from peritoneal cells isolated from latently infected mice, and this suppression was independent of LXR α expression (Fig. 5C). In summary, LXR α did not potentiate either IFN signaling in primary macrophages or the antiviral activity of IFN- γ during MHV68 reactivation.

LXR α does not promote overall IFN responses in the peritoneal cells of chronically infected mice. MHV68-specific CD4⁺ and CD8⁺ T cells represent a significant, but not the only, source of IFN- γ during chronic infection. In order to measure systemic IFN- γ production, levels of IFN- γ in serum were assessed at 14 and 28 days postinfection. As previously reported (47), IFN- γ levels in serum dramatically increased at 14 days postinfection of BL6 mice and returned to near baseline levels by 28 days postinfection (Fig. 5D). Interestingly, unlike what was observed in MHV68-specific T cells, systemic IFN- γ levels were decreased at both 14 and 28 days postinfection in LXR $\alpha^{-/-}$ mice.

Despite decreased IFN- γ levels in serum at 14 days postinfection, MHV68 latency and reactivation were well controlled in LXR $\alpha^{-/-}$ mice during early latency (Fig. 1A and B). This was likely due to the overall significant elevation in IFN- γ expression at 14 days postinfection, which is likely far above the threshold required to control MHV68 reactivation. However, because much lower levels of IFN- γ circulate in BL6 mice once

FIG 5 LXR α expression promotes neither signaling nor antiviral activity of IFN- γ . (A and B) Bone marrow-derived macrophages were treated with 10 U/ml IFN- β , 1 U/ml IFN- γ , or vehicle control for 30 min (A) or 7 h (B). (A) Total and phosphorylated STAT1 proteins were measured by Western blotting in two biological repeats for each condition. (B) MNDA expression was measured by qRT-PCR. The results of two independent experiments were pooled, and the symbols represent biological replicates. (C) Peritoneal cells isolated from mice of the indicated genotypes at 28 days postinfection were subjected to limiting-dilution analyses to assess the frequency of viral reactivation. Cultures were treated with 5 U/ml IFN- γ or vehicle control (Veh) immediately following plating to assess the ability of exogenous IFN- γ to suppress MHV68 reactivation. The data were pooled from two independent experiments. (D) Serum was collected before infection and at 14 and 28 days postinfection. IFN- γ concentrations were determined by ELISA. The data are pooled from at least two independent experiments, and each symbol represents an individual animal. (E and F) Mice of the indicated genotypes were intraperitoneally infected with MHV68 as for Fig. 1 or mock infected. At 28 days postinfection, RNA was isolated from peritoneal cells immediately following harvest. Each symbol represents an individual animal. The data are representative of the results of two independent experiments (A) or pooled from two or more independent experiments (B to F). Error bars represent the standard error of the mean.

an infection becomes chronic, a further decrease in IFN- γ observed in the absence of LXR α could translate into increased MHV68 reactivation. In addition to IFN- γ , MHV68 reactivation during long-term infection is also controlled by type I IFN (48), with the latter represented by a family of at least 17 different cytokines. Further, we showed that type I and type II IFNs cooperate to induce expression of antiviral ISGs in MHV68-infected primary macrophages and during acute MHV68 infection (49).

Because expression of ISGs represents the combined signaling outcome of type I and type II IFNs, with both types of IFN produced in infected mice, expression of MNDAs and MX-2 was measured in peritoneal cells, the site of poorly controlled MHV68 reactivation, at 28 days postinfection. The ISGs were chosen based on their demonstrated ability to restrict replication of MHV68 *in vitro* (44). There was no difference in the expression of MNDAs or MX-2 in peritoneal cells of infected BL6 mice and $LXR\alpha^{-/-}$ mice (Fig. 5E and F). Other ISGs identified by Liu et al. (44) were also measured; however, their expression level was below our limit of detection (data not shown). Thus, the combined *in vivo* signaling levels of IFN pathways were similar in the peritonea of long-term-infected BL6 and $LXR\alpha^{-/-}$ mice, suggesting that $LXR\alpha$ -dependent restriction of MHV68 reactivation is mediated by a mechanism that is independent of the known cellular and molecular factors that control chronic gammaherpesvirus infection.

$LXR\alpha$ expression dictates latent viral tropism in peritoneal cells. To address the possibility that $LXR\alpha$ expression may contribute to the tropism or latent gene expression of MHV68, BL6 and $LXR\alpha^{-/-}$ mice were infected with a recombinant MHV68 generated by Nealy et al. in which the viral gene ORF73 is fused to β -lactamase (28). In mice infected with MHV68. ORF73 β la, infected cells expressing the ORF73 product mLANA also exhibit β -lactamase activity, which can be detected via the use of the fluorescent β -lactamase substrate CCF2-AM. Consistent with the initial characterization of the MHV68. ORF73 β la virus (28), we were able to detect a small yet distinct population of mLANA⁺ cells in bulk splenocytes at 28 days postinfection (Fig. 6A). Further, nearly all mLANA⁺ splenocytes from BL6 and $LXR\alpha^{-/-}$ mice were B cells, as exhibited by expression of the B cell marker CD19 (Fig. 6A). Thus, in concordance with limiting-dilution viral assays (Fig. 1), $LXR\alpha$ expression did not affect splenic MHV68 latency.

Excitingly, using the MHV68. ORF73 β la virus, we were able to detect mLANA⁺ peritoneal cells at 28 days postinfection (Fig. 6B), which to our knowledge has not been demonstrated. Consistent with previous reports defining macrophages as the predominant peritoneal viral reservoir (41, 46), approximately 70% of mLANA⁺ peritoneal cells from BL6 mice stained positive for F4/80, a marker highly expressed on murine peritoneal macrophages. As expected, the majority of F4/80-negative peritoneal cells that expressed mLANA in BL6 mice were CD19⁺ (~20% to 25%), consistent with viral tropism for peritoneal B-1 and B-2 B cells (41). Remarkably, mLANA⁺ peritoneal cells from $LXR\alpha^{-/-}$ mice exhibited considerably different F4/80 and CD19 staining, with the vast majority of mLANA-expressing peritoneal cells staining positive for F4/80 and very little CCF2 signal in CD19⁺ B cells (Fig. 6B and C). Thus, $LXR\alpha$ was critical to support MHV68 latent gene expression in peritoneal B cells. These observations correlated with decreased frequency of MHV68 DNA-positive cells in sorted B2 and B1b B cells from $LXR\alpha^{-/-}$ mice (data not shown). Further, decreased MHV68 presence in peritoneal B cells of $LXR\alpha^{-/-}$ mice was not due to decreased abundance of these cells in the peritoneum in $LXR\alpha^{-/-}$ mice (Fig. 6B and C; see Fig. S2 in the supplemental material). In summary, $LXR\alpha$ expression promoted latent MHV68 infection of peritoneal B cells.

DISCUSSION

In this study, we demonstrate a novel antiviral role for $LXR\alpha$ in the control of chronic gammaherpesvirus infection. This is, to our knowledge, the first time a genetic approach, rather than synthetic LXR agonists, was used to define the physiological role of an individual LXR isoform during virus infection of an intact host. We found that $LXR\alpha$ was necessary to suppress MHV68 reactivation specifically in the peritoneal cavity, an anatomic site that houses primary effusion lymphoma driven by KSHV in humans. Increased gammaherpesvirus reactivation is associated with increased risk of viral lymphomagenesis (2–5); thus, attenuation of gammaherpesvirus reactivation by $LXR\alpha$ makes this host factor a potential candidate in the suppression of gammaherpesvirus-driven malignancies. In support of this hypothesis, expression of $LXR\alpha$, but not the related $LXR\beta$, is decreased in EBV-transformed primary human B cells (50). Intriguingly, our data rule out $LXR\alpha$ -dependent changes in the expression of lipogenic enzymes,

FIG 6 LXRα expression dictates latent viral tropism or gene expression in peritoneal cells. Mice of the indicated genotypes were intraperitoneally infected with MHV68. ORF73βla for 28 days. Splenocytes and peritoneal cells were isolated and stained for CD19 and F4/80 surface markers. The cells were then loaded with the fluorescent β-lactamase substrate CCF2-AM for 1 h at room temperature. (A and B) Bulk splenocytes (A) and peritoneal cells (B) were examined for mLANA expression via flow cytometry, as indicated by the fluorescent signal from cleaved CCF2. (C) mLANA⁻ and mLANA⁺ cells were gated and examined for CD19 and F4/80 expression. Data from two independent experiments are summarized.

generation and recruitment of virus-specific T cells, and IFN signaling, suggesting that LXR α suppresses long-term MHV68 reactivation via a novel antiviral mechanism.

We have previously demonstrated that deficiency of both LXR isoforms results in elevated viral replication in primary macrophage cultures (27). This antiviral activity was ascribed to the underappreciated ability of LXRs to repress the expression of target genes, thereby limiting the activity of proviral fatty acid and cholesterol synthesis pathways. However, a single deficiency of LXR α was not sufficient to increase the expression of fatty acid synthesis enzymes in peritoneal cells (Fig. 2), indicating compensation by the remaining LXR β , at least with regard to the regulation of peritoneal lipid homeostasis.

MHV68-specific CD8⁺ and CD4⁺ T cells are critical for the control of chronic MHV68 infection and reactivation (29, 30, 37, 51–53), and T cell deficiency is a well-defined risk factor for gammaherpesvirus-driven malignancies (8, 38, 54). To probe *trans*-acting LXR α mechanisms, we examined the generation of MHV68-specific CD8⁺ and CD4⁺ effector T cells and their recruitment to the peritoneal cavity and found no LXR α -dependent effects. We also found that MHV68-specific CD8⁺ T cells expressed similar levels of IFN- γ regardless of LXR α expression. Our finding is in contrast to a published study that showed increased IFN- γ expression in T cell lines following exposure to artificial LXR agonists (36). The difference in our findings could be the result of differences between the experimental systems (T cell lines *in vitro* versus primary T cells *in vivo*) or the off-target effects of synthetic LXR agonists. However, we also found a decrease in the circulating levels of IFN- γ in LXR α ^{-/-} MHV68-infected animals, suggesting that LXR α might facilitate IFN- γ expression in other cell types *in vivo*. Importantly, this decrease in serum IFN- γ did not translate into attenuated IFN responses in the peritoneal cells, suggesting that either similar levels of IFN- γ are produced in the peritoneal cavities of BL6 and LXR α ^{-/-} mice (levels that were also below the level of detection of commercially available enzyme-linked immunosorbent assays [ELISAs]; data not shown) or that increased production of cytokines from the type I IFN family compensated for the decreased levels of IFN- γ in the peritoneum in LXR α -deficient mice.

The peritoneal cavity harbors numerous cell types during MHV68 infection, including CD4⁺ and CD8⁺ T cells, B-1 and B-2 B cells, macrophages, NK cells, dendritic cells, and granulocytes (55). At 16 days post-intraperitoneal infection, macrophages represent the greatest proportion of latently infected cells in the peritoneal cavity, followed by B1 and B2 B cells (41). Gammaherpesvirus tropism for macrophages is not an artifact of the MHV68 animal model; instead, macrophages represent a physiologically relevant cell type for human and rodent gammaherpesvirus infection (41–43, 56–59). As the infection progresses into its long-term stage, MHV68 tropism gradually shifts to peritoneal B cells; however, macrophages continue to house a considerable proportion of latent MHV68 in the peritoneum in long-term-infected animals. Latently infected macrophages account for the majority of peritoneal reactivation in BL6 mice during early latency (16 days postinfection), as determined by macrophage and B cell depletion studies (46). While the cell types responsible for reactivating MHV68 at later times during chronic infection remain unknown, it is likely that macrophages continue to contribute to the viral reactivation from the peritoneal cells of long-term-infected BL6 mice.

Fascinatingly, through the use of an MHV68 reporter virus, we discovered a unique LXR α -dependent shift in peritoneal mLANA expression (Fig. 6). This finding likely reflects a shift in viral tropism within the peritoneal cavity, as most latently infected cells express mLANA, at least at some point during latency (28). The shift in viral tropism is further supported by our unpublished data demonstrating a reduced frequency of latently infected B-1b and B-2 B cells in the peritonea of LXR α ^{-/-} mice. Increased frequency of latent MHV68 infection of LXR α ^{-/-} peritoneal macrophages undoubtedly contributes to increased MHV68 reactivation, based on previous studies (46). However, a 15% increase in latently infected macrophages is unlikely to fully account for the 10-fold increase in the frequency of MHV68 reactivation observed in LXR α ^{-/-} peritoneal cells at 28 days postinfection, suggesting that multiple mechanisms contribute to increased viral reactivation. In one plausible scenario, LXR α may function in a

macrophage-intrinsic manner to restrict MHV68 reactivation. We entertained the possibility that LXR α directly interacts with the MHV68 genome to suppress viral gene expression. However, our bioinformatics analyses failed to uncover high-fidelity LXR binding sites in the MHV68 genome (data not shown), suggesting that direct LXR-based suppression of viral gene expression is unlikely. However, this does not rule out LXR α -dependent regulation of host gene expression that suppresses reactivation. Another intriguing possibility is that latently infected peritoneal B cells produce a signal that restricts viral reactivation from macrophages, a novel cross talk that will be tested in future studies. LXR activation with synthetic agonists is already known to limit signaling downstream of tumor necrosis factor (TNF), interleukin 1 (IL-1), Toll-like receptor 3 (TLR3), and TLR4 (reviewed by Bensinger and Tontonoz [60]). It would be beneficial to determine if these signaling changes occur *in vivo* during chronic gammaherpesvirus infection when the cells are exposed to only the endogenous LXR ligands and the implications of such changes for gammaherpesvirus infection. Further, defining the LXR α -regulated cellular and cytokine milieu may offer a novel mechanistic insight into not only the antiviral activity of this host factor, but also into the ability of LXR α to attenuate *Listeria* (15) and *Mycobacterium* (16) infections.

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 (MCW) (AUA971) and adhered to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the American Veterinary Medical Association Guidelines on Euthanasia.

Animals. All mouse strains used in this study were maintained at the MCW animal facility. BL6 mice were originally purchased from Jackson Laboratories (Bar Harbor, ME). LXR $\alpha^{-/-}$ mice (C57BL/6 genetic background) were a kind gift from Qing Miao (MCW). At 6 to 10 weeks of age, mice were intraperitoneally inoculated with 500 PFU of MHV68 (WUMS) in 200 μ l serum-free Dulbecco's modified Eagle medium (DMEM) or mock infected. The mice were euthanized by CO₂ inhalation from a compressed gas source in a nonovercrowded chamber as mandated by the NIH Guide for the Care and Use of Laboratory Animals and the American Veterinary Medical Association Panel on Euthanasia. Mice were bled at the indicated time points via the submandibular route, and serum was isolated using BD Microtainer blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ).

Limiting-dilution assays. The frequency of cells reactivating MHV68 *ex vivo* and the frequency of cells harboring viral genomes were determined as previously described (61). Briefly, to determine the frequency of cells reactivating virus *ex vivo*, serial 2-fold dilutions of splenocytes or peritoneal cell suspensions harvested from infected mice were plated onto monolayers of mouse embryonic fibroblasts (MEFs) at 24 replicates per dilution. In order to control for any preformed infectious virus, 2-fold serial dilutions of mechanically disrupted splenocytes or PECs were plated as described above. Cytopathic effect was scored at 21 days postplating. Because primary MEFs were used to amplify the virus, the sensitivity of the limiting-dilution reactivation assay was a single PFU of MHV68. Because the endpoint of viral amplification in MEFs was measured, the limiting-dilution reactivation assay was not susceptible to variability of titers released from primary cells upon viral reactivation *ex vivo*. Persistent viral replication was assessed similarly, with 6×10^6 splenocytes or 2.4×10^6 peritoneal cells suspended in hypotonic solution (primary cell culture medium diluted 1:3 in water), frozen at -80° , thawed (to release pre-formed infectious particles from the cells), and subjected to limiting-dilution assays as described above.

Flow cytometry. Splenocytes and peritoneal exudate cells were suspended in fluorescence-activated cell sorter (FACS) buffer, prestained with Fc block, and incubated with the indicated antibodies on ice. When necessary, following surface staining, cells were fixed and permeabilized with BD Cytofix/Cytoperm (Fisher Scientific, Hampton, NH) for the detection of intracellular antigens. Data acquisition was performed on an LSR II flow cytometer (BD Biosciences, Sparks, MD) and analyzed using FlowJo software (Tree Star, Ashland, OR). The following antibodies were purchased from BioLegend (San Diego, CA): CD3 ϵ (17A2), CD4 (GK1.5), CD8 α (53-6.7), IFN- γ (XMG1.2), and CD44 (IM7). Allophycocyanin-conjugated MHC class I tetramers specific for MHV68 epitopes D^b/ORF6_{487–495} (AGPHNDMEI) and K^b/ORF61_{524–531} (TSINF VKI) were obtained from the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Restimulation of virus-specific CD4⁺ and CD8⁺ T cells was performed by incubating PECs or splenocytes with 10 μ g/ml ORF6 and ORF61 or 2.5 μ g/ml gp150 peptides (Thermo Fisher Scientific, Waltham, MA) in the presence of 10 μ g/ml brefeldin A for 6 h at 37°C. Unstimulated cells were incubated with brefeldin A alone.

ELISA. IFN- γ concentrations were determined using the IFN- γ ELISA MAX Deluxe set (BioLegend, San Diego, CA) according to the manufacturer's instructions, using Nunc MaxiSorp flat-bottom plates (Thermo Fisher Scientific, Waltham, MA). Serum was diluted 1:2 in assay diluent prior to analysis. Horseradish peroxidase (HRP) enzymatic activity was stopped by the addition of 1 N HCl, and absorbance was read at 450 nm on a model 1420 Victor 3V multilabel plate reader (PerkinElmer, Waltham, MA).

Western analyses. Samples were collected in Laemmli buffer (0.1 M Tris, 4% SDS, 4 mM EDTA, 100 mM beta-mercaptoethanol, 3.2 M glycerol with 0.05% bromophenol blue), boiled for 10 min, and subjected to SDS-PAGE. The protein was transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Billerica, MA) using a Transblot transfer cell semidry system (Bio-Rad, Hercu-

TABLE 1 Primers used for qRT-PCR analyses of gene expression

Gene	Primer sequence (5'–3')	
	Forward	Reverse
LXR α	TAAGGGAGAGTCAACAGG	GGTCAACAAGGTCTTCAG
LXR β	GCAGTTGGCACTAGAAG	GGTAGGCTGAGGTGTAA
ACC	GGAGCACCTCAAGCAGATATT	GGCTCTGACTTCTCCGTATTG
FADS2	TCTCAGATCACCGAGGACTT	GGACAGGAGGAGAAAGAAGAAC
SCD2	ACCTTCCTCACTCAGAGATACA	AAGCCTGGGAGGGATAAGA
MNDA	GGAGTGGACAATGGCACAAACATCA	TGACGAAACTGTGGTCTCCACACA
MX-2	AGCAGAGTGACACAAGCGAGAAGA	AGCCCTTCTGTCCTGAATCACAA

les, CA). The membranes were blocked with 5% bovine serum albumin (BSA) or 5% nonfat milk in TBST buffer (150 mM NaCl, 10 mM Tris, and 0.2% Tween) for 1 hour at room temperature. The membranes were incubated with anti-pSTAT1 (1:500 in 1% BSA; clone D4A7; Cell Signaling Technology, Boston, MA), anti-STAT1 (1:10,000 in 5% nonfat milk; clone E-23; Santa Cruz Biotechnology, Santa Cruz, CA), or anti- β -actin (1:50,000 in 5% nonfat milk; clone 13E5; Cell Signaling Technology, Boston, MA) antibody at 4°C overnight. The membranes were washed three times for 10 min each time, followed by incubation with secondary HRP-conjugated antibodies (1:20,000; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. The membranes were again washed three times for 10 min each time and then developed with Immobilon Western chemiluminescent HRP substrate (Millipore Sigma, Burlington, MA) and imaged with an Azure C600 imaging system (Azure Biosystems, Dublin, CA).

Primary cell culture. Bone marrow was harvested from male and female mice that were between 3 and 10 weeks of age. Primary bone marrow-derived macrophages were generated as previously described (62). At least two independently derived batches of macrophages were used in each experiment. MEFs were generated from embryonic day 12 (E12) to E14 embryos and pooled at early passages.

Gene expression studies. Total RNA was harvested, reverse transcribed, and analyzed by quantitative reverse transcription (qRT)-PCR as previously described (63). cDNA was assessed in triplicate, along with corresponding reactions minus reverse transcriptase, by real-time PCR using a CFX Connect system (Bio-Rad, Hercules, CA). Analysis was performed using primers listed in Table 1. These primers were validated for sensitivity and specificity using bioinformatics and wet laboratory approaches; the slope and linear range were established for each primer pair, and all the primers were utilized within their linear ranges.

MHV68.ORF73 β la detection. BL6 and LXR $\alpha^{-/-}$ mice were infected intraperitoneally with 500 PFU MHV68.ORF73 β la. Mice were euthanized at 28 dpi, and then splenocytes and peritoneal cells were isolated and pooled from each group (4 or 5 mice/group). Approximately 2×10^7 cells from each group of mice were prestained with Fc block and then stained with α F4/80-allophycocyanin (APC)-Cy7 and α CD19-APC (BioLegend, San Diego, CA) for 30 min on ice. The cells were washed twice and then loaded with CCF2-AM (GeneBLAzer kit; ThermoFisher Scientific, Waltham, MA) at room temperature for 1 h. The cells were again washed twice and then suspended in FACS buffer and analyzed via flow cytometry.

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

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.02071-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

ACKNOWLEDGMENTS

Funding for this study was provided by CA183593 and CA203923 (V.L.T.) and by 18PRE33960455 (P.T.L.).

We thank Kyle Stoltz for his support of this study. We thank the members of the Corbett and Cui laboratory for lively discussions of the study and Bonnie Dittel and Daisy Sahoo for their thoughtful insights.

P.T.L. and V.L.T. wrote the manuscript. P.T.L., C.N.J., and V.L.T. designed the experiments. P.T.L., C.N.J., E.J.D., and K.E.J. performed and analyzed experiments.

We declare no competing interests.

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