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Expression of *Ifnlr1* on Intestinal Epithelial Cells Is Critical to the Antiviral Effects of Interferon Lambda against Norovirus and Reovirus

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ABSTRACT Lambda interferon (IFN-λ) has potent antiviral effects against multiple enteric viral pathogens, including norovirus and rotavirus, in both preventing and curing infection. Because the intestine includes a diverse array of cell types, however, the cell(s) upon which IFN-λ acts to exert its antiviral effects is unclear. Here, we sought to identify IFN-λ-responsive cells by generation of mice with lineage-specific deletion of the receptor for IFN-λ, *Ifnlr1*. We found that expression of IFNLR1 on intestinal epithelial cells (IECs) in the small intestine and colon is required for enteric IFN-λ antiviral activity. IEC *Ifnlr1* expression also determines the efficacy of IFN-λ in resolving persistent murine norovirus (MNoV) infection and regulates fecal shedding and viral titers in tissue. Thus, the expression of *Ifnlr1* by IECs is necessary for the response to both endogenous and exogenous IFN-λ. We further demonstrate that IEC *Ifnlr1* expression is required for the sterilizing innate immune effects of IFN-λ by extending these findings in *Rag1*-deficient mice. Finally, we assessed whether our findings pertained to multiple viral pathogens by infecting mice specifically lacking IEC *Ifnlr1* expression with reovirus. These mice phenocopied *Ifnlr1*-null animals, exhibiting increased intestinal tissue titers and enhanced reovirus fecal shedding. Thus, IECs are the critical cell type responding to IFN-λ to control multiple enteric viruses. This is the first genetic evidence that supports an essential role for IECs in IFN-λ-mediated control of enteric viral infection, and these findings provide insight into the mechanism of IFN-λ-mediated antiviral activity.

IMPORTANCE Human noroviruses (HNoVs) are the leading cause of epidemic gastroenteritis worldwide. Type III interferons (IFN-λ) control enteric viral infections in the gut and have been shown to cure mouse norovirus, a small-animal model for HNoVs. Using a genetic approach with conditional knockout mice, we identified IECs as the dominant IFN-λ-responsive cells in control of enteric virus infection *in vivo*. Upon murine norovirus or reovirus infection, *Ifnlr1* depletion in IECs largely recapitulated the phenotype seen in *Ifnlr1*$^{-/-}$ mice of higher intestinal tissue viral titers and increased viral shedding in the stool. Moreover, IFN-λ-mediated sterilizing immunity against murine norovirus requires the capacity of IECs to respond to IFN-λ. These findings clarify the mechanism of action of this cytokine and emphasize the therapeutic potential of IFN-λ for treating mucosal viral infections.

KEYWORDS innate immunity, interferons, mucosal immunity, norovirus, reovirus

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Norovirus and rotavirus are viral pathogens that infect at mucosal surfaces and induce gastroenteritis, characterized by vomiting, diarrhea, and malaise (1, 2). Viral gastroenteritis causes significant morbidity and mortality in children, the elderly, and immunocompromised persons, thus representing a substantial health care burden (3, 4). Treatments for these illnesses have been limited thus far to symptomatic care, including rehydration, because currently there is no specific antiviral therapy for these viral pathogens. Lambda interferon (IFN-λ; also called type III IFN) is an antiviral cytokine that regulates viral infection at mucosal surfaces and in the liver and brain (5–8). Administration of recombinant IFN-λ can prevent and resolve viral infections in the gastrointestinal tract (8, 9) and at other sites in mice (10). These effects are observed for murine norovirus (MNoV) in mice lacking adaptive immunity, thus representing sterilizing innate immunity in the intestine (8). These studies indicate the potential for IFN-λ as a therapeutic for viral infections, including those causing gastroenteritis, in humans, including immunocompromised hosts (11). Better understanding of the mechanisms by which this antiviral cytokine functions is essential to understanding basic mechanisms of intestinal control of viral infection and for potential therapeutic application in humans.

Binding of IFN-λ to its receptor, a heterodimer of interleukin-10R2 (IL-10R2) and IFNLR1 (12, 13), induces an antiviral gene expression program similar to that induced by type I IFN, with substantial overlap in gene sets *in vitro* (10, 14, 15). However, type I and III IFNs exhibit unique antiviral properties *in vivo*. *Ifnlr1*$^{-/-}$ mice exhibit elevated intestinal tissue replication and enhanced fecal shedding of a persistent strain of MNoV (8, 16), a model virus which allows for more tractable *in vitro* and *in vivo* analyses than human norovirus (reviewed in references 17 and 18). Recombinant IFN-λ treatment is sufficient to prevent and cure MNoV infection (8). In contrast, mice deficient for *Ifnar1* (the receptor for type I IFNs) show enhanced extraintestinal spread of virus, but levels of MNoV fecal shedding are comparable to those of wild-type mice (8, 16). Similarly, IFNLR1 restricts growth in the epithelium and fecal shedding of reovirus, while IFNAR1 instead regulates reovirus growth in the lamina propria (19). IFN-λ exhibits an antiviral role exclusive of type I IFNs against a murine rotavirus strain (9) but cooperates with type I IFNs to limit intestinal replication of a heterologous simian strain in neonatal but not adult mice (20). These findings indicate the likely importance of tissue compartment-, development-, and cell type-specific effects of type I and III IFNs *in vivo*. These effects may be secondary to unique virulence factors that counter specific IFNs or to differential expression of the IFN receptors (21, 22).

IFNAR1 is thought to be expressed ubiquitously and at especially high levels on cells of hematopoietic origin (reviewed in references 23 and 24), whereas expression of detectable IFNLR1 appears to be limited to mucosal epithelial cells (25), human hepatocytes (6), and neutrophils (26). Although IFNLR1 expression on peripheral leukocytes has also been reported, it does not appear to be functional (27). Upon IFN-λ treatment, IFN-stimulated genes accumulate in intestinal epithelial cells (IECs), indicating functional IFNLR1 expression (9, 19, 20). In contrast, in IECs of adult mice, IFNAR1 may be expressed at lower levels or alternately trafficked, such as only to the apical portion of the cell (9, 20). Differential receptor expression thus could account for complementary roles for different IFNs in protection against systemic infection (type I) and infection of mucosal (type III) sites. Importantly, however, it has been reported that cells that do not express detectably high levels of IFNLR1, such as the endothelial cells of the blood-brain barrier, may still respond to endogenous and exogenous IFN-λ with protective antiviral effects (10). Thus, to successfully identify the cell types required for the antiviral response to IFN-λ, analysis of receptor expression levels may be insufficient, and definitive resolution requires a genetic approach to selectively delete receptor expression in specific cell types.

To identify the cell types that respond to IFN-λ *in vivo* in the intestine, we generated mice with a conditional mutant allele for *Ifnlr1* and crossed them to mice expressing Cre recombinase via the action of different cell type-specific promoters (Table 1). *Ifnlr1* was targeted in cell types expected to express high receptor levels (intestinal epithelial cells

TABLE 1 Mouse lines, nomenclature, and cell types targeted by specific Cre lines

Ifnlr1 and Cre mouse line(s)	Line name[a]	Cell type(s) targeted (reference)
Ifnlr1^{tm1Palu}; no Cre line	*Ifnlr1*$^{-/-}$	All cells (28)
Ifnlr1$^{tm1a(EUCOMM)Wtsi}$; Villin-Cre	*Ifnlr1*$^{f/f}$*-Villincre*	Intestinal epithelial cells (29)
Ifnlr1$^{tm1a(EUCOMM)Wtsi}$; MRP8-Cre	*Ifnlr1*$^{f/f}$*-MRP8cre*	Neutrophils (30)
Ifnlr1$^{tm1a(EUCOMM)Wtsi}$; CD11c-Cre	*Ifnlr1*$^{f/f}$*-CD11ccre*	Dendritic cells and alveolar macrophages (31)
Ifnlr1$^{tm1a(EUCOMM)Wtsi}$; LysM-Cre	*Ifnlr1*$^{f/f}$*-LysMcre*	Macrophages, neutrophils, some dendritic cells (32, 33)
Ifnlr1$^{tm1a(EUCOMM)Wtsi}$; Deleter-Cre	*Ifnlr1*$^{f-/-}$	All cells (34)

[a] A conditional allele of *Ifnlr1* (*Ifnlr1*$^{f/f}$) was crossed to multiple different Cre lines for lineage-specific deletion of *Ifnlr1* in the specific cell types.

[25] and neutrophils [26]) and cells that are known to be permissive for MNoV replication in tissue culture (macrophages and dendritic cells [35]). Of all the cell types tested, only intestinal epithelial cells (IECs) required expression of *Ifnlr1* for the antiviral effects of IFN-λ against MNoV. To show the generality of our findings, we demonstrated the importance of IEC expression of this receptor for control of reovirus infection. This is the first study to genetically define IFN-λ-responsive cells *in vivo* in the context of two independent mucosal viral infections. This study also confirms that the cells required for responding to endogenous IFN-λ to attenuate MNoV infection are the same as those that respond to exogenous IFN-λ administration, including in the elicitation of sterilizing innate immunity.

RESULTS

***Ifnlr1* is expressed in the epithelial fraction along the length of the gastrointestinal tract.** Tissue from adult mice homozygous for a null mutation in *Ifnlr1* (28) or wild-type controls was collected from sites along the intestine, lung, mesenteric lymph node (MLN), or spleen (Fig. 1A). The small intestine was also dissociated into epithelial and lamina propria fractions as previously described (36), and RNA was isolated from

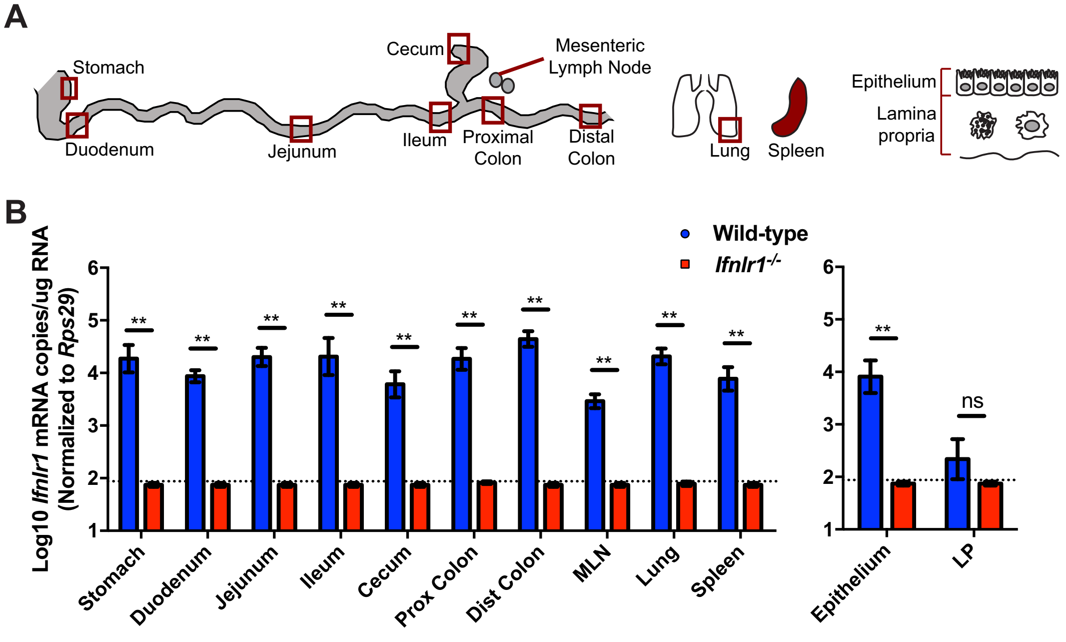

FIG 1 *Ifnlr1* is expressed in the epithelial fraction along the length of the intestine. (A) RNA was isolated from sites along the intestine and the lung, indicated by red boxes, whole mesenteric lymph node (MLN) and spleen, and epithelial and lamina propria (LP) fractions of the small intestine from *Ifnlr1*-sufficient and -deficient mice. (B) *Ifnlr1* expression was quantified by quantitative real-time PCR of RNA from sites depicted in panel A. $n = 4$ to 6 samples per group, from two independent experiments, analyzed by Mann-Whitney test. **, $P < 0.01$; ns, not significant. Prox, proximal; dist, distal.

these fractions and tissues. Expression of *Ifnlr1* was detected by quantitative real-time PCR of cDNA generated from these RNA samples. We found that *Ifnlr1* was expressed along the length of the intestine and in the lung, as well as in systemic tissues, including MLN and spleen (Fig. 1B). Intestinal *Ifnlr1* expression was substantially enriched (at least 30-fold; $P = 0.0381$) in the epithelial fraction compared to the lamina propria fraction (Fig. 1B), consistent with previous reports (9, 25). As expected, no transcript was detected in any tissue in *Ifnlr1*$^{-/-}$ mice (Fig. 1B).

***Ifnlr1* expression in the small and large intestine is significantly diminished in *Ifnlr1*$^{f/f}$*-Villincre* mice.** Embryonic stem (ES) cells targeted with a construct containing sequences homologous to *Ifnlr1*, an FLP recombinant target (FRT)-flanked *lacZ* and neomycin cassette, and loxP sites flanking exon 2 were provided by the Wellcome Trust Sanger Institute (Fig. 2A). Mice derived from these ES cells were crossed with mice expressing Flp recombinase for deletion of the FRT-flanked cassette (38), leaving a conditional allele of *Ifnlr1*, referred to as *Ifnlr1*^{f} (Fig. 2A). Following removal of the floxed region in cells expressing Cre, the resulting transcript is predicted to produce a truncated protein product (Fig. 2B). For disruption in specific cell lineages, *Ifnlr1*$^{f/f}$ mice were crossed with various Cre mouse lines (Table 1). For each line, Cre(+) *Ifnlr1*$^{f/f}$ mice were compared to Cre(−) *Ifnlr1*$^{f/f}$ littermates to assess the effects of cell type-specific deletion upon *Ifnlr1* expression along the intestine and in extraintestinal tissues (Fig. 1A). *Ifnlr1*$^{f/f}$*-Villincre* mice showed significantly diminished *Ifnlr1* expression in the small and large intestine (Fig. 2C). Fractionation of the small intestine into epithelial and lamina propria fractions revealed efficient deletion of *Ifnlr1* in the epithelium of these mice (Fig. 2C). In contrast, *Ifnlr1*$^{f/f}$*-MRP8cre*, *Ifnlr1*$^{f/f}$*-LysMcre*, and *Ifnlr1*$^{f/f}$*-CD11ccre* mice showed no alterations in intestinal *Ifnlr1* expression at the level of the whole tissues tested (Fig. 2D, E, and F). *Ifnlr1*$^{f/f}$*-MRP8cre*, *Ifnlr1*$^{f/f}$*-LysMcre*, and *Ifnlr1*$^{f/f}$*-CD11ccre* mice did exhibit substantial depletion of *Ifnlr1* in their respectively targeted cell types of neutrophils (~85%), macrophages (~91%), and dendritic cells (~85%), consistent with a previous report (39) (Fig. 2D, E, and F). Expression of *Ifnlr1* remained unchanged in lung, MLN, spleen, stomach, and duodenum in *Ifnlr1*$^{f/f}$*-Villincre* mice, indicating expression of Cre specific to distal small intestine and colon (Fig. 2C), consistent with previous reports (29, 40).

Expression of *Ifnlr1* in intestinal epithelium regulates MNoV shedding and response to recombinant IFN-λ. *Ifnlr1*$^{-/-}$ mice and wild-type controls were inoculated with CR6, a persistent strain of MNoV that replicates well in the intestine, is shed into the feces at readily detectable levels, and is sensitive to treatment with IFN-λ (8, 41). As described previously (8), *Ifnlr1*$^{-/-}$ mice allow higher levels of fecal MNoV shedding than do wild-type mice at early time points (Fig. 3A and G) and are insensitive to IFN-λ treatment, although this treatment terminates MNoV replication in wild-type mice (Fig. 3A). These results were also observed in a novel *Ifnlr1*-deficient mouse model (*Ifnlr1*$^{f-/-}$) (Table 1 and Fig. 3B). This assay was next applied to the four mouse strains with lineage-specific deletion of *Ifnlr1* (Table 1). *Ifnlr1*$^{f/f}$*-Villincre* mice phenocopied *Ifnlr1*$^{-/-}$ and *Ifnlr1*$^{f-/-}$ mice, exhibiting both elevated fecal shedding of MNoV and resistance to IFN-λ treatment (Fig. 3C). In contrast, *Ifnlr1*$^{f/f}$*-MRP8cre*, *Ifnlr1*$^{f/f}$-LysM*cre*, and *Ifnlr1*$^{f/f}$-CD11c*cre* mice exhibited viral loads and response to IFN-λ equivalent to those of *Ifnlr1*$^{f/f}$ controls (Fig. 3D, E, and F). At day 7 postinoculation, IFNLR1 regulated fecal shedding of MNoV, as seen by comparing wild-type and *Ifnlr1*$^{-/-}$ levels (Fig. 3G). *Ifnlr1*$^{f/f}$*-Villincre* mice allowed fecal shedding equivalent to *Ifnlr1*$^{-/-}$ and *Ifnlr1*$^{f-/-}$ mice, suggesting that control of MNoV fecal shedding can be fully accounted for by IFNLR1 in Villin-expressing cells (Fig. 3G). Similarly, *Ifnlr1*$^{f/f}$*-Villincre* mice exhibited no difference in comparison to *Ifnlr1*$^{-/-}$ and *Ifnlr1*$^{f-/-}$ mice along the full time course of infection (Fig. 3H).

Expression of *Ifnlr1* in intestinal epithelium is essential for induction of IFN-λ-mediated sterilizing innate immunity to MNoV infection. We previously reported that recombinant IFN-λ can cure persistently infected mice in the absence of adaptive immunity (8). To determine whether expression of *Ifnlr1* in IECs is required for IFN-λ-mediated sterilizing innate immunity to persistent MNoV infection, we established

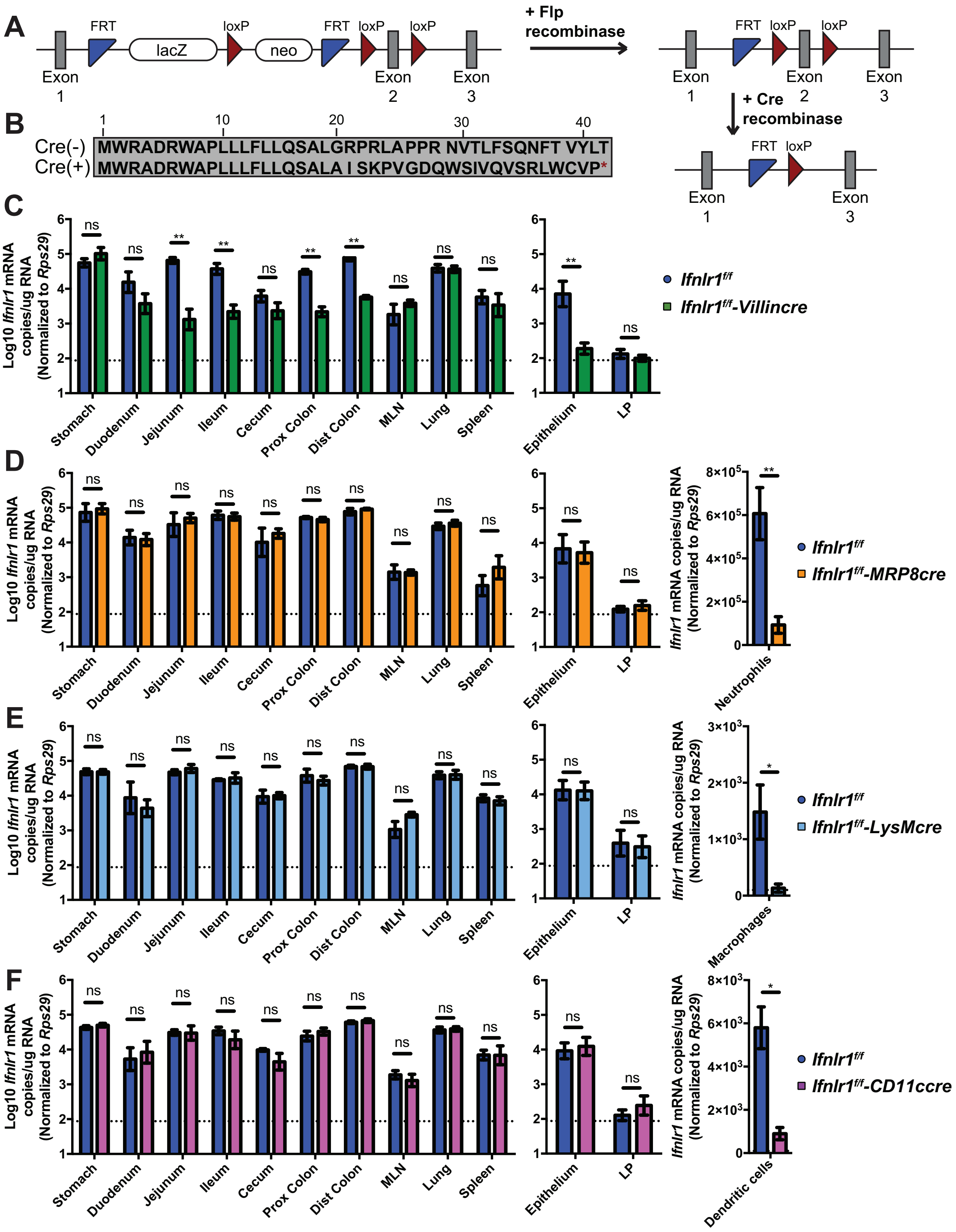

FIG 2 *Ifnlr1* expression is decreased in the small and large intestines of *Ifnlr1*$^{f/f}$-*Villincre* mice. (A) Schematic depicting the *Ifnlr1* gene locus in *Ifnlr1*$^{tm1a(EUCOMM)Wtsi}$ mice. After crossing with mice expressing Flp recombinase (+Flp recombinase), the region between the two FRT sites was deleted, leaving conditional-ready *Ifnlr1*$^{f/f}$ mice. In the absence of Cre, all exons are present. With the addition of Cre recombinase, the floxed exon 2 is deleted. (B) In the absence of Cre [Cre(−)], the IFNLR1 protein is expressed. In the presence of Cre [Cre(+)], the protein sequence is altered at amino acid 20 and a premature stop codon is introduced at amino acid 42. (C to F) *Ifnlr1* expression was assessed by quantitative real-time PCR of sites along the intestine and the lung, MLN and spleen, and epithelial and LP fractions from *Ifnlr1*$^{f/f}$-*Villincre* (C), *Ifnlr1*$^{f/f}$-*MRP8cre* (D), *Ifnlr1*$^{f/f}$-*LysMcre* (E), and *Ifnlr1*$^{f/f}$-*CD11ccre* (F) mice compared to their *Ifnlr1*$^{f/f}$ littermates. *Ifnlr1* expression was also assessed by quantitative real-time PCR of isolated bone marrow neutrophils from *Ifnlr1*$^{f/f}$-*MRP8cre* (D), splenic macrophages from *Ifnlr1*$^{f/f}$-*LysMcre* (E), and splenic dendritic cells from *Ifnlr1*$^{f/f}$-*CD11ccre* (F) mice compared to their *Ifnlr1*$^{f/f}$ littermates. $n = 4$ to 7 samples per group, from two independent experiments, analyzed by Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

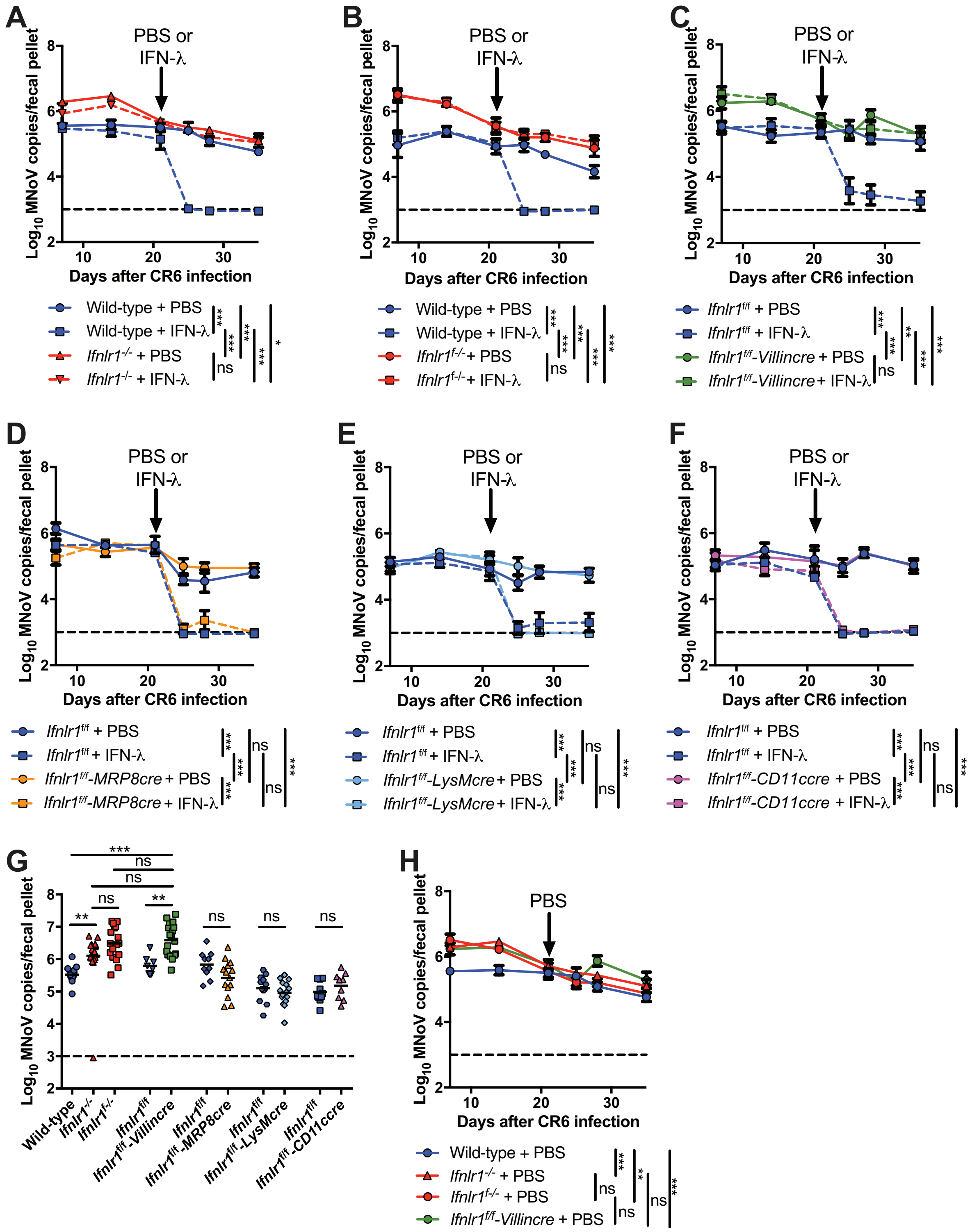

FIG 3 Expression of *Ifnlr1* on intestinal epithelial cells is required for the antiviral effects of endogenous and exogenous IFN-λ against MNoV. (A to E) Time course of MNoV genome copies shed into fecal pellets with time points at 7, 14, 21, 24, 28, and 35 days after CR6 infection. PBS or recombinant IFN-λ was injected intraperitoneally on day 21 into wild-type and *Ifnlr1*$^{-/-}$ (A), wild-type and *Ifnlr1*$^{f-/-}$ (B), *Ifnlr1*$^{f/f}$-*Villincre* (C), *Ifnlr1*$^{f/f}$-*MRP8cre* (D), *Ifnlr1*$^{f/f}$-*LysMcre* (E), or *Ifnlr1*$^{f/f}$-*CD11ccre* (F) mice and their *Ifnlr1*$^{f/f}$ littermates. $n = 6$ to 12 mice per group, from two to three independent experiments, analyzed by two-way ANOVA followed by Tukey's multiple-comparison test; a P value of <0.001 by ANOVA column factor was found for panels A to F. (G) Individual data points depicting MNoV genome copies shed into fecal pellets on day 7 from panels A to F. $n = 9$ to 21 mice per group, from two to three independent experiments, analyzed by one-way ANOVA followed by Tukey's multiple-comparison test; a P value of <0.001 was determined by ANOVA. (H) Fecal shedding data from PBS-treated mice in panels A to C is shown superimposed to facilitate comparison between strains. $n = 8$ to 11 mice per group, from two to three independent experiments, analyzed by two-way ANOVA followed by Tukey's multiple-comparison test; a P value of <0.001 was determined by ANOVA column factor. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

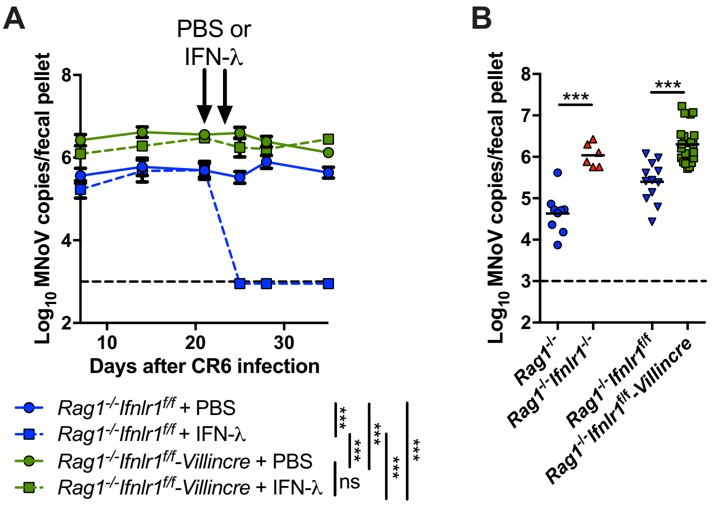

FIG 4 Expression of *Ifnlr1* on intestinal epithelial cells is required for the antiviral effects of IFN-λ against MNoV in the absence of adaptive immunity. (A) Time course of MNoV genome copies shed into fecal pellets with time points at 7, 14, 21, 24, 28, and 35 days after CR6 infection. PBS or recombinant IFN-λ was injected intraperitoneally on day 21 and day 23 into *Rag1*$^{-/-}$ *Ifnlr1*$^{f/f}$*-Villincre* or *Rag1*$^{-/-}$ *Ifnlr1*$^{f/f}$ mice. $n = 6$ to 14 mice per group, combined from three independent experiments, analyzed by two-way ANOVA followed by Tukey's multiple-comparison test; a P value of <0.001 was found by ANOVA column factor. (B) Individual data points depicting MNoV genome copies shed into fecal pellets on day 7 from *Rag1*$^{-/-}$, *Rag1*$^{-/-}$ *Ifnlr1*$^{-/-}$ double knockouts or mice depicted in panel A. $n = 6$ to 22 mice per group, combined from two to three independent experiments, analyzed by Mann-Whitney test. ***, $P < 0.001$; ns, not significant.

Rag1$^{-/-}$ *Ifnlr1*$^{f/f}$*-Villincre* conditional double knockout mice. *Rag1*$^{-/-}$ *Ifnlr1*$^{f/f}$*-Villincre* mice were orally inoculated with CR6, and viral shedding in the stool was quantified by quantitative PCR (qPCR). *Rag1*$^{-/-}$ *Ifnlr1*$^{f/f}$*-Villincre* mice showed increased viral shedding throughout the infection time course (Fig. 4A). Injection of recombinant IFN-λ terminated MNoV replication in *Rag1*$^{-/-}$ *Ifnlr1*$^{f/f}$ mice but did not affect MNoV loads in *Rag1*$^{-/-}$ *Ifnlr1*$^{f/f}$*-Villincre* mice (Fig. 4A). At 7 days postinoculation, *Rag1*$^{-/-}$ *Ifnlr1*$^{f/f}$*-Villincre* mice had significantly higher viral shedding than *Rag1*$^{-/-}$ *Ifnlr1*$^{f/f}$ mice, and the level of viral shedding in *Rag1*$^{-/-}$ *Ifnlr1*$^{f/f}$*-Villincre* mice was comparable to the level of viral shedding in *Rag1*$^{-/-}$ *Ifnlr1*$^{-/-}$ mice (Fig. 4B). Therefore, IFN-λ responses in IECs limited persistent MNoV infection in the absence of adaptive immunity, and IFN-λ signaling in IECs was essential for clearance of persistently infected MNoV by IFN-λ-mediated sterilizing innate immunity.

Control of reovirus in intestinal tissue by IFN-λ depends upon the expression of *Ifnlr1* in epithelial cells. To assess whether *Ifnlr1* expression on IECs was required for control of other enteric pathogens, *Ifnlr1*$^{-/-}$ and *Ifnlr1*$^{f/f}$*-Villincre* mice were orally inoculated with 10^8 PFU of reovirus strain type 1 Lang (T1L). At 4 days postinfection, viral titers in small intestinal tissues, including duodenum, jejunum, and ileum, as well as viral shedding in stools, were significantly higher in *Ifnlr1*$^{-/-}$ mice (Fig. 5A and B), consistent with a previous report using another strain of reovirus, type 3 Dearing (19). Control of reovirus was predominantly through the expression of IFNLR1 on IECs, as *Ifnlr1*$^{f/f}$*-Villincre* mice displayed increased titers of reovirus in small intestinal tissues as well as enhanced fecal shedding (Fig. 5A and B). These results demonstrate that expression of IFNLR1 in epithelial cells is essential for the control of reovirus infection by IFN-λ in the gut and indicate that IFN-λ signaling in IECs is an antiviral mechanism common to multiple enteric viral pathogens.

Interferon-stimulated gene expression in the intestine depends upon the expression of *Ifnlr1* in epithelial cells. Ileum and proximal colon tissues were isolated from wild-type (WT), *Ifnlr1*$^{-/-}$, *Ifnlr1*$^{f/f}$, and *Ifnlr1*$^{f/f}$*-Villincre* mice 1 day posttreatment with either PBS or IFN-λ. These tissues were then assessed for expression of canonical antiviral interferon-stimulated genes (ISGs) *Oas1a* (42), *Ifit1* (43), and *Ifi44* (44) (Fig. 6A

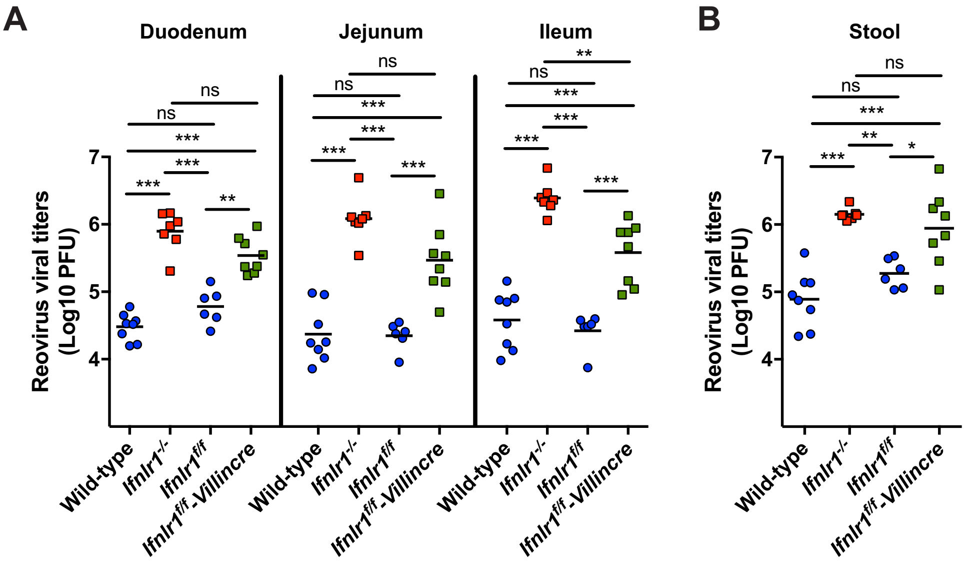

FIG 5 *Ifnlr1* expression on intestinal epithelial cells limits reovirus infection. (A and B) Titers of reovirus strain T1L were assessed at day 4 postinoculation in the different compartments of the small intestine (A) and stool (B) from wild-type, *Ifnlr1*$^{-/-}$, *Ifnlr1*$^{f/f}$-*Villincre*, and *Ifnlr1*$^{f/f}$ littermate control mice. The small intestine was resected from the pylorus to the cecum and sectioned into three equal parts, representing the duodenum, jejunum, and ileum. Titers are expressed as PFU per milliliter of tissue homogenate or gram of stool. $n = 6$ to 8 mice per group, combined from two independent experiments, analyzed by one-way ANOVA followed by Tukey's multiple-comparison test; a P value of <0.001 was determined by ANOVA column factor for all tissues and stool. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

to C). While intestinal tissues from WT and *Ifnlr1*$^{f/f}$ mice exhibited robust ISG induction in response to IFN-λ treatment, tissues from *Ifnlr1*$^{-/-}$ and *Ifnlr1*$^{f/f}$-*Villincre* mice failed to significantly upregulate these ISGs in response to IFN-λ. These data correlate with the impaired antiviral response against MNoV in *Ifnlr1*$^{-/-}$ and *Ifnlr1*$^{f/f}$-*Villincre* mice after IFN-λ treatment (Fig. 3A and C), consistent with a potentially critical role for IFNLR1 expression on epithelial cells for induction of antiviral ISGs in response to IFN-λ treatment.

DISCUSSION

In this study, we found that IECs are the predominant cell type expressing *Ifnlr1* in the small intestine and colon and that this cell type plays a major role in IFN-λ-mediated antiviral immunity in the intestine. Antiviral immunity elicited by IFN-λ to enteric reovirus and norovirus infection depends upon IFNLR1 signaling in Villin-positive IECs. Using four mouse strains with lineage-specific deletion of *Ifnlr1* to study persistent infection and IFN-λ-mediated clearance, we found that only *Ifnlr1*$^{f/f}$-*Villincre* mice exhibited a complete phenocopy of *Ifnlr1*$^{-/-}$ mice. Targeting *Ifnlr1* in other cells, including dendritic cells, macrophages, and neutrophils, had no detectable effect on basal levels of viral shedding or IFN-λ-mediated clearance of MNoV. The dominant IFN-λ-dependent antiviral contribution by IECs also was confirmed with reovirus infection. In studies using reovirus T1L, we observed viral titers in the small intestine of *Ifnlr1*$^{f/f}$-*Villincre* mice increased comparably to those detected in *Ifnlr1*$^{-/-}$ mice, although we cannot rule out a minor role for other IFN-λ-responding cells in the ileum. Therefore, IECs are the functionally dominant IFN-λ-responding cells for endogenous and exogenous IFN-λ control of viruses in the intestine. There is a clear correlation between IFN-λ-mediated induction of antiviral ISGs and IEC expression of IFNLR1, suggesting that induction of ISGs in IECs is the mechanism by which IFN-λ exerts its antiviral effects.

Expression of *Ifnlr1* mRNA throughout the gut and in other extraintestinal tissues (MLN, lung, and spleen) was quantified by qPCR analysis. In lamina propria cells, there were fewer than 500 copies of *Ifnlr1* mRNA per 1 μg total RNA. In contrast, IECs express

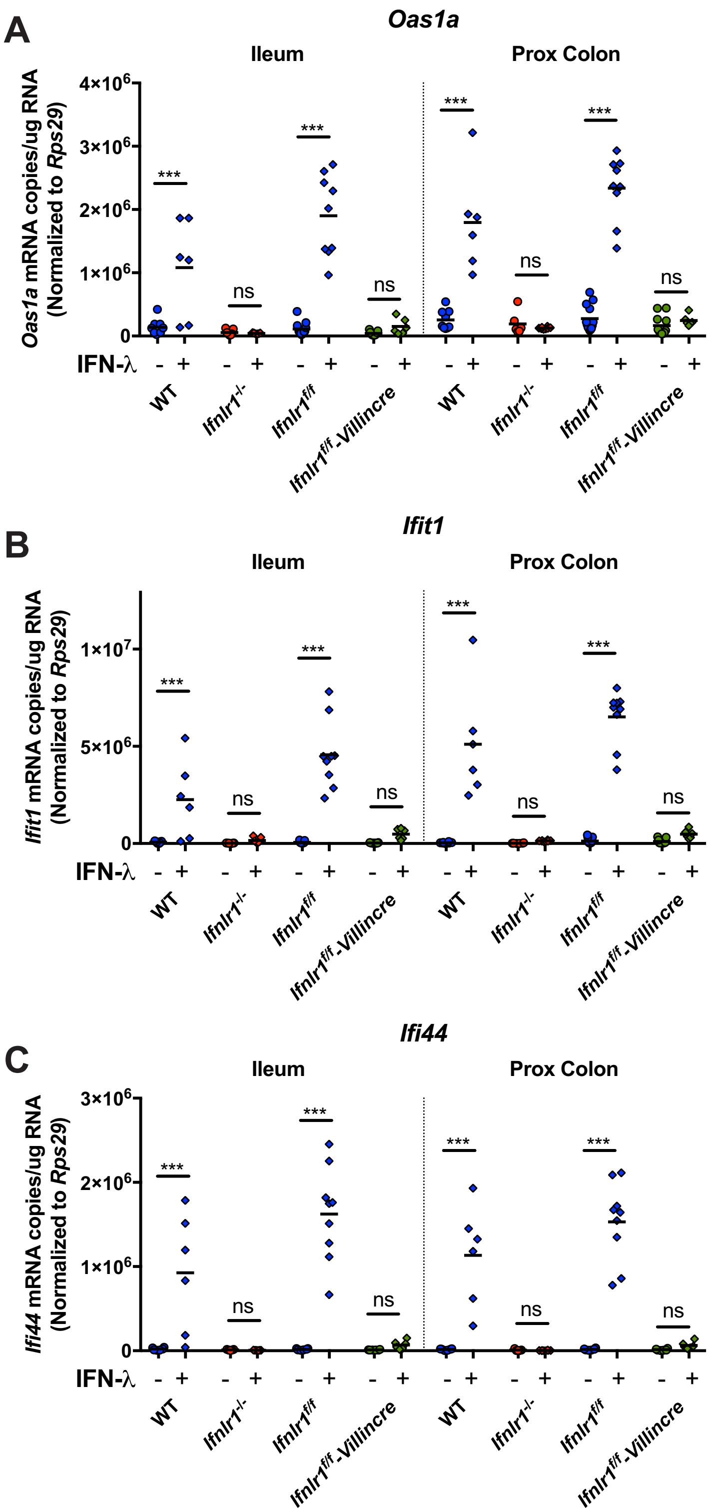

FIG 6 *Ifnlr1* expression on intestinal epithelial cells is necessary for induction of interferon-stimulated genes. *Oas1a* (A), *Ifit1* (B), and *Ifi44* (C) expression was assessed by quantitative real-time PCR of RNA from distal ileum and proximal colon tissue from wild-type (WT), *Ifnlr1*$^{-/-}$, *Ifnlr1*$^{f/f}$, and *Ifnlr1*$^{f/f}$*-Villincre* mice at 1 day posttreatment with PBS or recombinant IFN-λ. n = 5 to 9 mice per group, combined from two independent experiments, analyzed by one-way ANOVA followed by Tukey's multiple-comparison test; a P value of <0.001 was determined by ANOVA column factor for all tissues. ***, $P < 0.001$; ns, not significant.

more than 10,000 copies of *Ifnlr1* mRNA per 1 μg total RNA throughout the small intestine. Thus, IECs are the dominant *Ifnlr1*-expressing cells and function as the major IFN-λ-responding cells for antiviral immunity in the intestine. In MLN, spleen, and lung, we detected comparable expression of *Ifnlr1* mRNA. Villin-positive cells were not the major cell type responsible for *Ifnlr1* expression in these tissues, and neither were neutrophils, dendritic cells, or macrophages. Therefore, there may exist some other cell types that are important for IFN-λ responses in these tissues. Lung epithelial cells, which do not express Villin, likely reflect a major source of *Ifnlr1* in that tissue (29). B or T cells, which have been reported to express *Ifnlr1* even though they lack a robust response to IFN-λ, may account for *Ifnlr1* expression in the MLN and spleen (27). Another possible cellular source for this expression is endothelial cells, based on the report that blood-brain barrier endothelial cells respond to IFN-λ to restrict West Nile virus neuroinvasion (10). Thus, it would be interesting to study the role of IFN-λ in extraintestinal tissues in control of other pathogens and define the IFN-λ-responsive cell types in these contexts.

In some tissues, such as lung and vagina, there is redundancy between type I and III IFN-mediated antiviral responses. IFN-λ controls influenza virus, severe acute respiratory syndrome (SARS) coronavirus, respiratory syncytial virus infection in the lung (45–47), and herpes simplex virus 2 (HSV-2) infection in the genital tract (48), redundantly with type I IFNs. In the intestine, however, IFN-λ-mediated antiviral immunity does not redundantly overlap type I IFNs (8, 9, 19). Adult IECs have polarized apical IFNAR1 expression only at low levels (9), and although IECs in neonates exhibit robust STAT1 activation after type I IFN treatment, in adult mice they are largely unresponsive to type I IFN treatment *in vivo* (9, 19, 20). Moreover, the expression level of *Ifnlr1* mRNA is highly enriched in IECs but minimally detectable in other compartments of intestinal tissue (Fig. 1 and 2). This study, bolstering previous findings of alternate cellular expression patterns for type I and III IFN receptors, helps explain why IFN-λ-mediated immunity in the intestine is nonredundant with IFN-α/β in adult mice, even though they may stimulate transcription of highly overlapping sets of antiviral genes (7, 20). Our data support a role for IECs as sentinels for enteric virus infection via their response to compartment-specific IFN-λ signaling (19, 20).

One of the important features of IFN-λ-mediated immunity is its sterilizing activity against persistent MNoV infection in the absence of adaptive immunity (8). We observed that persistent MNoV infection of *Rag1*$^{-/-}$ *Ifnlr1*$^{f/f}$-*Villincre* mice was not resolved by IFN-λ treatment and showed increased viral titers in the stool, similar to our observations with *Rag1*$^{-/-}$ *Ifnlr1*$^{-/-}$ mice. Thus, IFN-λ-mediated sterilizing innate immunity requires IEC expression of the receptor. Since only macrophages, dendritic cells, and B cells are known to be susceptible to MNoV infection *in vitro* (35, 49), it is not clear how the IFN-λ response in IECs ablates persistent MNoV infection in the absence of adaptive immunity. One possible explanation is that there is a secondary *trans*-acting molecule induced by IFN-λ in IECs that clears MNoV in other cell types. A related study has demonstrated that rotavirus can be terminated by injecting IL-22 and IL-18 into *Rag1*$^{-/-}$ mice, but this IL-22- and IL-18-mediated viral clearance does not induce IFN-λ or Stat1 activation (50). Thus, there may be multiple innate immunological mechanisms to resolve persistent viral infection in the absence of adaptive immunity. Identifying the effectors of IFN-λ-mediated sterilizing immunity is an important area to pursue in IFN-λ immunology.

This study reveals that *Ifnlr1* expression in IECs is required for control of enteric MNoV and reovirus infections. Using a genetic approach with conditional knockout mice, we identified IECs as the dominant cell type that responds to endogenous and exogenous IFN-λ to control enteric viruses. Understanding the identity of IFN-λ-responsive cell types provides further insight into mechanisms that control enteric viruses and will enhance future development of IFN-λ-mediated antiviral therapeutics.

MATERIALS AND METHODS

Generation of MNoV stocks and determination of titers. Stocks of MNoV strain CR6 were generated from a molecular clone as previously described (51). Briefly, a plasmid encoding the CR6 genome was transfected into 293T cells to generate infectious virus, which was subsequently passaged on BV2 cells. After two passages, BV2 cultures were frozen and thawed to liberate virions. Cultures then were cleared of cellular debris and virus was concentrated by ultracentrifugation through a 30% sucrose cushion. Titers of virus stocks were determined by plaque assay on BV2 cells (51).

Generation of reovirus stocks and determination of titers. Spinner-adapted murine L929 (L) cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (SMEM; Lonza) supplemented to contain 5% fetal bovine serum (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), and 25 ng/ml amphotericin B (Sigma). BHK-T7 cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco) supplemented to contain 5% fetal bovine serum, 2 mM L-glutamine, 1 mg/ml Geneticin (Gibco), and nonessential amino acids (Sigma).

Recombinant reoviruses were generated using plasmid-based reverse genetics (52). Recombinant strain type 1 Lang (T1L) is a stock generated by plasmid-based rescue from cloned T1L cDNAs (53). After 3 to 5 days of incubation, cells were frozen and thawed three times, and virus was isolated by plaque purification using monolayers of L cells (54). Purified reovirus virions were generated from second- or third-passage L-cell lysate stocks (55). Viral particles were extracted from infected cell lysates using Vertrel XF (Dupont), layered onto 1.2- to 1.4-g/cm^3 CsCl gradients, and centrifuged at 62,000 × *g* for 16 h. Bands corresponding to virions (1.36 g/cm^3) were collected and dialyzed in virion storage buffer (150 mM NaCl, 15 mM $MgCl_2$, and 10 mM Tris-HCl [pH 7.4]) (56). The concentration of reovirus virions in purified preparations was determined from the following equivalence: one optical density (OD) unit at 260 nm equals 2.1×10^{12} virions (56). Viral titer was determined by plaque assay using L cells (54).

For analysis of reoviral titers in organs, mice were euthanized at various intervals postinoculation, and organs were harvested into 1 ml of PBS and homogenized by freeze-thaw and bead beating. For analysis of viral titer in stool, samples were harvested at various intervals, weighed, stored in 1 ml of PBS, and homogenized by freeze-thaw and bead beating. Viral titers in organs and stool homogenates were determined by plaque assay using L cells (54). Titers are expressed as PFU per milliliter of tissue homogenate or per gram of stool.

Mice, infections, and IFN-λ treatment. Wild-type (WT) C57BL/6J mice (stock number 000664) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at the Washington University School of Medicine under specific-pathogen-free conditions (57) according to university guidelines. *Ifnlr1*$^{-/-}$ (B6.Cg-*Ifnlr1*^{tm1Palu}) mice were obtained from Bristol-Myers Squibb (Seattle, WA) and backcrossed using speed congenics onto a C57BL/6J background (28).

To generate mice conditionally deficient for *Ifnlr1*, *Ifnlr1*$^{tm1a(EUCOMM)Wtsi}$ ES cells on a C57BL/6N background were provided by the Wellcome Trust Sanger Institute. A conditional-ready (floxed) allele in which exon 2 is flanked by loxP sites, designated *Ifnlr1*$^{f/f}$, was created (Fig. 2A) (38). *Ifnlr1*$^{f/f}$ mice were crossed to Villin-Cre (intestinal epithelial cells [29]), LysM-Cre (macrophages and neutrophils, as well as some dendritic cells [32, 33]), CD11c-Cre (dendritic cells and alveolar macrophages [31]), and MRP8-Cre (neutrophils [30]) lines for selective disruption of *Ifnlr1* in different cell types *in vivo*. *Ifnlr1*$^{f/f}$ mice were also crossed to a Deleter-Cre line (34) to generate an alternate *Ifnlr1*$^{-/-}$ line, here designated *Ifnlr1*$^{f-/-}$. *Ifnlr1*$^{f-/-}$ mice were backcrossed using speed congenics onto a C57BL/6J background. Mouse lines and naming conventions are summarized in Table 1.

For MNoV infections, mice were inoculated with a dose of 10^6 PFU of strain CR6 at 6 to 8 weeks of age by the oral route in a volume of 25 μl. For reovirus infections, mice were orally gavaged with a dose of 10^8 PFU of strain T1L virus at 6 to 8 weeks in a volume of 100 μl.

Recombinant IFN-λ was provided by Bristol-Myers Squibb (Seattle, WA) as a monomeric conjugate comprised of 20-kDa linear polyethylene glycol (PEG) attached to the amino terminus of murine IFN-λ, as previously reported (8). For treatment of mice, 25 μg of IFN-λ diluted in PBS was injected intraperitoneally.

Stool and tissues were harvested into 2-ml tubes (Sarstedt, Germany) with 1-mm-diameter zirconia/silica beads (Biospec, Bartlesville, OK). Tissues were flash frozen in a bath of ethanol and dry ice and either processed on the same day or stored at −80°C.

Isolation of epithelial and lamina propria fractions of small intestine. Epithelial and lamina propria fractions were prepared as previously described (36). In brief, after mice were euthanized, small intestines were collected. Intestinal tissues were washed with cold PBS twice and then chopped and transferred to new tubes. The tissues were incubated with stripping buffer (10% bovine calf serum, 15 mM HEPES, 5 mM EDTA, 5 mM dithiothreitol [DTT] in 1× Hanks' balanced salt solution [HBSS]) for 20 min at 37°C. The dissociated cells were collected as the epithelial fraction, consisting predominantly of IECs. The remaining tissue was used as the lamina propria fraction.

Isolation of neutrophils, macrophages, and dendritic cells. Neutrophils were isolated from *Ifnlr1*$^{f/f}$*-MRP8cre* mice and *Ifnlr1*$^{f/f}$ littermates by collecting bone marrow from femurs and tibias. Red blood cells were lysed using red blood cell lysis buffer (Sigma, St. Louis, MO), and neutrophils were isolated using the mouse neutrophil isolation kit (Miltenyi Biotec, Germany). Isolated neutrophils were confirmed to be 95 to 98% double positive for CD11b-allophycocyanin (APC) and Ly6G-fluorescein isothiocyanate (FITC) (BioLegend, San Diego, CA) (data not shown). Macrophages were isolated from *Ifnlr1*$^{f/f}$*-LysMcre* mice and *Ifnlr1*$^{f/f}$ littermates by collecting and homogenizing spleens, lysing red blood cells (RBCs), and enriching for macrophages using mouse anti-F4/80 UltraPure MicroBeads (Miltenyi Biotec). Isolated macrophages were confirmed to be 70 to 85% positive for F4/80-AF488

(Thermo Fisher Scientific) as well as CD11b-APC and CD45.2-phycoerythrin (PE) (BioLegend) (data not shown). Dendritic cells were isolated from *Ifnlr1*$^{f/f}$-*CD11ccre* mice and *Ifnlr1*$^{f/f}$ littermates by collecting and homogenizing spleens, lysing RBCs, and enriching for dendritic cells using the mouse pan-dendritic cell isolation kit (Miltenyi Biotec). Isolated dendritic cells were confirmed to be 70 to 85% CD11c-AF488 (BioLegend) single positive or CD11c-AF488 and B220-PE (BD Bioscience) double positive (data not shown).

Quantitative reverse transcription-PCR. RNA from stool was isolated using a ZR-96 viral RNA kit (Zymo Research, Irvine, CA). RNA from tissues or cells was isolated using TRI Reagent (Invitrogen) and a direct-zol-96 RNA kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. Five microliters of RNA from stool or 1 μg of RNA from tissue was used as a template for cDNA synthesis with the ImPromII reverse transcriptase system (Promega, Madison, WI). DNA contamination was removed using the DNAfree kit (Life Technologies).

MNoV TaqMan assays were performed, using a standard curve for determination of absolute viral genome copies, as described previously (58). Quantitative PCR for housekeeping gene *Rps29* was performed with forward primer 5′-GCAAATACGGGCTGAACATG-3′, reverse primer 5′-GTCCAACTTAATG AAGCCTATGTC-3′, and probe 5′-/5HEX/CCTTCGCGT/ZEN/ACTGCCGGAAGC/3IABkFQ/-3′ (where 3IABkFQ is 3′ Iowa Black fluorescence quencher; Integrated DNA Technologies), each at a concentration of 0.2 μM, using AmpliTaq gold DNA polymerase (Applied Biosystems). Quantitative PCRs for *Ifnlr1* (Mm.PT.58.10781457), *Oas1a* (Mm.PT.58.30459792), *Ifi44* (Mm.PT.58.12162024), and *Ifit1* (Mm.PT.58.32674307) were similarly performed using PrimeTime qPCR assays (Integrated DNA Technologies). Standard curves for quantitative PCR assays were generated to facilitate absolute quantification of transcript copy numbers. For *Rps29*, the PCR product using the above-described primers was cloned into the p-ENTR/D-TOPO vector (Thermo Fisher Scientific), and for *Ifnlr1* a full-length *Ifnlr1* cDNA clone (5036481; Open Biosystems) was used. Plasmids were Sanger sequenced to confirm the identity of the inserts. For *Oas1a*, *Ifit1*, and *Ifi44*, absolute transcripts were quantitated based on target sequence-containing gBlocks (Integrated DNA Technologies). Cycling parameters for *Rps29*, *Ifnlr1*, *Oas1a*, *Ifit1*, and *Ifi44* were identical to those for MNoV TaqMan. Absolute values of *Ifnlr1*, *Oas1a*, *Ifit1*, and *Ifi44* per microgram of RNA were normalized to the within-tissue average of housekeeping gene *Rps29*. No significant changes in absolute copy number of *Rps29* were detected between comparison groups (data not shown).

Statistical analysis. Data were analyzed with Prism 7 software (GraphPad Software, San Diego, CA). In all graphs, three asterisks indicate a P value of <0.001, two asterisks indicate a P value of <0.01, one asterisk indicates a P value of <0.05, and ns indicates not significant ($P > 0.05$) as determined by Mann-Whitney test, one-way analysis of variance (ANOVA), or two-way ANOVA with Tukey's multiple-comparison test, as specified in the relevant figure legends.

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