A homeostatic interferon-lambda response to bacterial microbiota stimulates preemptive antiviral defense within discrete pockets of intestinal epithelium

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Abstract

Interferon-lambda (IFN-λ) protects intestinal epithelial cells (IECs) from enteric viruses by inducing expression of antiviral IFN-stimulated genes (ISGs). Here, we find that bacterial microbiota stimulate a homeostatic ISG signature in the intestine of specific pathogen-free mice. This homeostatic ISG expression is restricted to IECs, depends on IEC-intrinsic expression of IFN-λ receptor (Ifnlr1), and is associated with IFN-λ production by leukocytes. Strikingly, imaging of these homeostatic ISGs reveals localization to pockets of the epithelium and concentration in mature IECs. Correspondingly, a minority of mature IECs express these ISGs in public single-cell RNA sequencing datasets from mice and humans. Furthermore, the bacterial component, lipopolysaccharide, partially restores localized ISGs in mice lacking bacterial microbiota. Lastly, IECs lacking Ifnlr1 are hyper-susceptible to initiation of murine rotavirus infection. These observations indicate that bacterial microbiota stimulate ISGs in localized regions of the intestinal epithelium at homeostasis, thereby preemptively activating antiviral defenses in vulnerable IECs to improve host defense against enteric viruses.
Introduction

Interferons (IFNs) are a family of cytokines produced in response to infection that signal IFN receptor-bearing cells to induce transcription of hundreds of interferon-stimulated genes (ISGs). These ISGs perform diverse functions, but many cooperate to induce an antiviral state (Sadler and Williams, 2008; Schoggins and Rice, 2011). There are three types of IFNs: type I IFNs (IFN-αs, IFN-β, others), type II IFN (IFN-γ), and type III IFNs (IFN-λs). These three types are differentiated by receptor usage (type I IFN receptor: Ifnar1/Ifnar2; type II IFN receptor: Ifngr1/Ifngr2; type III IFN receptor: Ifnlr1/Il10rb), but all three receptor complexes signal through Janus-kinase (JAK) and signal transducer and activator of transcription (STAT) factors to stimulate ISG transcription (Ingle et al., 2018; Schneider et al., 2014). Type I and III IFNs are directly stimulated by host detection of microbe-associated molecular patterns (MAMPs) such as viral nucleic acids, and the prominent contribution of these IFN types to antiviral defense is reflected by the breadth of evasion strategies used by diverse viral families to prevent their production or action (Levy and García-Sastre, 2001; Rojas et al., 2021; Taylor and Mossman, 2013). The type I IFN receptor is expressed broadly across most cell types, whereas the type III IFN receptor, Ifnlr1, is primarily restricted to epithelial cells (Kotenko and Durbin, 2017; Sommereyns et al., 2008). Accordingly, IFN-λ is of particular importance in effective antiviral defense of barrier tissues.

Interestingly, previous studies in mice have noted that intestinal epithelial cells (IECs) are hyporesponsive to type I IFN (Lin et al., 2016; Mahlakõiv et al., 2015; Pott et al., 2011; Van Winkle et al., 2020). Additionally, infection of mice deficient in IFN receptors (Ifnar1, Ifngr1, Ifnlr1; single or double knockouts) with enteric viruses indicates that IFN-λ is the predominant antiviral IFN type that controls viral replication in the gastrointestinal epithelium (Mahlakõiv et al., 2015; Nice et al., 2015; Pott et al., 2011). IECs can robustly respond to IFN-λ with upregulation of canonical antiviral ISGs and increased resistance to infection by enteric viruses, such as rotaviruses and noroviruses.
Mouse rotavirus (mRV) is a natural pathogen of mice that infects enterocytes located at the tips of villi in the small intestine (Burns et al., 1995). Rotaviruses have developed mechanisms to antagonize the induction of IFN by infected cells, suggesting that evasion of the IFN response is necessary for efficient epithelial infection (Arnold et al., 2013). Indeed, prophylactic administration of IFN-λ significantly reduces the burden of mRV infection, demonstrating its potential for mediating epithelial antiviral immunity to this pathogen (Hernández et al., 2015; Lin et al., 2016; Pott et al., 2011; Van Winkle et al., 2020). However, a protective role of the endogenous IFN-λ response to mRV infection is less clear, perhaps reflecting the success of mRV evasion mechanisms.

Epithelial immunity in the gut must be appropriately balanced to protect the intestinal epithelium while preventing loss of barrier integrity and intrusion by microbes that are abundant within the gastrointestinal tract. The bacterial microbiota in the intestine perform critical functions, aiding in host metabolism (Krajmalnik-Brown et al., 2012), providing a competitive environment to defend against pathogens (Kim et al., 2017), and initiating and maintaining host immune function during homeostasis (Honda and Littman, 2016; Rooks and Garrett, 2016). In this complex environment host epithelial and immune cells detect bacteria and viruses using a suite of pattern recognition receptors (PRRs) that sense the presence of MAMPs. Stimulation of PRRs, such as the toll-like receptor (TLR) family, activates antimicrobial and antiviral defenses providing local protection in many tissues (Chu and Mazmanian, 2013; Iwasaki and Medzhitov, 2015; Thompson et al., 2011). TLR-dependent pathways induce production of IFNs, primarily by signaling through TIR-domain-containing adapter-inducing interferon-β (TRIF) and myeloid differentiation primary response 88 (MYD88) adapter proteins (Monroe et al., 2010; Odendall et al., 2017), providing a mechanism by which bacterial MAMPs can initiate IFN responses.

Signals from the bacterial microbiota have been shown to elicit a steady-state type I IFN response in systemic tissues and cell types that can prime antiviral immunity by several independent studies (Abt et al., 2012; Bradley et al., 2019; Ganal et al., 2012; Steed et al., 2017;
Additionally, a steady-state ISG signal has been observed in the intestine of uninfected mice (Baldridge et al., 2015; Lin et al., 2016; Stockinger et al., 2014), but this intestinal response remained poorly characterized. Together with the observed hypo-responsiveness of IECs to type I IFN, these findings suggested that bacterial microbiota may stimulate a local IFN-λ response in the gut. To explore this interaction, we undertook the present study to assess the role of bacterial microbiota in induction of enteric ISGs at homeostasis using a combination of broad-spectrum antibiotics (ABX) and genetically modified mice.

In this study, we uncovered an ISG signature in IECs that was dependent on the presence of bacteria and IFN-λ signaling (hereafter referred to as ‘homeostatic ISGs’). This panel of genes was present in WT mice with conventional microbiota and was reduced in WT mice treated with ABX and in mice lacking Ifnlr1. We revealed that homeostatic ISG expression is i) restricted to the intestinal epithelium across both the ileum and colon, ii) independent of type I IFN signaling, and iii) associated with IFN-λ transcript expression by epithelium-associated CD45+ leukocytes. Surprisingly, we found that homeostatic ISGs are not expressed uniformly by all IECs; rather, expression is concentrated in localized pockets of IECs and in differentiated IECs relative to crypt-resident progenitors. These patterns of localized ISG expression are corroborated by independently-generated single-cell RNA sequencing data from mouse and human IECs. We also found that ISG expression can be partially rescued by reconstitution of bacterial microbiota or administration of bacterial lipopolysaccharide (LPS) to ABX-treated mice. Finally, we found that this microbiota-stimulated ISG signature provides protection from initiation of mRV infection. Cumulatively, this study found that bacteria initiate preemptive IFN-λ signaling in localized areas to protect IECs from enteric viruses.
Results

Bacterial microbiota stimulate IFN-λ response genes in the ileum at homeostasis

To determine the effect of bacterial microbiota on homeostatic IFN-λ responses, we compared gene expression in whole ileum tissue for the following experimental groups: i) wild-type C57BL/6J (WT) mice intraperitoneally-injected with IFN-λ as compared to unstimulated WT mice, ii) WT mice with conventional microbiota as compared to WT mice treated with an antibiotic cocktail (ABX) to deplete bacteria, iii) WT mice with conventional microbiota as compared to Ifnlr1−/− mice, and iv) Ifnlr1−/− mice with conventional microbiota compared to Ifnlr1−/− mice treated with ABX (Figure 1A). We performed gene-set-enrichment-analysis (GSEA) to test for enrichment or depletion of ‘IFN-λ response genes’ that were defined in a prior study of IFN-λ-stimulated ileum tissue (Lee et al., 2019). As expected, GSEA comparing IFN-λ-treated WT mice to unstimulated WT mice showed significant enrichment of IFN-λ response genes (Figures 1B-C). Therefore, GSEA of these genes is indicative of IFN-λ responses in the ileum and GSEA can be used to assess the relative differences in IFN-λ responses between experimental conditions.

To deplete bacterial microbiota, we administered a cocktail of broad-spectrum antibiotics, and demonstrated that this treatment reduced bacterial 16S gene copies in stool to below the limit of detection (Figure S1). GSEA of WT mice treated with ABX showed a significant depletion of IFN-λ response genes in the ileum relative to WT mice with conventional microbiota (Figure 1B, 1D). These data indicate that IFN-λ response genes are present at steady-state in the ileum of specific-pathogen-free mice with conventional microbiota and suggest that microbiota stimulate expression of these genes in the ileum at homeostasis.

Type I, II, and III IFN responses have substantial gene expression overlap; therefore, to prove that the IFN-λ receptor was necessary for the homeostatic expression of IFN-λ response genes, we analyzed gene expression in the ileum of Ifnlr1−/− mice. Indeed, GSEA showed significant reduction of IFN-λ response genes in the ileum of Ifnlr1−/− mice compared to WT mice.
Furthermore, expression of IFN-λ response genes was not significantly decreased in *Ifnlr1*⁻/⁻ mice treated with ABX compared to *Ifnlr1*⁻/⁻ mice with conventional microbiota (Figures 1B, 1E). Together, these findings suggest that *Ifnlr1* is necessary for the bacterial microbiota-dependent expression of IFN-λ response genes in the ileum at homeostasis.

To define a core set of bacterial microbiota-dependent, *Ifnlr1*-dependent ‘homeostatic ISGs’, we determined the overlap of differentially expressed genes (DEGs) by defining genes with: i) increased expression upon IFN-λ stimulation in WT mice, ii) decreased expression upon ABX treatment in WT mice, and iii) decreased expression in *Ifnlr1*⁻/⁻ mice relative to WT mice. The DEGs shared by each of these comparisons comprised a set of 21 genes that are decreased upon treatment with ABX and loss of *Ifnlr1*, and are induced in response to IFN-λ (Figure 1G). This set of homeostatic ISGs includes antiviral genes that are dependent on bacterial microbiota and the IFN-λ pathway. Hierarchical clustering of mice based on expression of these homeostatic ISGs distinctly grouped mice treated with IFN-λ based on high expression of all 21 identified genes (Figure 1H). However, clustering also separates WT mice from *Ifnlr1*⁻/⁻ mice and ABX-treated mice of both genotypes, indicating modest but significant expression of ISGs at homeostasis that was lost with *Ifnlr1* deficiency or ABX treatment. Together, these analyses revealed a homeostatic signature of ISGs in the ileum that depends upon the presence of bacterial microbiota and on intact IFN-λ signaling.
Figure 1. Bacterial microbiota stimulate IFN-λ response genes in the ileum at homeostasis.  

A. Depiction of experimental treatments and comparison groups. Following the indicated treatments, a segment of whole ileum tissue was harvested and analyzed by RNA sequencing for differentially expressed genes between paired conditions. B-E. Gene-set enrichment analysis of IFN-λ response genes was performed with the following comparisons: WT mice treated with 25µg of IFN-λ relative to WT mice treated with PBS (B), WT mice treated with ABX relative to untreated WT mice (C), Ifnlr1−/− mice relative to WT mice (D), and Ifnlr1−/− mice treated with ABX relative to untreated Ifnlr1−/− mice (E). F. Statistics from gene-set enrichment analysis in B-E with significant nominal p-values highlighted (red). G. A Venn diagram depicting the total number of differentially expressed genes that are i) increased with IFN-λ stimulation (orange), ii) decreased with ABX treatment (green), or iii) decreased in Ifnlr1−/− mice relative to WT (blue). An overlapping subset of 21 genes was shared among all three comparisons (red box). H. A heatmap with hierarchical clustering of mice based on the relative expression of 21 genes that overlap in all experimental groups in G (‘homeostatic ISGs’).

Figure S1. Treatment with antibiotics reduces enteric 16S gene copies to below the limit of detection. rDNA was isolated from luminal contents of mice after 14 days of ABX-treatment. 16S
gene copies were assessed by qPCR and normalized to input. Limit of detection: dashed line.

Statistical significance was determined by Mann Whitney, where *** = p < 0.001.

**Homeostatic ISGs are primarily expressed in intestinal tissues**

To complement the results of the RNA-sequencing and extend this analysis to other tissue sites, we quantified tissue-level expression of a panel of three ISGs by qPCR: *Ifit1*, *Oas1a*, and *Stat1*. *Ifit1* and *Stat1* were present among the 21 homeostatic ISGs in the preceding analysis and *Oas1a* was included as a representative canonical ISG that we hypothesized would be present in the homeostatic signature, but did not meet the statistical criteria used to define the core set of 21 homeostatic ISGs (Figure 1G and 1H). We assessed absolute abundance of these ISG transcripts in the ileum, colon, mesenteric lymph nodes (MLN), and spleen of WT and *Ifnlr1*−/− mice with or without ABX treatment (Figures 2A-C). Consistent with our RNA-seq data, these ISGs were reduced in the ilea of *Ifnlr1*−/− mice and ABX-treated WT mice compared to WT mice with conventional microbiota (Figure 2A). Secondly, homeostatic ISGs were expressed in WT colonic tissue and were significantly decreased in colonic tissue of *Ifnlr1*−/− mice and ABX-treated WT mice (Figure 2B), indicating that homeostatic ISGs in both the ileum and colon were dependent on *Ifnlr1* and the bacterial microbiota. To confirm that treatment with ABX does not ablate the ability of the intestine to respond to IFN-λ, we stimulated WT and ABX-treated mice with intraperitoneal IFN-λ and harvested ileum tissue. Stimulation with small amounts of IFN-λ rescued ISG expression in whole tissue (Figure S2), indicating that that reduction of homeostatic ISG expression upon treatment with ABX is not due to an inability for the intestinal epithelium to respond to IFN-λ.

Enteric colonization by bacteria was shown to stimulate systemic type I IFN responses (Abt et al., 2012; Bradley et al., 2019; Ganal et al., 2012; Steed et al., 2017; Stefan et al., 2020; Winkler et al., 2020), so we assessed whether the decreases in ISGs upon ABX treatment or loss of *Ifnlr1* in the ileum were recapitulated in systemic immune tissues. We quantified *Ifit1*, *Stat1*,
and *Oas1a* expression in the mesenteric lymph nodes (MLN) spleen and found that ABX treatment and *Ifnlr1* deletion did not reduce ISGs in these tissues (Figure 2C). Although we observe increases in *Ifit1* expression in MLN upon treatment with ABX, these results are not recapitulated by *Stat1* and *Oas1a* expression, and no significant changes in ISG expression were detected in the spleen. Cumulatively, these data indicate that homeostatic ISGs include genes beyond the core signature identified in Figure 1 (e.g. *Oas1a*), that homeostatic ISGs are present in colonic tissue, and that homeostatic IFN-λ stimulated genes are most prominent in enteric tissues.
Figure 2. Homeostatic ISGs are primarily expressed in intestinal tissues. A segment of ileum or colon tissue from WT or Ifnlr1−/− mice was harvested following H2O or ABX treatment and the
ISGs *Ifit1*, *Stat1*, and *Oas1a* were analyzed by qPCR. Transcripts were quantified in ileum (A), colon (B), or MLN and spleen (C) with normalization to untreated WT mice. Data points represent individual mice. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparisons. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

**Figure S2.** Treatment with antibiotics does not ablate responsiveness to IFN-λ. *Ifit1*, *Stat1*, and *Oas1a* expression was quantified in the ileum of WT mice treated with or without ABX and was normalized to untreated mice. Increasing quantities of IFN-λ were intraperitoneally injected into ABX-treated mice to rescue basal ISG expression. Statistical significance was determined by Kruskal-Wallis test with Dunn’s multiple comparisons with *** = p < 0.001, and **** = p < 0.0001.

**Homeostatic ISG expression in the intestine is independent of type I IFN**

To determine whether detection of enteric bacteria by TLRs stimulates homeostatic ISGs, we measured tissue ISG expression in mice that were deficient in TRIF or MYD88. Signaling through TRIF results in activation of interferon regulatory factors (IRFs), such as IRF3 and IRF7, that commonly contribute to IFN induction (Honda et al., 2005a; Osterlund et al., 2007; Schmid et al., 2010). Additionally, signaling through MYD88 can aid initiation of IFN expression, through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor family members (Osterlund et al., 2007) and IRF7 (Honda et al., 2005b; Tomasello et al., 2018). *Ifit1* expression in the ileum and colon of Trif−/− mice was not significantly different than in WT mice,
and \textit{Ifit1} expression was reduced with ABX in \textit{Trif}^−/− mice (\textbf{Figure 3A}). However, we found that mice lacking \textit{Myd88} exhibited tissue-specific decreases in \textit{Ifit1} expression, with significant decreases in the ileum, but not in the colon, relative to WT mice (\textbf{Figure 3A}). These data are consistent with a previous report (Stockinger et al., 2014) and suggest that signaling through MYD88, but not TRIF, is necessary for homeostatic ISG expression in the ileum, whereas other factors may dominate in the colon.

To expand these findings, we assessed the role of IRF3 and IRF7 transcription factors that are commonly activated downstream of MYD88 and TRIF. We did not observe significant decreases in \textit{Ifit1} expression in the ileum or the colon of \textit{Irf3}^−/− mice as compared to WT (\textbf{Figure 3B}), indicating that IRF3 is not required for homeostatic IFN-λ induction. However, we observed a modest (two-fold) decrease in \textit{Ifit1} expression in both the ileum and colon of \textit{Irf7}^−/− mice when compared to WT mice (\textbf{Figure 3B}). Although IRF7 is implicated by these data, it does not appear to be strictly required for homeostatic expression of \textit{Ifit1} because expression is further reduced by ABX treatment (\textbf{Figure 3B}). Therefore, these data suggest that IRF7 cooperates with other factors in the homeostatic ISG response to the bacterial microbiota.

Type I and III IFNs stimulate overlapping ISG responses that are both dependent on STAT1. Therefore, to further investigate the contribution of type I IFN signaling to homeostatic ISG expression, we quantified \textit{Ifit1} expression in ileum and colon tissue from \textit{Ifnar1}^−/− or \textit{Stat1}^−/− mice. \textit{Ifit1} expression was not significantly different in either ileum of colon tissue of \textit{Ifnar1}^−/− mice compared to WT (\textbf{Figure 3C}). However, \textit{Ifit1} expression was significantly lower in ileum and colon tissue of \textit{Stat1}^−/− mice compared to \textit{Ifnar1}^−/− and WT mice. Importantly, treatment of \textit{Stat1}^−/− mice with ABX did not further reduce \textit{Ifit1} expression, emphasizing the necessity of STAT1 for this homeostatic ISG response.

Lastly, to determine whether type I IFN signaling plays a compensatory role in homeostatic ISG expression in the absence of \textit{Ifnlr1}, we bred mice with heterozygous expression of both \textit{Ifnlr1} and \textit{Ifnar1} (\textit{Ifnlr1}^Het/\textit{Ifnar1}^Het) with mice that lack \textit{Ifnlr1} and \textit{Ifnar1} (\textit{Ifnlr1}^{KO}/\textit{Ifnar1}^{KO}). This breeding
scheme produced littermate-matched mice that were \( \text{Ifnrl}^{\text{Het}}/\text{Ifnar}^{\text{Het}} \), \( \text{Ifnrl}^{\text{KO}}/\text{Ifnar}^{\text{KO}} \), and mice singly deficient in \( \text{Ifnrl} \) (\( \text{Ifnrl}^{\text{KO}}/\text{Ifnar}^{\text{Het}} \)) or \( \text{Ifnar} \) (\( \text{Ifnrl}^{\text{Het}}/\text{Ifnar}^{\text{KO}} \)). We found that \( \text{Ifit}1 \) expression was not significantly different in \( \text{Ifnrl}^{\text{Het}}/\text{Ifnar}^{\text{KO}} \) relative to \( \text{Ifnrl}^{\text{Het}}/\text{Ifnar}^{\text{Het}} \) controls in stripped epithelium (Figure 3D). \( \text{Ifit}1 \) expression in \( \text{Ifnrl}^{\text{KO}}/\text{Ifnar}^{\text{Het}} \) was significantly lower compared to \( \text{Ifnrl}^{\text{Het}}/\text{Ifnar}^{\text{Het}} \) controls, but was not significantly different from \( \text{Ifnrl}^{\text{KO}}/\text{Ifnar}^{\text{KO}} \) mice (Figure 3D). Cumulatively, these data indicate that homeostatic ISG expression in the intestine is partly dependent on MYD88 and IRF7, and is independent of type I IFN signaling.
Figure 3. Homeostatic ISG expression is independent of type I IFN. *I*fit1 expression levels were assessed by qPCR from the ileum or colon of (A) *Trif*−/− and *Myd88*−/−, (B) *Irf3*−/− and *Irf7*−/−, or (C) *Ifnar1*−/− and *Stat1*−/− mice treated with or without ABX normalized to untreated, WT mice. Some WT controls are shared across experiments in (A-C). D. *Ifit1* expression was measured by qPCR in stripped epithelium of indicated genotypes. Statistical significance was determined by one-way ANOVA with Dunnett’s multiple comparisons in A-D, where * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

Homeostatic ISG expression in the intestine is restricted to epithelial cells

Given the primarily epithelial expression of *Ifnlr1*, we assessed which compartment of the ileum expresses homeostatic ISGs by isolating a stripped intestinal epithelial fraction and digested underlying lamina propria. We assessed ISG expression in these two fractions from WT and *Ifnlr1*−/− mice treated with or without ABX. Treatment with ABX or loss of *Ifnlr1* reduced homeostatic ISGs in the IEC fraction, but the lamina propria had low expression of these ISGs relative to the epithelium of untreated WT mice, regardless of treatment or genotype (Figure 4A).

IECs express abundant *Ifnlr1*, but other intra-epithelial cell types do not (Mahlakõiv et al., 2015; Sommereyns et al., 2008). To determine whether *Ifnlr1* expression by IECs was required for homeostatic ISG expression, we used mice with IECs that are conditionally deficient in *Ifnlr1* (*Ifnlr1ΔIEC*) and littermates that retain normal *Ifnlr1* expression (*Ifnlr1*^{flox/flox}) (Baldridge et al., 2017). *Ifit1* and *Oas1a* expression in the ileum (Figure 4B) and colon (Figure 4C) of *Ifnlr1*^{flox/flox} mice was decreased upon treatment with ABX, consistent with the phenotype observed in WT mice. Conditional deletion of *Ifnlr1* in IECs reduced *Ifit1* and *Oas1a* to a similar extent as the reduction observed in *Ifnlr1*−/− animals, above (Figures 2A and 2B). Together, these findings indicate that homeostatic ISGs are dependent on *Ifnlr1*-expression by IECs.
**A**

Ileum

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**B**

Ileum

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Figure 4. Homeostatic ISG expression in the intestine is restricted to epithelial cells. A. Ifit1, Stat1, and Oas1a expression was quantified in rough epithelial fractions or in the lamina propria digested ileum. Comparisons were performed between WT and Ifnlr1−/− mice with or without ABX treatment, and ISG expression was normalized to WT values. B and C. Ifit1 and Oas1a expression from the (B) ileum or (C) colon of mice with conditional presence (flox) or absence (ΔIEC) of Ifnlr1 in intestinal epithelial cells. Statistical significance was determined by one-way ANOVA with Dunnett’s multiple comparisons. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Bacterial microbiota stimulate expression of Ifnl2/3 by CD45-positive cells

To determine which cellular compartment produces IFN-λ in response to bacterial microbiota at homeostasis, we enriched cell subsets from epithelial or lamina propria fractions of the ileum for quantification of IFN-λ transcripts (Ifnl2/3) by qPCR. We treated WT mice with control, ABX, or stimulation with a synthetic dsRNA analogue (poly I:C) as a positive-control. We then magnet-enriched EpCAM+ or CD45+ cells from stripped intestinal epithelium or digested lamina propria ileal tissue (Figure S3). We found that CD45+ cells from the intestinal epithelial and lamina propria fraction expressed detectable Ifnl2/3 at homeostasis, but EpCAM+ cells did not, which is consistent with a previous study (Mahlakõiv et al., 2015). However, in ABX-treated mice, we found that expression of Ifnl2/3 in CD45+ cells from the epithelial fraction was significantly reduced, and CD45+ cells from lamina propria had a marginal (p = 0.0523) reduction in Ifnl2/3 expression (Figure 5A). Therefore, we conclude that epithelium-associated CD45+ leukocytes are the likely source of homeostatic IFN-λ in response to bacterial microbiota.

Similar to Ifnl2/3, we found that CD45+ cells of the epithelial fraction modestly expressed Ifnb1 at homeostasis. Additionally, CD45+ cells of the lamina propria robustly expressed Ifnb1 at homeostasis. However, unlike Ifnl2/3, Ifnb1 was not decreased in mice treated with ABX (Figure 5B). These data indicate that Ifnb1 expression by CD45+ cells in the intestine is less dependent
on stimulation by bacterial microbiota relative to *Ifnl2/3*, consistent with the dominant role of IFN-λ responses in driving homeostatic ISG expression in the epithelium.

**Figure 5. Bacterial microbiota stimulate expression of *Ifnl2/3* by CD45-positive cells.** A–B. The ileal epithelium and lamina propria was harvested from WT mice treated with H₂O, ABX, or stimulated with poly I:C. Resulting cell suspensions were enriched for EpCAM-positive and CD45-positive cells by magnetic separation. (A) *Ifnl2/3* expression and (B) *Ifnb1* expression was quantified from each enriched cellular fraction by qPCR. Statistical significance was determined by two-way ANOVA with Dunnett’s multiple comparisons, where * = p < 0.05 and **** = p < 0.0001.
Figure S3. Enrichment of EpCAM+ and CD45+ cells from the intestinal epithelium and lamina propria. A-C. The ileal epithelium and lamina propria was harvested from WT mice treated with H2O, ABX, or stimulated with poly I:C. Resulting cell suspensions were enriched for EpCAM-positive and CD45-positive cells and the purity of cell populations pre- and post-enrichment were quantified by flow cytometry. The percentage of cells with surface expression of (A) EpCAM or (B) CD45 prior to- and following- enrichment from stripped intestinal epithelium. C. The percentage of cells with surface expression of CD45 prior to and following CD45 enrichment from isolated lamina propria. Mean fold enrichment for each condition is noted above each column.

Homeostatic ISG expression in the small intestine is highly localized

Homeostatic ISG expression in the ileum was of relatively low magnitude when compared to IFN-λ treatment (Figures 1-2). Therefore, we hypothesized that a low abundance of homeostatic ISG expression would be uniformly distributed between IECs of the intestinal epithelium, and we sought to assess the distribution of the homeostatic ISG, Ifit1, using in situ hybridization (RNAscope) (Figures 6A-G). Contrary to our hypothesis, RNAscope staining of the ileum from untreated WT mice revealed localized pockets of robust Ifit1 expression rather than ubiquitously low expression (Figure 6A). Additionally, Ifit1 localization was skewed away from the crypt and towards the tips of individual villi within the ileum, and this localization was not specific to Ifit1 because the distinct ISG Usp18 was colocalized with Ifit1 (Figure 6A). These data indicate
that homeostatic ISGs are co-expressed primarily in mature enterocytes that are most distally located in villi. We determined that localized ISG expression was not due to a localized capacity for IEC IFN-λ response because stimulation with exogenous IFN-λ resulted in Ifit1 expression throughout the entire intestinal epithelium (Figure S4). Additionally, the non-uniform distribution of homeostatic Ifit1 expression was ablated in the ileum of mice treated with ABX (Figures 6B-D), consistent with a dependency on bacterial microbiota. To determine whether localized Ifit1 expression was dependent on IEC expression of Ifnlr1, we assessed the distribution of Ifit1 expression in the ilea of littermate Ifnlr1^flox/flox and Ifnlr1^ΔIEC mice. We found that the localized Ifit1 expression observed in untreated WT mice was recapitulated in Ifnlr1^flox/flox mice (Figure 6E), but these areas of ISG expression were ablated in Ifnlr1^ΔIEC mice (Figures 6F and 6G). Lastly, we found that this discrete localization of the homeostatic ISG response is not limited to the ileum, as localized Ifit1 staining is also observed in the colonic epithelium (Figure S5). Together, our analyses indicated that homeostatic ISGs are expressed in a highly localized manner within the intestinal epithelium.
**Figure 6. Homeostatic ISG expression in the small intestine is highly localized.** The ilea of WT, *Iflr1*fl/fl, or *Iflr1*ΔIEC mice were harvested, processed into a Swiss rolls, and stained by in situ hybridization for the ISGs, *Ifit1* (red) and *Usp18* (green), with a DAPI (blue) counterstain. **A.** Representative high-magnification images of co-localized *Ifit1* (red) and *Usp18* (green) expression in the ileum of WT mice, see arrows. **B-D.** WT mice treated with H2O control or ABX with quantification of *Ifit1* area relative to the total area of the section across replicate mice. **E-G.** *Iflr1*fl/fl or *Iflr1*ΔIEC mice at homeostasis with quantification of *Ifit1* area relative to the total area.
of the section across replicate mice. Scale bar = 100µm in A and 500µm in B-G and. Statistical significance was calculated by unpaired t-test where ** = p < 0.01. Each data point in D and G represents an individual mouse from 2 replicate experiments, with n = 7-8 for each condition.

**Figure S4. The entire intestinal epithelium is responsive to IFN-λ.** Wild-type mice were injected with PBS or 3µg of pegylated IFN-λ3. The ileum was harvested after four hours and a small section was assessed for expression of the ISGs: Ifit1 (A), Isg15 (B), and Mx2 (C) by qPCR. The remaining tissue was stained by RNAscope for the ISG, Ifit1 (red), with a DAPI (blue) counterstain. D-E. Representative images from the ileum of mice injected with PBS (D) or IFN-λ (E). F. Quantification of Ifit1 area relative to the total area of the section across replicate mice. Scale bar = 500µm. Statistical significance was determined by unpaired t-test with ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.
Figure S5. Homeostatic ISG expression is also highly localized in the colon. The colon of Ifnlr1Het/Ifnar1Het, Ifnlr1Het/Ifnar1KO, and Ifnlr1KO/Ifnar1Het mice was harvested and stained by RNAscope for the ISG, Ifit1 (red), with a DAPI (blue) counterstain. Representative images from Ifnlr1Het/Ifnar1Het (A), Ifnlr1Het/Ifnar1KO (B), and Ifnlr1KO/Ifnar1Het (C) mice. Scale bar = 500µm.

Mature enterocytes express homeostatic ISGs in public single-cell datasets from mouse and human

To determine the extent of conservation for homeostatic ISG expression by IECs, we performed orthogonal analyses of publicly available single-cell RNA-seq (scRNA-seq) datasets from mouse (Haber et al., 2017) and human (Elmentaite et al., 2020) IECs. Recently, Haber et al.
published a large single-cell RNA sequencing dataset that profiled sorted IECs from the small intestine of specific-pathogen-free mice and defined 15 distinct IEC subtypes (Haber et al., 2017). We analyzed IECs from this dataset, and found that of the 21 homeostatic ISGs identified in Figure 1D, 19 were present in the Haber et al. single-cell dataset. We determined the percentage of each epithelial cell subtype that expresses each individual homeostatic ISG, and generated a heatmap with hierarchical clustering to group IEC subtypes that have similar ISG expression patterns (Figure 7A). Homeostatic ISGs were predominantly expressed in mature enterocyte subtypes, which clustered separately from crypt-resident progenitor IECs such as transit amplifying (TA) cells and stem cells (Figure 7A). To compare homeostatic ISG expression between polar extremes of the crypt-villus axis, we grouped IEC subtypes that represented enterocytes (mature enterocyte cells) and crypt cells (TA cells and stem cells) and compared the overall proportions of these cells with homeostatic ISG expression. We found that a significantly higher percentage of enterocytes express homeostatic ISGs than crypt cells, but that these homeostatic ISGs were expressed in a relatively small proportion of enterocytes (< 20%) (Figure 7B). Notably, Ifit1 (highlighted in red) was present in ~5% of enterocytes by scRNA-seq, which is consistent with our observation of 1-4% Ifit1-positive area by imaging the mouse small intestine (Figures 6B-G). Furthermore, the relative absence of ISG-positive crypt cells in scRNA-seq data is consistent with our observation that crypt cells lacked Ifit1 and Usp18 expression by imaging.

We expanded our investigation to a scRNA-seq dataset from the ileum of healthy, human, pediatric patients that was previously described (Elmentaite et al., 2020). IECs from this dataset were previously clustered into eight subtypes. For our analysis of homeostatic ISG expression, we excluded IEC sub-types with fewer than 20 constituent cells, which retained five subtypes: enterocytes, early enterocytes, goblet cells, TA cells, and crypt. Of the 21 homeostatic ISGs identified in Figure 1D, 14 homologous human genes were present in these data. Similar to analysis in Figure 7A, we determined the percentage of each cell type that expresses each individual homeostatic ISG and generated a heatmap of these data with hierarchical clustering to
group IEC subtypes that have similar ISG expression patterns (Figure 7C). Enterocyte, early enterocyte, and goblet subtypes clustered separately from TA and crypt cells, with the highest proportion of homeostatic ISGs being present in the enterocyte subtype (Figure 7C). As with mouse IEC data, above, we grouped cell subtypes that localize in the crypt (TA cells and crypt cells) and compared overall proportions of homeostatic ISG expression with the mature enterocyte subtype (Figure 7D). Similar to mice, homeostatic ISGs were present in significantly more enterocytes than crypt cells, and most homeostatic ISGs in this human dataset were present in a relatively small proportion of cells (< 20%). These data suggest that ISGs are present in a small proportion of IECs from healthy, human ileal tissue at homeostasis and may share the localization observed in our murine analyses. Together, our analyses of these public scRNA-seq datasets support our observations that homeostatic ISGs are not ubiquitously expressed throughout the intestinal epithelium; rather, they are expressed in a minority of IECs and skewed towards mature enterocytes along the crypt-villus axis.
Figure 7. Mature enterocytes express homeostatic ISGs in public single-cell datasets from mouse and human. A-B. A mouse IEC single-cell transcriptional dataset (Haber et al., 2017) (GSE92332) was analyzed to determine the percentage of each epithelial cell subtypes that express homeostatic ISGs. A. Heatmap depicting the proportion of each epithelial cell type expressing nineteen of the twenty-one homeostatic ISGs identified in Figure 1. B. Enterocyte subtypes (blue text) and crypt-resident progenitor subtypes (green text) cells were grouped and the percentage of cells that express each homeostatic ISG was compared. C-D. A human IEC single-cell transcriptional dataset (Elmentaite et al., 2020) (E-MTAB-8901) was analyzed for the percentage of epithelial cell subtypes that express homeostatic ISGs. C. A heatmap depicting the percentage of IEC subtypes that express human orthologs of murine homeostatic ISGs identified in Figure 1. D. The mature enterocyte subtype (blue text) and crypt-resident progenitor subtypes
(green text) were grouped and the percentage of cells that express each homeostatic ISG was compared. Lines in B and D link paired ISGs in each IEC subset. Statistical significance in B and D was calculated by Wilcoxon test where * = p < 0.05, and *** = p < 0.001.

Homeostatic ISG expression is partially restored by peroral LPS in ABX-treated mice

To further define the relationship between bacteria and homeostatic ISGs, we assessed whether oral administration of fecal contents or purified LPS (a bacterial MAMP and TLR4 agonist) could restore localized ISG expression in mice with depleted bacterial microbiota. Control groups of Ifnlr1^{fox/fox} mice with conventional microbiota (Figure 8A) retained a localized Ifit1 expression pattern, whereas ABX-treated Ifnlr1^{fox/fox} mice (Figure 8B) and Ifnlr1^{ΔIEC} mice (Figure 8C) lacked Ifit1 expression. Oral LPS administered to conventional Ifnlr1^{fox/fox} mice did not significantly alter the distribution or frequency of localized Ifit1 expression (Figure 8D). However, localized Ifit1 expression was restored in a subset of ABX-treated Ifnlr1^{fox/fox} mice administered LPS (Figure 8E). Fecal transplant of conventional microbes to ABX-treated Ifnlr1^{fox/fox} mice was similarly capable of restoring localized expression of Ifit1 in a subset of mice (Figure 8F). Importantly, we did not observe restoration of localized Ifit1 expression in Ifnlr1^{ΔIEC} mice following LPS administration (Figure 8G), indicating that restoration depends on IEC expression of Ifnlr1.

We noted that restoration of localized Ifit1 signal following LPS administration or fecal transplant appeared largely binary (i.e. present or absent) in our imaging data (Figures 8E and 8F, representative Ifit1-positive and Ifit1-negative images). Using quantification of Ifit1 area (Figure 8H), we stratified mice into Ifit1-postive and Ifit1-negative groups (Figure 8I-J) based on a cut-off set at the maximal Ifit1 area value of Ifnlr1^{ΔIEC} mice (dashed line in Figure 8H). This analysis indicated 8/8 conventional Ifnlr1^{fox/fox} mice, 0/8 ABX-treated control mice, 4/12 LPS-treated mice, and 4/8 fecal transplant mice were Ifit1-positive (Figure 8I-J). Statistical analysis by Fisher’s exact test indicated that LPS administration modestly increased (p = 0.1022) the proportion of mice that were Ifit1-positive, whereas fecal reconstitution of ABX-treated mice
significantly increased \( (p < 0.05) \) the likelihood of these mice being \( \text{Ift1} \)-positive (Figure 8J).

Importantly, mice that received fecal transplant had full restoration of 16S gene copies (Figure 8K) despite having partial restoration of homeostatic ISGs (4/8). These findings indicate that reconstitution of homeostatic ISG signal has incomplete penetrance, underscoring the partial restoration by LPS administration to ABX-treated mice.

To corroborate these findings, we performed orthogonal analyses of \( \text{Ift1} \), \( \text{Stat1} \), and \( \text{Oas1a} \) expression in ileum tissue of WT mice treated with ABX followed by fecal transplant, LPS administration, or administration of TLR9 agonist, CpG DNA (Figure S6). These data exhibited high variance, but were consistent with imaging data in Figure 8, indicating a partial restoration of homeostatic ISGs in 20-50\% of ABX-treated mice by LPS or fecal transplant, but not CpG DNA (Figure S6). Together, these data indicate that LPS is sufficient for localized, homeostatic ISG expression to a similar extent as total microbiota, suggesting that localized exposure to bacterial MAMPs is the basis for localized, homeostatic ISG expression.
Figure 8. Homeostatic ISG expression is partially restored by peroral LPS in ABX-treated mice. The ilea of treated WT, \textit{Ifnlr1}\textsuperscript{flox/flox}, and \textit{Ifnlr1}\textsuperscript{ΔIEC} mice were harvested, processed into Swiss rolls, and stained by \textit{in situ} hybridization for the ISG, \textit{Ifit1} (red), with a DAPI (blue) counterstain.

A-C. Representative images from \textit{Ifnlr1}\textsuperscript{flox/flox} mice treated with H\textsubscript{2}O control followed by PBS stimulation (A), ABX followed by PBS stimulation (B), or from \textit{Ifnlr1}\textsuperscript{ΔIEC} mice (B). D. Representative images of \textit{Ifnlr1}\textsuperscript{flox/flox} mice treated with H\textsubscript{2}O control followed by LPS stimulation.

E-F. Two representative images of \textit{Ifnlr1}\textsuperscript{flox/flox} mice treated with ABX followed by LPS stimulation (E) or ABX followed by fecal transplantation (F). H. Quantification of \textit{Ifit1} area relative to the total area of each tissue section with a dashed line at the highest \textit{Ifnlr1}\textsuperscript{ΔIEC} value. The proportion of \textit{Ifit1}-positive mice (above dashed line) and \textit{Ifit1}-negative mice (below dashed line) are tabulated (I) and graphed (J) for \textit{Ifnlr1}\textsuperscript{flox/flox} mice of each condition. K. rDNA was isolated from the luminal contents of mice at endpoint harvest. 16S gene copies were assessed by qPCR and normalized to input. Limit of detection: dashed line. Where depicted, scale bar = 500\textmu m. Statistical significance was determined by one-way ANOVA with Dunnett’s multiple comparisons (H, K) and by Fisher’s exact test (J) where * = p < 0.05, ** = p < 0.01, and **** = p < 0.0001.
Figure S6. Homeostatic ISG expression is partially restored by peroral LPS in ABX-treated mice. The ileum of treated WT, Ifnlr1^{flox/flox}, and Ifnlr1^ΔIEC mice was harvested and Ifit1 (A), Stat1 (B), and Oas1a (C) was quantified by qPCR. Delineation of positive ISG expression was

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conservatively selected as one standard deviation above the mean of ABX-treated mice (dashed line). Mice were binned into ISG-positive (above dashed line) or ISG-negative (below dashed line) groups and are tabulated for each condition in D. The proportion of mice with expression of Ifit1 (E), Stat1 (F), and Oas1a (G) are graphed for each condition. Statistical significance was determined by one-way ANOVA with Dunnett’s multiple comparisons (A-C) and by Fisher’s exact test (E-G) where * = p < 0.05, ** = p < 0.01, and **** = p < 0.0001.

**The homeostatic IFN-λ response preemptively protects IECs from murine rotavirus infection**

To assess the capacity of homeostatic ISGs to protect IECs from viral infection, we utilized infection with murine rotavirus (mRV), an IEC-tropic pathogen. Prior studies of rotaviruses have identified viral immune evasion genes that block IFN induction through multiple mechanisms (Arnold et al., 2013). However, we reasoned that pre-existing ISG expression stimulated by the bacterial microbiome at homeostasis may preemptively protect IECs from the infection before viral gene expression is initiated. To determine the role of an epithelial IFN-λ response over the course of mRV infection in adult mice, we monitored daily shedding of viral genomes in the stool of Ifnlr1floxflox mice and Ifnlr1ΔIEC littermates. We first detected mRV shedding in the stool on day two after inoculation, and at this early timepoint, Ifnlr1ΔIEC shed 20-fold more mRV genomes into their stool than Ifnlr1floxflox mice (Figure 9A). However, at the peak of viral shedding between days three and five, there were no significant differences between Ifnlr1floxflox and Ifnlr1ΔIEC littermates (Figure 9A). This similarity at peak of viral shedding was consistent with an ability of mRV to evade the host IFN response once infection is established. Together, these findings suggest that Ifnlr1ΔIEC mice have defects in protection against initiation of mRV infection and that the protective capacity of endogenous IFN-λ signaling against mRV is primarily prophylactic in nature.

To more stringently assess the capacity of Ifnlr1 to protect IECs against the earliest stages of mRV infection, we inoculated mice with 5000 SD50 (50% shedding dose) of mRV to maximize
the likelihood of uniform viral exposure throughout the intestine. At 12 and 24 hours post-ino- 
culation, we quantified mRV genomes in the ileum and the proportion of infected IECs (live, 
EpCAM-positive, CD45-negative, and mRV-positive) by flow cytometry. At 24 hours post- 
ino- 
oculation, we found that Ifnlr1ΔIEC mice had 20-fold more mRV genomes than Ifnlr1flox/flox mice 
(Figure 9B). In addition, we found that a three-fold greater proportion of IECs were infected with 
mRV in Ifnlr1ΔIEC mice than Ifnlr1flox/flox mice at 24 hours post-infection (Figures 9C-F). However, 
the median fluorescence intensity (MFI) of mRV antigen was equivalent in mRV-infected IECs 
from Ifnlr1flox/flox and Ifnlr1ΔIEC mice (Figure 9G) and the MFI of mRV did not correlate (r² = 0.0003) 
with the percentage of infected IECs (Figure 9H). This equivalent mRV antigen burden in infected 
cells from Ifnlr1flox/flox and Ifnlr1ΔIEC mice in combination with the lack of correlation between antigen 
burden and percentage of infected cells suggests that the protective role of Ifnlr1 is to prevent 
infection of IECs rather than to limit replication within IECs after they are infected. Similar trends 
toward an increased proportion of IEC infection in Ifnlr1ΔIEC relative to Ifnlr1flox/flox littermates were 
observed at 12 hours post-inoculation, but the extent of infection was 10-100 fold lower and near 
the limit of detection (Figure S7). Thus 12- and 24-hour timepoints capture the earliest detectable 
infection of IECs by mRV. Together these data indicate that Ifnlr1 expression by IECs reduces 
the proportion of cells initially infected by mRV, but does not limit the extent of replication once 
infection is established, consistent with an early protective role for homeostatic ISG expression 
that preempts viral IFN evasion mechanisms.
Figure 9. The homeostatic IFN-λ response preemptively protects IECs from murine rotavirus infection. Ifnlr1^flox/flox and Ifnlr1^ΔIEC mice were infected with 100 SD50 (A) or 5000 SD50 (B-G) of mRV and stool (A) or stripped ileal intestinal epithelial cells (B-G) were assessed for mRV infection. A. Timecourse of mRV genome copies detected in the stool of Ifnlr1^flox/flox and Ifnlr1^ΔIEC mice by qPCR. B-G. Mechanically stripped intestinal epithelial cell fractions were...
analyzed by qPCR for mRV genome copies (B) or flow cytometry for RV antigen-positive IECs (C-G). Representative flow cytometry plots of naïve (C), Ifnlr1\textsuperscript{flox/flox} (D), and Ifnlr1\textsuperscript{ΔIEC} (E) mice infected with mRV with quantification in (F). G. The MFI of mRV antigen in infected cells relative to uninfected cells. H. A linear correlation plot of mRV+ cells and mRV MFI with 95% confidence intervals (dashed lines). Where designated in A-G, dashed lines = LOD as set by naïve mice. Statistical significance was determined by two-way ANOVA with Sidak’s multiple comparisons (A), by Mann Whitney (B, F), or unpaired t-test (G) where * = p < 0.05, *** = p < 0.001.

Figure S7. The homeostatic IFN-λ response preemptively protects IECs from murine rotavirus infection. Ifnlr1\textsuperscript{flox/flox} and Ifnlr1\textsuperscript{ΔIEC} mice were infected with 5000 SD50 of mRV by oral gavage and stripped ileal intestinal epithelial cell fractions were analyzed by qPCR for mRV genome copies (A) or flow cytometry for RV antigen-positive IECs (B-F). Representative flow cytometry plots of naïve (B), Ifnlr1\textsuperscript{flox/flox} (C), and Ifnlr1\textsuperscript{ΔIEC} (D) mice infected with mRV. F. The MFI of mRV antigen in infected cells relative to uninfected cells. G. A linear correlation plot of mRV+
cells and mRV MFI with 95% confidence intervals (dashed lines). Statistical significance was calculated by Mann Whitney (A, E), or unpaired t-test (F) where * = p < 0.05.
Discussion

Here, we report that bacterial microbiota induce an enteric IFN-λ response in IECs at homeostasis (Figures 1-4). This IEC-centric response is dependent on the IFN-λ receptor and independent of type I IFN signaling (Figure 3), consistent with the dominant role of IFN-λ signaling in the gastrointestinal epithelium. Furthermore, we find minimal changes in ISG expression within the spleen and MLN after ABX treatment, suggesting that homeostatic ISGs are predominantly expressed in the intestine. These findings differ slightly from prior descriptions of systemic type I IFN responses that are dependent on bacterial microbiota (Abt et al., 2012; Bradley et al., 2019; Ganal et al., 2012; Steed et al., 2017; Stefan et al., 2020; Winkler et al., 2020). However, differences in the specific tissues and cell types analyzed make it difficult to draw direct comparisons across these studies. Therefore, we conclude that homeostatic ISGs are substantially present in IECs, but do not dispute the prior findings that relatively low homeostatic ISG expression induced by type I IFN plays an important role in extra-intestinal tissues and non-epithelial cell types.

Prior studies by Mahlakõiv et al. noted the presence of Ifnl2/3 transcripts in CD45+ cells but not EpCAM+ IECs within the intestine at homeostasis. We have confirmed these findings and have extended them to show that this homeostatic expression of Ifnl2/3, but not homeostatic expression of Ifnb1, is dependent on bacterial microbiota (Figure 5). However, we have been unable to detect IFN-λ transcripts by RNA scope in situ hybridization in mice at homeostasis, consistent with recently published data (Ingle et al., 2021). This suggests that the production of IFN-λ is below our limit of detection by imaging or highly transient in nature. Swamy et al. showed that T cell receptor stimulation led intraepithelial lymphocytes to produce IFN-λ (Swamy et al., 2015) and Mahlakõiv et al. suggested that the primary producers of IFN-λ at steady-state are intraepithelial lymphocytes. However, myeloid cells also reside near the intestinal epithelium and can sample luminal contents by various mechanisms (Chieppa et al., 2006; McDole et al., 2012; Niess et al., 2005). Thus, it remains to be determined which CD45+ cell types are responsible for
producing IFN-λ in response to stimulation by luminal MAMPs at homeostasis, but is of distinct interest for future studies.

We initially anticipated that the distribution of homeostatic ISGs would be uniform among IECs. Instead, we found that this IFN-λ response is highly localized within enteric tissues. Homeostatic ISGs are observed in a minority of small intestinal villi and are primarily present in mature epithelium towards the villus tips (Figure 6). Likewise, homeostatic ISGs are present within patches of the mature epithelium in the colon (Figure S5). These surprising findings are supported by analysis of independently-generated scRNA-seq datasets from mouse and human small intestinal IECs that depict expression of homeostatic ISGs in a minority of cells with predominant expression in mature enterocytes (Figure 7). The basis for this pattern is unknown; however, it may reflect the distribution of cells capable of sensing bacterial microbiota or microenvironments within the gastrointestinal tract that are particularly exposed to luminal bacteria. Further delineation of the factors that render specific regions “responsive” will be of great interest for follow-up studies.

Our finding that LPS administration or fecal transplant can partially restore the localized expression of homeostatic ISGs in the small intestine (Figures 8 and S6) supports the concept that these ISG-positive regions are uniquely exposed or responsive to bacterial MAMPs. Indeed, we find that MYD88 is required for WT levels of homeostatic ISG expression in the small intestine (Figure 3). However, MYD88 is dispensable for homeostatic ISG expression in the colon, and TRIF is not required in either small intestine or colon (Figure 3). These data suggest that the bacterial microbiota broadly stimulates homeostatic IFN-λ through multiple, redundant PRRs. Furthermore, localized restoration of ISGs following LPS administration (Figure 8) suggests that localization is an intrinsic property of homeostatic ISG stimulation. This localization may be indicative of regional differences in access of luminal bacterial MAMPs to IFN-λ-producing cells.

One host mechanism to limit bacterial interactions with the intestinal epithelium is the presence of mucus layers (Atuma et al., 2001; Johansson et al., 2011). Intriguingly, the single mucus layer
in the small intestine is much less adherent than the mucus layers present in the colon (Johansson et al., 2011), which might allow occasional direct bacterial interactions with IECs or other cells near the intestinal epithelium. However, soluble components from enteric bacteria may also readily diffuse through mucus layers. In this case, there may be sporadic defects in tight junction proteins that are required to maintain the intestinal epithelium. Tight junction remodeling is essential to maintain intestinal integrity during apoptosis and extrusion of IECs that are regularly shed from the intestinal epithelium (Williams et al., 2015). Future studies will be necessary to determine whether defects in epithelial barrier integrity during extrusion events are linked to the local ISG responses that we observe.

The signature of homeostatic ISGs in ileum tissue included well-characterized antiviral-ISGs (Figures 1-2). These findings suggested that the homeostatic ISG response in IECs would provide protection against IEC-tropic viruses, such as mRV. To investigate this question, we used Ifnlr1ΔIEC mice that lack homeostatic ISGs (Figure 4 and 6) rather than using ABX-treatment, which introduces pleiotropic effects on rotavirus infection (Uchiyama et al., 2014) and dramatically increases transit time through the intestine (Baldridge et al., 2015). Using Ifnlr1ΔIEC mice, we found increase in IEC infection by mRV at early stages of infection compared to Ifnlr1flox/flox littermates (Figure 9). However, the protection offered by IEC expression of Ifnlr1 was lost by the middle and late stages of infection, consistent with the ability of mRV to antagonize induction of IFN responses once infection is established (Arnold et al., 2013). Our observations during initiation of infection may provide important context to observations in other studies that report differing capacity for infection-induced IFN-λ to protect against mRV infection (Lin et al., 2016; Pott et al., 2011). Ultimately, it is clear that prophylactic administration of exogenous IFN-λ protects against mRV infection (Lin et al., 2016; Pott et al., 2011; Van Winkle et al., 2020), indicating that homeostatic IFN-λ is also protective when induced by bacterial microbiota prior to infection. In sum, these findings indicate that preexisting, homeostatic ISGs present in Ifnlr1-sufficient mice are protective during initiation of mRV infection, but that endogenous IFN-λ does not reduce mRV
burden in infected cells. These data highlight the possibility that detection of bacterial microbiota in particularly exposed areas may preemptively activate homeostatic ISGs as a form of anticipatory immunity to protect the intestinal epithelium from enteric viruses.
Methods

Mice

All mice were bred using the C57BL/6 background and used within the age of 8-12 weeks; C57BL/6J mice (stock # 000664) were purchased from Jackson Laboratories (Bar Harbor, ME) and used as wild-type. Genetically modified mice included Ifnlr1^{-/-} and Ifnlr1^{floxed/floxed} (generated from Ifnlr1^{tm1a(EUCOMM)Wtsi} as published (Baldridge et al., 2017)), Ifnar1^{-/-} (B6.129.1fnar1^{tm1}), Trif^{-/-} (JAX C57BL/6J-Ticam1^{Lps2/J}, stock #005037), Myd88^{-/-} (JAX B6.129P2(SJL)-Myd88^{tm1.1Defr/J}, stock #009088), Irf3^{-/-} (B6.129S/SvEv-Bcl2l12/Irf3^{tm1Ttg}), Irf7^{-/-} (B6.129P2-Irf7tm1Ttg/TtgRbrc), Stat1^{-/-} (B6.129.-Stat1^{tm1Dlv}), and Villin-cre (B6.Cg-Tg(Vil1-cre)997Gum/J) mice. Ifnlr1^{-/-} and Ifnar1^{-/-} mice were bred to Ifnlr1^{Het}/Ifnar1^{Het} mice to generate littermate Ifnlr1^{Het}/Ifnar1^{Het}, Ifnlr1^{Het}/Ifnar1^{KO}, Ifnlr1^{KO}/Ifnar1^{Het}, Ifnlr1^{KO}/Ifnar1^{KO} offspring.

All mice were maintained in specific-pathogen-free facilities at Oregon Health & Science University (OHSU) and Washington University in St. Louis (WUSTL). Animal protocols were approved by the Institutional Animal Care and Use Committee at OHSU (protocol #IP00000228) and WUSTL (protocol #20190162) in accordance with standards provided in the Animal Welfare Act.

Mouse Treatments

Mice were administered an ad libitum antibiotic cocktail consisting of: 1 g/L ampicillin, 1 g/L metronidazole, 1 g/L neomycin, and 0.5 g/L vancomycin (Sigma, St. Louis, MO) in autoclaved H2O (OHSU) or in 20 mg/mL grape Kool-Aid (Kraft Foods, Northfield, IL) (WUSTL). Sterile H2O (OHSU) or Kool-Aid (WUSTL) alone was used as a control. Mice were maintained on antibiotics or control for 2 weeks prior to harvest.

Recombinant IFN-λ was provided by Bristol-Myers Squibb (New York City, NY) as a monomeric conjugate comprised of 20kDa linear PEG attached to the amino-terminus of murine
Mice were injected intraperitoneally with IFN-λ or an equal volume of PBS vehicle as indicated in figure legends at the indicated time prior to analysis. Mice were stimulated with 100µg of the synthetic dsRNA analogue, poly I:C (R&D, #4287) or PBS by intraperitoneal injection in a 200µL volume, 2 hours prior to harvest. 25µg of the bacterial product lipopolysaccharide (Sigma #L4391) or CpG (Invivogen #tlrl-1585; Class A CpG oligonucleotide) were perorally administered to mice in 25µL of sterile PBS. Mice were treated on days 15 and 16 of antibiotic treatment or H2O control prior to harvest on day 17.

For transplantation of fecal material, antibiotic treatment was stopped and mice were fed 25uL of fecal mixture by pipet for two consecutive days. Fecal mixture was prepared by collecting fecal samples from control mice; a single stool pellet was resuspended in 200uL of sterile PBS, stool was broken apart by pipetting, and large particulate was allowed to settle for several minutes prior to administration.

**Cell Isolation**

Epithelial fractions were prepared by non-enzymatic dissociation as previously described (Nice et al., 2016). Briefly, mouse ileum was opened longitudinally and agitated by shaking in stripping buffer (10% bovine calf serum, 15 mM HEPES, 5 mM EDTA, and 5 mM dithiothreitol [DTT] in PBS) for 20 min at 37°C. Lamina propria fractions were prepared by enzymatic digestion and dissociation with the Lamina Propria Dissociation Kit and GentleMacs Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Dissociated cells were collected for use in qPCR analysis, flow cytometry, and magnet enrichment.

**Rotavirus infection of mice**

Mouse rotavirus (EC strain) was graciously provided by Andrew Gewirtz (Georgia State University). Viral stocks were generated by inoculating 4- to 6-day-old neonatal BALB/c mice and harvesting the entire gastrointestinal tract upon presentation of diarrheal symptoms 4 to 7 days
later. Intestines were freeze-and-thawed, suspended in PBS, and homogenized in a bead beater using 1.0-mm zirconia-silica beads (BioSpec Products). These homogenates were clarified of debris, aliquoted, and stored at -70°C. The 50% shedding dose (SD50) was determined by inoculation of 10-fold serial dilutions in adult C57BL/6J mice. For stool time-course studies, mice were inoculated by peroral route with 100 SD50 and a single stool pellet was collected daily for viral quantitation by qPCR. For protection studies, mice were inoculated by intragastric gavage with 5000 SD50, and ileum was isolated and mechanically stripped 12- and 24-hours later for quantitation of viral burden by qPCR and flow cytometry.

RNA isolation, rDNA isolation, qPCR, and analysis

RNA from tissue and stripped IECs was isolated with TRIzol (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. RNA from magnet-enriched cells was purified by Zymo Quick-RNA Viral Kit (Zymo Research, Irvine, CA). The larger of either 1μg of RNA or 5μL of RNA were used as a template for cDNA synthesis by the ImProm-II reverse transcriptase system (Promega, Madison, WI) after DNA contamination was removed with the DNAfree kit (Life Technologies). 16S bacterial rDNA was isolated from stool and intestinal contents with a ZymoBIOMICS DNA kit (Zymo Research, Irvine, CA) kit. Quantitative PCR was performed using PerfeCTa qPCR FastMix II (QuantaBio, Beverly, MA) and the absolute quantities of transcript were determined using standard curves composed of gBlocks (IDT) containing target sequences. Absolute copy numbers from tissue samples were normalized to the housekeeping gene, ribosomal protein S29 (Rps29). Taqman assays for selected genes were ordered from IDT (Coralville, IA): Rps29 (Mm.PT.58.21577577), Ifit1 (Mm.PT.58.32674307), Oas1a (Mm.PT.58.30459792), Mx2 (Mm.PT.58.11386814), Stat1 (Mm.PT.58.23792152), Ifn15 (Mm.PT.58.41476392.g). Taqman assays for Ifnl2/3 and Ifnb1 were designed previously (Van Winkle et al., 2020) and consisted of the following primer-probe sequences: Ifnl2/3 (Primer 1 – GTTCTCCCAGACCTTCAGG, Primer 2 – CCTGGGACCTGAAGCG, Probe –
CCTTGCAAGCTAGGGG; *Ifnb1* (Primer 1 – CTCCAGCTCAAGAGAAC, Primer 2 – GCCCTGTAGGTAGGGAT, Probe – CAGGAGCTCCTGGAGCAGCTGA). Murine rotavirus was detected using Taqman primer-probe sets specific for 422-521 of GeneBank sequence DQ391187 as previously described (Fenaux et al., 2006) with the following sequences: Primer 1 – GTTCGTTGTGCCTCATTCG, Primer 2 – TCGGAACGTACTTCTGGAC, Probe – AGGAATGCTTCAGCGCTG; and universal bacterial 16S rDNA was detected using Taqman primer-probe sets with previously designed sequences (Nadkarni et al., 2002): Primer 1 – GGACTACCAGGGTATCTAATCCTGTT, Primer 2 – TCCTACGGGAGGCAGCAGT, Probe – CGTATTACCAGGGCTGCTGGAC.

**RNAscope**

Swiss rolls of intestinal tissue were fixed in 10% neutral-buffered formalin for 18-24hr and paraffin-embedded. Tissue sections (5μm) were cut and maintained at room temperature with desiccant until staining. RNA *in situ* hybridization was performed using the RNAscope Multiplex Fluorescent v2 kit (Advanced Cell Diagnostics, Newark, CA) per protocol guidelines. Staining with anti-sense probes for detection of *Ifit1* (ACD, #500071-C2) and *Usp18* (ACD, #524651) was performed using ACDBio protocols and reagents. Slides were stained with DAPI and mounted with ProLong Gold antifade reagent (ThermoFisher), and imaged using a Zeiss ApoTome2 on an Axio Imager, with a Zeiss AxioCam 506 (Zeiss).

**Flow cytometry and Magnet Enrichment**

Dissociated cells were collected and stained for flow cytometry. Cells were stained with Zombie Aqua viability dye (BioLegend), Fc receptor-blocking antibody (CD16/CD32; BioLegend), anti-EpCAM (clone G8.8; BioLegend), and anti-CD45 (clone 30-F11; BioLegend). For analysis of murine rotavirus infection, cells were stained with anti-rotavirus (polyclonal; ThermoFisher, #PA1-7241) followed by goat anti-rabbit secondary (ThermoFisher). All data were analyzed using FlowJo software (BD Biosciences). Gates were set based on unstained and single-fluorophore
stains. IECs were selected by gating on live, EpCAM-positive, CD45-negative cells. Gates for murine rotavirus infection were set based on naïve samples.

Where indicated, dissociated cells were enriched using MojoSort Mouse anti-APC Nanobeads (BioLegend, #480072) after flow cytometry staining for anti-EpCAM and anti-CD45 with APC fluorophores by following manufacturer protocols.

**RNA sequencing and expression analysis**

Wild-type C57BL/6J or Ifnlr1−/− mice were administered *ad libitum* Kool-aid or ABX for two weeks, or wild-type mice were administered 25µg recombinant IFN-λ for one day, then ileal segments lacking Peyer’s patches were harvested and RNA sequencing was performed as prior (Park et al., 2016). mRNA from ilea was purified with oligo-dT beads (Invitrogen, Carlsbad, CA) and cDNA was synthesized using a custom oligo-dT primer containing a barcode and adaptor-linker sequence, degradation of RNA-DNA hybrid following single-strand synthesis, and ligation of a second sequencing linker with T4 ligase (New England Biolabs, Ipswich, MA). These reactions were cleaned up by solid phase reversible immobilization (SPRI), followed by enrichment by PCR and further SPRI to yield strand-specific RNA-seq libraries. Libraries were sequenced with an Illumina HiSeq 2500 with three to four mice were included in each group. Samples were demultiplexed with second mate, reads were aligned with STAR aligner and then counted with HT-Seq. Differentially expressed genes were identified using DESeq2 (Love et al., 2014) based on cutoffs of 2-fold change, and an inclusive p-value < 0.5. Standard gene set enrichment analysis was performed to identify enrichments in IFN-λ response genes. RNA-seq data were uploaded to the European Nucleotide Archive (accession #: PRJEB43446).

**Statistical Analyses**

Data were analyzed with Prism software (GraphPad Prism Software), with specified tests as noted in the figure legends.
Data availability.

RNA sequencing data obtained in this study have been deposited in the European Nucleotide Archive (accession #: PRJEB43446).

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Author Contributions


Disclosure

We declare no competing interests.
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