



Lymphatic Type 1 Interferon Responses Are Critical for Control of Systemic Reovirus Dissemination

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ABSTRACT Mammalian orthoreovirus (reovirus) spreads from the site of infection to every organ system in the body via the blood. However, mechanisms that underlie reovirus hematogenous spread remain undefined. Nonstructural protein $\sigma 1s$ is a critical determinant of reovirus bloodstream dissemination that is required for efficient viral replication in many types of cultured cells. Here, we used the specificity of the $\sigma 1s$ protein for promoting hematogenous spread as a platform to uncover a role for lymphatic type 1 interferon (IFN-1) responses in limiting reovirus systemic dissemination. We found that replication of a $\sigma 1s$ -deficient reovirus was restored to wild-type levels in cells with defective interferon- α receptor (IFNAR1) signaling. Reovirus spreads systemically following oral inoculation of neonatal mice, whereas the $\sigma 1s$ -null virus remains localized to the intestine. We found that $\sigma 1s$ enables reovirus spread in the presence of a functional IFN-1 response, as the $\sigma 1s$ -deficient reovirus disseminated comparably to wild-type virus in IFNAR1^{-/-} mice. Lymphatics are hypothesized to mediate reovirus spread from the intestine to the bloodstream. IFNAR1 deletion from cells expressing lymphatic vessel endothelium receptor 1 (LYVE-1), a marker for lymphatic endothelial cells, enabled the $\sigma 1s$ -deficient reovirus to disseminate systemically. Together, our findings indicate that IFN-1 responses in lymphatics limit reovirus dissemination. Our data further suggest that the lymphatics are an important conduit for reovirus hematogenous spread.

IMPORTANCE Type 1 interferons (IFN-1) are critical host responses to viral infection. However, the contribution of IFN-1 responses to control of viruses in specific cell and tissue types is not fully defined. Here, we identify IFN-1 responses in lymphatics as important for limiting reovirus dissemination. We found that nonstructural protein $\sigma 1s$ enhances reovirus resistance to IFN-1 responses, as a reovirus mutant lacking $\sigma 1s$ was more sensitive to IFN-1 than wild-type virus. In neonatal mice, $\sigma 1s$ is required for reovirus systemic spread. We used tissue-specific IFNAR1 deletion in combination with the IFN-1-sensitive $\sigma 1s$ -null reovirus as a tool to test how IFN-1 responses in lymphatics affect reovirus systemic spread. Deletion of IFNAR1 in lymphatic cells using Cre-lox technology enabled dissemination of the IFN-1-sensitive $\sigma 1s$ -deficient reovirus. Together, our results indicate that IFN-1 responses in lymphatics are critical for controlling reovirus systemic spread.

KEYWORDS dissemination, interferon, mouse, pathogenesis, reovirus

Systemic dissemination is a fundamental step in viral pathogenesis. To spread within the host, viruses need to replicate in multiple cell and tissue types. Viruses also must overcome a variety of physical and physiological barriers, including host antiviral defenses. Mammalian orthoreovirus (reovirus) is a member of the *Reoviridae* family of nonenveloped, double-stranded RNA (dsRNA) viruses that infects its hosts via respiratory or enteric routes. Following replication at the portal of entry, reovirus traffics to secondary organs and tissues, including the heart and central nervous

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system (CNS) (1, 2). Reoviruses primarily disseminate via the blood, although serotype 3 (T3) reoviruses can also spread by neural routes (3). In the intestine, reovirus infects intestinal epithelial cells (IECs) and Peyer's patch (PP) cells (4–6) and is hypothesized to traffic through the mesenteric lymph node (MLN) to the blood via the lymphatics (7). However, the functional route of reovirus systemic spread is not defined.

Reovirus dissemination is influenced by a combination of host and viral factors (8). A key host determinant of reovirus spread is junctional adhesion molecule A (JAM-A), a cell surface receptor for reovirus (9, 10). JAM-A is a tight junction protein that promotes polarization and barrier formation by epithelial and endothelial cells and also is expressed on monocytes, lymphocytes, dendritic cells, and platelets, where it aids in cell migration and extravasation (11–14). JAM-A is dispensable for reovirus replication in the intestine but required for hematogenous spread (10). JAM-A on endothelial cells is required for establishment of viremia as well as egress of reovirus from the bloodstream into organs (15).

Reovirus nonstructural protein $\sigma 1s$ is also required for reovirus systemic spread (16). Like JAM-A, $\sigma 1s$ is dispensable for reovirus replication in the intestine but is essential for spread through the blood (16, 17). The $\sigma 1s$ protein is not needed for reovirus to traffic from the PP to the MLN (16). However, $\sigma 1s$ is required for reovirus replication in the MLN, which is hypothesized to facilitate viral spread through intestinal lymphatics to the bloodstream for the establishment of viremia and systemic dissemination (17). In culture, $\sigma 1s$ enhances reovirus replication in numerous cell lines, including simian virus 40 (SV40) immortalized endothelial cells (SVECs) and murine embryonic fibroblasts (MEFs) (16–19). In these cell lines, $\sigma 1s$ functions as a replication accessory factor that promotes reovirus protein synthesis (19). Therefore, $\sigma 1s$ may promote efficient viral replication in cells that are required for reovirus dissemination.

Type 1 interferons (IFN-1) are critical for host control of viral infections (20). IFN-1 (IFN- α/β) is produced in response to viruses and signals in an autocrine or paracrine manner to induce expression of hundreds of IFN-stimulated genes (ISGs) that function to limit viral replication (20–22). Adult mice are normally refractory to reovirus disease (23, 24). However, reovirus infection is lethal in adult mice that lack interferon- α receptor subunit 1 (IFNAR1) and cannot respond to IFN-1 (25). In adult IFNAR1^{-/-} mice, IFNAR1 expression on hematopoietic cells is required for protection from reovirus (25). Neonatal IFNAR1^{-/-} mice also succumb more rapidly to reovirus and have higher viral loads than wild-type (WT) mice (24, 26). Although IFN-1 is an important host response against reoviruses *in vivo*, the cell types responsible for IFN-1-mediated protection against reoviruses are not defined.

Here, we used tissue-specific IFNAR1 deletion in combination with the IFN-1-sensitive reovirus as a tool to identify a role for lymphatics in reovirus dissemination. We found that $\sigma 1s$ is a viral determinant of reovirus resistance to IFN-1 responses in cultured cells and *in vivo*, as $\sigma 1s$ -deficient reovirus disseminates efficiently in IFNAR1^{-/-} mice. Using *Cre-lox* technology, we found that the IFN-1-sensitive $\sigma 1s$ -deficient reovirus disseminated in mice with lymphatic endothelial cell-specific deletion of IFNAR1. Together, our results indicate that IFN-1 responses in lymphatics are a critical barrier that reovirus must overcome to spread systemically.

RESULTS

$\sigma 1s$ facilitates reovirus replication in the presence of IFN-1 responses. Previous work indicates that $\sigma 1s$ does not affect the induction of IFN-1 responses to reovirus (19). To further explore the relationship between $\sigma 1s$ and IFN-1, we tested whether $\sigma 1s$ promotes reovirus replication in cells when IFN-1 signaling was blocked. Murine SV40 immortalized endothelial cells (SVEC4-10; SVECs), a mouse lymphatic endothelial cell line, were infected with the T1L or T1L $\sigma 1s$ -null strain in the presence of isotype control or anti-IFNAR1 antibodies, and viral spread in culture was assessed over a 7-

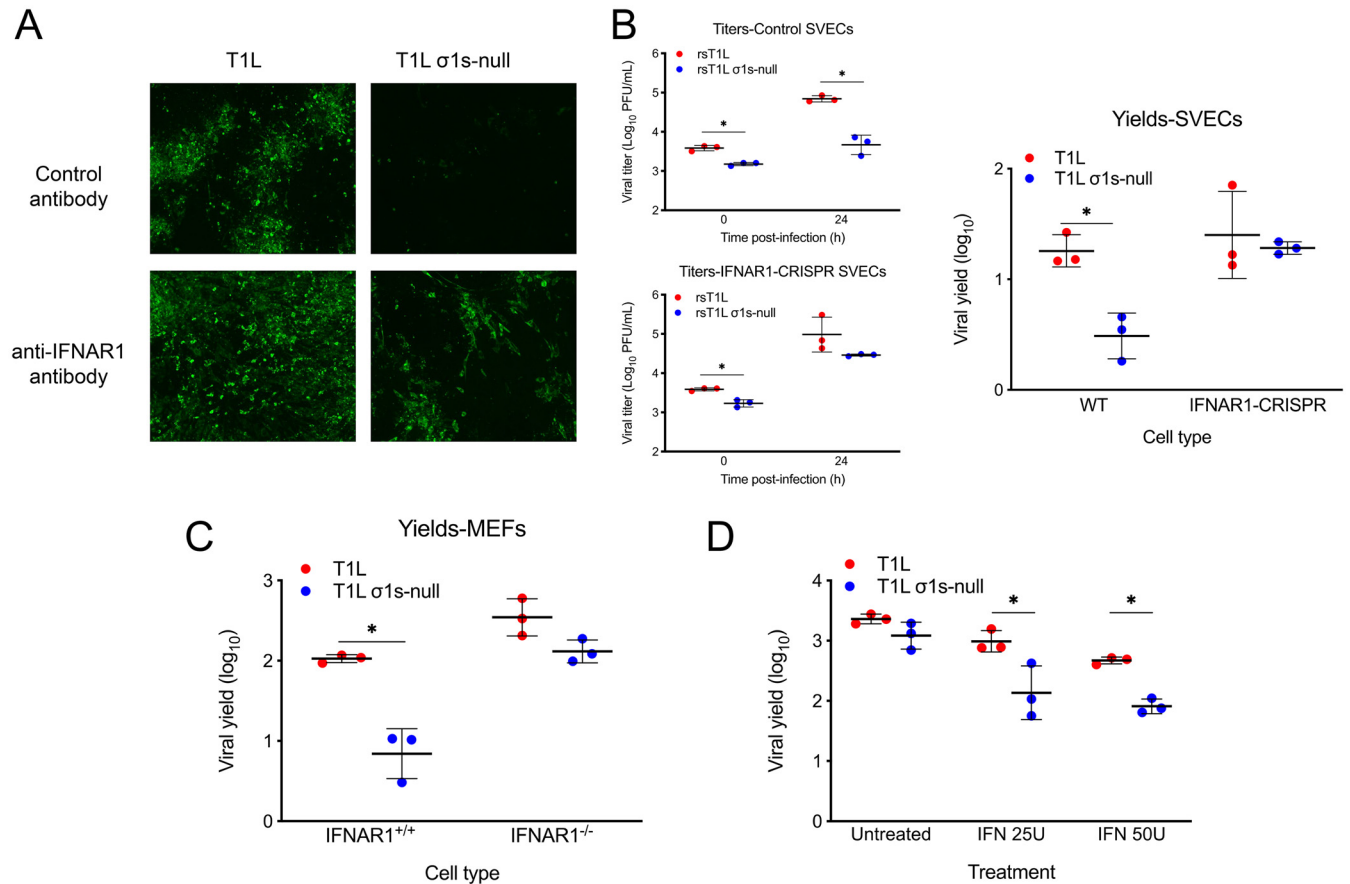


FIG 1 The σ 1s protein facilitates reovirus replication in the presence of IFN-1 responses. (A) SVECs were infected with the T1L or T1L σ 1s-null strain at an MOI of 10 PFU/cell and treated with control or anti-IFNAR1 antibodies. At 7 days, infected cells were identified via indirect immunofluorescence using reovirus polyclonal antiserum. (B) Wild-type or IFNAR1-CRISPR SVECs were infected with the T1L or T1L σ 1s-null strain at an MOI of 1 PFU/cell. Viral titers were determined at 0 and 24 h. Results are presented as (left) mean viral titers or (right) mean viral yields from three independent experiments. (C) Wild-type (IFNAR1^{+/+}) or IFNAR1^{-/-} MEFs were infected with the T1L or T1L σ 1s-null strain at an MOI of 1 PFU/cell. Viral titers were determined at 0 and 24 h, and results are presented as the mean viral yield from three independent experiments. (D) L929 cells were left untreated or treated with 25 or 50 units of recombinant IFN- α / β for 6 h prior to infection with the T1L or T1L σ 1s-null strain at an MOI of 1 PFU/cell. Viral titers were determined at 0 and 24 h, and results are presented as the mean viral yield. Error bars represent standard deviation (SD). *, $P < 0.05$ as determined by Student's t test.

day period (Fig. 1A). T1L produced large foci and spread throughout the culture in the presence of the control antibody. In contrast, the T1L σ 1s-null strain was limited to individual cells with few apparent multicellular foci. Treatment with anti-IFNAR1 antibodies enhanced spread of the T1L and T1L σ 1s-null strains. To quantitatively assess the effect of IFN-1 responses on viral replication, we measured replication in wild-type SVECs and SVECs with IFNAR1 deleted using CRISPR-Cas9 editing (IFNAR1-CRISPR SVECs). Consistent with our previous work (19), the T1L strain produced approximately 10-fold more virus than the T1L σ 1s-null strain in wild-type SVECs. In contrast, T1L and T1L σ 1s-null strains replicated to equivalent levels in IFNAR1-CRISPR SVECs (Fig. 1B). Similarly, T1L replicated to significantly higher levels than the T1L σ 1s-null strain in IFNAR1^{+/+} MEFs, but T1L and T1L σ 1s-null strains produced comparable yields in IFNAR1^{-/-} MEFs (Fig. 1C). These results indicate that σ 1s facilitates efficient reovirus replication in the presence of IFN-1 responses.

To determine whether σ 1s enhances reovirus sensitivity to IFN-1, replication of T1L and T1L σ 1s-null strains was measured in L929 cells treated with recombinant IFN- β prior to infection (Fig. 1D) (27). Unlike SVECs or MEFs, σ 1s is not required for reovirus replication in L929 cells and allows assessment of the relationship between σ 1s and IFN-1 independent of σ 1s effects on viral replication (19, 28). Consistent with previous work, T1L and T1L σ 1s-null strains replicated equivalently in untreated L929 cells. However, T1L replication was modestly impaired by IFN- β , and the reduction in T1L

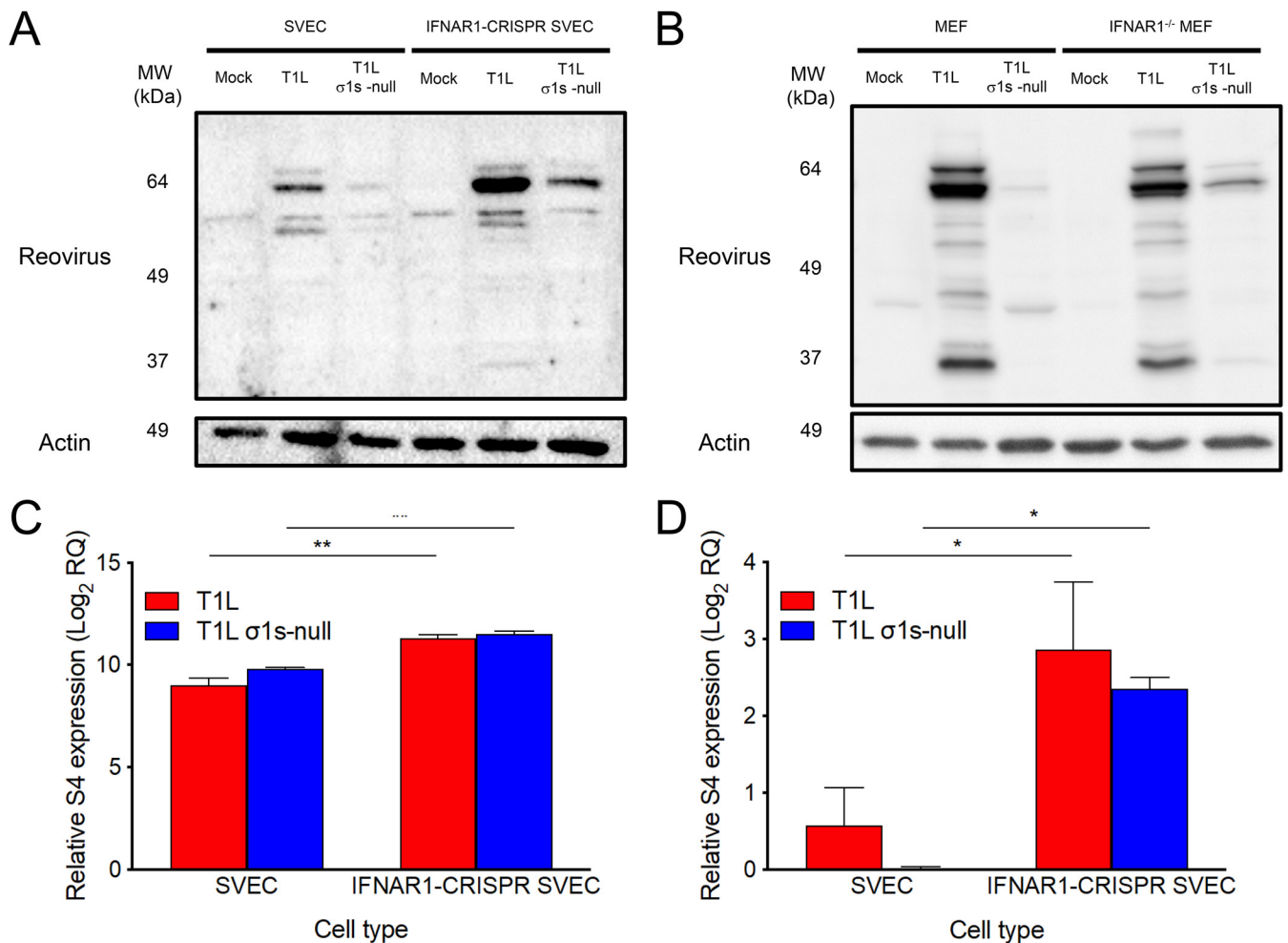


FIG 2 The σ 1s protein does not counteract IFN-1-mediated inhibition of reovirus protein synthesis. Wild-type or IFNAR1-CRISPR SVECs (A) or wild-type (*IFNAR1*^{+/+}) or *IFNAR1*^{-/-} MEFs (B) were infected with the T1L or T1L σ 1s-null strain at an MOI of 10 PFU/cell. At 18 h, whole-cell lysates were collected and separated by SDS-PAGE. Reovirus proteins and β -actin were detected by Western blot. (C and D) WT or IFNAR1-CRISPR SVECs were infected with the T1L or T1L σ 1s-null strain at an MOI of 10 PFU/cell. At 0 or 18 h, total RNA was collected and relative expression of mRNA (C) or negative-sense RNA (D) was determined compared to 0 h. The RQ of positive- or negative-sense RNA was determined with reference to the quantity at the 0 h. The RQ of reovirus mRNA was determined by subtracting the RQ of negative-sense viral RNA (representing genomic RNA production) from the RQ of positive-sense RNA. Data are presented as the mean log₂ RQ from three independent experiments. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.005$ as determined by Student's *t* test.

σ 1s-null strain yields were significantly more pronounced. Together, these findings indicate that σ 1s enhances reovirus resistance to IFN-1.

The σ 1s protein enhances reovirus replication in SVECs and MEFs by promoting viral protein production (19). Inhibition of viral protein synthesis is a key mechanism by which IFN-1 responses combat viral infections (20). To determine whether σ 1s allows reovirus to overcome IFN-1-mediated protein synthesis inhibition, viral protein production was assessed in wild-type and IFNAR1-CRISPR SVECs (Fig. 2A) and *IFNAR1*^{+/+} and *IFNAR1*^{-/-} MEFs (Fig. 2B). Consistent with previous results (19), T1L produced more viral protein than the T1L σ 1s-null strain in wild-type SVECs and MEFs. Although both viruses produced more protein in IFNAR1-CRISPR SVECs than in wild-type cells, protein expression by the T1L σ 1s-null strain remained substantially lower than that of T1L in both cell types. No difference in T1L protein levels was observed between wild-type and *IFNAR1*^{-/-} MEFs. However, the T1L σ 1s-null strain produced more protein in *IFNAR1*^{-/-} MEFs than wild-type MEFs. These data indicate that σ 1s does not directly counteract the inhibition of reovirus protein synthesis caused by IFN-1.

We next quantified viral RNA to determine the effect of IFN-1 responses on viral RNA synthesis by T1L and T1L σ 1s-null strains. Consistent with previous results (19),

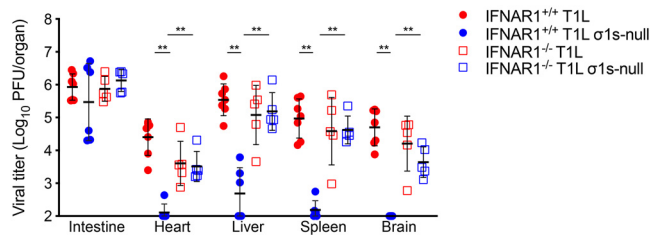


FIG 3 The $\sigma 1s$ protein is required for reovirus dissemination in the presence of IFN-1 responses. Three- to four-day-old wild-type ($IFNAR1^{+/+}$) or $IFNAR1^{-/-}$ neonatal mice were infected orally with 10^4 PFU of the T1L or T1L $\sigma 1s$ -null strain. At 4 days, the indicated organs were resected and homogenized, and viral titer was determined by plaque assay. Error bars represent SD. **, $P < 0.005$ as determined by Mann-Whitney test.

T1L and T1L $\sigma 1s$ -null S4 mRNA levels (Fig. 2C) were comparable in wild-type SVECs (\log_2 9.0 and \log_2 9.8, respectively). In contrast, T1L and T1L $\sigma 1s$ -null strains produced more S4 mRNA in $IFNAR1$ -CRISPR SVECs (\log_2 11.3 and \log_2 11.5, respectively). Negative-sense RNA was detected in wild-type SVECs infected with T1L (Fig. 2D), but the T1L $\sigma 1s$ -null strain produced little, if any, negative-sense RNA (Fig. 2D). Negative-sense RNA levels increased in $IFNAR1$ -CRISPR SVECs for T1L and T1L $\sigma 1s$ -null strains. These data are consistent with previous findings that $\sigma 1s$ is dispensable for reovirus RNA synthesis (19). These results indicate that while IFN-1 responses limit reovirus RNA synthesis, $\sigma 1s$ does not specifically modulate antiviral responses that prevent viral RNA accumulation.

The $\sigma 1s$ protein facilitates reovirus dissemination in the face of IFN-1 responses *in vivo*. Our data and published studies (29) indicate that $\sigma 1s$ contributes to reovirus IFN-1 resistance in cultured cells. To determine whether $\sigma 1s$ is required for reovirus to overcome IFN-1 responses *in vivo*, we assessed T1L and T1L $\sigma 1s$ -null strains spread in wild-type and $IFNAR1^{-/-}$ mice (Fig. 3). T1L replicated in the intestine and spread systemically in wild-type mice. Consistent with previous findings (16), the T1L $\sigma 1s$ -null strain produced viral titers similar to those of T1L in the intestine of wild-type mice but T1L $\sigma 1s$ -null titers in target organs (brain, heart, liver, and spleen) were near or below the limit of detection. As in wild-type mice, T1L and T1L $\sigma 1s$ -null strains produced comparable intestinal titers in $IFNAR1^{-/-}$ mice. However, equivalent titers of T1L and T1L $\sigma 1s$ -null strains were recovered from peripheral sites in $IFNAR1^{-/-}$ mice. These data indicate that the IFN-1 response acts as a barrier to reovirus hematogenous dissemination. These findings further suggest that $\sigma 1s$ is required for reovirus to spread systemically in the presence of IFN-1 responses.

The $\sigma 1s$ protein is required for efficient reovirus replication in lymphatic endothelial cells. Reovirus is hypothesized to spread via the lymphatics, which are largely formed from lymphatic endothelial cells (LECs) (30). Previous work revealed that $\sigma 1s$ was required for efficient reovirus replication in SVECs, an immortalized lymphatic endothelial cell line (19). To determine whether $\sigma 1s$ is required for reovirus replication in primary LECs, we quantified T1L and T1L $\sigma 1s$ -null progeny yields produced by LECs derived from C57BL/6 mice (Fig. 4A). We found that T1L generated significantly higher progeny yields than the T1L $\sigma 1s$ -null strain in primary LECs at both multiplicity of infection (MOI) values tested. In cells where $\sigma 1s$ is required for reovirus replication, $\sigma 1s$ also mediates optimal viral protein production (19). In primary LECs, we observed that differential replication of wild-type and $\sigma 1s$ -deficient viruses correlated with differences in viral protein production, as T1L produced substantially more viral protein than the T1L $\sigma 1s$ -null strain (Fig. 4B). Together, these data indicate that $\sigma 1s$ promotes reovirus replication in primary LECs.

Lymphatics facilitate reovirus dissemination. If lymphatics function as conduits for reovirus dissemination, we hypothesized that ablating IFN-1 responses specifically in LECs would enable dissemination of $\sigma 1s$ -deficient reovirus. To test this hypothesis, we used the IFN-1-sensitive $\sigma 1s$ -null reovirus in combination with lymphatic-specific

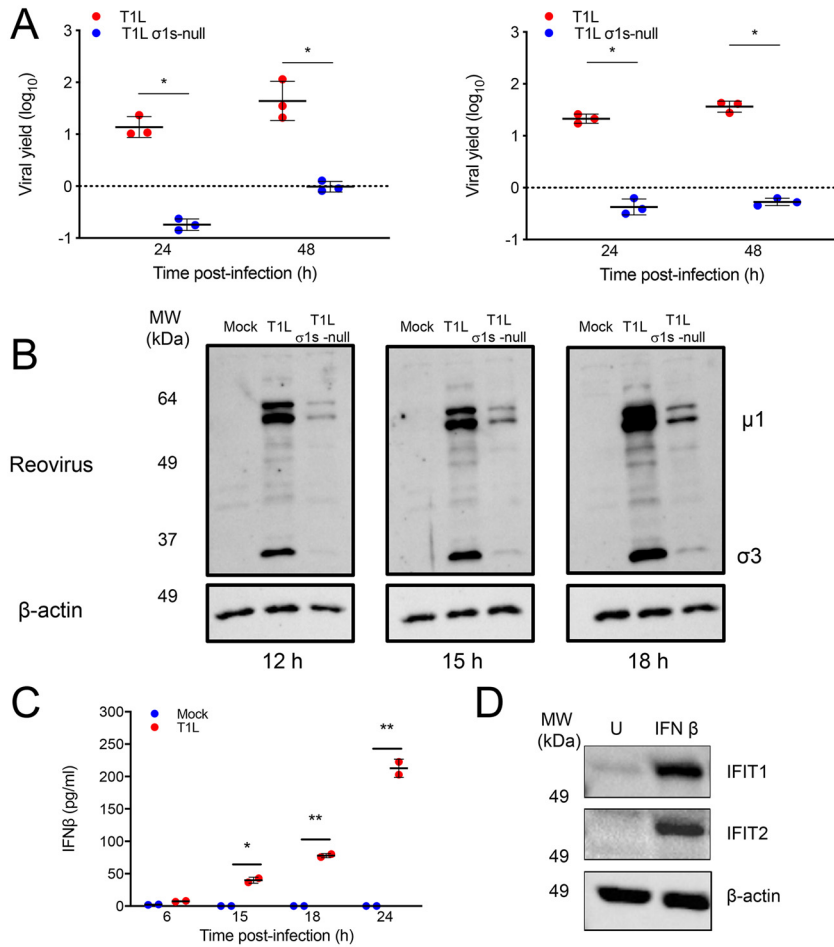


FIG 4 The $\sigma 1s$ protein facilitates reovirus protein synthesis and replication in primary lymphatic endothelial cells. (A) Primary LECs from C57BL/6 mice were infected with the T1L or T1L $\sigma 1s$ -null strain at MOIs of 1 (left) or 10 (right) PFU/cell. At 0, 24, and 48 h, viral titers were determined by plaque assay. Results are presented as the mean viral yield from two independent experiments. Error bars represent SD. *, $P < 0.05$ as determined by Student's t test. (B) Primary LECs were mock infected or infected with the T1L or T1L $\sigma 1s$ -null strain at an MOI of 5 PFU/cell. At the indicated times, whole-cell lysates were collected and separated by SDS-PAGE. Reovirus proteins and β -actin were detected by Western blot. (C) Primary LECs were mock infected or infected with the T1L strain at an MOI of 100 PFU/cell. At the indicated times, IFN- β levels in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA). (D) Primary LECs were treated with 200 U IFN- β or left untreated. At 6 h, whole-cell lysates were collected and separated by SDS-PAGE. IFIT1, IFIT2, and β -actin were detected by Western blot.

deletion of *IFNAR1*. We first confirmed that primary LECs secrete IFN- β in response to reovirus infection (Fig. 4C) and produce ISGs following IFN-1 treatment (Fig. 4D). To generate lymphatic-specific *IFNAR1* deletion mice, *IFNAR1^{fl/fl}* mice were crossed with *Lyve1-Cre* mice (31, 32). Lymphatic vessel endothelium receptor 1 (LYVE-1) is a receptor for hyaluronan that promotes LEC proliferation (33, 34). LYVE-1 is a commonly used marker for the lymphatic endothelium but is also expressed on liver sinusoid, some tissue-resident macrophages, and a subset of hematopoietic stem cells (31, 35–37). T1L disseminated in the parental *IFNAR1^{fl/fl}* (Fig. 5A) and *Lyve1-Cre* (Fig. 5B) strains, similar to results obtained with C57BL/6 mice (16). In contrast, the T1L $\sigma 1s$ -null strain did not spread efficiently in either parental mouse strain.

The F1 progeny resulting from crossing *IFNAR1^{fl/fl}* and *Lyve1-Cre* strains were bred to *IFNAR1^{fl/fl}* mice. The resulting progeny (*Lyve1-Cre-IFNAR1^{+fl}*, *IFNAR1^{+fl}*, *IFNAR1^{fl/fl}* littermate controls, and *Lyve1-Cre-IFNAR1^{fl/fl}* LEC *IFNAR1* deletions) were infected with the

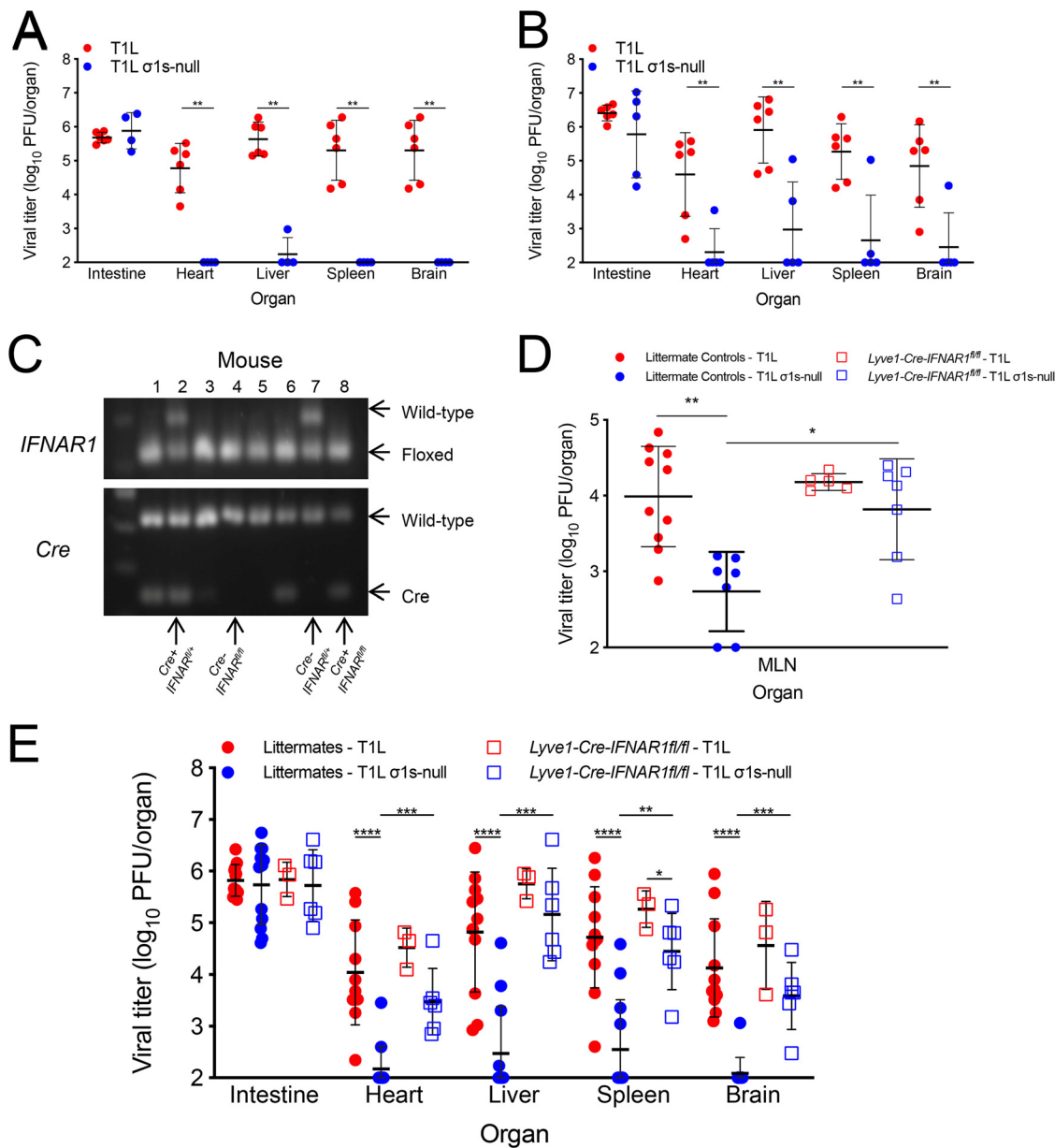


FIG 5 IFN-1 responses in lymphatics limit reovirus dissemination. *IFNAR1^{fl/fl}* (A) or *Lyve1-Cre* (B) mice were orally infected with 10^4 PFU T1L or T1L σ 1s-null virus. At 4 days, the indicated organs were resected and homogenized, and viral titer was determined by plaque assay. (C) The genotype of transgenic mice was determined by performing PCR analysis of chromosomal DNA using primers specific for *IFNAR1* and *Cre*. The floxed *IFNAR1* alleles and *Cre* were differentiated based on migration of the PCR products in 1% agarose gel and stained with ethidium bromide (EtBr). (D and E) Littermate control (*IFNAR1^{fl/fl}*, *IFNAR1^{+/+}*, *Lyve1-Cre-IFNAR1^{fl/fl}* or *Lyve1-Cre-IFNAR1^{fl/fl}*) mice were orally infected with 10^4 PFU T1L or T1L σ 1s-null virus. At 4 days, MLNs (D) or the indicated organs (E) were resected and homogenized, and viral titer was determined by plaque assay. Error bars represent SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0005$; ****, $P < 0.0001$ as determined by Mann-Whitney test.

T1L or T1L σ 1s-null strain, and at 4 days, viral tissue titers were determined. Mice were genotyped by PCR at the time of harvest to determine their *Cre* and *IFNAR1* status (Fig. 5C). Following oral infection, we found that T1L titers were substantially higher on day 4 than the T1L σ 1s-null strain in the MLNs of littermate control animals (Fig. 5D). In contrast, T1L and T1L σ 1s-null strains produced comparable titers in MLNs from *Lyve1-Cre-IFNAR1^{fl/fl}* mice. These data indicate that IFN-1 responses in the lymphatics impair spread of σ 1s-deficient reovirus to the MLN.

We next assessed reovirus spread in mice with lymphatic *IFNAR1* deletion. In littermate control mice, T1L and T1L σ 1s-null strains produced comparable titers in the

intestine, but only T1L had high titers in peripheral organs. T1L σ 1s-null titers in organs from littermate control mice were near or below the level of detection (Fig. 5E). These data are consistent with observations in wild-type, *IFNAR1^{fl/fl}*, and *Lyve1-Cre* mice that σ 1s is required for efficient reovirus dissemination. In contrast, T1L and T1L σ 1s-null strains produced largely comparable titers in all organs of *Lyve1-Cre-IFNAR1^{fl/fl}* mice. Thus, deletion of *IFNAR1* in lymphatics enables dissemination of σ 1s-deficient reovirus. Together, these results indicate that lymphatic IFN-1 responses are critical for controlling reovirus dissemination.

DISCUSSION

Here, we identified a role for lymphatic IFN-1 responses in controlling hematogenous reovirus dissemination. In the intestine, reovirus is transcytosed by microfold cells (M-cells) in the gut-associated lymphoid tissue (GALT) where it infects the basolateral surface of IECs (8). Replication in IECs mediates reovirus release into the stool for spread to future hosts (8, 38, 39). To disseminate systemically, reovirus is taken up by cells in the Peyer's patch (4, 10, 16) and hypothesized to traffic through the MLN via the lymphatics and then to the bloodstream (8). However, the operant route of reovirus dissemination is not known. Our data provide support for the hypothesis that the lymphatics function in hematogenous reovirus dissemination, as *IFNAR1* deletion in LYVE-1-expressing cells allowed spread of the IFN-1-sensitive σ 1s-deficient reovirus.

Global and conditional deletion of JAM-A revealed that endothelial cells, but not hematopoietic cells, mediate establishment of reovirus viremia and egress from the blood into tissues (10, 15). It is possible that σ 1s promotes reovirus replication in LECs that line lymphatic vessels and lymph nodes to provide a reservoir that seeds virus for trafficking through the lymphatics to the blood. Consistent with this hypothesis, we found that σ 1s was required for reovirus protein expression and replication in primary LECs (Fig. 4). LYVE-1 is predominantly expressed on LECs but also on a small subset of fetal and adult hematopoietic stem cells, liver sinusoidal endothelial cells, and adult tissue-resident macrophages (31, 35–37). Conditional expression of JAM-A on hematopoietic cells is insufficient to restore reovirus hematogenous spread in JAM-A-deficient mice, indicating that hematopoietic cells do not mediate reovirus dissemination (15). Liver sinusoidal epithelial cells are not reported to harbor reovirus (40). In the liver, reovirus is taken up by Kupffer cells and is detected in hepatocytes (40). Treatment with silica dioxide or carrageenan to prevent macrophage uptake reduced reovirus levels in bile following intravenous inoculation (40). In contrast, carrageenan increased viral blood titers, indicating that macrophages restrict systemic spread when virus is administered intravenously (40). However, the role tissue-specific macrophages, including Kupfer cells, play in reovirus dissemination remains to be determined.

It is also possible that loss of IFN-1 signaling in LYVE-1-positive cells increases the permeability of the lymphatic endothelium, thereby allowing reovirus to escape the lymphatic vessels. IFN-1 controls LEC expansion in response to viral infection (41) and also modulates vascular endothelial barrier function, particularly at the blood-brain barrier (42, 43). Lack of IFN-1 signaling in the lymphatic endothelium could allow the σ 1s-null virus to leak into the lymphatic vessels. Loss of IFN-1 signaling in the LECs also could alter the transport dynamics of the lymphatics. In the skin, IFN-1 signaling blocks fluid transport to the regional lymph node and limits poxvirus dissemination (44). If IFN-1 has similar effects on the dynamics of gut lymphatics, removing *IFNAR1* from LECs could prevent the interruption of lymphatic flow intended to impede viral spread.

Why σ 1s is dispensable for reovirus replication in the intestine remains an open question. One possibility is that reovirus replication in the intestine is largely controlled by interferon λ (IFN- λ) as opposed to IFN-1 (38, 39, 45). Like IFN-1, IFN- λ provokes ISG expression, but IFN-1 induces ISGs with more rapid kinetics and to a greater magnitude than IFN- λ (46). IFN- λ limits reovirus replication in the intestine, as mice lacking *IFNLR1* or *IFN- λ 2/3* have elevated reovirus IEC infection and shedding (38, 39, 47). We found

no difference in viral intestinal titers between wild-type and $\sigma 1s$ -deficient viruses in wild-type or IFNAR1-knockout mice. These data are consistent with IFN- λ as the primary means of controlling reovirus replication in the intestine. It is possible that $\sigma 1s$ is more important for resisting IFN-1 than IFN- λ responses due to the lower potency of IFN- λ compared to that of IFN-1. However, the relationship between $\sigma 1s$ and IFN- λ remains unexplored.

Like most viruses, reovirus activates cellular mechanisms that function to impair viral protein synthesis, including the dsRNA-dependent protein kinase (PKR) that phosphorylates α subunit of eukaryotic initiation factor 2 (eIF2 α) to block translation (48–50) and the 2'-5' oligoadenylate synthase (OAS)-RNase L system that degrades viral RNA (48). Reovirus must produce viral proteins in the face of host translational shutoff in order to replicate efficiently. It is also hypothesized that reovirus benefits from host shutoff, as viral replication is decreased in MEFs lacking PKR or expressing a constitutively active form of eIF2 α (50). Reovirus uses multiple mechanisms to evade host translational shutoff, including outer capsid protein $\sigma 3$ binding dsRNA to blunt PKR activation (51) and IFN-1 signaling (52). Nonstructural protein σNS also facilitates escape from host shutoff by mediating dissolution of stress granules (53). However, reovirus has other means to circumvent host translational arrest. It is possible that $\sigma 1s$ promotes reovirus protein expression by counteracting the function of one or more ISGs that block host translation (20, 54). Although $\sigma 1s$ is required for reovirus replication in the presence of IFN-1 responses, $\sigma 1s$ does not function as a classical IFN-1 antagonist (19), as IFN-1 secretion, IFNAR signaling, and ISG induction are comparable between wild-type and $\sigma 1s$ -deficient viruses (19). We observed that viral protein expression by the $\sigma 1s$ -deficient virus is only partially restored in the absence of IFNAR1 signaling. This result suggests that $\sigma 1s$ promotes reovirus protein expression via an IFN-1-independent mechanism.

The $\sigma 1s$ protein is required for systemic reovirus spread (16, 17). Here, we found that $\sigma 1s$ is important for reovirus resistance to IFN-1 in cell culture and *in vivo*. We used the IFN-1-sensitive $\sigma 1s$ -deficient reovirus in combination with tissue-specific deletion of IFNAR1 in lymphatic endothelial cells to identify a role for IFN-1 responses in lymphatics in controlling reovirus spread. Together, our findings provide new insight into mechanisms that control reovirus dissemination and further define how reovirus spreads from mucosal sites of infection to target organs and tissues.

MATERIALS AND METHODS

Cells and viruses. Murine L929 fibroblasts were maintained in Joklik's modified Eagle medium (JMEM; Sigma) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin-100 μ g/ml streptomycin (Invitrogen), and 250 ng/ml amphotericin B (Sigma). SV40 immortalized endothelial cells (SVECs; ATCC), C57BL/6 murine embryonic fibroblasts (MEFs), IFNAR1^{-/-} MEFs, and human embryonic kidney 293 cells (HEK293T) were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented to contain 10% heat-inactivated FBS and 2 mM L-glutamine. SVECs lacking the IFN- α/β receptor (IFNAR1-CRISPR) were maintained in the same medium as SVECs with 2 μ g/ml puromycin (Gibco). Primary LECs (Cell Biologicals) were maintained in endothelial cell medium (Cell Biologicals) supplemented to contain 5% FBS, 2 mM L-glutamine, antibiotic-antimycotic solution (100 U/ml penicillin-100 U/ml streptomycin-50 ng/ml amphotericin B), and each of the following according to the manufacturer's instructions: vascular endothelial growth factor (VEGF), heparin, endothelial cell growth supplement (ECGS), epidermal growth factor (EGF), and hydrocortisone.

Reoviruses were generated using plasmid-based reverse genetics as described (8, 16, 17, 55, 56). Purified reovirus stocks were obtained from second- or third-passage L929 cell lysates from twice-plaque-purified reovirus (57). Vertrel was used to extract reovirus particles, which were separated on a 1.2- to 1.4-g/cm³ CsCl density gradient and exhaustively dialyzed in virion storage buffer (150 mM NaCl, 15 mM MgCl₂, 10 mM Tris-HCl, pH 7.8). The titers of the viral stocks were determined by plaque assay on L929 cells (58).

CRISPR-Cas9 deletion of IFNAR1. IFNAR1 was deleted in SVECs using the CRISPR-Cas9 system (59). The plentiCRISPRv2 (Addgene) was digested with BsmBI and ligated with guide RNA sequences specific for IFNAR1, (IFNAR1⁺) 5'-CACCGGCTCGCTGTCTGTGGCGCGG-3' and (IFNAR1⁻) 5'-AAACCCGCGCCCACGACAGCGAGCC-3'. The cloned plasmids were transfected in to HEK293T cells in combination with pSPAX2 and pCMV-G plasmids using Lipofectamine 2000 (Invitrogen). Supernatants were collected at 24 and 48 h posttransfection, passed through 0.45- μ m syringe filters, and applied to SVECs in 6-well plates (~50% confluent). At 48 h posttransduction, puromycin (Invitrogen; 2 μ g/ml) was added to the medium.

Puromycin-selected SVECs were tested for IFNAR1 deletion by treatment with IFN- β (PBL) followed by reverse transcriptase quantitative PCR (RT-qPCR) to measure ISG expression.

Viral replication assays. Monolayers of cells in 24-well plates (1×10^5 cells/well) were infected with the T1L or T1L σ 1s-null strain at an MOI of 1 PFU/cell at 4°C for 1 h. Cells were washed twice with cold phosphate-buffered saline with calcium and magnesium (PBS+/+; Invitrogen), and fresh medium was added. Infected cells were freeze-thawed twice at the indicated times in the figure legends, and viral titers were determined by plaque assay on L929 cells (58). Viral yields were calculated using the following formula: $\log_{10} \text{yield}_{tx} = \log_{10}(\text{PFU/ml})_{tx} - \log_{10}(\text{PFU/ml})_{t0}$, where tx is the time postinfection.

Immunoblotting. Monolayers of cells in 6-well plates (1×10^6 cells/well) were mock infected or infected with reovirus or treated with recombinant IFN- β (PBL Assay Science) as indicated in the figure legends. Whole-cell lysates were collected in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, 1% desoxycholate, and 1/100 IGEPAL [NP-40]) at the indicated times. Total protein in each sample was quantified using the DC protein assay (Bio-Rad), and 10 μ g protein was separated by 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and incubated in blocking buffer (5% milk in $1 \times$ Tris-buffered saline [TBS] with 0.05% Tween 20 [TBS-T]) for 1 h. Membranes were incubated in blocking buffer containing reovirus-specific rabbit polyclonal antiserum (1:2,000 dilution), IFIT1 antibody (Abcam 111821; 1:1,000 dilution), or IFIT2 antibody (Thermo Fisher PA3-834; 1:1,000 dilution) overnight at 4°C. Membranes were washed three times with TBS-T followed by incubation in blocking buffer containing horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunolabs; 1:2,000 dilution) for 1 h with rocking. Following three TBS-T washes, proteins were detected using SuperSignal West chemiluminescent substrate (Thermo Fisher) and imaged using a ChemiDoc imaging system (Bio-Rad). Blots were stripped for reprobing by washing membranes three times with TBS followed by incubation in Restore Western blot stripping buffer (Thermo Scientific) for 15 min at room temperature (RT). Following three washes in TBS, membranes were blocked as described above, and β -actin was detected using mouse β -actin-specific monoclonal antibody (Sigma; 1:10,000 dilution) and peroxidase-conjugated goat anti-mouse IgG (Jackson Immunolabs; 1:2,000 dilution).

RT-qPCR. Monolayers of cells in 6-well plates (1×10^6 cells/well) were infected with the T1L or T1L σ 1s-null strain at an MOI of 10 PFU/cell. Total RNA was collected using the RNeasy Plus kit (Qiagen). Reovirus S4 RNA was quantified using the TaqMan fast virus one-step master mix (Applied Biosystems), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected as an endogenous control using the predeveloped TaqMan assay reagent kit for mouse GAPDH (Applied Biosystems) as described previously (19). The relative quantity (RQ) of reovirus positive- or negative-sense RNA was quantified using $t = 0$ h postinfection as the reference sample. The $\Delta\Delta C_T$ was calculated for each sample using the following formula: $\Delta\Delta C_T = (\text{unknown } C_{Tx} - \text{GAPDH } C_{Tx}) - (\text{unknown } C_{T0} - \text{GAPDH } C_{T0})$ where tx = time postinfection. The $\Delta\Delta C_T$ was then used to calculate RQ using the following formula: $\text{RQ} = 2^{-\Delta\Delta C_T}$.

Mouse experiments. Animal husbandry, housing, and experiments were performed according to the guidelines of the Division of Laboratory Animal Medicine (DLAM) at University of Arkansas for Medical Sciences (UAMS). C57BL/6 (JAX stock number 000664), C57BL/6 *IFNAR1*^{-/-} (JAX stock number 028288), C57BL/6 *IFNAR1*^{fl/fl} (JAX stock number 028256), and C57BL/6 *Lyve1-Cre* (JAX stock number 012601) mice were obtained from Jackson Laboratory. *IFNAR1*^{fl/fl} and *Lyve1-Cre* mice were crossed to obtain *IFNAR1*^{+fl}/*Lyve1-Cre* heterozygous mice. *IFNAR1*^{+fl}/*LYVE1-Cre* heterozygous mice were crossed to *IFNAR1*^{fl/fl} mice for experiments, yielding litters of *IFNAR1*^{fl/fl}, *IFNAR1*^{+fl}, *IFNAR1*^{+fl}/*Lyve1-Cre*, and *IFNAR1*^{fl/fl}/*Lyve1-Cre* mice. Three- to four-day-old mice were infected orally with 10^4 PFU T1L or T1L σ 1s-null strain diluted in PBS as previously described (10, 60). At 4 days postinfection, organs were resected and homogenized, and viral titer was determined by plaque assay. Infected mice were genotyped after experiments using the KAPA HotStart mouse genotyping kit (KAPA Biosystems) and primers for the floxed *IFNAR1* allele and the *Lyve1-Cre* gene from the Jackson Laboratory website.

Statistics. Differences in viral replication were determined by an unpaired Student's *t* test. Differences in viral titer from mouse experiments were determined by Mann-Whitney test. Statistical tests were performed using Prism software (GraphPad Software, Inc.). *P* values of <0.05 were considered significant.

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