INTERFERON-λS: NEW REGULATORS OF INFLAMMATORY PROCESSES

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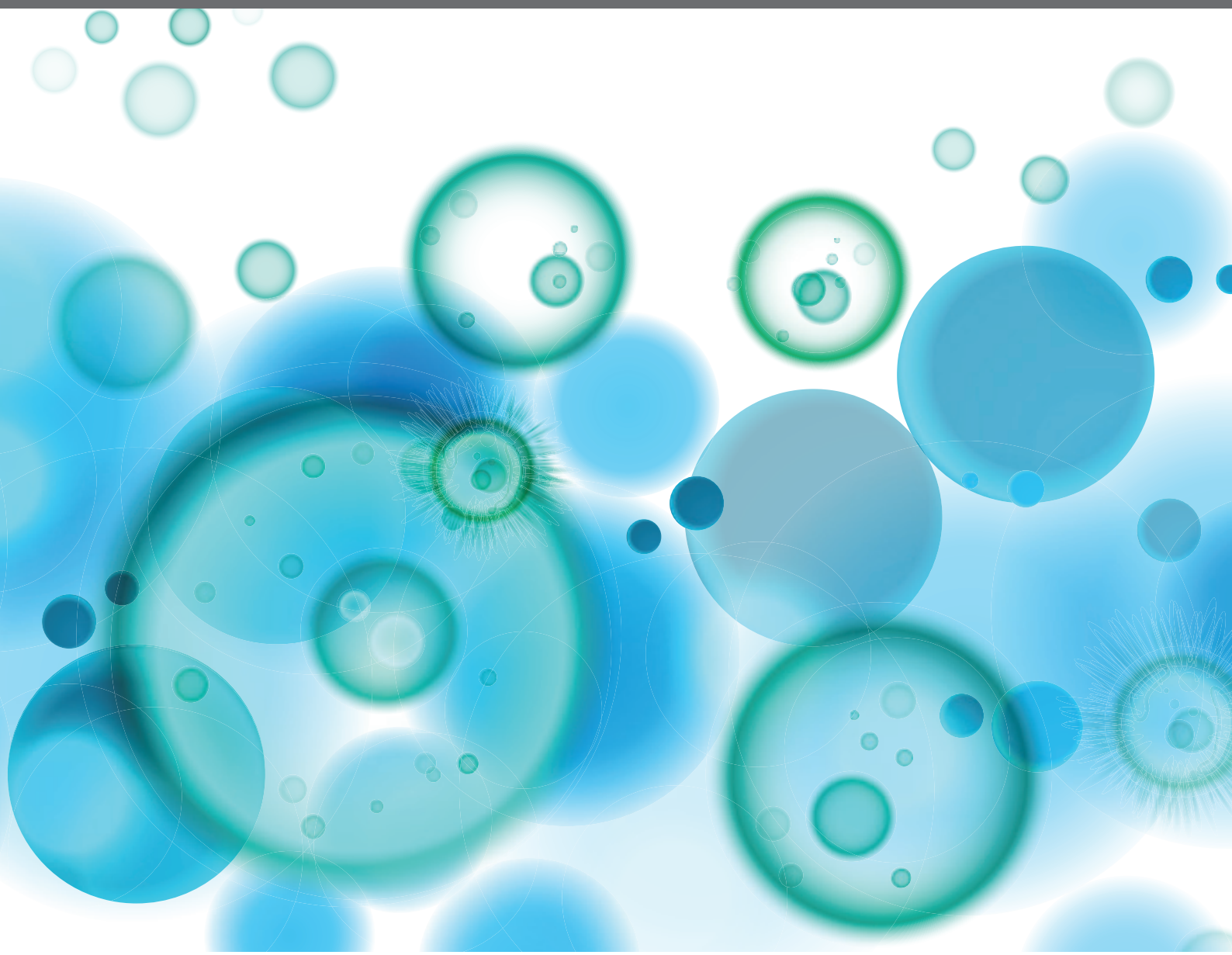

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INTERFERON-λS: NEW REGULATORS OF INFLAMMATORY PROCESSES

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EDITORIAL
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Editorial: Interferon-λs: New Regulators of Inflammatory Processes

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Keywords: interferon, type III IFNs, barrier, inflammation, IFN lambda

Editorial on the Research Topic

Interferon-λs: New Regulators of Inflammatory Processes

Interferons (IFN) were the first family of cytokines to be discovered, with type I IFNs in 1957 followed closely by type II IFN in 1965. Type I IFNs are a large family comprised of IFNαs, β, and other subtypes while IFNγ is the sole type II family member. Our understanding of IFN function was binary for a long time, with type I IFNs considered mainly antiviral while IFNγ was classified as the antibacterial IFN. It isn't until 2003 that a third family emerged, type III IFNs also known as IFNλ1-4 or IL29, Il28A-C. Type III IFNs are functionally closer to type I IFNs as they have potent antiviral functions and induce a largely overlapping family of interferon stimulated genes (ISGs). These functions are best described in epithelial cells, the primary target of type III IFNs, but more recent immunomodulatory roles of type III IFNs are emerging. This topic discusses several aspects of type III IFN biology, from its similarities and differences with type I IFNs, to functions in different tissues and models.

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ANTIVIRAL ACTIVITIES OF TYPE III IFNS AT MUCOSAL SITES

One of the distinct features of type III IFN biology is the restricted expression of its receptor. The IFNλ receptor (IFNLR) is composed of two chains including a unique chain, IL28Rα (IFNLR1), whose expression is most predominant on cells of epithelial lineage. As a consequence, the function of type III IFNs are most important at mucosal surfaces and type III IFNs have been emerging as critical regulators of immunity at barrier sites. In this topic Hemann et al. deliver a very nice overview of the regulation of IFNλ gene expression in response to viral infections. This review also compares the different functions of type I and III IFNs in different models. Lee and Baldridge dives deeper in the antiviral functions of IFNλs in the intestine. Both these review articles include comprehensive tables outlining the functions of IFNλs in response to different viral challenges. Andreakos et al. take a closer look at the roles of type III IFNs in the respiratory tract. IFNλs are "front-line guardians" and contribute to immunity against acute viral infections but also chronic respiratory diseases. This theme is also tackled by Sopel et al. that specially discuss the role of IFNλ in asthma.

DIFFERENCES BETWEEN TYPE I AND III IFN SIGNALING IN POLARIZED EPITHELIAL CELLS

The antiviral state activated by type I and III IFNs is mediated by the induction of a large family of ISGs. In contrast to IFNγ that induces genes regulated by gamma activated sequence (GAS) promoters, type I and III IFNs both induce genes containing IFN stimulated response elements

(ISRE). The ISGs induced by type I and III IFNs were therefore considered to be largely identical. Here, Selvakumar et al. use a mouse polarized intestinal epithelial cell line to identify a subset of ISGs uniquely induced by IFNλ2. Interestingly these genes are only strongly induced in polarized gut epithelial cells, but not in unpolarized cells, bone-marrow derived dendritic cells or primary lung epithelial cells. Polarization of intestinal epithelial cells increased expression of both chains of the IFNLR, while the type I IFN receptor (IFNAR) chains remained unchanged.

In a parallel study Bhushal et al. compare the strength and frequency of ISG expression upon type I and III IFN treatment. They show that almost all unpolarized cells in culture respond to high concentrations of IFNβ while IFNλ responsiveness plateaus below 50% of cells. Histone deacetylase inhibitors (HDAC) restore frequency of responsiveness upon IFNλ treatment revealing the role of epigenetic regulatory mechanisms in ISG expression downstream of type III but not type I IFN stimulation. As seen above, upon cell polarization, full IFNλ responsiveness was restored, but this response was no longer enhanced by HDAC inhibition. It would be interesting to investigate if the expression IFNλ-specific ISGs identified by Selvakumar et al. is particularly regulated by epigenetic modifications.

In another study investigating signaling downstream of type I and III IFNs in intestinal epithelial cells, Pervolaraki et al. use human mini-gut organoids and polarized human cell lines. They find that both IFN classes induce ISGs and control virus infection but that they do so via separate pathways. In addition to STAT1, 2, and 3, both IFNs phosphorylated MAP kinases (MAPKs). However, inhibition of MAPKs only affected the ability of IFNλs to control viral infection. Interestingly this effect was independent of STAT1 phosphorylation showing that the two pathways are probably independent. Whether these findings are linked to the differential ISG regulation observed in the previously mentioned studies will need to be addressed in the future.

IMMUNOMODULATORY ROLES OF TYPE III IFNS

As discussed in most papers in this topic, in contrast to IFNAR which is ubiquitously expressed, IFNLR expression is limited to a small number of lineages. It is widely accepted that IFNLR is most strongly expressed on cells of epithelial origin. Recently, neutrophils also appeared as target cells, while there is some debate as to natural killer (NK) cells or dendritic cells (DCs) were IFNλ-responsive. In this topic, Zanoni et al. review the roles of IFNλs on immune cells. IFNλs function on neutrophils to halt their migratory capabilities, or inhibit reactive oxygen species production and neutrophil extracellular trap release. They also discuss that while type III IFNs do affect the functions of NK cells, it is most likely indirect as there is limited evidence that NK cells express the IFNLR or are IFNλ responsive. Conversely, conventional mouse DCs and human plasmacytoid dendritic cells (pDCs) do respond to type III IFN stimulation. IFNλ treatment of human pDCs leads to JAK/STAT activation and induction of ISGs, as well as up regulation of certain cytokines and surface markers, and increased survival. For a deeper look, the different functions of IFNλs on pDCs is synthesized by Finotti et al. in this topic.

TREATMENT OPTIONS WITH TYPE III IFNS

Type I IFN are an accepted treatment strategy for a number of inflammatory or infectious diseases including viral hepatitis. However, the efficacy of these treatments is not optimal and they come with many debilitating side effects. The emergence of type III IFNs as potent antiviral cytokines opened new treatment options for several afflictions including patients with chronic viral infections. In this topic, Phillips et al. report a clinical trial using pegylated IFNλ in chronic hepatitis B (CHB) patients. They find that in combination with directly acting antiviral therapy, peg-IFNλ treatment increased antiviral cytokines in the serum, and enhanced NK cell function while maintaining HBV-specific CD8+ T cell functions. Overall a better control of viral replication was observed.

CONCLUSION

Type III IFNs were identified over 17 years ago and are almost reaching majority. While work in the infant years of IFNλs mostly listed the similarities between the type I and III IFN systems, more recent work has revealed important differences. Type III IFNs have specific functions on epithelial and immune cells that make them key actors in immunity and regulators of inflammation at mucosal sites. As IFNλs continue to attract more interest from infection biologists and immunologists, many more important discoveries can be expected about this fascinating family of cytokines.

AUTHOR CONTRIBUTIONS

CO and IZ wrote the manuscript.

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Type I and Type III Interferons Display Different Dependency on Mitogen-Activated Protein Kinases to Mount an Antiviral State in the Human Gut

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Intestinal epithelial cells (IECs) are constantly exposed to commensal flora and pathogen challenges. How IECs regulate their innate immune response to maintain gut homeostasis remains unclear. Interferons (IFNs) are cytokines produced during infections. While type I IFN receptors are ubiquitously expressed, type III IFN receptors are expressed only on epithelial cells. This epithelium specificity strongly suggests exclusive functions at epithelial surfaces, but the relative roles of type I and III IFNs in the establishment of an antiviral innate immune response in human IECs are not clearly defined. Here, we used mini-gut organoids to define the functions of types I and III IFNs to protect the human gut against viral infection. We show that primary non-transformed human IECs, upon viral challenge, upregulate the expression of both type I and type III IFNs at the transcriptional level but only secrete type III IFN in the supernatant. However, human IECs respond to both type I and type III IFNs by producing IFN-stimulated genes that in turn induce an antiviral state. Using genetic ablation of either type I or type III IFN receptors, we show that either IFN can independently restrict virus infection in human IECs. Importantly, we report, for the first time, differences in the mechanisms by which each IFN establishes the antiviral state. Contrary to type I IFN, the antiviral activity induced by type III IFN is strongly dependent on the mitogen-activated protein kinases signaling pathway, suggesting a pathway used by type III IFNs that non-redundantly contributes to the antiviral state. In conclusion, we demonstrate that human intestinal epithelial cells specifically regulate their innate immune response favoring type III IFN-mediated signaling, which allows for efficient protection against pathogens without producing excessive inflammation. Our results strongly suggest that type III IFN constitutes the frontline of antiviral response in the human gut. We propose that mucosal surfaces, particularly the gastrointestinal tract, have evolved to favor type III IFN-mediated response to pathogen infections as it allows for spatial segregation of signaling and moderate production of inflammatory signals which we propose are key to maintain gut homeostasis.

Keywords: interferon-lambda, interferon-β, intestinal epithelial cells, mitogen-activated protein kinases, human gut microbiota, antiviral immunity, mucosal immunity

INTRODUCTION

Intestinal epithelial cells (IECs), lining the surface of the intestine, assemble as a continuous monolayer of tightly juxtaposed cells. Their primary functions are to permit nutrient absorption and to balance electrolytes and water levels. They also act as a barrier separating the interior from the exterior milieu that enteric pathogens have to face to establish a productive infection. The lumen of the intestine is in constant contact with the "ever-present" microbiota and their various pro-inflammatory associated products (e.g., LPS). Surprisingly, this microbial load does not elicit constant inflammation in the intestine under physiological conditions. Several mechanisms have been reported to participate in the tolerance of the commensal flora. Evidence suggests that IECs generate an innate immune response in the gut that is specifically and uniquely tailored with a perfect responsive balance to flare up and control pathogens in the lumen of the gut without causing excessive local inflammation (1, 2).

Interferons (IFNs) are a class of cytokines that are often produced and secreted upon infection, in particular by viruses. IFNs bind to the infected and uninfected bystander cells to induce JAK/STAT-dependent signaling cascades that lead to the production of IFN-stimulated genes (ISGs). ISGs alert cells against the presence of pathogens conferring them an antiviral state. There are three classes of IFNs: type I, II, and III. While type II IFNs are mostly specific to immune cells, type I and III IFNs are expressed by both immune cells and epithelium cells making them very relevant for viral infection of epithelium surfaces. Type I IFNs are composed of IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω in humans. All type I IFNs bind to the interferon alpha receptor (IFNAR) complex, which is composed of a heterodimer of IFNAR1 and IFNAR2 (2–4). There are four subtypes of type III IFNs: IFN-λ1–3 (also called IL28a, IL28b, IL29) (5, 6) and IFN-λ4 (7). Type III IFNs bind to a heterodimeric receptor complex composed of the interferon-lambda receptor (IFNLR1) and the interleukin-10 receptor beta (6, 8, 9). Type I and III IFNs, as well as IFNAR1 and IFNAR2, are expressed by most cells. By contrast, the type III IFN-specific receptor IFNLR1 is expressed mainly on epithelial cells (i.e., respiratory tract, intestinal tract, and hepatocytes) (9–12). The functions of type I (β) and III (λ1–3) IFNs has been intensely studied in murine systems (13). However, to date, there are only few reports describing how and whether these two IFNs act differently in human organs.

The current view is that both type I and III IFNs are redundant by inducing very similar signaling pathways that lead to the expression of a comparable set of ISGs (14). This model is supported by work in the lower respiratory tract during influenza A virus (IAV) infection where multiple lines of evidence suggest that both type I and III IFNs participate in the protection against IAV (12, 15–18). On the contrary, studies focusing on the mouse gastrointestinal tract have shown an age-restricted dependence on IFNs. Neonatal mice have epithelium cells that respond to both type I and III IFNs (19), but adult mice are insensitive to type I IFNs. In adult animals, type III IFN controls local viral infection of the epithelial layer, while type I IFN controls systemic viral spread (20–23). Similarly, human hepatocytes become refractive to type I IFN treatment but never lose their ability to respond to type III IFNs (24). These examples of differential regulation of type I and type III IFNs signaling and the epithelium specificity of type III IFN-mediated immunity strongly suggest major functional and regulatory differences between the IFNs at mucosal surfaces.

Here, we use human mini-gut organoids and human IEC lines to study the relative roles of type I and type III IFNs in protecting the human gut against viral infection. We show that primary non-transformed human IECs respond to both type I and type III IFNs by producing ISGs. Using genetic ablation of either type I or type III IFN receptors, we show that either IFN can independently restrict virus infection in human IECs. However, contrary to type I IFN, the antiviral activity induced by type III IFN is strongly dependent on the mitogen-activated protein kinases (MAPKs) signaling pathway, suggesting a pathway used by type III IFNs that non-redundantly contributes to the antiviral state.

RESULTS

Type III IFN Is Produced during Viral Infection of Human Mini-Gut Organoids

The roles of type I and III IFNs at mucosal surfaces and in epithelial cells have been extensively studied in mice (12, 15, 16, 25, 26). Whether and how these two IFN types have antiviral activity in human epithelial cells remains much less characterized, particularly in human intestinal epithelial cells (hIECs). To investigate the functions of both IFN types in the context of primary untransformed human cells, we used human colon and intestinal mini-gut organoids. This *ex vivo* human model for the gut fully reproduces the structural architecture of the human intestinal tract and contains all major intestinal cell lineages (27, 28). Mini-gut organoids were formed by isolating intestinal crypts containing stem cells from human gut (colon or small intestine) resections from multiple donors. Single crypts were grown in Matrigel and 24 h post-isolation, opened crypts started to re-seal with the evident formation of a lumen within these organoids at 3–5 days of culture. At 7–10 days, the organoids were significantly increased in size (**Figure 1A**). After differentiation, human colon organoids displayed the typical organization with a clear and developed lumen, localization of E-cadherin at the basolateral side of the cells, tight junctions located at the apical side, as well as presence of mucin-secreting goblet cells (Mucin-2) and enteroendocrine cells (Syn) (**Figure 1B**). To address whether type I and/or type III IFNs protect the human gut against viral infection we used mammalian reovirus (MRV). MRV is a well-known virus model that induces immune response in infected cells and is sensitive to type I and III IFNs (29). In the following, we use the terms type I and type III IFNs to describe IFN β1 and IFN λ1–3, respectively. Colon organoids were infected with MRV with a multiplicity of infection (MOI) of 0.5, harvested 16 h post-infection (hpi), and viral replication was assessed by immunostaining of the reovirus non-structural protein μNS and by quantification of viral replication using quantitative real-time (qRT)-PCR. As shown in **Figure 1C**, MRV efficiently infects human mini-gut organoids as evidenced by the presence of MRV-infected cells (**Figure 1C**, left panels) and potently replicates

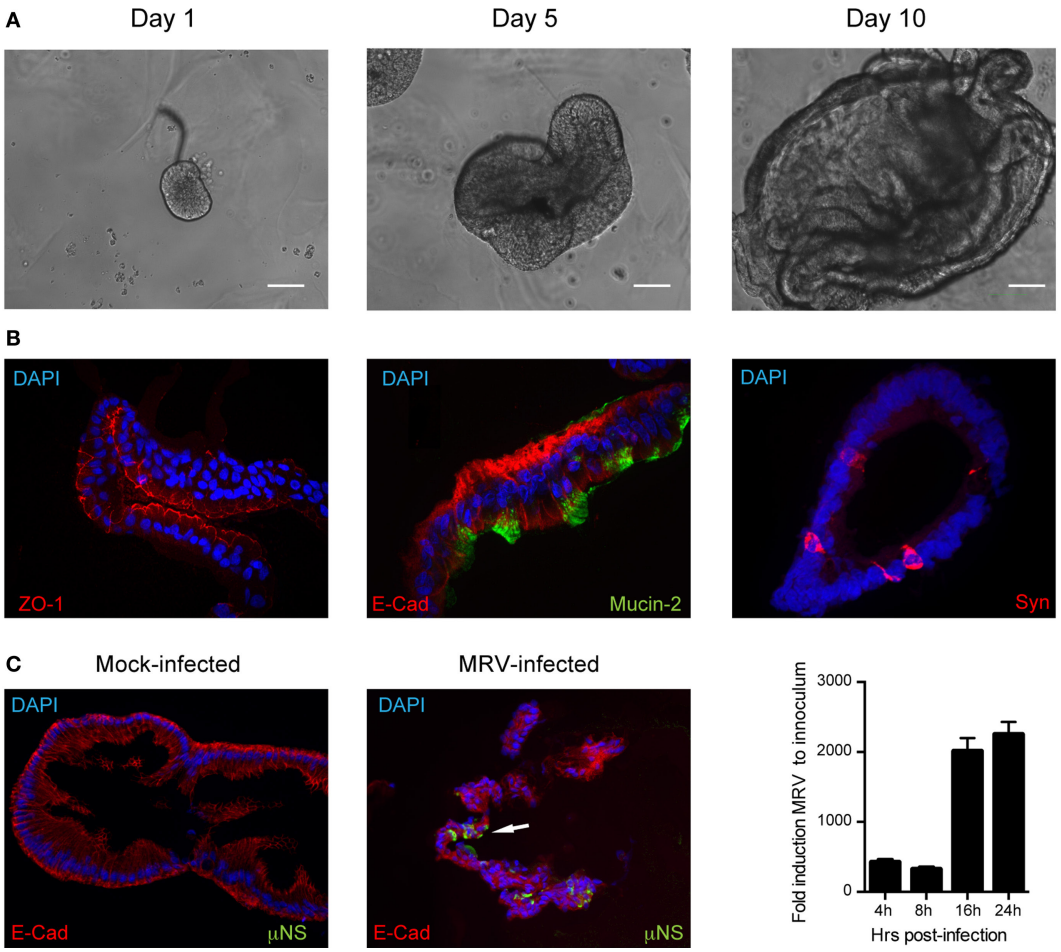

FIGURE 1 | Infection of human mini-gut organoids with mammalian reovirus (MRV). **(A)** Human colon organoids were prepared according to methods. Representative images of human colon organoids grown over 10 days from intestinal crypts. **(B)** Five days post-differentiation, organoids were mock or MRV infected (multiplicity of infection = 0.5). 16 hpi, organoids were fixed, cryosectioned and immunostained for adherent junctions E-cadherin (E-cad), tight junctions (ZO-1), Goblet cells (Mucin-2), and Enteroendocrine cells (synaptophysin, Syn). **(C)** MRV-infected cells were detected using an antibody against the MRV non-structural protein μNS. Representative images are shown. White arrow indicates infected cells. Organoids were infected with MRV and virus replication was monitored by qRT-PCR over a timecourse of 24 h. Data represent the mean values of three independent experiments. Error bars indicate the SD.

over the course of infection (**Figure 1C**, right panel). Of note, MRV infection severely disrupted the structural integrity of the human mini-gut organoids (**Figure 1C**, also confirmed later in **Figure 3B**), which were mostly fragmented pieces of cellular monolayers, compared to the intact structures observed in mock-infected organoids. To characterize the innate immune response generated by organoids, we monitored the upregulation of both type I (IFN-β) and III (IFN-λ2–3) IFNs and of two representative ISGs (Viperin and IFIT1) over the course of MRV infection. We found that viral infection of organoids induces the transcriptional upregulation of type III IFN and to a lesser extent type I IFN (**Figure 2A**). This was congruent with the detection of only type III IFN in the supernatant of infected organoids (**Figure 2B**). Additionally, viral infection of organoids was associated with the upregulation of ISGs. The transcriptional upregulation of two representative ISGs (Viperin and IFIT1) over the course of MRV infection is shown in **Figure 2C**.

Type I and III IFNs Protect Human Mini-Gut Organoids against Viral Infection

We found that viral infection of organoids induces the upregulation of both type I and III IFNs. To address whether both IFNs can in turn induce the expression of ISGs, mini-gut organoids were stimulated with a broad range of IFN concentrations. Results revealed that both type I and III IFNs induce the upregulation of ISGs in a dose-dependent manner (**Figure 3A**). Interestingly, type I IFN appears to be more potent as it induces higher expression of the Viperin and IFIT1 ISGs compared to type III IFN (**Figure 3A**). Similar results were found with multiple ISGs (Mx-1, ISG15, ISG54, data not shown). We also observed a continuous increase in ISG mRNA levels as the concentration of type I IFN increased, whereas ISG transcript levels quickly reached a plateau in type III IFN-treated cells.

To evaluate whether type I and/or III IFN protect primary human IECs from viral infection, organoid cultures were treated

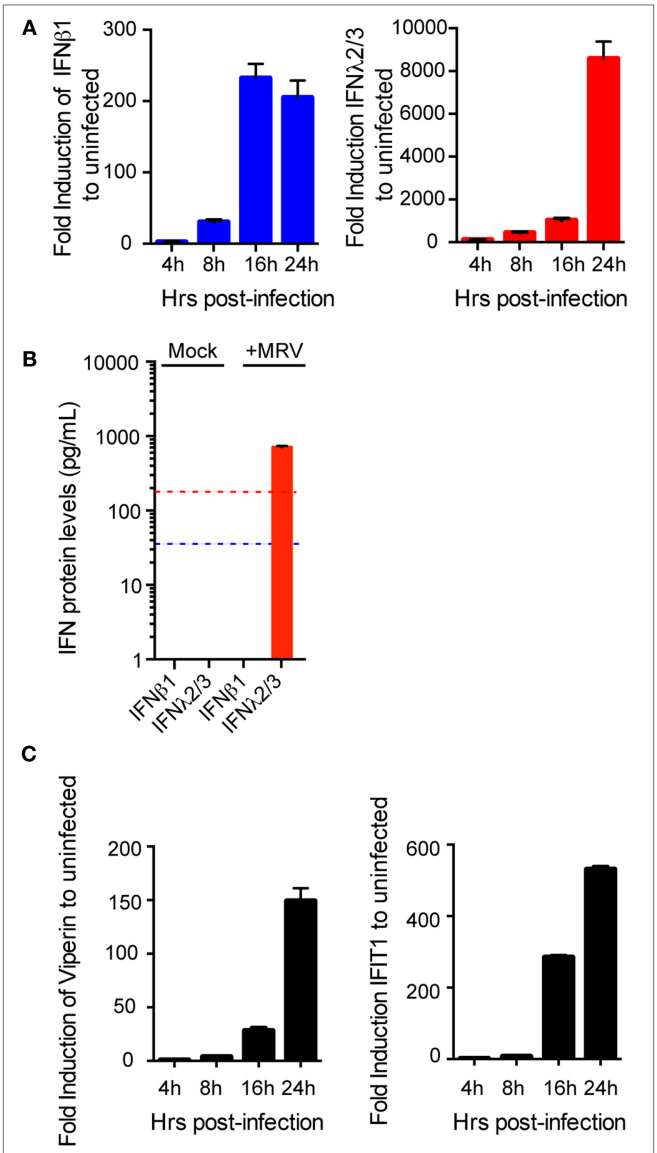

FIGURE 2 | Induction of immune response in human mini-gut organoids after mammalian reovirus (MRV) infection. Organoids were infected with MRV (multiplicity of infection = 0.5), quantitative real-time (qRT)-PCR and ELISA were used to detect **(A)** a time course of transcriptional upregulation of both type I interferons (IFNs) (β) and type III IFN (λ2/3) IFNs **(B)** 24 hpi the production and secretion of IFN proteins in the supernatant of infected organoids and **(C)** a time course of transcriptional upregulation of the IFN-stimulated genes Viperin and IFIT1. qRT-PCR data were normalized to TBP and HPRT1 (housekeeping genes) and are expressed relative to uninfected organoids at each time point. qRT-PCR data and ELISA data represent the mean values of three independent experiments. Error bars indicate the SD. The blue and red lines in **(B)** demarcate the limit of detection of our ELISA for type I and type III IFNs, respectively.

with 8 ng/mL of type I IFN (IFN-β) (equivalent 2,000 RU/mL, see Materials and Methods) or 300 ng/mL of type III IFN (IFN-λ1−3) prior to exposure to MRV. Organoids were harvested 16 hpi for analysis of viral infection/replication by immunostaining and immunoblotting against the reovirus non-structural protein μNS as well as by quantification of viral replication using qRT-PCR. Compared to mock-treated cells, pre-treatment of colon organoids with either IFN significantly reduced both the number of MRV-infected cells (**Figures 3B,C**, immunostaining and quantification) and the viral antigen levels within these organoids (**Figure 3D**). Complementarily, viral replication was severely impaired when organoids were treated with either IFNs as assayed by qRT-PCR (**Figure 3E**). To ensure that these findings were neither donor nor colon specific, colon organoids from different donors (colon D2–D3) and organoids derived from ileum or jejunum were similarly pre-treated with type I or III IFNs and infected with MRV. Reduced viral infection characterized by the lower expression levels of the MRV μNS protein and the decreased MRV replication was observed in colon organoids generated from different donors (Figure S1A in Supplementary Material) and in ileum (Figure S1B in Supplementary Material) and jejunum (Figure S1C in Supplementary Material) derived organoids. Similar results were found using vesicular stomatis virus (VSV), an unrelated model virus whose replication is also sensitive to both type I and III IFNs (29, 30). Pre-treatment of human organoids with either IFNs resulted in a significant inhibition of VSV replication as measured by the significant decrease of bioluminescence when using VSV-expressing luciferase (VSV-luc) as a reporter of viral replication (Figure S2 in Supplementary Material). All together, these results demonstrate the antiviral protective role of both type I and III IFNs in colon, ileum, and jejunum derived hIECs.

Human IEC Lines Express Type I and III IFNs upon MRV Infection

Human mini-gut organoids are very difficult to modify genetically. Therefore, in order to better characterize the functions and the mechanisms by which type I and III IFNs confer hIECs an antiviral state, we used the human colon carcinoma-derived cell line T84. T84 cells were infected with MRV and harvested at different time points post-infection to evaluate the transcriptional upregulation of both type I and III IFNs. Viral infection of T84 cells induces the upregulation of type I and type III IFNs (**Figure 4A**). Similar to human mini-gut organoids (**Figure 2**), viral infection induces a higher transcriptional upregulation of type III IFNs compared to type I IFN (**Figure 4A**; Figure S3A in Supplementary Material). To address whether both IFNs were made at the protein level and secreted by infected T84 cells, we measure the amount of both IFNs in the supernatant of infected T84 cells using ELISA. As observed for viral infection of mini-gut organoids (**Figure 2B**), only type III IFN was found in the supernatant (**Figure 4B**). However, type I IFN can be detected in the supernatant if added exogenously to inhibit viral infection (data not shown).

To address whether T84 IECs respond to either type I and III IFNs, we treated T84 IECs with type I or III IFN and measured the expression levels of ISGs at different time points post-IFN treatment. Like human mini-gut organoids (**Figure 3A**), we found that type I IFN induces higher expression of the ISGs Viperin and IFIT1 compared to type III IFN (Figure S3B in Supplementary Material). Transcriptome analysis of T84 cells treated with either type I or III IFN revealed that type I IFN consistently induced

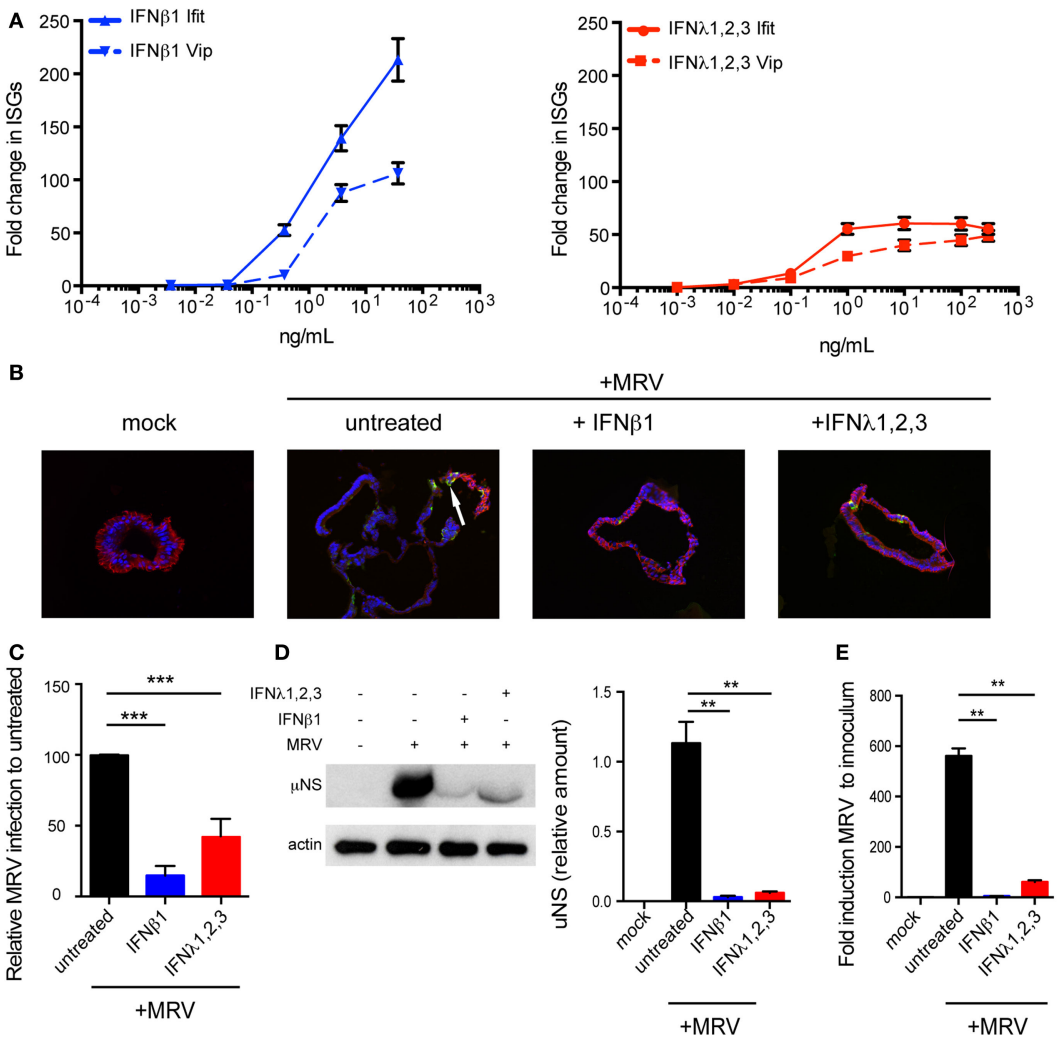

FIGURE 3 | **Both type I and type III interferons (IFNs) confer human mini-gut organoids protection against viral infection**. **(A)** Colon organoids were treated with increasing concentrations of type I IFN (β) and type III IFN (λ1–3) IFN. Six hours posttreatment, organoids were harvested and the transcriptional upregulation of the IFN-stimulated genes Viperin (Vip) and Ifit1 was measured using qRT-PCR. Data were normalized to TBP and HPRT1. **(B–E)** Colon organoids were treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for 2.5 h prior to infection with mammalian reovirus (MRV) (multiplicity of infection = 0.5) for 16 h. **(B)** MRV-infected organoids were analyzed by μNS-specific immunofluorescence (green). The cells were stained against E-cadherin (red) and the nuclei were stained with Dapi (blue). Representative data from triplicate experiments are shown. White arrow indicates infected cells. **(C)** The fluorescence intensity of MRV μNS per organoid was measured and expressed relative to untreated organoids (set as 100). **(D)** MRV-infected organoids were analyzed for μNS production by Western blot. Actin was used as loading control. Production of μNS was quantified by densitometer. **(E)** The protective effect of type I or type III IFN was assayed by monitoring viral replication by qRT-PCR normalized to inoculum. Data represent the mean values of three independent experiments. Error bars indicate the SD. $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired *t*-test).

higher transcript levels across all induced ISGs (Figure S3C in Supplementary Material).

To determine the antiviral potency of type I and III IFNs in T84 cells, we pre-treated T84 cells with increasing concentrations of each IFN prior infection with MRV. *De novo* production of viral proteins was monitored by blotting for the MRV non-structural protein μNS. **Figure 5A** shows that both type I and III IFNs inhibit viral infection in a dose-dependent manner. In addition, cells were fixed 16 hpi and the fraction of μNS-expressing cells was determined by immunofluorescence, which demonstrates that either type I or III IFN decreased both the number of MRV-infected cells and the level of viral antigen per cell (**Figure 5B**). To confirm that this observation was not virus-specific, T84 cells were treated with increasing concentrations of either type I or III IFNs and subsequently infected with VSV-luc as a reporter of viral replication. Measurement of viral infection by bioluminescence showed that similar to MRV, either type I or III IFNs are capable of inhibiting VSV infection in hIECs in a dose-dependent manner (**Figure 5C**). All together these results show that either type I or III IFNs confer T84 cells lines an antiviral state and that T84 cells phenocopy the antiviral response generated by primary hIECs in the context of mini-gut organoid.

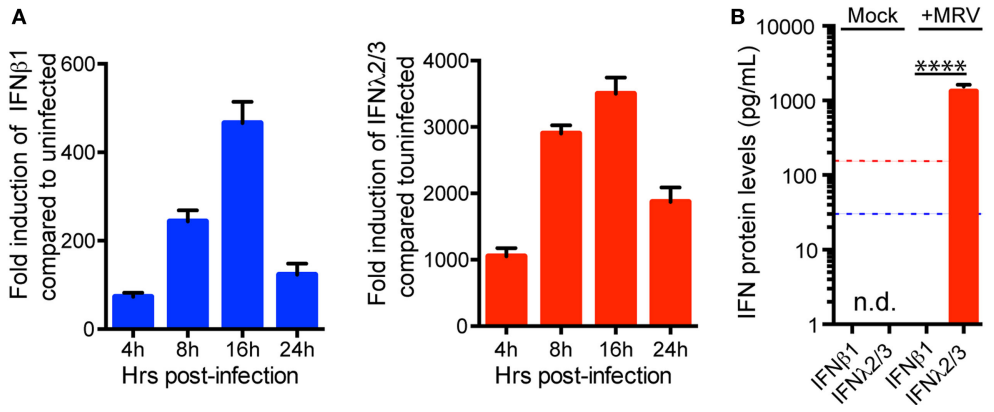

FIGURE 4 | Expression pattern of interferon (IFN) mRNA and protein in human intestinal epithelial cells upon viral infection (A). Relative quantification of type I IFN (β) and type III IFN (λ2/3) transcripts during the course of mammalian reovirus (MRV) (multiplicity of infection = 1) infection of T84 cells. Data are normalized to TBP and HPRT1 and are expressed relative to uninfected cells at each time point. **(B)** Quantification of type (IFNβ) and type III (IFN λ2/3) protein levels by ELISA in supernatants of uninfected or MRV-infected T84 cells. The blue and red dashed lines demarcate the limit of detection of our ELISA for type I and type III IFNs, respectively. n.d., not detectable. Data represent the mean values of three independent experiments. Error bars indicate the SD. ****$P < 0.0001$ (unpaired *t*-test).

Type I and III IFN Signaling Pathways Independently Mediate an Antiviral State in Human IECs

To test whether types I and III IFNs act in combination or separately in establishing the antiviral state of IECs, we generated T84 cell lines deficient for either the IFN alpha (IFNAR) or the IFN lambda (IFNLR) receptor using CRISPR/Cas9 technology. Inactivation of the IFN receptors was confirmed by sequencing of the knockout (KO) cell lines, which revealed nucleotide deletions and changes of open reading frame in IFNAR−/− and IFNLR−/− genes (data not shown). As shown in **Figures 6A,B**, IFNAR−/− cells were no longer able to phosphorylate pSTAT1 and induce ISGs after type I IFN treatment, but remained fully responsive to type III IFN, indicating a selective disruption of the type I IFN signaling pathway. Conversely, IFNLR−/− cells were insensitive to type III IFN but responded to type I IFN. These results were consistent across multiple IFNAR−/− and IFNLR−/− cell clones (Figures S4A,B in Supplementary Material).

To evaluate whether deletion of IFNAR or IFNLR renders human IECs more susceptible to viral infection, cells lacking functional receptors for type I or III IFN were infected by either MRV or VSV and compared to wild-type or scrambled guide RNA-exposed cells. Immunofluorescence analysis revealed that loss of IFNAR slightly increased the number of MRV-infected cells compared to control cells (**Figure 6C**), but did not affect the average fluorescent intensity of MRV antigen per infected cell (**Figure 6D**). Interestingly, IFNLR−/− cells appeared to be more susceptible to VSV infection. The number of VSV-infected cells and the amount of viral antigens in each cells were significantly increased in IFNLR−/− cells compared to control cells (**Figures 6E,F**). To confirm the protective role of type I and III IFN against viral infection in human IECs, IFNAR−/− and IFNLR−/− cells were pre-treated with either type I or III IFNs and subsequently infected with MRV or VSV. Type I or III IFN could efficiently inhibit infection by both MRV and VSV in control cells (**Figures 6G,H**). As expected, the protective effect of type I IFN against MRV (**Figure 6G**, left panel) and VSV (**Figure 6H**) was no longer observed in IFNAR1−/− cells, but was preserved in IFNLR1−/− cells. Conversely, disruption of IFNLR1−/− specifically abolished the protective effect of type III IFN, but not of type I IFN. Similar results were obtained with several KO clones (Figure S4C in Supplementary Material). All together these data demonstrate that in human T84 cells, either type I or III IFNs are capable of independently mediating antiviral protection.

MAP Kinases Are Required for Type III but Not Type I IFN Antiviral Activity in hIECs

Type I and III IFN signaling and antiviral activity are dependent on the JAK/STAT pathway, and inhibition of STAT1 phosphorylation blocks the production of ISGs and inhibits IFN-mediated antiviral protection (31–33). Several MAPKs have also been reported to be activated (34) and contribute to ISG upregulation in type I or III IFN-stimulated cells (35, 36), but the role of the MAPK pathways in the antiviral functions of type III IFN remains unclear. We found that both type I or III IFN treatment induced the phosphorylation of STAT1, STAT2, and STAT3 with similar kinetics in T84 cells (data not shown). Type I or III IFN treatment did not induce STAT5A, STAT5B, or STAT6 phosphorylation (data not shown). We next addressed whether type I and III IFNs activate the MAPKs. We found that both IFNs induce the phosphorylation of the MAPKs, p38, ERK, and JNK to the same extent and with similar kinetics (Figure S5A in Supplementary Material). To determine the role of the STAT and MAPK pathways in the antiviral activity of IFNs, we used specific pharmacological inhibitors in combination with IFN treatment. The specificity of these inhibitors and their toxicity were tested in T84 cells by Western blot analysis (Figure S6 in Supplementary Material) and cell viability assay (Figure S5B in Supplementary Material). Inhibiting the JAK/STAT pathway with a pan-JAK inhibitor almost fully blocked phosphorylation of STAT1 (Figure

S6 in Supplementary Material) and strongly impaired the antiviral activity of either type I or III IFNs on both VSV and MRV (**Figure 7A**). Interestingly, inhibition of the MAPKs with specific inhibitors, had no effect on the phosphorylation kinetics of STAT1 (Figure S6 in Supplementary Material) but strongly affected the antiviral protection conferred by type III IFN only

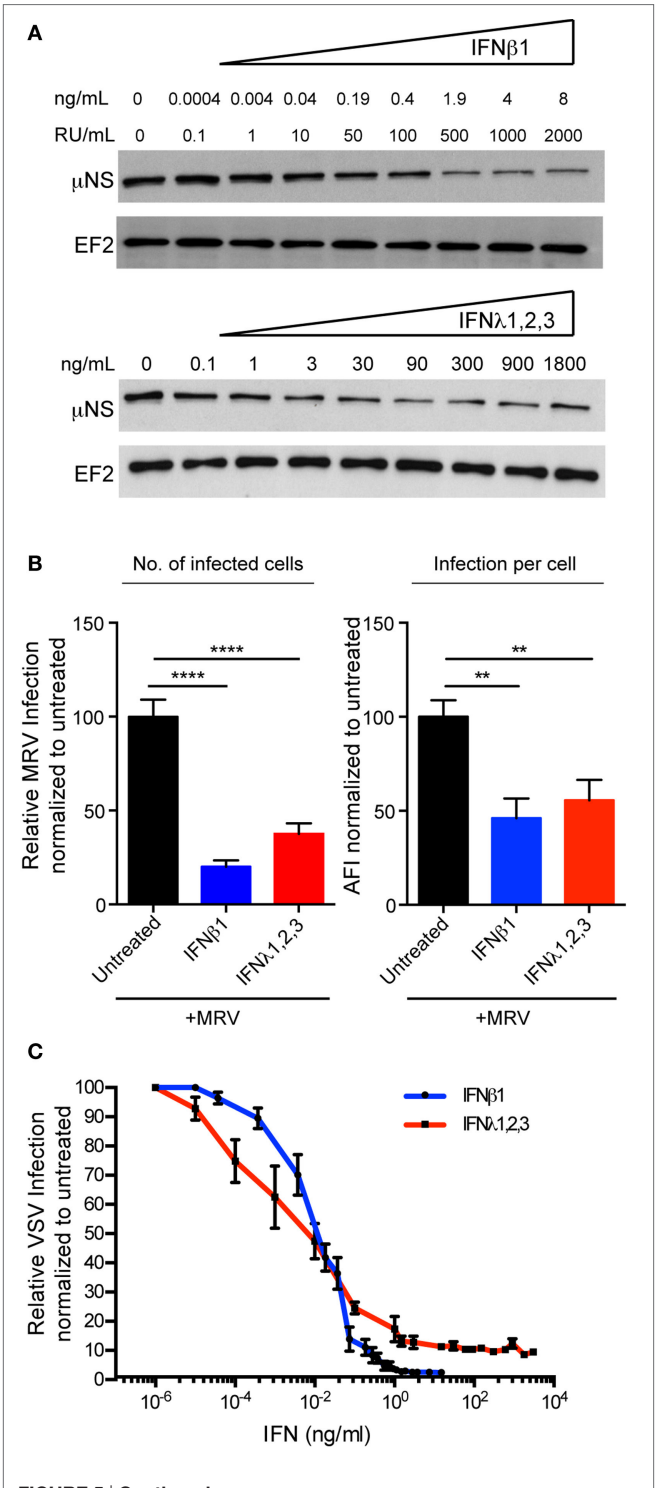

FIGURE 5 | Continued

FIGURE 5 | Continued
Both type I and type III interferons (IFNs) mediate antiviral protection in human T84 cells. (A) T84 cells were pre-treated for 2.5 h with the indicated concentrations of type I IFN (β) and type III IFN (λ1–3) IFNs and then subsequently infected with mammalian reovirus (MRV) [multiplicity of infection (MOI) = 1]. Sixteen hours post-infection, the protective effect of type I or III IFN was assayed by immunoblotting for the viral non-structural protein μNS. EF-2 is used as a loading control. A representative immunoblot out of three independent experiments is shown. **(B)** T84 cells were treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for 2.5 h prior to infection with MRV for 16 h. MRV-infected cells were analyzed by μNS-specific immunofluorescence. (Left panel) The number of infected cells was quantified and is expressed relative to untreated cells (set to 100). (Right panel) MRV uNS staining intensity was measured to obtain the average fluorescent intensity per cell and is expressed relative to untreated cells (set to 100). Data represent the mean values of three independent experiments. **(C)** T84 cells were pre-treated with the indicated concentrations of type I or III IFNs for 2 h prior to infection with vesicular stomatis virus (VSV) expressing Firefly luciferase VSV expressing luciferase (MOI = 1). Viral replication was assayed by measuring the luciferase activity. For each sample luciferase activity was measured in triplicates and is expressed as the percentage of the activity present in VSV-infected cells without IFN treatment (set to 100). The mean value obtained from three independent experiments is plotted. Error bars indicate the SD. $^{**}P < 0.005$, $^{****}P < 0.0001$ (unpaired *t*-test).

(**Figure 7A**). This specific inhibition is seen across a range of concentrations (**Figure 7B**). Of note, a partial inhibition of the antiviral activity of type I IFN was observed at high concentration of JNK inhibitor (**Figure 7B**) but at this concentration cell viability was severely affected (Figure S5B in Supplementary Material). This type III IFN restricted dependence on MAPKs was independent of IFN concentration (Figure S5C in Supplementary Material), validating that the non-dependence of type I IFN for MAPKs was not the result of differences in the IFN concentration used to stimulate the cells. Altogether, these results demonstrate the fundamental role of STAT-dependent signaling in conferring both type I and type III IFNs antiviral activity, and in addition demonstrate a unique role for MAPKs toward inducing the antiviral state induced by type III IFN but not type I IFN.

Although it has been shown in multiple cell lines that IFNs can induce the activation of MAPKs (34–36), the importance of these kinases in the IFN-mediated antiviral state has never been reported to our knowledge. This suggests that dependency on MAPKs might be cell type specific. To ensure that the antiviral activity of type III IFN in primary non-transformed hIECs depends on MAPKs, we used our mini-gut organoid culture system. Colon organoids were treated with pharmacological inhibitors of the JAK/STAT or MAPKs signaling pathways. Following pre-treatment with type I or type III IFNs, organoids were infected with VSV. Eight hpi, organoids were harvested and the impact of the pharmacological inhibitors on the antiviral activities of both IFNs was measured. As expected, inhibition of the JAK/STAT signaling pathway fully restores VSV infection to a level similar to infected organoids in the absence of IFNs (**Figure 8**). This confirms that the JAK/STAT signaling pathways is key for both type I and type III IFN activity in primary hIECs. Interestingly and similar to T84 cells, inhibition of either p38 or JNK MAPKs partially impairs only the antiviral activity of type III IFNs in human mini-gut organoids (**Figure 8**). No significant effect of MAPK inhibition

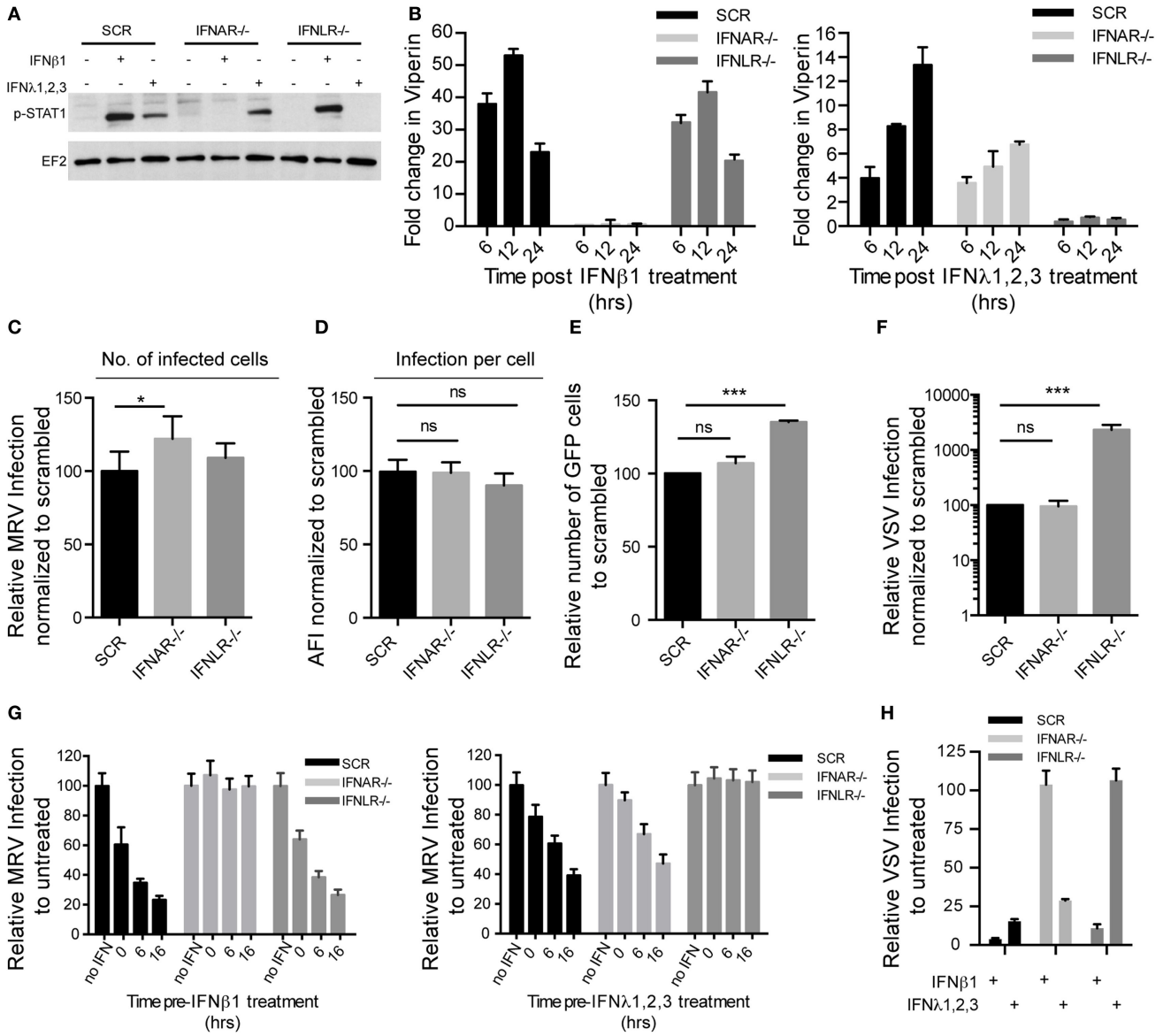

FIGURE 6 | Type I and type III interferons (IFNs) independently confer intestinal epithelial cells antiviral protection. T84 IFNAR1 and IFNLR1 knockout cell lines were generated using the CRISPR/Cas9 system. **(A)** T84 cell lines were treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for 1 h and IFN signaling was measured by immunoblotting for pSTAT1 Y701. EF-2 is used as a loading control. A representative immunoblot out of three independent experiments is shown. **(B)** Same as **(A)**, except that induction of IFN-stimulated genes was monitored by relative qRT-PCR quantification of Viperin at indicated times post-IFN treatment. Data were normalized to TBP and HPRT1 and are expressed relative to untreated cells of each time point. **(C,D)** T84 cell lines were infected with mammalian reovirus (MRV) for 16 h (multiplicity of infection (MOI) = 1) and MRV-infected cells were analyzed by μNS-specific immunofluorescence. **(C)** The number of infected cells is expressed relative to scramble control cells (set to 100). **(D)** MRV μNS staining intensity was measured to obtain the average fluorescence intensity per cell and expressed relative to scramble control cells (set to 100). **(E)** T84 cell lines were infected with vesicular stomatis virus (VSV)-GFP (MOI = 1) for 8 h and the number of VSV-infected cells were analyzed by FACS. The percentage of infected cells is expressed relative to scramble control cells (set to 100). **(F)** Same as **(E)**, except that T84 cell lines were infected with VSV expressing luciferase (VSV-luc) (MOI = 1) and viral replication was assayed by measuring the luciferase activity. For each cell line luciferase activity was measured in triplicates and is expressed relative to scramble control cells (set to 100). **(G)** Same as **(C)**, except that T84 cell lines were treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) at indicated time points prior to infection with MRV. **(H)** Same as **(F)**, except that T84 cell lines were treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for 2 h prior to infection with VSV-luc. Data **(B–H)** represent the mean values of three independent experiments. Error bars indicate the SD. $^*P < 0.05$, $^{***}P < 0.001$, ns, not significant (unpaired *t*-test).

on type I IFN-mediated antiviral activity was observed. The effect of inhibiting ERK-dependent signaling on the antiviral activity of both IFNs was not determined (n.d.) since treatment of mini-gut organoids with ERK inhibitor induced disruption and death of the organoid culture (**Figure 8** and data not shown). Altogether, these results confirm that MAPK signaling pathways participate in the establishment of the antiviral state mediated by type III IFN in primary non-transformed hIECs.

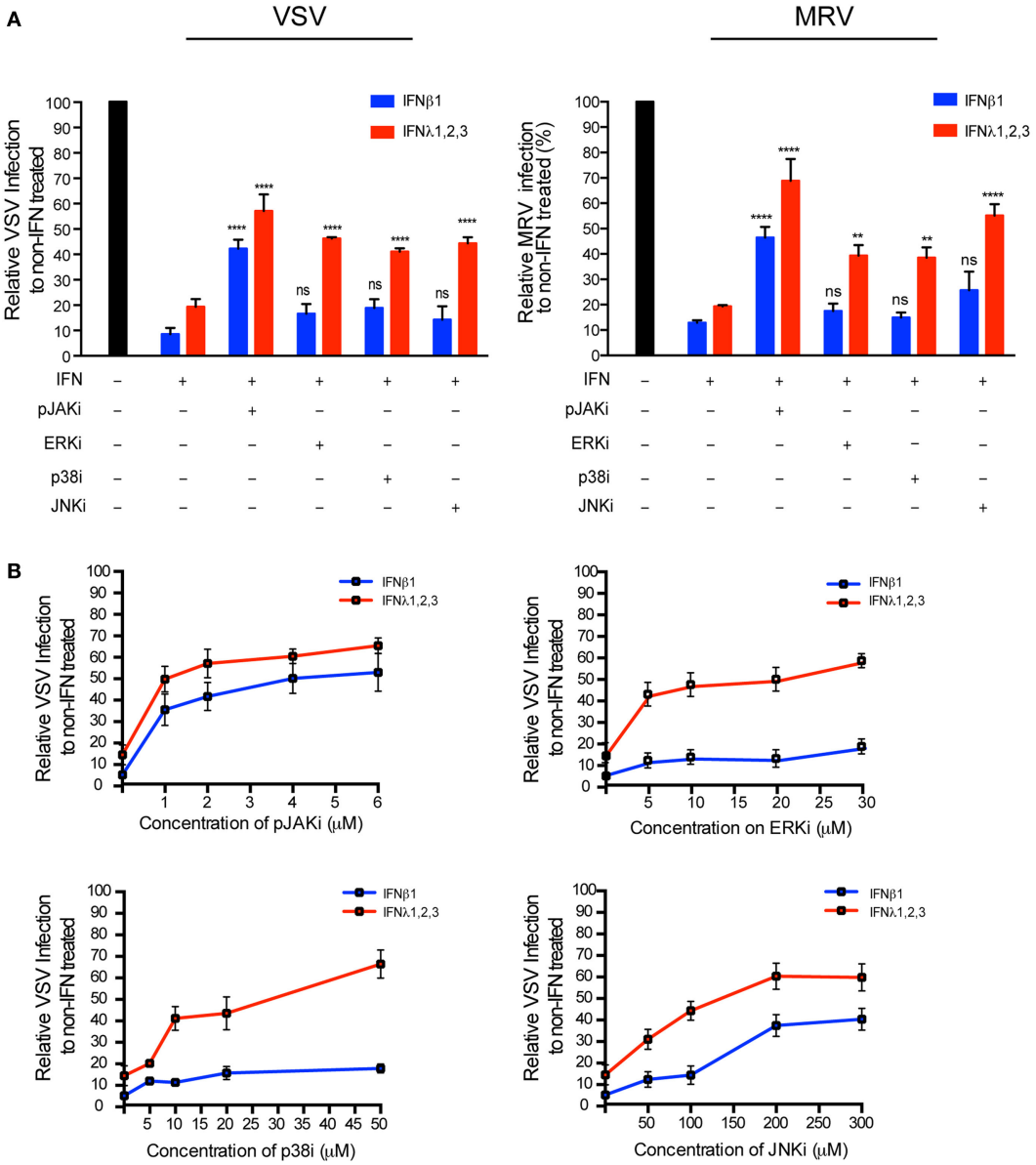

FIGURE 7 | Type III interferons (IFNs) require mitogen-activated protein kinases for their antiviral response. (A) T84 cells were mock incubated (black bar) or pre-incubated for 30 min with 2 μM Pyridone 6 (pan-JAK inhibitor), 10 μM U0126 (ERK inhibitor), 10 μM SB202190 (p38 inhibitor), or 100 μM SP600125 (JNK inhibitor). Then, T84 cells were mock treated (black bar) or treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) in the presence the inhibitor. Two hours post-IFN treatment cells were infected with a multiplicity of infection of 1 with VSV expressing luciferase (left panel) or mammalian reovirus (right panel). Viral replication was assayed by measuring the luciferase activity or by relative quantification of viral genome using qRT-PCR. Data were normalized to non-IFN-treated sample for each inhibitor (set to 100). **(B)** Same as **(A)**, except T84 cells were pre-incubated with increasing concentrations of JAK or MAP kinase inhibitors prior to treatment with IFNs. The mean value obtained from three independent experiments, is plotted. Error bars indicate the SD. $^{****}P < 0.0001$, $^{**}P < 0.005$, ns, not significant (unpaired *t*-test).

DISCUSSION

In recent years, there has been a large interest in uncovering the specific roles of type III IFNs in epithelial cells including lung epithelium, gastrointestinal tract epithelium and in hepatocytes. In this work, by exploiting human mini-gut organoids, we performed a functional characterization of both type I and III IFNs in a human primary intestinal cell context. We found that, upon viral infection, human IECs strongly upregulate both type I and III IFNs at the transcriptional level. Although only type III IFN was found to be secreted by IECs, we demonstrated that either type I or III IFNs induce the production of ISGs and that this production is associated with the establishment of an antiviral state that efficiently protects IECs from viral infection. Importantly, we revealed that type III IFN-mediated signaling allows for efficient protection against viral infection while limiting ISG production.

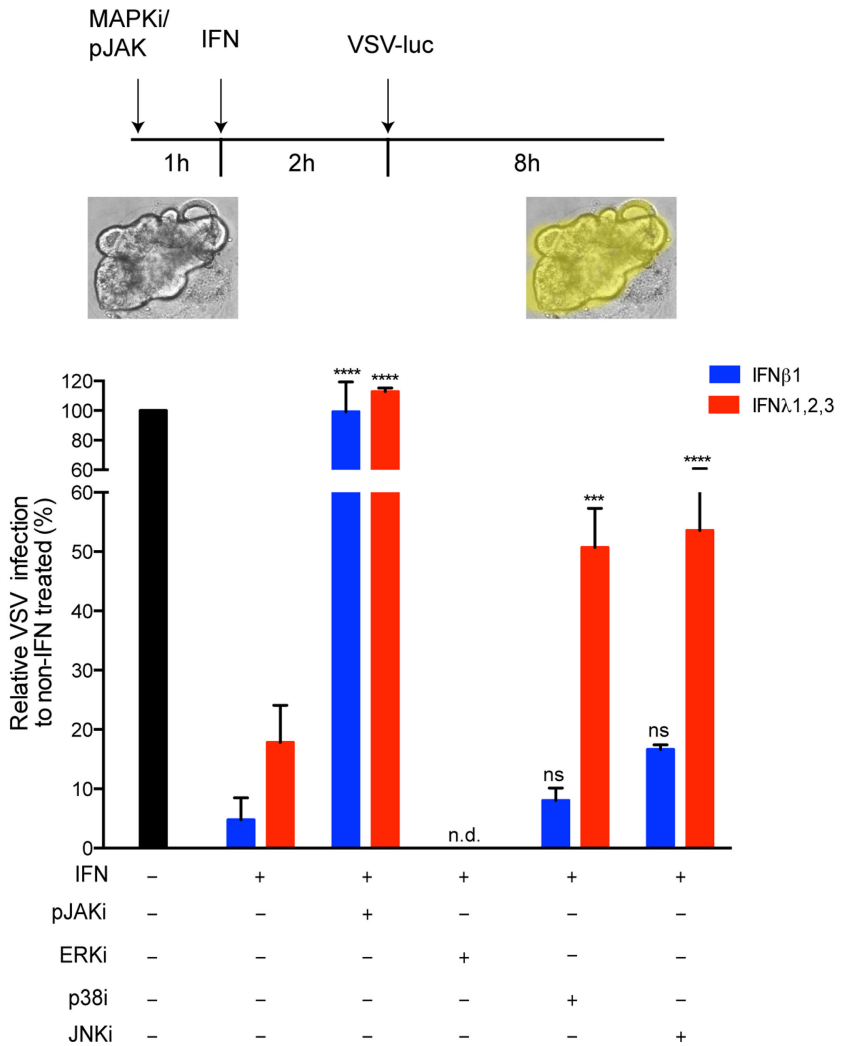

FIGURE 8 | The antiviral activity of type III interferons (IFNs) strongly dependent on mitogen-activated protein kinases in the contact of primary human intestinal epithelial cells. Human colon organoids were mock incubated (black bar) or pre-incubated with 2 μM Pyridone 6 (pan-JAK inhibitor), 10 μM U0126 (ERK inhibitor), 10 μM M SB202190 (p38 inhibitor) and 100 μM SP600125 (JNK inhibitor). One hour posttreatment, organoids were mock treated (black bar) or co-treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for 2 h. Organoids were then infected with VSV expressing luciferase (multiplicity of infection = 1). Eight hpi, viral replication was assayed by measuring the luciferase activity. Data are normalized to non-IFN-treated sample for each inhibitor (set to 100). The mean value obtained from three independent experiments is plotted. Error bars indicate the SD. $^{****}P < 0.0001$, $^{***}P < 0.001$, ns, not significant (unpaired *t*-test), n.d. (not determined).

We propose that this represents a mechanism to limit inflammation in the gut while remaining responsive to pathogens. Additionally, genetic ablation of IFN signaling using CRISPR/Cas9-mediated KO of IFN receptors further demonstrated that type I and III IFNs independently mediate an antiviral activity. Comparative analyses revealed that both IFNs induce the same set of ISGs and that both antiviral states depend on the JAK/STAT signaling pathway. Importantly, we discovered that the type III IFN-mediated, but not the type I IFN-mediated antiviral activity depends on MAPK signaling pathways. This work establishes that both type I and III IFNs provide potent antiviral protection in the human gut, and identifies, for the first time, fundamental differences in the mechanism by which these two IFN types establish the antiviral state in primary hIECs.

Since the implementation of organoid cultures, these systems have gained substantial and increasing interest in the fields of cellular biology and medicine (27, 28). More recently, these organoids have been also used to study and describe infectious diseases (37–41). In the present study, we have exploited organoids not only to describe the response of hIECs upon pathogen challenges but also to perform a functional characterization of both type I and III IFNs in the context of primary non-transformed hIECs.

In contrast to data in murine IECs (20), our results demonstrate that hIECs can mount an antiviral state in response to either type I or III IFN treatment. Similar observations have been made in lung epithelium, where both type I and type III IFNs participate in the protection against IAV (12, 15–18). Our functional characterizations performed in both mini-gut organoids and colon

carcinoma-derived cell lines revealed that although these cells transcriptionally upregulate both type I and III IFNs, they secrete very little to no type I IFN. A favored type III IFN response over type I IFN has been observed in other epithelial cells stimulated with various viruses and pathogen-associated molecular patterns (16, 30). It was shown that although both IFNs can protect airway epithelial cells against viral infection, type III IFNs were preferentially made in response to influenza infection (15). Similarly, it was shown that upon stimulation of IECs with the double strand RNA structural analog poly-inosinic:cytidylic acid (poly I:C), only type III IFN was secreted by the cells although both type I and III IFNs were upregulated at the transcriptional level (42). Additionally, it has been reported that human hepatocytes can become refractive to type I IFN, while maintaining their responsiveness to type III IFN (24). Consequently, favoring type III IFN signaling appears to be a common strategy developed at epithelial surfaces (airway, hepatocytes, intestinal tract) to mount an antiviral response.

It remains unclear whether translation or secretion of type I IFN is restricted in hIECs. To date, very little is known about the mechanisms that lead to type I and III IFN secretion. It has been shown that signaling downstream of mitochondrial-associated MAVS (mitochondrial antiviral-signaling protein) induces the secretion of type I IFN that can be inhibited by brefeldin A. On the contrary, the antiviral activity generated following activation of peroxisomal-associated MAVS was insensitive to brefeldin A (29). It was later demonstrated that this briefeldinA-insensitive antiviral state was mediated by type III IFN, which was secreted following activation of peroxisomal MAVS (30). These observations strongly suggest that type I and III IFNs are secreted from cells by two distinct mechanisms.

Although type III IFN stimulation of hIECs results in significantly less induction of ISGs compared to type I IFN (**Figure 3**; Figure S3 in Supplementary Material), we found that type III IFN was only slightly less potent in protecting the cell against viral infection (**Figures 3** and **5**). This lower induction of ISGs is not cell type specific as recent publications addressing the role of these IFNs in hepatocytes also reported that type III IFN induces less ISGs compared to type I IFN (43–45). However, in these studies, the antiviral potency of both IFNs was not addressed side-by-side. As such, type III IFN could be considered a milder IFN favored at epithelial surfaces (at least intestinal epithelium) due to its ability to confer an antiviral state without inducing excessive amounts of ISGs, which might result in the induction of local pro-inflammatory signals. The molecular mechanisms by which type III IFN signaling modulates ISG expression remain unknown. Desensitization is a possible mechanism through which IFN signaling is reduced following stimulation. Different negative regulators of IFN signaling might be used to regulate or turn off ISG expression. Signal transduction strongly depends on the amount of receptor and the affinity of the ligand for its receptor. It is known that type I IFN (IFNβ) has a very strong affinity for its receptor. Differences in the affinity of type I and III IFNs for their respective receptor or differences in the amount of type I and III IFN receptors at the surface of hIECs might be partially responsible for the observed differences in the magnitude of ISG expression.

Functional characterization of type III IFN and comparison to type I IFN suggests that both cytokines are functionally redundant by inducing the same set of ISGs (8, 35, 46). However, the restriction of type III IFN receptor to epithelium cells suggests that type III IFN might have unique functions or provide specific advantages at epithelial surfaces. Several studies have tried to characterize functional differences both in human (hepatocytes) (43, 44) and in murine model systems (lung and intestinal tract) (16, 19, 20, 26). To date, the main difference between both IFNs has been explained by the spatial restriction of type III IFN receptor at epithelial surfaces. In this work, we demonstrate that type III IFN induces less ISGs compared to type I IFN. Most importantly, we unravel, for the first time, fundamental differences in the mechanisms by which both IFN mount the antiviral state in hIECs. We demonstrate that the antiviral activity of type III IFN partially depends on MAPKs, which is not the case for type I IFN. Interestingly, inhibition of MAPKs did not influence the expression of both IFIT1 and Viperin ISGs (data not shown). As such, it remains an important task for future work to dissect how signaling downstream of MAPKs participates in the antiviral activity of type III IFN only. As both IFNs have been reported to activate MAPKs (34–36), it will be interesting to address whether the dependency of type III IFN for MAPKs is epithelium cell specific or intestinal epithelium cell specific.

It is not known whether hIECS can protect themselves against viral infection by secreting and responding to their own IFNs. It was proposed that, during rotavirus infection, IFNs are produced by immune cells and not by epithelial cells (42). Indeed, during rotavirus infection of hIECs, multiple strategies are developed by the virus to inhibit innate immune response particularly the inhibition of both type I and III IFNs production (47). Additionally, blocking IFN signaling in hIECs does not lead to an increased rotavirus replication (42). Our data clearly show, for the first time, that when primary hIECs are infected with viruses that do not block IFN synthesis, hIECs produce and secrete at least type III IFN (maybe some type I IFN but under the detection limit of our ELISA assay) in order to protect themselves. Complementarily, KO of IFNLR renders hIECs more susceptible to viral infection (**Figure 6**).

Considering our results that only type III IFN is secreted by hIECs, it is tempting to propose that IECs have evolved to favor type III IFN over type I IFN, as it allows for similar protection against pathogens while limiting production of ISGs. From the perspective of an epithelium, which is always exposed to the extracellular environment and commensal challenges, this might represent a "smart strategy" to regulate the immune response in order to achieve the balance between responsiveness to pathogens versus tolerance of commensals. Restricting signaling to type III IFNs allows for response compartmentalization because type III IFN signaling is limited mostly to epithelial cells (11, 12, 35), thereby limiting systematic inflammation. From our findings in epithelium cells of the gastrointestinal tract, we can speculate that the first response to pathogen threats will be generated by hIECs. This response will be characterized by type III IFN-mediated signaling, therefore limiting ISGs and pro-inflammatory cytokine production. This first wave response of type III IFN produced by IECs, alone might be enough to clear enteric virus infection

(22, 48). A second wave response might be generated through recruitment of immune cells at the site of epithelium infection, which in turn will produce various cytokines including type I IFN. This IFN will then mediate a strong induction of ISGs and pro-inflammatory signals to powerfully combat pathogen at the infected mucosa and also will provide systemic protection. This uniquely tailored response would be fundamental for the maintenance of human gut homeostasis.

MATERIALS AND METHODS

Cells, Viruses, and Viral Infection

T84 human colon carcinoma cells (ATCC CCL-248) were maintained in a 50:50 mixture of Dulbecco's modified Eagle's medium and F12 (GibCo) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco). Reovirus MRV strains Type 3 clone 9 derived from stocks originally obtained from Bernard N. Fields were grown and purified by standard protocols (49). VSV-luc was a kind gift from Sean Whelan (Harvard Medical School) and was produced as described in Ref. (50). An MOI of 1 was used to infect T84 cells and organoids. Titers were determined as described in Ref. (51). For T84 cell MRV infections, MRV were purified on CsCl-gradient and stocks were titred by fluorescence foci forming assay (express in FFU) in T84 cells. Titers were calculated by determining the 50% tissue culture infective dose and expressed in FFU/mL. T84 cells were infected as described in Ref. (49). The MOI was determined as the ratio of infected cells (determined by fluorescence foci forming assay)/total number of cells. An MOI of 1 was used in T84 based experiments resulting in about 50–60% of infected as determined by fluorescence assay. For mini-gut organoids MRV infection, Organoids were removed from Matrigel by adding cold-PBS for 5 min, liquefied Matrigel and organoids were separated by centrifugation (400 g 5 min), the total number of cells per organoid samples was measured using an haematocytometer. Organoids were resuspended in culture medium containing or not MRV. When using an MOI of 1 (as determined in T84 cells) to infect mini-gut organoids, very few infected cells were detected per organoid. This discrepancy between T84 and organoid infectivity might be due to the 3-dimensional nature of the organoids and to residual Matrigel that might absorb and neutralize MRV. As such MRV stocks were titred directly in organoids by serial dilution infection and subsequent immunostaining. The MOI was calculated by the ratio of the number of infected/total number of cells/organoid. An MOI of 0.5 was used to infect organoids.

Human Organoid Cultures

Human colon tissue was received from colon resection (52–54) from the University Hospital Heidelberg under the approved study protocol S-024/2003 and human ileum and jejunum were purchased from Baylor University and transferred by signed MTA. Stem cells containing crypts were isolated following 2 mM EDTA dissociation of tissue sample for 1 h at 4°C. Crypts were spun and washed in ice-cold PBS. Fractions enriched in crypts were resuspended in Matrigel and maintained in basal culture media (53) Advanced DMEM/F12, supplemented with 1% penicillin/streptomycin, 10 mM HEPES, 50% v/v Wnt3A conditioned media, 1× B-27 (Life technology), 1× N-2 (Life technology), 2 mM GlutaMax (Gibco), 50 ng/mL EGF (Invitrogen), 1 µg/mL Spondin (Peprotech), 100 ng/mL Noggin (Peprotech), 10 nM Gastrin (Sigma), 1 mM *N*-acetyl-cysteine (Sigma), 10 mM nicotinamide (Sigma), and 500 nM A-83-01 (Tocris). Differentiation media is the same as above except without Wnt3A, nicotinamide and 50% reduced levels of R-Spondin and Noggin. Organoids were stained after cryo-sectioning of embedded organoids in Tissuetek.

Antibodies/Reagents

Rabbit polyclonal antibody against reovirus µNS was used at a 1/1,000 dilution for immunostaining and Western blots (49). Commercially available primary antibodies were goat polyclonal antibody recognizing EF-2 (Santa Cruz Biotechnology # sc-13004), rabbit polyclonal anti-Mucin-2 (Santa Graz Biotechnology# sc-15334), mouse monoclonal antibodies recognizing phospho-STAT1 or STAT1 (BD Transductions #612233 or #610115, respectively), ZO-1 (Invitrogen #339100) or E-cadherin (BD Transductions #610181). Rabbit polyclonal anti-phospho p38 (#4511), anti-p38 (#8690), anti-phospho-SAPK/JNK (#4668), anti-SAPK/JNK(#9258), anti-phospho ERK1/2 (#4370), and anti-ERK1/2 (#4695) antibodies were obtained from Cell Signaling. Secondary antibodies were conjugated with AF568 (Molecular Probes) or horseradish peroxidase (HRP) (Sigma-Aldrich) and directed against the animal source. Anti-mouse (GE Healthcare # NA934V), anti-rabbit (GE Healthcare #NA931V) and anti-goat (Jackson Immunoresearch # 705-035-147) antibodies, each coupled with HRP, were used as secondary antibodies for Western blot at a 1:5,000 dilution. Human recombinant IFN-beta1a (IFNβ) was obtained from Biomol (#86421). Recombinant human IFNλ 1 (IL-29) (#300-02L), IFNλ 2 (IL28A) (#300-2K), and IFNλ 3 (IL-28B) (#300-2K) were purchased from Peprotech. The pharmacological inhibitors used were 2 µM Pyridone 6 (Calbiochem #420099-500), 10 µM SB202190 (Tocris Bioscience #1264) for p38, 100 µM SP600125 (Tocris Bioscience #1496) for JNK, and 10 µM U0126 (Cell signaling #9903) for MEK-1/2.

RNA Isolation, cDNA, and qPCR

RNA was harvested from cells using the NucleoSpin RNA extraction kit (Machery-Nagel) and following the manufacturer's instructions. cDNA was made using iSCRIPT reverse transcriptase (Bio-Rad) from 250 ng of total RNA as per the manufacturer's instructions. qRT-PCR was performed using SsoAdvanced SYBR green (Bio-Rad) as per the manufacturer's instructions. TBP and HPRT1 were used as normalizing genes. Type I IFN was analyzed using primers specific for human IFNβ, and type III IFN was analyzed using primers specific for human IFNλ 2/3. The expression levels (fold of induction) of the investigated genes were calculated as ΔΔCq, normalizing to untreated or mock samples and to normalizing genes.

Western Blot

At time of harvest, media was removed, cells were rinsed once with 1× PBS and lysed with 1× RIPA (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium

dodecyl sulfate (SDS), 50 mM Tris, pH 8.0 with phosphatase, and protease inhibitors (Sigma-Aldrich)) for 5 min at room temperature (RT). Lysates were collected and equal protein amounts were separated by SDS-PAGE and blotted onto a PVDF membrane by wet-blotting (Bio-Rad). Membranes were blocked with 5% milk or 5% BSA in TBS containing 0.1% Tween 20 (TBS-T) for 1 h at RT. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. Membranes were washed 3× in TBS-T for 5 min at RT. Secondary antibodies were diluted in blocking buffer and incubated at RT for 1 h with rocking. Membranes were washed 3× in TBS-T for 5 min at RT. HRP detection reagent (GE Healthcare) was mixed 1:1 and added to the membrane, which was then incubated at RT for 5 min. Membranes were exposed to film and developed.

Indirect Immunofluorescence Assay

T84 cells were seeded in a 24-well plate. Cells were fixed in 2% paraformaldehyde for 20 min at RT, washed with PBS and permeabilized using 0.5% Triton X-100 for 15 min. After blocking with 3% BSA in PBS for 1 h at RT, cells were incubated with primary antibodies in 3% BSA for 1 h at RT. After washing with PBS, cells were stained with secondary antibodies in 3% BSA for 45 min at RT. To stain mini-gut organoids, 10 µm cryosections were fixed in 80% ethanol for 10 min at RT, followed by 2 min incubation in ice-cold acetone. After blocking in 5% goat serum in PBS containing 1% Triton for 1 h at RT, sections were incubated with primary antibodies in blocking solution for 2 h at RT or overnight at 4°C. After washing in PBS, sections were stained with secondary antibodies in 1% BSA in PBS containing 0.5% Triton for 2 h at RT. Nuclear DNA was stained with ProLong Gold DAPI (Molecular Probes). Slides were imaged by epifluorescence using a Nikon Eclipse Ti-S (Nikon) microscope or by confocal tile scans on a Zeiss LSM 780 (Zeiss) microscope. Image processing was performed using the Fiji software. For infection experiments, the percentage of infected cells was determined by counting at least 600–1,000 cells detected in 10 fields of view for each condition.

VSV Luciferase Assay

T84 cells were seeded in a white bottom 96-well plate. Cells were pre-treated prior to infection as indicated with increasing concentrations of type I or type III IFNs. VSV-luc (MOI = 1) was added to the wells and the infection was allowed to proceed for 8 h. At the end of the infection, media was removed, cells were washed 1× with PBS and lysed with Cell Lysis Buffer (Promega) at RT for 5 min. 1:1 dilution of Steady Glo (Promega) and PBS were added to the cells and incubated at RT for 7 min. Luminescence was read using an Omega Luminometer.

Microarray

Total RNA was purified as described above from T84 cells treated with 2,000 RU/mL of type I IFN (β) or 100 ng/mL of each type III IFN (λ1−3) for 6 hr. Microarray data were processed using the software package R. Differentially expressed probe sets were determined by comparing the triplicate stimulated samples with the three unstimulated samples. Significance was defined by a minimum absolute of twofold change in expression and a q-value (false discovery rate) <0.05.

ELISA

IFNβ and IFNL2/3 contained in the supernatant of cells were quantified using the human IFN-beta ELISA kit and DIY IFNLR 2/3 ELISA kit both from PBL-Interferon Source, per manufacturer's instructions.

Human KO Cell Lines

Knockout of IFNAR1 and IFNLR1 in T84 cells were achieved by using the CRISPR/Cas9 system. Three different single-guide RNAs (sgRNAs) per gene were used targeting the coding region of IFNAR1 and IFNLR1 and inserted into the lentiviral vector lentiCRISPR v2 (Addgene #52961) also encoding the Cas9 nuclease. The following sgRNAs were used: IFNAR1 (#1) 5′ GCGGCTGCGGACAACACCCA 3′, (#2) 5′ GACCCTAGTGCTCGTCGCCG 3′, (#3) 5′ CTAGGGTCGTCGCGCCCAGG 3′, IFNLR1(#1) 5′ ACTGGATCTGAAGTATGAGG 3′, (#2) 5′ CC TGGTGCTCACCCAGACGG 3′ (#3) 5′ TGAGGTGGCATTCTG GAAGG 3′. Lentiviruses were produced and T84 cells were transduced two times using 1:2 diluted stocks of lentiviral particles encoding sgRNA #1, 2 or 3. All shown data were obtained by using a cell clone treated with the sgRNA #2 for IFNAR1 and IFNLR1, but analogous results were obtained with cell clones generated with the other sgRNAs. To establish IFNAR1 and IFNLR1 KO cells, clonal selection was performed via single-cell dilution in a 96-well plate. KOs were confirmed by functional tests.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "University hospital Heidelberg" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the "Ethic commission of University hospital Heidelberg" under the approved study protocol S-024/2003.

AUTHOR CONTRIBUTIONS

MS, KP, and SB designed the experiments. KP, MS, SM, and DA performed most experiments. LR and RR performed IFN-specific qRT-PCR. SK and JS-B assisted with organoid preparation. ES and DG designed CRISPR/cas experiments. MS, RR, DG, and SB wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00459/full#supplementary-material.

FIGURE S1 | **Type I and III interferons (IFNs) confer protection against mammalian reovirus (MRV) infection to all sections of the gut**. Organoids from multiple colon donors and multiple intestinal sections were treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for 2.5 h prior to infection with MRV (multiplicity of infection = 0.5) for 16 h. **(A)** MRV-infected colon organoids (donor 2 and 3) were analyzed by μNS-specific immunofluorescence (green). The cells were stained against E-cadherin (red) and the nuclei were stained with Dapi (blue). Representative image of triplicate experiments are shown. The fluorescence intensity of MRV μNS per organoid was measured and expressed relative to untreated organoids (set to 100). **(B,C)** MRV-infected organoids were analyzed for μNS production by Western blot. Actin was used as loading control. Production of μNS was quantified by densitometer. The protective effect of type I IFN (β) and III IFN (λ1–3) was assayed by monitoring the relative viral genome copies by quantitative real-time PCR normalized to inoculum. **(B)** Ileum. **(C)** Jejunum. Data represent the mean values of three independent experiments. Error bars indicate the SD. $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired *t*-test).

FIGURE S2 | **Type I and III interferons (IFNs) confer protection against vesicular stomatis virus (VSV) infection in mini-gut organoids**. Colon organoids were treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for 2 h prior to infection with VSV-expressing luciferase (multiplicity of infection = 1). Eight hpi, VSV replication was assayed by measuring the luciferase activity. The mean values obtained from three independent experiments are plotted. Error bars indicate the SD.

FIGURE S3 | **T84 cells respond to both type I and III interferons (IFNs) by upregulating IFN-stimulated genes (ISGs)**. **(A)** T84 cells were infected with mammalian reovirus (multiplicity of infection = 1) and 16 hpi, cells were harvested and the copy number of the expression of 13 human type I and three type III IFNs by quantitative real-time (qRT)-PCR (55) were determined by qRT-PCR analysis. The geometric means of the peak responses in mock and infected intestinal epithelial cells are shown in a log10 scale as copy numbers per μg RNA. **(B)** T84 cells were stimulated with indicated concentrations of type I (β) or III IFN (λ1–3) for different times and the transcript levels of the ISGs Viperin (Vip) and IFIT1 were analyzed by qRT-PCR. Data are normalized to TBP and HPRT1 and are expressed relative to untreated cells at each time point. A representative experiment out of three independent experiments is shown. Mean values and SD are shown. **(C)** T84 cells were treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for 6 h and identification of the IFN-induced ISGs was performed by transcript profiling using an Illumina microarray.

FIGURE S4 | **Characterization of different T84 IFNAR and IFNLR knockout (KO) cell clones generated using the CRISPR/Cas system**. **(A)** IFNAR and IFNLR KO clones were treated with type I interferons (IFN) (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for 1 h and IFN signaling was measured by immunoblotting for pSTAT1 Y701. EF-2 is used as a loading control. A representative immunoblot out of three independent experiments is shown. **(B)** Same as **(A)**, except that IFN signaling was evaluated by monitoring induction of IFN-stimulated genes by relative quantification of Viperin at indicated times post-IFN treatment using qRT-PCR. Data are normalized to TBP and HPRT1 and are expressed relative to untreated control cells of each time point. A representative experiment, out of three independent experiments is shown. **(C)** T84 cell lines were treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for 2 h prior to infection with VSV-expressing luciferase (multiplicity of infection = 1) and viral replication was assayed by measuring the luciferase activity. Results are expressed relative to mock-IFN-treated control cells generated with a scrambled control gRNA (set to 100). The mean value obtained from two independent experiments is plotted. Error bars indicate the SD.

FIGURE S5 | **Role of MAP kinase pathway in type I and III interferon (IFN) antiviral activity**. **(A)** T84 cells were treated with type I IFN (β) (2,000 RU/mL equivalent 8 ng/mL) or type III IFN (λ1–3) (300 ng/mL) for the indicated time points. The levels of phosphorylation of MAPkinases p38, ERK, and JNK were assessed by Western blot analysis. p38, ERK, JNK, and EF-2 were used as loading control. The phosphorylation of the MAP kinases was quantified and expressed relative to untreated cells (right panel). Data represent the mean values of three independent experiments. **(B)** T84 cells were treated with increasing concentrations of JAK and MAP kinase inhibitors. 24 h posttreatment of inhibitors the cell viability was assessed by MTT assay in triplicates. **(C)** T84 cells were pre-incubated for 30 min with 2 μM Pyridone 6 (pan-JAK inhibitor), 10 μM U0126 (ERK inhibitor), 10 μM SB202190 (p38 inhibitor) and 100 μM SP600125 (JNK inhibitor). Then the indicative concentrations of type I or III IFN were added in parallel to the inhibitor. Two hours post-IFN treatment cells were infected with VSV expressing luciferase (multiplicity of infection = 1) and viral replication was assayed by measuring the luciferase activity. Data were normalized to in no IFN-treated samples for each inhibitor. The mean value obtained from three independent experiments is shown. Error bars indicate the SD.

FIGURE S6 | **Specific inhibition of MAP kinases phosphorylation**. T84 intestinal epithelial cells were pre-treated with JAK and MAPK inhibitors for 30 min prior to interferon treatment. Cells were harvested at different times posttreatment and the extent of JAK or MAPK inhibition was addressed by Western blot analysis. The specificity of each inhibitor was controlled by monitoring the phosphorylation status of JAK and all MAPKs. **(A)** 2 μM Pyridone 6 (pan-JAK inhibitor). **(B)** 10 μM U0126 (ERK inhibitor). **(C)** 10 μM SB202190 (p38 inhibitor).

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Peg-Interferon Lambda Treatment Induces Robust Innate and Adaptive Immunity in Chronic Hepatitis B Patients

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IFN-lambda (IFNλ) is a member of the type III IFN family and is reported to possess anti-pathogen, anti-cancer, and immunomodulatory properties; however, there are limited data regarding its impact on host immune responses *in vivo*. We performed longitudinal and comprehensive immunosurveillance to assess the ability of pegylated (peg)-IFNλ to augment antiviral host immunity as part of a clinical trial assessing the efficacy of peg-IFNλ in chronic hepatitis B (CHB) patients. These patients were pretreated with directly acting antiviral therapy (entecavir) for 12 weeks with subsequent addition of peg-IFNλ for up to 32 weeks. In a subgroup of patients, the addition of peg-IFNλ provoked high serum levels of antiviral cytokine IL-18. We also observed the enhancement of natural killer cell polyfunctionality and the recovery of a pan-genotypic HBV-specific $CD4^+$ T cells producing IFN-γ with maintenance of HBV-specific $CD8^+$ T cell antiviral and cytotoxic activities. It was only in these patients that we observed strong virological control with reductions in both viral replication and HBV antigen levels. Here, we show for the first time that *in vivo* peg-IFNλ displays significant immunostimulatory properties with improvements in the main effectors mediating anti-HBV immunity. Interestingly, the maintenance in HBV-specific $CD8^+$ T cells in the presence of peg-IFNλ is in contrast to previous studies showing that peg-IFNα treatment for CHB results in a detrimental effect on the functionality of this important antiviral T cell compartment.

Clinical Trial Registration: ClinicalTrials.gov NCT01204762.

Keywords: peg-interferon lambda, direct antiviral, hepatitis B, immunity, *in vivo*

INTRODUCTION

Type I and type III interferons are the primary mediators of antiviral protection and the main therapeutic protagonists include IFN-alpha (IFN-α) and IFN-lambda (IFNλ; IL-29), respectively. The immune-mediating properties of IFN-α have been extensively described both *in vivo* and *in vitro* in the context of many diseases (1–5). However, little is understood about the immunomodulatory properties of IFNλ in different disease states.

Both type I and III interferons have been shown to play an important role in control of HBV replication (6). Indeed, IFN-α has been used as a treatment strategy for chronic hepatitis B (CHB) for over 40 years; however, its efficacy is suboptimal with resolution of infection being achieved in <7% patients (7, 8). This is marginally improved during combination treatment with potent directly acting antiviral agents, such as entecavir (ETV) or tenofovir, but still remains inadequate with functional cure being achieved in only 15% of patients (9–11). The root cause of this may be immunological in nature. IFN-α has a dual mechanism of action in CHB, first, a direct antiviral effect achieved through inhibiting the synthesis of viral DNA, virus particles, and activation of antiviral enzymes, and second, an augmentation of antiviral host immunity (8). In CHB, IFN-α treatment induces narrowly focused immune responses restricted to activation of the innate immunity with little impact on reactivating stagnant HBV-specific adaptive immune responses which are central to long-term control of infection (12–15).

The precise role and activity of IFNλ as an immunomodulator is unknown *in vivo* in humans and remains unclear in *in vitro* experiments. Indeed, the immune potentiating functions of IFNλ are slowly starting to emerge (16–20). Early data suggests that although IFNλ activates the same signaling pathway as IFN-α, their temporal activation of ISGs as well as the induction of an antiviral response is different (6, 21, 22). There is also some discrepancy regarding the direct impact of IFNλ on immunocytes. Some studies find little or no expression of IFNλR on immune cells, while others show IFNλR expression on both natural killer (NK) and T cells (16, 18, 19, 23, 24). Further to this, IFNλ is also reported to be unable to directly activate NK cell function, influence T cell differentiation, or induce cytokine production by T cells (25–27). In other studies, however, IFNλ stimulates a significant antitumor immunity in murine models (28) and directly modulates T cell activity with promotion of Th1 and inhibition of Th2 responses (16, 29, 30). These discrepancies are likely to be due to differences in the cellular, tissue, and animal models utilized and are compounded by a paucity of studies investigating the relationship between IFNλ and the host immune response *in vivo* (17, 18, 25, 27). Defining whether IFNλ acts as a broad or narrow immunostimulant *in vivo* in the context of a chronic disease will allow its appropriate therapeutic application in infection and disease.

In this study, we have comprehensively analyzed the impact of IFNλ treatment on antiviral immunity in CHB patients. This is an ideal model infection to study the immunostimulatory effects of a therapeutic agent, as persistence of this virus is fundamentally associated with a weak antiviral immune response, characterized by defective NK cells and impaired virus-specific T cell responses (31–38). Moreover, there is strong evidence demonstrating that the development and re-establishment of innate and adaptive host immunity in CHB is associated with control of infection (39–43). Therefore, using CHB infection as a model, we have for the first time utilized the structured platform of a clinical trial to dissect the relationship between the innate and adaptive host immune response and IFNλ.

MATERIALS AND METHODS

Study Design and Patients

We performed longitudinal immuno-surveillance of a subgroup of patients participating in a phase 2b clinical study to evaluate the safety, efficacy and tolerability of pegylated IFNλ (PegIFNλ) in combination with ETV in Hepatitis B e Antigen positive ($HBeAg^+$) CHB patients [sponsored by Bristol-Myers Squibb, Wallingford (BMS), CT, USA]. Treatment naïve, $HBeAg^+$ CHB patients were recruited in 12 centers world-wide (Portland, California, Frankfurt, Hamburg, Hannover, Rotterdam, Taipei, Tainan, Seoul, and Taichung). The 13 patients (patient characteristics described in **Table 1**) received 12 weeks of ETV monotherapy (0.5 mg/day) followed by up to 32 weeks of combination therapy ETV (0.5 mg/day)/PegIFNλ (180 μg/weekly) (**Figure 1**). Clinical parameters [HBV-DNA, HBeAg, hepatitis surface antigen (HBsAg), and alanine aminotransferase (ALT)] were measured in the serum at central laboratories. This study was approved by the Ethics Committee at each recruitment site and informed consent was obtained from all patients before enrollment. The isolation and cryopreservation of peripheral blood mononuclear cells (PBMC) was standardized by supplying each site with a written and video protocol. Prior to patient enrollment, each site performed PBMC isolation dry run which were shipped to the Institute of Hepatology for testing and once PBMCs met

TABLE 1 | Patients characteristics at baseline.

Characteristics	Entecavir and IFN-λ
Age (years)[a]	31.2 (21, 41)
Gender (male/female ratio)	10:3
Racial group (no. of patients)	
White	1
Asian*	12
Asian Indian	1
Chinese	4
Korean	4
Other	3
Alanine aminotransferase (IU/ml)[a]	88.2 (38, 297)
HBV-DNA (Log_{10} copies/ml)[a]	8.3 (6.4, 9.7)
HBV genotype (no. of patients)	
B	7
C	5
D	1
qHBeAg (Log_{10} copies/ml)[a]	2.4 (0.1, 2.8)
qHBsAg (Log_{10} copies/ml)[a]	4.6 (3.9, 5.4)
IL-28B (no. of patients)	
CC	9
CT	4
Non-cirrhotic	13

[a]*The data are shown as mean (range). *Asian subgroups are detailed below.*

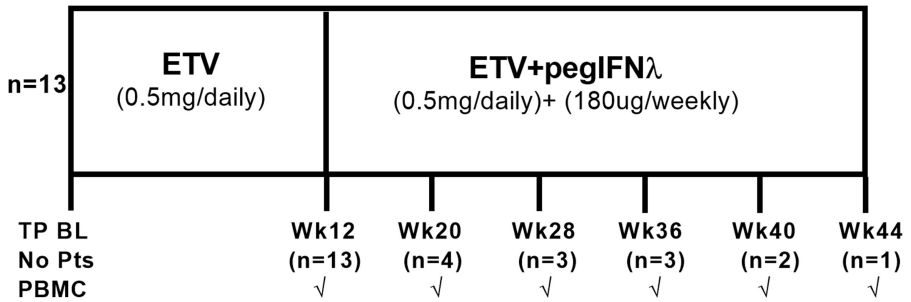

FIGURE 1 | **Study design**. All 13 patients were treated with entecavir (ETV) for 12 weeks and subsequently received ETV + pegIFN-λ. The weeks of treatment reached by the patients are shown. Peripheral blood mononuclear cells (PBMC) collection are also indicated.

standardized criteria of >95% viability and >80% recovery did the sites initiate recruitment and collection of PBMC locally.

PBMC Isolation

Peripheral blood mononuclear cells were isolated from heparinized blood by lymphoprep gradient centrifugation as described previously (33, 34, 37). The cryopreserved PBMC were stored at −80°C at each site and subsequently batch shipped to the Institute of Hepatology for immunological analysis.

Antigens

Commercially available recombinant HBV nucleocapsid protein (HBcAg) and purified HBsAg were purchased from American Research products, Belmont, MA. HBV genotype A, B, C, and D 15-mers overlapping peptides covering the entire HBcAg and HBsAg region (Proimmune, Oxford, UK) were mixed in pools of five adjacent peptides. The pools were reconstituted at 8 mg/ml in dimethyl sulfoxide (DMSO). Recall antigen, tuberculin purified protein derivate (PPD) (Statens Seruminstitut, Copenhagen, Denmark), PMA, inomycin, and phytohemagglutinin (PHA) (Sigma, Poole, UK) were used as positive controls.

Flow Cytometry

All antibodies used for flow cytometry were purchased from BD Biosciences except when mentioned differently. Cells were acquired after staining on FACSCanto II flow cytometer (BD) and analyzed using FACS DIVA software.

NK Analysis

Peripheral blood mononuclear cells were surface stained with CD3-BV510, CD16-FITC, CD56-V450, NKG2D-PerCP-eFluor710 (eBioscience), and TRAIL-PE (R&D systems) as previously described (44, 45). To measure the frequency of IFN-γ-producing NK cells and NK cell degranulation, PBMC were incubated for 6 h with rhIL-12 and rhIL-18 (R&D systems) and CFSE-stained K562 (E:T 5:1), respectively. CD107a-APC was added 2 h after the start of the culture to the PBMC: K562 cultures. A protein inhibitor cocktail (eBioscience) was added to all cultures 3 h from the start of the culture. PBMC were then surface stained as described above with CD3, CD16, and CD56 antibodies. The rhIL-12- and rhIL-18-stimulated wells were stained intracellularly with IFN-γ-PE-Cy7 as previously described (44, 45). PBMC were also stimulated with PMA/ionomycin as a positive control. The gating strategy to assess the *ex vivo* frequency of cytokine-producing ($CD56^{bright}$, $CD16^{-}$) and cytotoxic ($CD56^{dim}$, $CD16^{+}$) NK subsets is described in Figure S1 in Supplementary Material.

Frequency of HBV-Specific Producing T Cells IFN-γ

The frequency was assessed by ELISpot assays. PBMC from patients and from a quality control PBMC batch (inter-assay control) were thawed, washed, and resuspended in RPMI1640/10% AB serum. The cell viability was assessed with propidium iodide using an automated cell counter. ELISPOT assays were performed as previously described (34). PBMC were incubated in the presence of HBcAg (1 μg/ml), HBsAg (2 μg/ml), peptide pools (4 μg/ml), PPD (10 μg/ml), and PHA (2 μg/ml).

Functions of HBV-Specific T Cells

Peripheral blood mononuclear cells were stimulated with HBV antigens and genotype-specific peptide pools for 7 days. On day 6, PBMC were subjected to a second round of stimulation with the HBV antigens and the overlapping HBV peptides and stained overnight with CD107a-APC and protein inhibitor cocktail. On day 7, PBMC were surface stained with CD3-BV510, CD4-V450, and CD8-APC-eFluor780 antibodies and stained intracellularly for IFN-γ as described above. PBMC stimulated with PMA/ionomycin were used as a positive control.

For T-regulatory cell staining, PBMC were surface stained with CD3, CD4, and CD25-FITC, fixed and permeabilized with FoxP3 buffer (eBioscience) and stained intracellularly with FoxP3-PerCPCy5.5 antibody as per the manufacturer's instructions.

Determination of Serum Cytokine Profiles

The serum levels of IL-2, IL-6, IL-8, IL-10, IL-12p70, IL-18, IP-10, IFN-γ, TNF-α, Granzyme B, and MIP-1α were quantitated using cytometric bead array (BD Biosciences) in accordance with manufacturer's instructions. The levels of IL-15, IL-17, IFN-α, and IFN-β were determined by ELISA (R&D systems).

Statistics Analysis

Statistical significance was assessed during course of the treatment before and after segregation of patients into groups using repeated measure one-way ANOVA and two-way ANOVA, respectively. Multiple comparisons tests were performed only when the null hypothesis was rejected with the ANOVA test. Pearson's correlation was used for correlation analyses. Analyses were conducted with the GraphPad Prism software version 6.05 for Windows (La Jolla, CA, USA). $p < 0.05$ was considered statistically significant.

RESULTS

Clinical Responses and Group Stratification

This clinical study was terminated early for commercial reasons based on results from a parallel trial showing that non-inferiority of IFNλ to IFNα was not met at week 24. This early curtailment was not related to any safety concerns (46). The 13 patients initially received the full 12 weeks of ETV alone. Due to the truncated nature of this study, four patients received ETV plus Peg-IFNλ for 8 weeks, three for 16 weeks, three for 24 weeks, two for 28 weeks, and one for 32 weeks. Patients' responses were not significantly different between consecutive time points and were therefore grouped during ETV and ETV plus Peg-IFNλ treatments.

Treatment with ETV alone led to a mean drop of −3.72 Log_{10} copies/ml in HBV-DNA levels during the first 12 weeks of therapy (**Table 2**) in line with previous studies (47, 48). A further reduction in viral replication was observed with the addition of Peg-IFNλ (−1.8 Log_{10} copies/ml) (**Table 2**). A significant drop in HBsAg levels (−0.63Log_{10} IU/ml) and in the% HBsAg decline (ETV: 7.8% vs ETV + Peg-IFNλ: 13.2%) was also greater when Peg-IFNλ was administered. HBeAg levels did not fall during ETV alone but the addition of Peg-IFNλ did induce a significant reduction (−0.73Log_{10} IU/ml) and a drop in the % HBeAg decline (ETV + Peg-IFNλ: 31.5%) (**Table 2**). No HBsAg or HBeAg loss or seroconversion occurred during the course of this truncated study, and no significant changes overall were observed in serum ALT (**Table 2**). Two patients experienced an ALT flare (ALT greater than 2× baseline and 5× the upper limit of normal) during add-on Peg-IFNλ.

Analysis of the clinical data revealed two distinct groups of patients based on the rates of decline of the viral antigen levels (HBeAg and HBsAg) when Peg-IFNλ was introduced. Nine patients (Group 1) had a greater and significant reduction in HBsAg and HBeAg compared to the remaining four patients (Group 2) who showed no change in viral antigen levels during the addition of Peg-IFNλ (**Table 3**). In Group 1, HBsAg and HBeAg declined by −0.73 and −0.95 Log_{10} IU/ml, respectively (**Table 3**). Furthermore, the difference in HBsAg and HBeAg levels between Group 1 and Group 2 was greater than 1 Log (Group 1 − Group 2: HBsAg: −1.07 Log_{10}; HBeAg: −1.08 Log_{10}) (**Table 3**).

Reductions in viremia were also different between these two groups. Significant reductions were observed in Group 1 between ETV alone and ETV plus Peg-IFNλ (−3.84 Log_{10}; −1.9 Log_{10}, respectively), whereas HBV-DNA decline was less pronounced in Group 2 (ETV alone: −3.45 Log_{10}; ETV + Peg-IFNλ: −1.57 Log_{10}).

TABLE 2 | Changes in patients virological parameters during treatment.

Patients (n = 13)	HBV-DNA (Log_{10} copies/ml)			Hepatitis surface antigen (HBsAg) (Log_{10} IU/ml)			HBeAg (Log_{10} IU/ml)			Alanine aminotransferase (ALT) (U/l)		
	Baseline	Entecavir (ETV)	ETV + pegIFN-λ	Baseline	ETV	ETV + pegIFN-λ	Baseline	ETV	ETV + pegIFN-λ	Baseline	ETV	ETV + pegIFN-λ
M	8.37 ± 0.25	4.65 ± 0.26	2.85 ± 0.27	4.59 ± 0.16	4.23 ± 0.20	3.67 ± 0.35	2.37 ± 0.27	2.32 ± 0.18	1.59 ± 0.32	107.7 ± 29.4	75.82 ± 13.83	114.2 ± 25.84
Δ		−3.72 ± 0.17			−0.29 ± 0.09			−0.05 ± 0.16			−31.83 ± 25.47	
		($p < 0.0001$)			($p = 0.029$)			(p = ns)			(p = ns)	
Δ1			−5.5 ± 0.23			−0.92 ± 0.23			−0.78 ± 0.28			6.53 ± 27.54
			($p < 0.0001$)			($p = 0.009$)			(p = ns)			(p = ns)
Δ2			− 1.8 ± 0.20			−0.63 ± 0.20			−0.73 ± 0.23			38.37 ± 29.86
			($p < 0.0001$)			($p = 0.024$)			($p = 0.027$)			(p = ns)

The data are shown as mean ± SEM at baseline (BL), ETV, and ETV + pegIFN-λ. (M) Mean change in HBV-DNA, HBsAg, HBeAg, and ALT from: (Δ) BL to ETV, (Δ1) BL to ETV + pegIFN-λ, (Δ2) ETV to ETV + pegIFN-λ. (repeated measures one-way ANOVA was performed).

TABLE 3 | Changes in patients' virological parameters after segregation in two groups during treatment.

		HBV-DNA (Log_{10} copies/ml)			Hepatitis surface antigen (HBsAg) (Log_{10} IU/ml)			HBeAg (Log_{10} IU/ml)			Alanine aminotransferase (ALT) (U/l)		
		Baseline	**Entecavir (ETV)**	**ETV + pegIFN-λ**	**Baseline**	**ETV**	**ETV + pegIFN-λ**	**Baseline**	**ETV**	**ETV + pegIFN-λ**	**Baseline**	**ETV**	**ETV + pegIFN-λ**
Patient Group 1	M	8.14 ± 0.30	4.30 ± 0.30	2.40 ± 0.26	4.39 ± 0.17	4.08 ± 0.19	3.35 ± 0.35	2.21 ± 0.37	2.19 ± 0.24	1.23 ± 0.37	118.5 ± 41.35	70 ± 10.85	112.5 ± 30.87
	Δ		−3.84 ± 0.17 ($p < 0.0001$)			−0.31 ± 0.10 (p = ns)			−0.013 ± 0.22 (p = ns)			−48.53 ± 35.47 (p = ns)	
($n = 9$)	Δ1			−5.74 ± 0.24 ($p < 0.0001$)			−1.04 ± 0.32 ($p = 0.005$)			−0.97 ± 0.38 ($p = 0.019$)			−6.07 ± 26.02 (p = ns)
	Δ2			− 1.9 ± 0.23 ($p = 0.0002$)			−0.73 ± 0.28 ($p = 0.042$)			−0.95 ± 0.28 ($p = 0.020$)			40.47 ± 27.41 (p = ns)
Patient Group 2	M	8.88 ± 0.34	5.43 ± 0.21	3.86 ± 0.21	5.05 ± 0.18	4.81 ± 0.40	4.42 ± 0.28	2.75 ± 0.00	2.65 ± 0.10	2.31 ± 0.32	82.75 ± 26.09	88.25 ± 41.49	117.8 ± 54.54
	Δ		−3.45 ± 0.41 ($p < 0.0001$)			−0.24 ± 0.23 (p = ns)			−0.10 ± 0.10 (p = ns)			5.5 ± 16.03 (p = ns)	
($n = 4$)	Δ1			−5.02 ± 0.46 ($p < 0.0001$)			−0.63 ± 0.11 (p = ns)			−0.44 ± 0.16 (p = ns)			35.03 ± 73.78 (p = ns)
	Δ2			−1.57 ± 0.40 (p = ns)			−0.39 ± 0.13 (p = ns)			−0.33 ± 0.16 (p = ns)			29.53 ± 84.19 (p = ns)
M Group 1 – M Group 2	Δ3	−0.74 ± 0.47 (p = ns)	−1.13 ± 0.47 ($p = 0.023$)	−1.46 ± 0.47 ($p = 0.041$)	−0.65 ± 0.44 (p = ns)	−0.73 ± 0.44 (p = ns)	−1.07 ± 0.44 ($p = 0.022$)	−0.54 ± 0.46 (p = ns)	−0.45 ± 0.46 (p = ns)	−1.08 ± 0.46 ($p = 0.026$)	35.77 ± 47.83 (p = ns)	−18.26 ± 47.83 (p = ns)	−7.21 ± 47.83 (p = ns)

The data are shown as mean ± SEM at BL, ETV, and ETV + pegIFN-λ. (M) Mean change in HBV-DNA, HBsAg, HBeAg, and ALT from (Δ) BL to ETV, (Δ1) BL to ETV + pegIFN-λ, (Δ2) ETV to ETV + pegIFN-λ. (Δ3) Mean difference in HBV-DNA and viral antigens levels between group 1 and group 2 at BL, ETV, and ETV + pegIFN-λ. Repeated measures two-way ANOVA was performed.

There was a difference in HBV-DNA levels greater than 1 Log between the two groups (ETV alone: −1.13 Log_{10}; ETV + Peg-IFNλ: −1.46 Log_{10}) (**Table 3**). Serum ALT levels were not different between Group 1 and 2.

As it has been previously shown that declining HBsAg levels denote activation of the host immunity and control of infection (49–52), the impact of IFNλ on the host immunity was analyzed in the two groups identified: those who did (Group 1) or did not (Group 2) experience changes in antigen levels during combination treatment.

Addition of Peg-IFNλ Induces a Poly-Functional NK Response

During CHB, NK cells exhibit profound impairments in their ability to eliminate HBV by non-cytolytic and cytolytic mechanisms. They notably also lose the ability to orchestrate key players of the adaptive immune response (36, 53–55). Using standardized protocols (44), we analyzed the impact of Peg-IFNλ on the phenotype and functionality of NK cells. In Group 1 patients (i.e., those with the greatest reduction in antigenaemia), we found an expansion in the frequency of these cells expressing the cytotoxic marker tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) during combination Peg-IFNλ (**Figure 2A**). Importantly, this pattern was not observed in Group 2 patients. We also assessed the relationships between the NK cell population and viral parameters and found the increase in TRAIL-positive NK cells to correlate positively with serum ALT levels in Group 1 patients ($r = 0.848$; $p < 0.0001$). This was reflected in two patients who showed the greatest increase in these NK cells and had the greatest elevation in serum ALT, denoting a cytolytic clearance of infected hepatocytes during the Peg-IFNλ phase of treatment. This observation was in line with previously described findings that in CHB, TRAIL expression increases together with ALT levels in patients treated with Peg-IFNα and denotes the elimination of infected hepatocytes (53). Interestingly, TRAIL-positive cytokine-producing NK cells of Group 1 patients were found to correlate negatively with HBsAg levels ($r = -0.699$; $p = 0.0025$).

The data also clearly demonstrate that NK functionality is modulated by the addition of Peg-IFNλ *in vivo* and is different between the "responding" and "non-responding" groups. The cytotoxic potential of NK cells increased significantly in Group 1 but remained unchanged in Group 2 during the presence of Peg-IFNλ (**Figure 2B**). The frequency of IFN-γ-producing NK cells also changed during treatment and increased with Peg-IFNλ in Group 1, although the difference between ETV alone and ETV + Peg-IFNλ was not statistically significant (**Figure 2C**). Nevertheless, the increase in the frequency of IFN-γ-producing NK cells was found to correlate negatively with HBV viral load ($r = -0.545$; $p = 0.030$). In contrast, the expression of the inhibitory marker, NKG2D did not change on any of the NK cells subsets during treatment (**Figures 2D,E**).

Peg-IFNλ Augments HBV-Specific T Cells Responses

We comprehensively assessed the HBV-specific T cell response to recombinant HBV core, HBV surface antigen proteins, and overlapping genotype-specific core and surface peptides pools. Significant expansion was observed in the frequency of HBV-specific T cells producing IFN-γ during the add-on Peg-IFNλ phase of treatment in Group 1 but not in Group 2 (**Figure 3A**). We observed an increase in the percentage of patients who responded to HBV antigens and peptides pools initiated by treatment with ETV alone and this was augmented further when Peg-IFNλ was added (**Figure 3B**). Further characterization of this reactive T cell population revealed that the increased virus-specific response observed in Group 1 was predominantly driven by the $CD4^+$ T cell population (**Figure 3C**). We also assessed the relationships between $CD4^+$ T cell population and viral parameters and found this cell population to be negatively correlated with HBV-DNA ($r = -0.752$; $p = 0.019$) and HBsAg ($r = -0.795$; $p = 0.010$) and positively correlated with ALT ($r = 0.824$; $p = 0.006$). A small but significant increase in the IFN-γ-producing HBV-specific $CD8^+$ T cell population was also detected in 40% of patients in Group 1, during ETV and maintained during the addition of Peg-IFNλ (**Figure 3D**). The change in this cell population was found to correlate negatively with HBsAg ($r = -0.676$; $p = 0.045$) and positively with serum ALT ($r = 0.770$; $p = 0.015$). We also evaluated the cytotoxic potential of HBV-specific $CD8^+$ T cells during the study by assessing their ability to degranulate and found that CD107a-positive HBV-specific $CD8^+$ T cells were maintained through the treatment period, reflecting the steady levels of ALT observed in most of these patients (**Figure 3E**). There were, however, higher frequencies of this subset in Group 1 than Group 2. Indeed, during add-on PegIFNλ, 50% of subjects in Group 1 had more than 10% of HBV-specific $CD8^+$ T cells expressing CD107a in contrast to none of the patients in Group 2. The frequency of T-regulatory cells was assessed and found to be low in all patients at baseline and did not change during treatment or between the groups (**Figure 3F**).

Peg-IFNλ Alters Serum IL-18 Levels

Finally, we examined the impact of Peg-IFNλ add-on on a panel of antiviral and pro/anti-inflammatory serum cytokines. Notably, we found that IL-18 levels significantly increased during treatment in Group 1 (**Figure 4A**). Although this increase is statistically significant, we recognize that it is quite small and the biological relevance needs to be further studied. This change in IL-18 was found to correlate positively with serum ALT ($r = 0.432$; $p = 0.024$). The levels of IL-8, IL-15 IL-17, and IP-10 did not change during the course of treatment in the two groups (**Figures 4B–E**). Type I IFNs, IFN-β, and IFN-α could be detected but only in Group 1; however, their levels did not change during treatment (**Figures 4F,G**). The other cytokines measured, IL-2, IL-6, IL-10, IL-12p70, IFN-γ, TNF-α, Granzyme B, and MIP-1α were undetectable in both groups at all time points assessed.

DISCUSSION

The present data show for the first time that *in vivo,* IFNλ displays immunostimulatory properties and provokes anti-HBV immunity in both the innate and adaptive compartments but only in patients that achieve the greatest decline in viral replication rates. This is of much relevance in CHB, as reductions in, or loss of

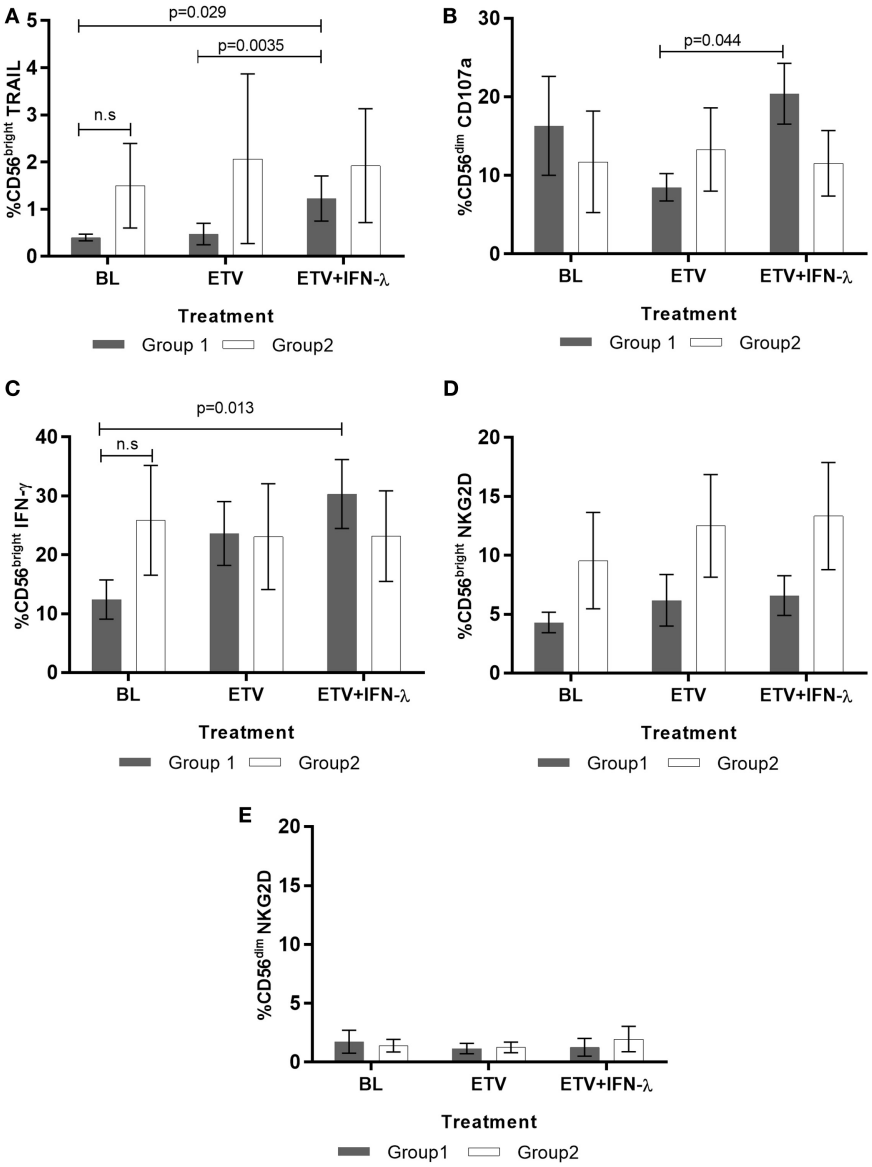

FIGURE 2 | Effect of treatment on natural killer (NK) cells response in Group 1 and Group 2 patients ($n = 13$). Percentage of **(A)** tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-positive $CD56^{bright}$ NK cells **(B)** CD107a-producing cytotoxic $CD56^{dim}$ NK cells, **(C)** IFN-γ producing $CD56^{bright}$, **(D)** NKG2D-positive $CD56^{bright}$, and **(E)** NKG2D-positive $CD56^{dim}$ were measured by flow cytometry. A total of 100,000 events were collected during FACS acquisition and the subsequent analysis was performed using FACS DIVA software. Data are shown as mean ± SEM. Two-way ANOVA followed by multiple comparison tests were performed for statistical analysis.

viremia in the serum does not equate to cure or viral eradication as the HBV genome persists as an integrated genome and/or as episomal covalently closed circular DNA for life (56). Long-term off-treatment control is only likely to be achieved *via* the activation strong antiviral host immunity, as seen in patients who resolve the infection spontaneously (56).

The findings from this study supports our previous reports demonstrating that overcoming immune hyporesponsiveness and development of immune-modulating therapies for CHB can only be achieved in patients who have low viral replication rates (33, 37). This observation is also supported by previous findings from Webster et al. showing that a HBV-DNA load less than 10^7 copies/ml is the threshold below which circulating multi-specific HBV-specific T cells can be consistently detected (57).

Natural killer cell and virus-specific T cell responses represent the main effectors of a favorable antiviral immune response and are critical in the long-term control of HBV infection (58, 59); functional impairments in their response to HBV have been widely shown to be correlated with an inability of the host to control replication and the persistence of infection (37, 38). In this study, we show that therapeutic administration of IFNλ can induce a functional restoration of NK cells and virus-specific

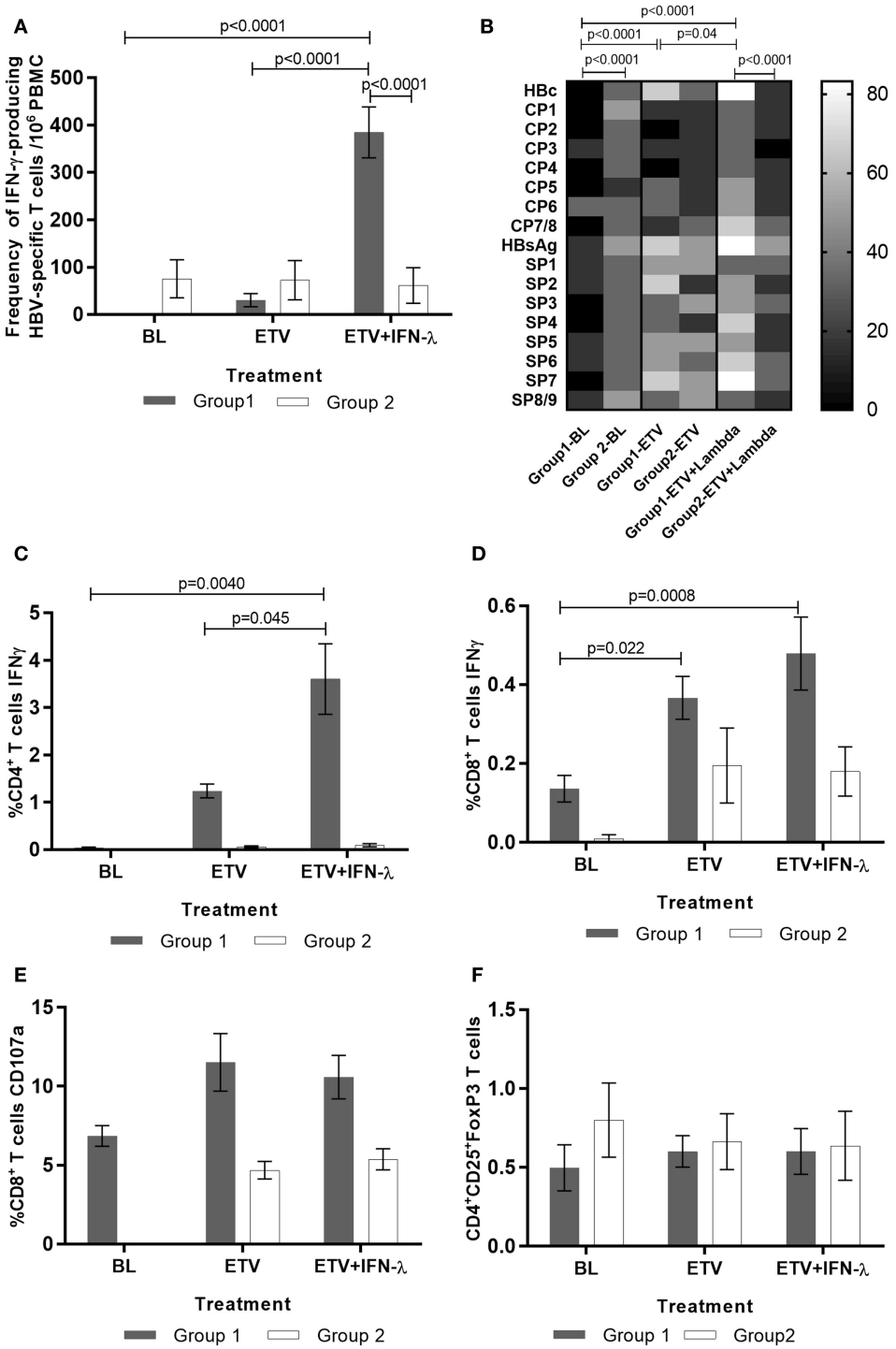

FIGURE 3 | **Effect of treatment on HBV-specific T cells and T regs response in Group 1 and Group 2 patients (n = 13)**. The frequency of IFN-γ-producing HBV-specific T cells was evaluated by ELISPOT following peripheral blood mononuclear cells (PBMC) stimulation with HBV antigens and HBV-specific overlapping peptides. PBMC stimulation with recall antigen purified protein derivate and mitogen phytohemagglutinin elicited a measurable strong response which did not change significantly during the course of the treatment. **(A)** ELISPOT quantitation of frequency of IFN-γ-producing HBV-specific T cells. **(B)** Heat map representation of the percentage of patients reacting to each individual HBV antigen and peptide pool in ELISPOT. The assessment of the functionality of T cells was performed by FACS following two rounds of stimulation with HBV antigens and HBV-specific overlapping peptides covering HBV core and HBV surface regions. **(C)** IFN-γ-producing HBV-specific $CD4^+$ T cells, **(D)** IFN-γ-producing HBV-specific $CD8^+$ T cells, **(E)** CD107a-producing HBV-specific $CD8^+$ T cells, and **(F)** T regulatory cells were quantitated by FACS. A total of 100,000 events were collected during FACS acquisition and the subsequent analysis was performed using FACS DIVA software. Data are shown as mean ± SEM. Two-way ANOVA followed multiple comparison tests were performed for statistical analysis.

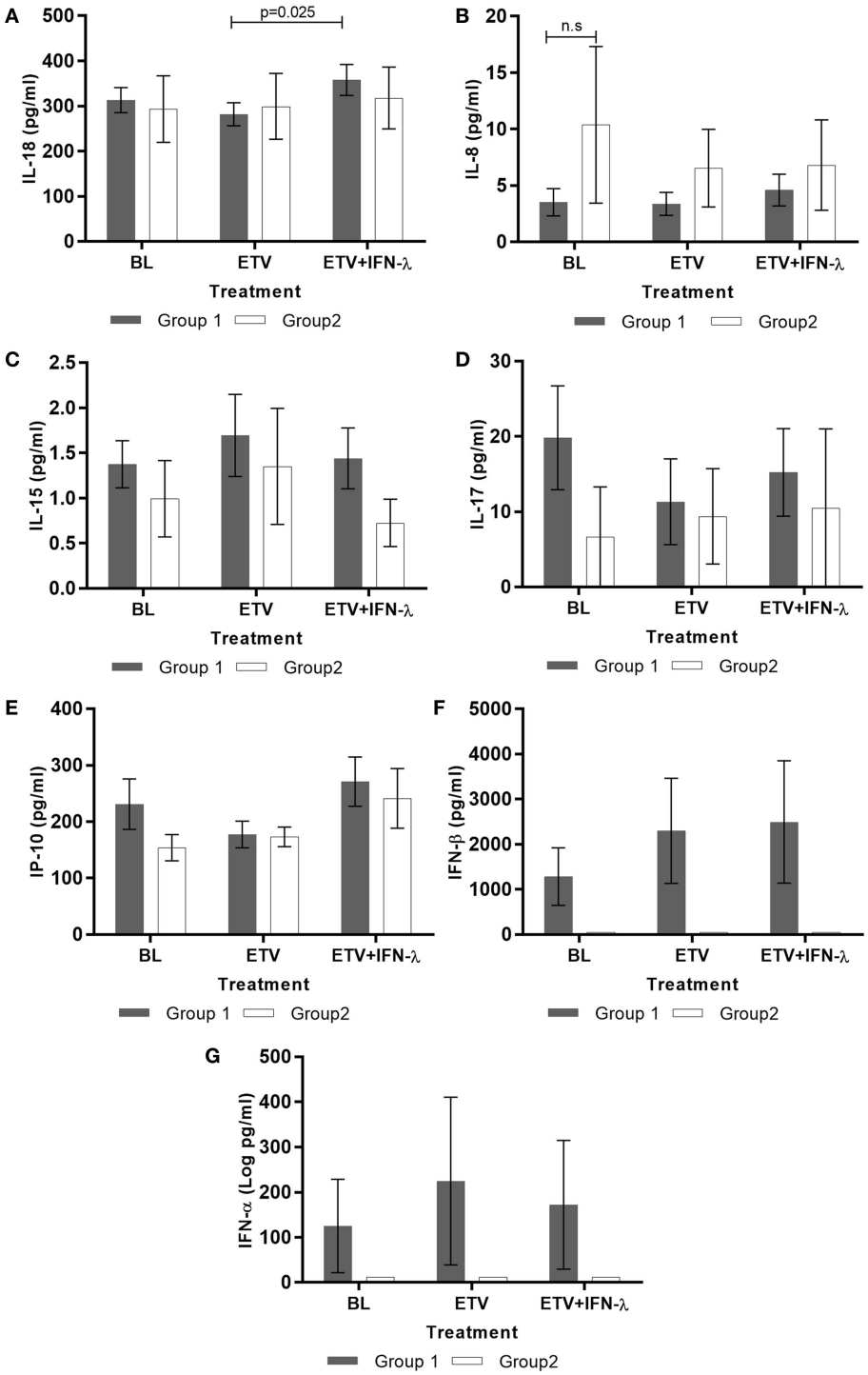

FIGURE 4 | **Effect of treatment on serum cytokines production during treatment in patients group 1 and group 2 (*n* = 13)**. Cytokines **(A)** IL-18, **(B)** IL-8, **(C)** IL15, **(D)** IL-17, **(E)** IP-10, **(F)** IFN-α, and **(G)** IFN-β were measured in the sera of patients by cytometric bead array or ELISA. Data are shown as mean ± SEM. Two-way ANOVA followed by multiple comparison tests were performed for statistical analysis.

T cells antiviral abilities. Further to this, we show that the spectrum of changes observed with IFNλ treatment are far wider than that observed with conventional IFN-α treatment in CHB patients.

Natural killer cells display two main effector functions that directly contribute to HBV infection control, direct killing of infected cells and the production of a variety of cytokines including the potent anti-HBV cytokine IFN-γ, which has directly

antiviral activities and activates and promotes downstream antigen-specific adaptive immune responses (60). In our study, we find an improvement in these two functions of NK cells when Peg-IFNλ is introduced and observe a notable increase of NK cells expressing TRAIL, an activation marker which induces target-cell apoptosis. Studies that investigated the modulation of NK cells during directly acting antiviral therapy have shown no effect on IFN-γ producing $CD56^{bright}$ NK cells; which allow us to conclude that the increase in IFN-γ-producing NK cells observed during add-on Peg-IFNλ is directly the result of IFNλ administration (36, 61). IFNλ mediated activation of cytolytic and non-cytolytic NK functionality is found in our study to be closely associated with reduction in viral replication rates and HBsAg levels. We therefore conclude that IFNλ induces a significant expansion of both cytotoxic and IFN-γ-producing NK cells in patients with the greatest decline in viral replication during ETV.

In addition to the activation of NK cell functionality during IFNλ therapy in Group 1 patients, we also observed significant restoration of the virus-specific T cell responses which are widely known to be pivotal to the host control of HBV replication long-term (43, 62). We observed an increase in the frequency of HBV-specific $CD4^+$ and $CD8^+$ T cells producing IFN-γ, which correlated strongly with the reduction of viremia and HBsAg. Further to this, we report an increase in the percentage of patients recognizing HBV antigens and peptide pools suggesting a diversification of epitope recognition and T cell activation. This is key for long-term control, as the ability of the immune system to attack multiple targets on a given pathogen has obvious advantages (63). Previous studies of ETV treatment of HBeAg-positive patients have reported partial restoration of HBV-specific $CD8^+$ T cells and may explain the small increase in IFN-γ producing HBV-specific $CD8^+$ T cells during ETV alone (64, 65). IFN-γ producing HBV-specific $CD4^+$ T cells, however, are not susceptible to this ETV-driven immune improvement. In parallel, during IFNλ treatment, we observe a temporal relationship between HBV-specific $CD8^+$ T cells and mild elevations of liver transaminases denoting destruction of infected hepatocytes, suggesting the mobilization of activated cytotoxic immune cells into the liver. We have previously shown that this equilibrium between cytolytic and non-cytolytic $CD8^+$ T cells functions is critical in control of infection without excessive exacerbation of inflammation and liver injury and this study reveals that IFNλ favorably maintains this balance (66).

It was not possible to delineate the direct mechanisms by which IFNλ activated NK and virus-specific T cell responses. We did explore whether this was mediated *via* the programmed death-1 pathway and found no modulation of the expression of this inhibitory pathway on NK or T cells *ex vivo* during the study period (data not shown). While further work will need to be performed to identify the specific pathways of IFNλ-mediated immune activation, our data does reveal a novel relationship between IFNλ and IL-18, particularly in patients that showed greatest decline in HBsAg levels during IFNλ treatment. This increase in IL-18 levels was however quite small and further studies are needed. The lack of changes in IFN-α and IFN-β plasma levels during IFNλ administration, is in line with previous *in vitro* work by Ank et al (67), suggests that this is also not the mechanism by which host antiviral immunity was induced.

The root cause for lack of immune reactivation in Group 2 could not be fully delineated due to the lack of sample availability. We had hypothesized that hyperexpression of the checkpoint inhibitor programmed death-1 may be partly responsible but this was not substantiated experimentally. This does not preclude the possibility of overexpression of other immune checkpoint inhibitors, on immune cells of group 2 patients, such as Tim-3 and CTLA-4 which have been documented to impair immune function in CHB (68). Further to this, multiple reports have suggested that mutations and splice variants in the HBV genome and lower pregenomic/precore RNA could negatively influence the response to interferon treatment (69–71) and this may also be responsible for lack of response observed in Group 2. Finally and possibly most likely, IFNλ intracellular signaling may have been disrupted by HBV-induced elevated levels of the suppressor of cytokine signaling SOCS 1 and 3 in Group 2 patients, thereby rendering IFNλ treatment ineffective (72, 73). Further in-depth studies addressing these possibilities are required to characterize and confirm the mechanisms underlying IFNλ non-responsiveness *in vivo*.

In this study, we have highlighted several differences in the immunoregulatory activities of IFNλ when compared to IFN-α. The dysregulation of the adaptive immune response, a hallmark of CHB, cannot be overcome by treatment with IFN-α (13). In fact, studies have shown that treatment with IFN-α actively results in the suppression of HBV-specific $CD8^+$ T cells (12, 14). It has been hypothesized that this is consequent to the known potent anti-proliferative effects of IFN-α. This suppressive effect of IFN-α is not confined to CHB and has been demonstrated in several other chronic viral infections (74–77). We show that add-on IFNλ treatment does not lead to the suppression but to the maintenance in the frequency of HBV-specific $CD8^+$ T cells producing IFN-γ. Their negative strong correlation with HBsAg levels further highlights the importance of these cells in the control of HBV infection. In further contrast, IFN-α does not seem to activate the cytotoxic capacity of NK cells to kill target cells (13), whereas in this investigation we reveal the ability of IFNλ to improve this important effector function. Our data also suggest that IFNλ mediates improvement of anti-HBV immunity *via* IL-18. In contrast, IFN-α is believed to activate NK responses *via* IL-15 (13).

Anti-HBs seroconversion, the marker of functional cure in CHB, was not seen in this study. It is well described that a decline of >1 Log HBsAg is predictive of sustained HBsAg loss in HBeAg-positive CHB patients (78, 79) and we would suggest that given the steady decline of HBsAg levels seen in Group 1 patients during IFNλ treatment, in concert with improvement in innate and adaptive immune responses in Group 1, we may have observed HBsAg loss, possibly followed by anti-HBs seroconversion post-treatment. Additionally, IFNλ was only administered for a truncated 32 weeks and treatment for at least 48 weeks might be needed to observe an on-treatment HBsAg loss and anti-HBs seroconversion especially due to the restricted distribution of IFNλ receptor. Regrettably, there was no posttreatment follow-up

due to the early cessation of the clinical trial, due to commercial reasons based on results from a parallel trial showing that non-inferiority of IFNλ to IFN-α was not met at week 24 (46).

In conclusion, this study has demonstrated for the first time a dual immunomodulatory effect of IFNλ on both the innate and adaptive arms of the immune response *in vivo* during chronic viral infection. When IFNλ is administered in patients with suppressed HBV replication rates, it can induce broad immune stimulatory properties and drive activation of cytokine-producing and cytotoxic NK cells, IFN-γ-producing HBV-specific $CD4^+$ T and maintenance of the antiviral and cytotoxic functions of HBV-specific $CD8^+$ T cells.

ETHICS STATEMENT

The study protocol was approved by the Institutional review board/Independent Ethics Committee at each recruitment site. An informed consent was obtained from all patients before enrolment at each site in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SP performed study design, performed experiments, analyzed results, and manuscript preparation. SM set up assays, conducted the immunological experiments, and acquired data. AR performed statistical analysis. HC performed experiments. SZ, CW, P-JC, C-YP, T-TC, SL, RG, M-SC, HW, MD, C-WK, MS, SP, KK, TH-L, and H-CC recruited patients, collected blood and serum samples, and isolated PBMC. MW-R and EC performed clinical and immunological trial design and manuscript preparation. RW funded the research and edited the manuscript. SC performed study design, analyzed results, and manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00621/full#supplementary-material.

FIGURE S1 | **Flow cytometry gating strategy for analysis of $CD56^{bright}$, $CD56^{dim}$ NK cells subsets**. **(A)** The lymphocyte population was gated on forward and side scatter. **(B)** The NK cells population was identified with the CD56 and CD3 markers. **(C)** CD56 and CD16 markers were used to identify $CD56^{bright}$ NK cells ($CD56^{bright}/CD16^-$) and $CD56^{dim}$ NK cells ($CD56^{dim}/CD16^+$) subpopulations. A total of 250,000 events were acquired. Analysis was performed using FACS diva software.

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Cell Polarization and Epigenetic Status Shape the Heterogeneous Response to Type III Interferons in Intestinal Epithelial Cells

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Type I and type III interferons (IFNs) are crucial components of the first-line antiviral host response. While specific receptors for both IFN types exist, intracellular signaling shares the same Jak-STAT pathway. Due to its receptor expression, IFN-λ responsiveness is restricted mainly to epithelial cells. Here, we display IFN-stimulated gene induction at the single cell level to comparatively analyze the activities of both IFN types in intestinal epithelial cells and mini-gut organoids. Initially, we noticed that the response to both types of IFNs at low concentrations is based on a single cell decision-making determining the total cell intrinsic antiviral activity. We identified histone deacetylase (HDAC) activity as a crucial restriction factor controlling the cell frequency of IFN-stimulated gene (ISG) induction upon IFN-λ but not IFN-β stimulation. Consistently, HDAC blockade confers antiviral activity to an elsewise non-responding subpopulation. Second, in contrast to the type I IFN system, polarization of intestinal epithelial cells strongly enhances their ability to respond to IFN-λ signaling and raises the kinetics of gene induction. Finally, we show that ISG induction in mini-gut organoids by low amounts of IFN is characterized by a scattered heterogeneous responsiveness of the epithelial cells and HDAC activity fine-tunes exclusively IFN-λ activity. This study provides a comprehensive description of the differential response to type I and type III IFNs and demonstrates that cell polarization in gut epithelial cells specifically increases IFN-λ activity.

Keywords: epithelial cell line, interferon-lambda, heterogeneous gene expression, cell polarization, small intestinal organoids

INTRODUCTION

The innate defense against viral infection in mammals is based on the coordinated action of type I and type III interferons (IFNs), which are produced by virus-infected and bystander cells (1–3). IFNs induce antiviral mechanisms within virus-infected and uninfected cells and contribute to the adaptive immune responses against viral pathogens (4, 5). Both IFN types reprogram gene expression through the same signal transduction pathway involving the formation of the ternary ISGF3 complex, composed of STAT1, STAT2, and IRF9 (6). Following nuclear translocation, the ISGF3 complex binds to the promoters of IFN-stimulated genes (ISGs) and regulates gene transcription. Thus, type

I and type III IFNs induce the expression of a highly overlapping set of genes and share biological activities in the affected cells (2, 7–10). A major difference between the type I and type III IFN-mediated antiviral systems is their engagement of different receptor chains. Whereas all type I IFNs utilize a heterodimeric receptor complex composed of IFN-αR1 and IFN-αR2 subunits, type III IFNs engage the IFN-λR1 (also known as IL28R) and IL10R2 receptor chains for signaling (2, 3). This allows a tissue- and cell-type-specific response. While the type I IFN receptor is found ubiquitously, expression of the IFN-λ receptor is mainly restricted to the epithelium of mucosal surfaces and also to a few other cell types such as hepatocytes in humans (11). Because most pathogens enter the host through mucosal surfaces, the IFN-λ-based antiviral response is the determining factor to establish the first line of defense against invading pathogens (12–14).

Apart from graded responses toward different concentrations of external stimuli, cells can adopt a metastable state with respect to the initiation of signaling events and show bimodal forms of responses. This generates a heterogeneous response within a cell population. This heterogeneity is a hallmark of embryonic cells and was shown to correlate with cell-specific patterns of transcription factor expression and chromatin modifications. While this heterogeneity has been extensively studied in stem cells during embryonic development (15), bimodal responses toward external signals were also found in differentiated cells as exemplified by immune responses to PAMPS or cytokines (16–18). Rand et al. demonstrated that type I IFNs, in particular at low concentrations, lead to the induction of ISGs and subsequent establishment of an antiviral state only in a fraction of cells of a clonal population, whereas others do not respond at all (19).

Although the type I and type III receptor complexes induce the same Jak-STAT signaling, they are structurally distinct and might thus exhibit differences in their signal propagation (20). Since the strength and kinetics of gene induction from type I and type III IFNs differ, we aimed at comparing signal transduction and gene activation in a controlled setting. We employed a recently established murine intestinal epithelial cell line (IEC) (21) and gut stem cell organoids generated from a transgenic fluorescent IFN response reporter mouse. Both culture systems are responsive to both types of IFNs and show properties such as cell polarization and differentiation that reflect critical functional aspects of the gut epithelium *in vivo* (21, 22). The use of the fluorescent reporter allowed us to monitor ISG induction at the cellular level and record the heterogeneity of responses to both IFNs in real time. Indeed, both types of IFNs installed a bimodal distribution of ISG expression within a clonal population. The extent of intrinsic heterogeneity was strongly manifested at low IFN concentrations and depended for IFN-λ on the cellular polarization status. The digital response was based on stochastic decisions downstream of STAT1 nuclear translocation, presumably at the transcriptional level within individual cells. Further experiments highlighted the importance of histone deacetylase (HDAC)-mediated epigenetic modifications during IFN-λ but not during type I IFN induction. Our results demonstrate significant differences in the response toward type I and type III IFNs and identify cell polarization and epigenetic modifications as underlying responsible mechanisms.

MATERIALS AND METHODS

Generation of the Bacterial Artificial Chromosome (BAC) Mx2tRFP

The BAC clone RP24-71I6 containing the murine Mx2 locus was obtained from BACPAC resource center. Homologs recombination was performed using the bacteriophage λ recombination system (23). Thereby, the open reading frame of the murine Mx2 gene was replaced by a linear fragment containing the amplified reporter TurboRFP (Evrogen) followed by an SV40 polyadenylation signal and an FRT (FLP recognition target) flanked cassette harboring a prokaryotic promoter, the PGK-promoter, a gene encoding for kanamycin/neomycin phosphotransferase and the bovine growth hormone polyadenylation signal. Primers used: Mx2Phom+Fluc2: 5′-TTA TAA TAT TCA TTT CCC ACA GAG TAC CCA ACT GAG AGA AGA AAT AAA AGA TGG AAG ATG CCA AAA ACA TTA AGA-3′ and Mx2Exon14hom+*Bam*HI: 5′-AAA GAA AAG TGG TTT ATT AAG GAA TGC AAC AGG CAG CTC CCA TTT GTA CAC TCA AGG GCA TCG GTC GAC GGA TCC-3′. Modified BAC DNA was isolated using NucleoBond BAC100 (Macherey-Nagel).

Cell Lines, Virus Infection, and Reagents

The intestinal epithelial cell line IEC-Mx2Luc-10 was generated from a transgenic mouse containing the firefly luciferase gene under the transcriptional control of the Mx2 promoter region as described earlier (21). The cell line IEC Mx2tRFP was established by transfecting the BAC Mx2tRFP into IEC-Mx2Luc-10. After selection, clones were picked and tested for similarity in morphology, barrier formation, and reactivity to type I and type III IFNs compared to those of the parental cell line. A representative cell clone showing stable expression of the reporter and efficient barrier formation indicated by an increase in the trans-epithelial electrical resistance (TEER) was selected. IECs were stimulated with IFN-β or IFN-λ3 (PBL Assay Science) and treated with the HDAC inhibitors valproic acid (VPA) (750 μM, Sigma-Aldrich), TSA (2 μM, Sigma-Aldrich), and MS275 (0.51 or 1.7 μM, Selleckchem) and the Bromodomain inhibitor I-BET151 (250, 500, or 800 nM, Cellagentech) as described in the figure legends. IECs were pre-stimulated with IFN-β or IFN-λ3 for 24 h. Infection with vesicular stomatitis virus (VSV) containing an EGFP reporter (24) was performed after washing with serum-free medium. After 1 h of infection, residual virus was removed by washing three times with serum-containing medium.

Barrier Formation and Polarization

Intestinal epithelial cell lines were grown until fully polarized in transwell cultures as already described earlier (21). 3×10^5 cells were grown on 0.4 μM pore sized transwell inserts (Costar). The culture medium was renewed every third day. The TEER was measured by a chopstick electrode with Volt/Ohm meter (World Precision Instruments). TEER values are reported as $\Omega^{*}cm^2$, i.e., the resistance in Ohm multiplied by the surface area of the transwell insert. The resistance value for the transwell insert without cells was subtracted as the basal resistance.

Organoid Derivation and Cultivation

Organoids were cultured from crypt-enriched jejunal and ileal fractions from 6- to 12-week-old Mx2tRFP mice as previously described (22). Briefly, a 10 cm midsection of the small intestine was excised and flushed with ice cold PBS. After removal of mucus and villi, the intestine was cut into 1–2 cm pieces and washed extensively with cold PBS. The epithelium was dissociated for 30 min at 4°C in a solution of 2 mM EDTA in PBS. Afterward, the crypts were suspended in 10% FCS in PBS and passed through a 70-mm cell strainer (BD Biosciences), centrifuged at 200 *g* (5 min, 4°C), and resuspended in 10 ml Ad-DF medium [advanced DMEM/F12 supplemented with 1% Glutamax (Invitrogen), 10 mM HEPES, and 100 U/ml of Penicillin/Streptomycin]. After centrifugation, the crypts were resuspended in Matrigel (BD Biosciences) at a desired crypt density. 20 µl Matrigel was seeded per well on a pre-warmed 48-well flat-bottom plate and incubated for 30 min at 37°C and 5% CO_2 atmosphere. Then, 300 µl of Intesticult organoid growth medium (Stemcell Technologies) was added. The passaging was performed every 1–2 weeks with a split ratio of 1:3 by harvesting the organoids, mechanic disruption into single crypt domains, and seeding with fresh Matrigel.

Antibodies and Western Blotting

Primary antibodies for Western blot analysis were purchased from Cell Signaling Technology (STAT1 Antibody #9172; Phospho-STAT1 (Tyr701) (58D6) Rabbit mAb #9167) and from Santa Cruz Biotechnology (β-Actin (ACTBD11B7) sc-81178). For generation of whole cell extracts, cells were lysed in RIPA buffer (10 mM Tris–HCl, pH 7.5, 150 mM Sodium chloride, 1% Triton X-100, 0.1% Sodium dodecyl sulfate, 1% Sodium deoxycholat, 1 mM Dithiothreitol, 1 mM Sodium orthovanadate, 1 mM Sodium fluoride, 1× HALT™ Protease Inhibitor Cocktail). Whole cell extracts were diluted in 4× NuPAGE® LDS Sample Buffer (Invitrogen), and proteins were separated by denaturing SDS-PAGE in a 10% separation gel (10% Acrylamide/Bis (37.5:1), 0.375 M Tris pH 8.8, 0.1% Sodium dodecyl sulfate, 0.001% TEMED, 0.1% Ammonium persulfate). Proteins were transferred to an activated PVDF membrane, and the membranes were washed three times in TBST, blocked with TBST containing 5% milk powder, and probed by incubation with primary antibodies, followed by incubation with a horse-radish peroxidase-conjugated antibody (Amersham). Luminescence signal was detected by either ECL Advance® (Amersham) or ECL Prime® (Amersham) according to the manufacturer's instructions. Luminescence was measured using the ChemiDoc XRS system and quantified with Quantity One (Bio-Rad) or ImageJ.

Luciferase Assay

Cells were washed once in cold PBS and incubated with adjusted amounts of reporter lysis buffer (RLB, Promega) at −70°C for 20 min. Cell lysates were assayed for luciferase activity using standard reaction buffer (20 mM glycylglycine, 12 mM $MgSO_4$, 1 mM ATP) containing luciferin (Promega) and a single tube luminometer (Lumat LB 9507, Berthold Technologies).

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation analysis was performed using the ChIP-IT High-Sensitivity Kit (Active Motif) in accordance with the manufacturer's protocol. Cells were grown to near-confluency in a 100-mm culture dish. In brief, after crosslinking, the cell pellet was suspended in Lysis buffer (20 mM Tris–HCl pH8.0, 85 mM KCl, 0.5% NP-40, PMSF, Protease Inhibitor cocktail, 1 µM TSA) and incubated for 10 min on ice. Following centrifugation, nuclei were lysed in Nuclei Lysis buffer (50 mM Tris–HCl pH8.0, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% Na-Deoxycholate, PMSF, Protease Inhibitor cocktail, 1 µM TSA). Bioruptor™ sonicator (Diagenode) was used to shear chromatin. Immunoprecipitation was performed overnight at 4°C with H3K9ac antibody (Active Motif, #39137). Real-time quantitative PCR was performed using SYBR Green I Master Mix (Roche Applied Sciences) in a LightCycler480 II (Roche Applied Sciences) with specific primers. Primers were designed to amplify proximal promoters containing ISRE site(s). IFIT1, 5′-GTCTGTATCCGTTTCAGAGC-3′ (forward), 5′-GAACAGG GAAATCCTTACCC-3′ (reverse); IRF7, 5′-GAAGGGCAGTGA AGAGAAGC-3′ (forward), 5′-GTCACAGGTGTTAATCCAGC-3′ (reverse); Rsad2, 5′-TCACTGCCTTTCCTTGGCTT-3′ (forward), 5′-GCCTGCAAGGATGCAGCTAT-3′ (reverse). Input C_T values were adjusted for dilution and used to calculate % input values for immunoprecipitated samples.

qRT-PCR

RNA was isolated from IECs using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and quantified with an ND-1000 spectrophotometer (NanoDrop Technologies). Total RNA from intestinal organoids was isolated using Trizol LS reagent (Life Technologies) according to the manufacturer's instructions. 2 µg of total RNA was used for cDNA synthesis using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). RT-PCR was run at 58°C annealing temperature using SYBR Green I Master Mix (Roche Applied Sciences) in a LightCycler480 II (Roche Applied Sciences). Data were processed using Light Cycler 480 Software 1.5. The mRNA levels were normalized to those of β-Actin gene. Murine PCR primers for β-Actin (forward primer, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and reverse primer, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′), IRF-7 (forward primer, 5′-GAA GAC CCT GAT CCT GGT GA-3′ and reverse primer, 5′-CCA GGT CCA TGA GGA AGT GT-3′), Mx2 (forward primer, 5′-TCA CCA GAG TGC AAG TGA GG-3′ and reverse primer, 5′-CAT TCT CCC TCT GCC ACA TT-3′), Rsad2 (forward primer, 5′-GTC CTG TTT GGT GCC TGA AT-3′ and reverse primer, 5′-GCC ACG CTT CAG AAA CAT CT-3′), Usp18 (forward primer, 5′-CAT CCT CCA GGG TTT TCA GA-3′ and reverse primer, 5′-AAG GAC CAG ATC ACG GAC AC-3′), IFI44 (forward primer, 5′-AAC TGA CTG CTC GCA ATA ATG T-3′ and reverse primer, 5′-GTA ACA CAG CAA TGC CTC TTG T-3′), IFIT1 (forward primer, 5′-TGT TGA AGC AGA AGC ACA CA-3′ and reverse primer, 5′-TCT ACG CGA TGT TTC CTA CG-3′), IL28R (forward primer, 5′-CCC TGT TTC CTG ACA CTC CC-3′ and reverse primer, 5′-TCA GAA AAG TCC AGT GCC CG-3′), and IFNAR2 (forward primer, 5′-CTA TCG TAA TGC TGA AAC GG-3′ and reverse primer, 5′-CGT AAT TCC ACA GTC TCT TCT-3′).

Flow Cytometry and Immunofluorescent Staining

Intestinal epithelial cell lines were seeded in 12-well plates and treated as previously described (21). Flow cytometry analysis was performed on an LSR-II SORP and FACS-Calibur (BD Biosciences). Fluorescence-activated cell sorting was performed on an ARIA-II SORP (BD Biosciences). Data were processed using FlowJo v7.6.5 (Tree Star, Inc.). For immunofluorescent staining, IECs were seeded in an 8-well chamber slide (ibidi) and treated as described. After fixation with 4% formaldehyde, cells were washed with PBST (0.02% Tween in PBS), blocked with 1% BSA in PBS, and stained with Phospho-STAT1 (Tyr701) (Cell Signaling Technology, 58D6, Rabbit mAb) antibody for 1 h at room temperature. After washing, the samples were incubated with FITC-labeled goat anti-Rabbit antibody for 1 h at room temperature. Fluoroshield with DAPI was added for nuclear staining and fluorophore protection. The samples were examined under a Zeiss 510 Laser Scanning confocal microscope.

Image and Statistical Analysis

Microscopic picture series were analyzed using ImageJ (NIH, Bethesda, MD, USA) and built-in plugins as well as MTrackJ (E. Meijering). All data analyses were performed using GraphPad Prism v5.04 (Graph Pad Software, La Jolla, CA, USA). Results were presented as mean value ± SEM from triplicates or from numbers indicated in the figure legend. Statistical significance was tested using one-way ANOVA, followed by Tukey's Multiple Comparison test, the non-parametric unpaired Mann–Whitney *U* test, and Student's *t*-test. The statistical test used for each analysis is mentioned in the respective figure legend. *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

Bimodality of Gene Induction in Responses to Type I and Type III IFNs

In order to investigate the differences in type I and III IFN-mediated signaling, we used a recently described IEC that was derived from a transgenic mouse expressing a firefly luciferase reporter under the transcriptional control of the IFN-dependent mouse Myxovirus resistance gene 2 (Mx2) promoter (21). Stimulation of IECs by either IFN-β or IFN-λ3 revealed a significant increase in total luciferase activity over time (Figure S1A in Supplementary Material). However, IFN-β-mediated Mx2Luc gene induction was rapid and peaked between 6 and 9 h after stimulation, followed by a steady decrease to half-maximal activity at 48 h. In contrast, IFN-λ3 induced a delayed but gradual increase in Mx2-driven luciferase activity for up to 48 h. Maximal Mx2 promoter activity following IFN-λ3 stimulation was 5–6 times lower than that induced by IFN-β (Figure S1B in Supplementary Material).

To investigate the timing and dynamic of gene expression in individual cells of a given population, we next generated a clonal IEC line harboring a BAC encoding TurboRFP under the control of the Mx2 promoter region (Mx2tRFP). Mx2tRFP cells were stimulated with increasing concentrations of either type of IFN, and flow cytometric analysis was performed. Stimulation with IFN-β concentrations above 100 U/ml induced Mx2tRFP expression on average in 90% of the cells (**Figures 1A,B**). In contrast, stimulation with low doses (between 2 and 10 U/ml) of IFN-β left the majority of cells unresponsive and induced Mx2-tRFP expression only in a small fraction of cells. Of note, clonal cell populations were employed for these experiments, indicating that a portion of cells did not properly respond at the time point of IFN stimulation. Mean fluorescence intensity (MFI) determination of the tRFP-positive population indicated a continuous rise in the mean Mx2 promoter activity in individual cells with increasing IFN-β concentrations (**Figure 1D**). Thus, both the number of cells and the individual cell response increased with increasing IFN-β concentrations. This was also reflected by the continuous increase in Mx2-driven total luciferase activity upon stimulation with increasing doses of IFN-β (Figure S1B in Supplementary Material). Strikingly, stimulation with IFN-λ3 induced the same pattern of digital Mx2tRFP expression characterized by a concentration-dependent gradual increase in the fraction of responding cells. Yet, the tRFP-positive cell fraction even at the highest IFN concentration did not exceed 55% of the total population (**Figures 1A,C**). Interestingly, only a marginal increase in the MFI of the tRFP-positive population could be observed after IFN-λ3 stimulation (**Figure 1E**). The reduced ability of high-dose IFN-λ compared to that of IFN-β to induce gene expression in individual cells was confirmed for other ISGs by qRT-PCR (Figure S1C in Supplementary Material). Thus, stimulation of IECs with IFN-λ resulted in Mx2 promoter induction, characterized by a limited number of responding cells and lower levels of gene expression. This is in contrast to type I IFN stimulation, where no such limitations could be observed. As published earlier (8, 25), we observed that the kinetic of ISG induction differs for both IFN types as IFN-λ induces a delayed expression of the Mx2tRFP reporter (Figure S1D in Supplementary Material). Importantly, stimulation with low doses of either IFN-β or IFN-λ resulted in a heterogeneous pattern of Mx2tRFP induction with highly responsive and completely non-responsive subpopulations. This is reminiscent of the IRF-7-mCherry reporter induction by IFN-β in mouse fibroblasts (19).

Mx2 is one out of many IFN-induced proteins that protect cells against viral infection. To confirm that the observed bimodality of Mx2tRFP expression correlates with the induction of an antiviral state, i.e., reflects the induction of ISGs, IFN-pretreated IECs were subjected to VSV infection. IECs harboring Mx2tRFP were stimulated with either 10 U/ml IFN-β or 25 ng/ml IFN-λ3 for 20 h and subsequently infected with a recombinant VSV constitutively expressing eGFP (VSV-GFP). Fluorescence microscopy revealed that Mx2tRFP-positive cells were protected against infection with VSV, whereas tRFP-negative cells remained susceptible to viral infection (**Figure 2A**). This inverse correlation between Mx2tRFP expression and VSV-GFP replication was observed for both types of IFNs, IFN-β and IFN-λ3, arguing for comparable thresholds of antiviral activity. Thus, the Mx2 reporter reflects the coordinated induction of at least a group of ISGs sufficient to provide protection against viral replication. These results also indicate that the antiviral state is an unpredictable all-or-nothing

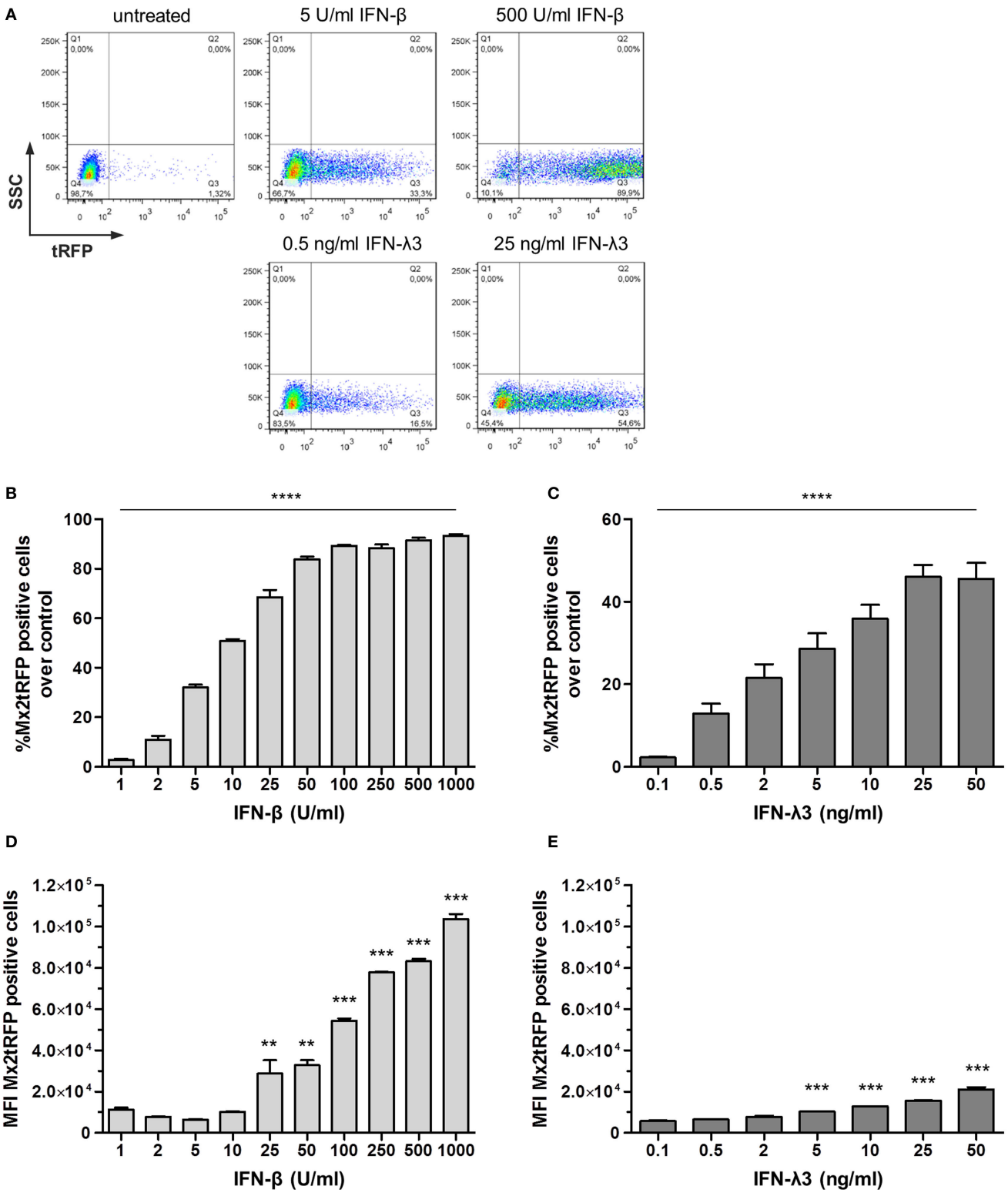
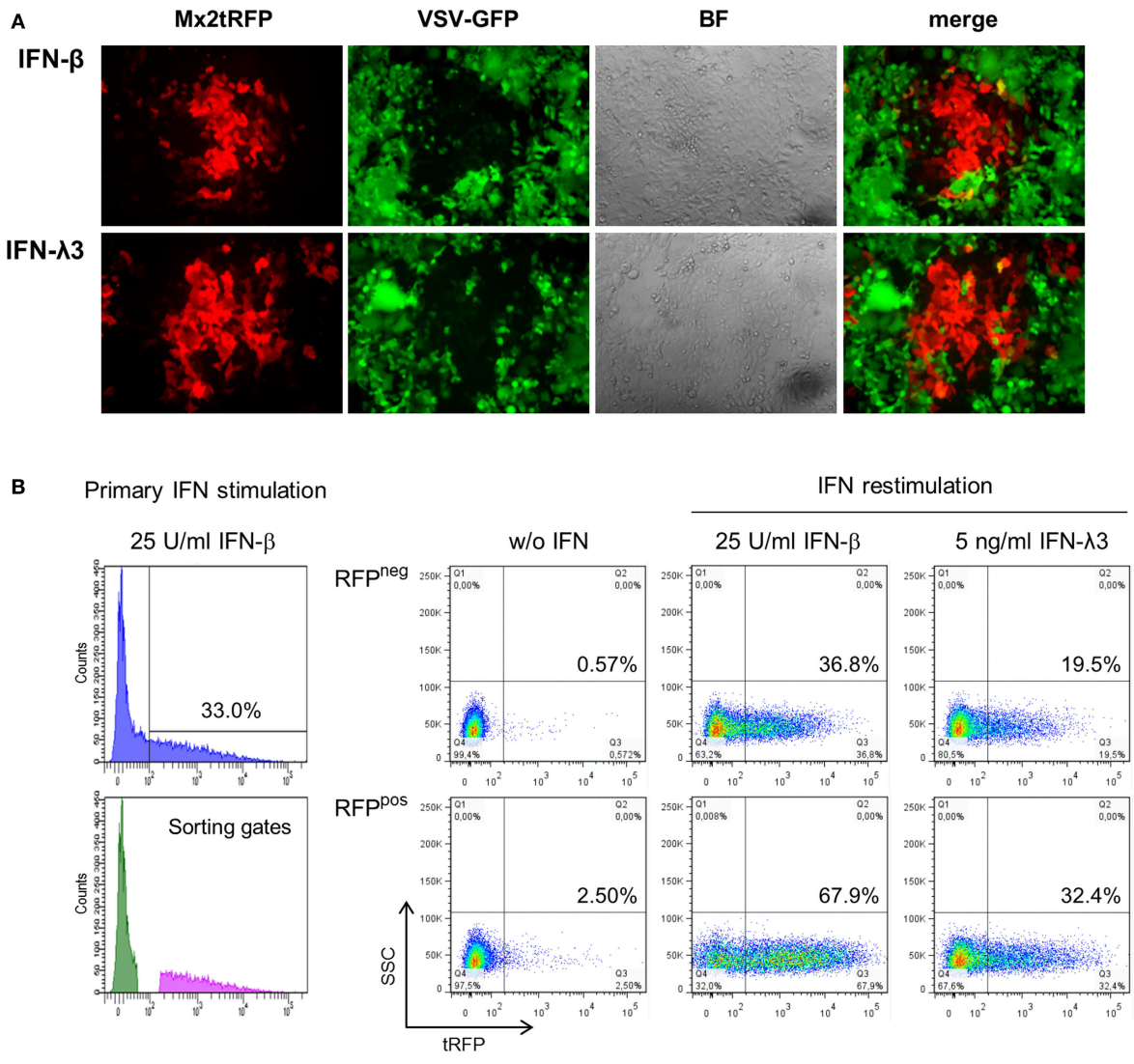


FIGURE 1 | Bimodal nature of gene expression toward type I and type III interferon (IFN) stimulation. Intestinal epithelial cell lines harboring the bacterial artificial chromosome construct Mx2tRFP were stimulated with different concentrations of IFN-β and IFN-λ3 for 20 h. Expression of the tRFP reporter was measured by flow cytometry (n = 5–7, mean ± SEM). **(A)** Representative dot plots show Mx2tRFP expression at high and low concentrations of IFN-β and IFN-λ3. **(B,C)** Percentage of Mx2tRFP-positive cells for all used concentrations of IFN-β and IFN-λ3. P values were calculated by one-way ANOVA. **(D,E)** Mean fluorescence intensity (MFI) of Mx2tRFP-postive cells. P values were calculated by one-way ANOVA, followed by Tukey's Multiple Comparison Test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). P values are given for differences among each stimulated group and control group.

decision of individual cells. While higher concentrations of IFN-β lead to a protection of the vast majority of cells within a culture, saturating amounts of IFN-λ3 induced Mx2tRFP expression only in a maximum of 50% of the cells and failed to protect the whole IEC population from viral replication (**Figure 2A**).

To test whether the bimodal ISG expression pattern during stimulation with low doses of IFN was stable or showed short-term variability, we performed stimulation-sort-restimulation experiments. First, IECs harboring Mx2tRFP were stimulated with a low dose of IFN-β and sorted into non-responding ("non-responder," tRFP-negative) and responding ("responder," tRFP-positive) populations (Figure S2A in Supplementary Material). Both populations were cultivated separately for 48 h in the absence of IFN to allow the reporter signal to decrease to baseline levels. Subsequently, cells were restimulated with both types of IFNs, and the Mx2tRFP expression was monitored by flow cytometry.

When restimulated, none of the two populations maintained Mx2tRFP expression pattern in the first stimulation. Cells from the non-responder population behaved like naïve cells following primary stimulation with approximately 35% Mx2tRFP-positive cells upon IFN-β exposure (**Figures 2B,C**). In contrast, the frequency of Mx2tRFP expression and the mean strength of the tRFP signal in the responder cell fraction were highly enhanced upon secondary IFN stimulation. However, approximately 35% of the responder population showed no detectable Mx2tRFP induction upon IFN-β restimulation. Notably, the responsiveness toward restimulation with IFN-λ also increased strongly in the responder population (**Figures 2B,C**). Restimulation experiments were also conducted with IFN-λ as a primary inducer. Again, upon IFN-λ3 restimulation, Mx2tRFP expression was induced in the non-responding population to frequencies comparable to those of naïve cells, whereas "responder" cells showed



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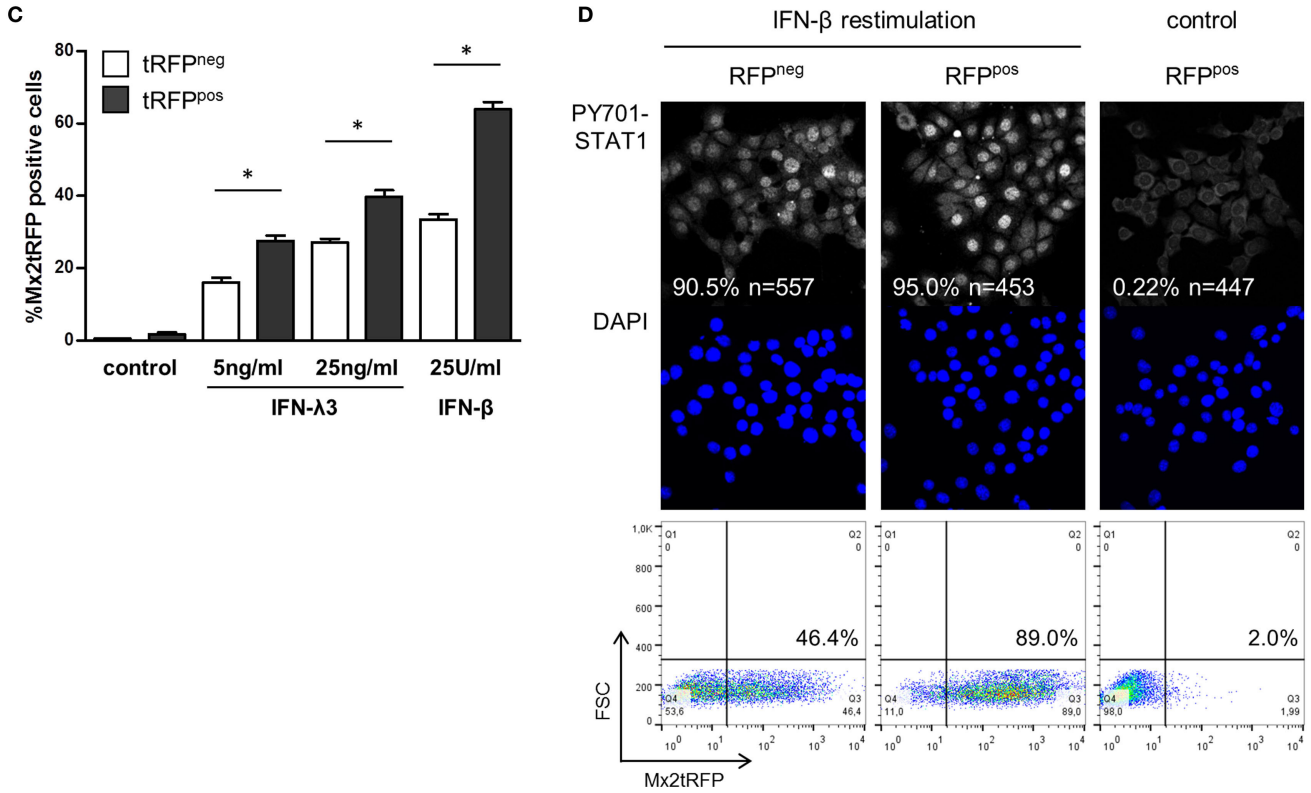


FIGURE 2 | "Memory" effect in IFN-stimulated cells. **(A)** Intestinal epithelial cell lines (IECs) harboring Mx2tRFP were stimulated with either 10 U/ml interferon (IFN)-β or 25 ng/ml IFN-λ3 for 20 h. Cells were infected with vesicular stomatitis virus-GFP (MOI 0.02) for 1 h and imaged by confocal microscopy 20 h post-infection. Representative pictures are shown. Data are representative of three independent experiments. **(B)** IECs were stimulated for 24 h with 25 U/ml IFN-β, and Mx2tRFP-positive and -negative populations were separated by cell sorting. Cells were cultured for 48 h in the absence of IFN, and responder and non-responder populations were restimulated for 20 h with the indicated concentrations of IFN-β and IFN-λ3. Mx2-driven tRFP expression was measured by flow cytometry. Representative FACS dot plots are shown. **(C)** Frequencies of Mx2tRFP expression in responder and non-responder populations upon IFN restimulation were calculated from two independent experiments ($n = 4$, mean ± SEM). *P* values were calculated by Mann–Whitney U test (*$P \leq 0.05$). **(D)** Sorted Mx2tRFP-negative and Mx2tRFP-positive cell populations were cultured for 48 h in the absence of IFN. Both populations were stimulated for 1 h with 50 U/ml IFN-β, fixed, and processed by immunofluorescence staining against P-Y701 STAT1. Control column represents Mx2tRFP-positive cells without IFN treatment. Images were obtained by confocal microscopy, and the percentage of cells showing activated STAT1 in the nucleus was calculated (*n* indicates the number of analyzed cells). Representative pictures are shown. DAPI staining indicates localization of cell nuclei. Dot plots show representative Mx2tRFP expression analysis from (B) after restimulation with 50 U/ml IFN-β for 20 h.

a much higher percentage of reporter gene induction (Figure S2B in Supplementary Material). In summary, we conclude that the ability to realize ISG induction in response to low amounts of IFN depends on short-term variables, and we further exclude the existence of a stable fraction of IFN-λ-unresponsive cells within the propagated (clonal) IEC line.

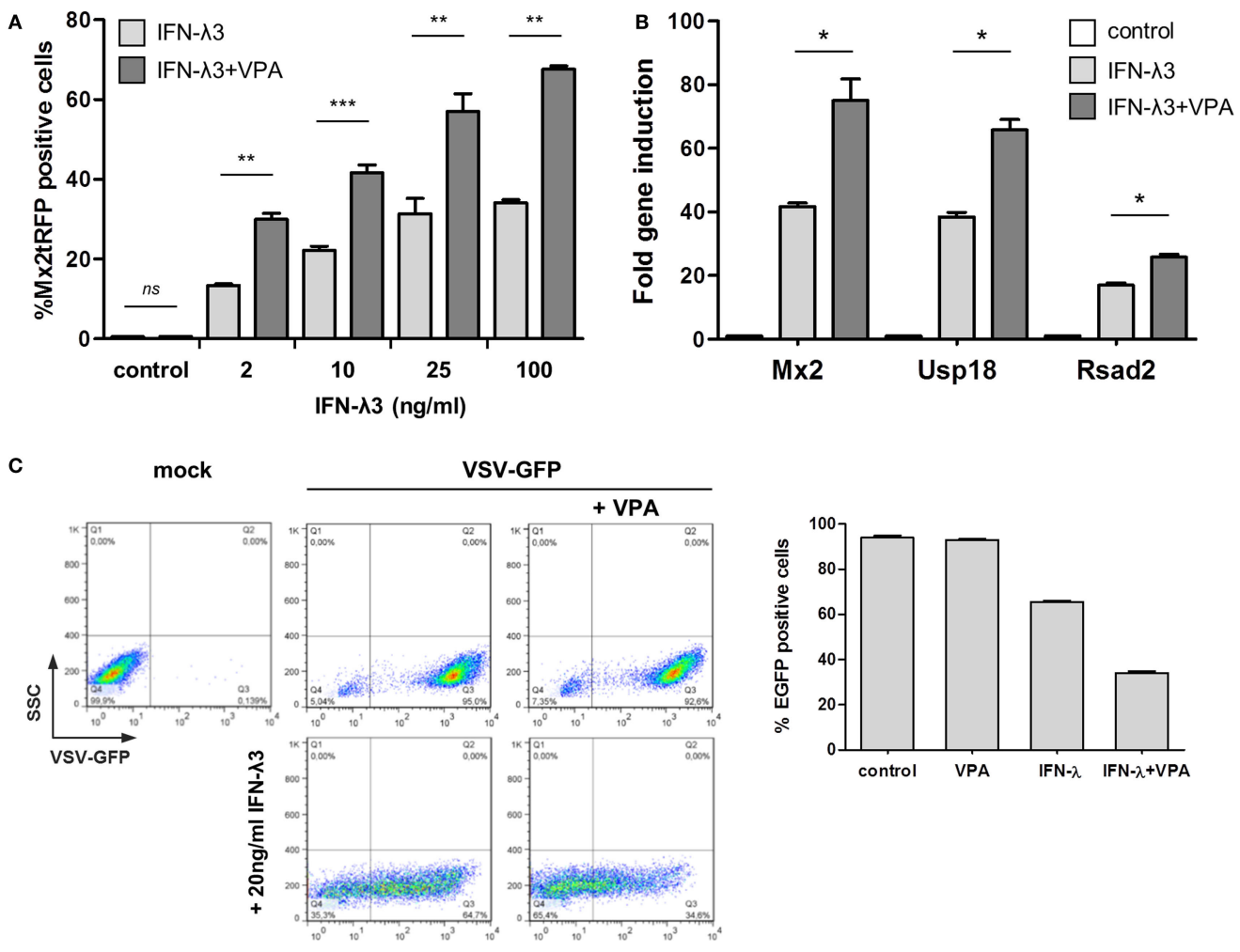
Thus, an intrinsic alteration in the "responder cells" has the capacity to increase the sensitivity toward further IFN stimulation. To address the nature of this intrinsic priming, we determined the frequency of cells with STAT1 nuclear accumulation in each of the responder and non-responder cell populations upon IFN restimulation. The responder and non-responder cell populations were restimulated with 50 U/ml IFN-β for 1 h, fixed, and stained for STAT1-Y701 phosphorylation. Using confocal microscopy, the number of cells staining positive for activated STAT1 in the nucleus was evaluated (**Figure 2D**). Unexpectedly, the frequency of nuclear phospho-STAT1-positive cells was similar between the tRFP-positive ("responder") and tRFP-negative ("non-responder") populations. In both populations, IFN-β stimulation was sufficient to induce nuclear accumulation of Y701-phosphorylated STAT1 in more than 90% of the cells. Thus, IFNAR-dependent STAT1 activation is not significantly influenced by prior IFN stimulation and can thereby be excluded as the underlying mechanism for bimodal cell responses. Instead, it appears likely that the cellular heterogeneity results from alterations at the level of gene transcription, suggesting that epigenetic differences account for the differential reaction toward IFN.

HDAC Inhibition Enhances the Frequency of Responsiveness upon IFN-λ Stimulation

To test the possible influence of epigenetic alterations on the cellular response to IFNs, the effect of HDAC inhibitors on the frequency of ISG induction upon IFN stimulation was examined.

As shown above, even saturating concentrations of IFN-λ3 failed to induce Mx2tRFP expression in all cells and to establish protection from VSV infection. However, addition of VPA, which inhibits class I and II HDACs (with a high potency for class I HDACs), was able to significantly increase the frequency of responding cells for all tested concentrations of IFN-λ3 (**Figure 3A**). This effect was confirmed for other ISGs by qRT-PCR (**Figure 3B**). Finally, VPA added together with IFN-λ3 elevated antiviral activity (**Figure 3C**). We also determined Mx2-driven Luciferase activity in the cell line IEC-Mx2Luc-10 upon HDAC inhibition. Stimulation with IFN-λ3 in the presence of VPA led to a strongly increased Mx2-Luciferase expression (Figure S3A in Supplementary Material). In contrast, VPA addition did not modulate Mx2 reporter gene activity of IFN-β to the same extent (Figure S3A in Supplementary Material). Accordingly, the frequency of Mx2tRFP expression in cells stimulated with different doses of IFN-β was not affected by the presence of VPA (Figures S3B,C in Supplementary Material). Of note, the effect of VPA was only observed when administered simultaneously with type III IFN; pretreatment of cells with VPA did not change the frequency of Mx2tRFP induction upon subsequent IFN-λ stimulation (Figure S3D in Supplementary Material). In addition, other HDAC inhibitors such as MS275 and TSA induced a similar increase in the number of Mx2tRFP-expressing cells upon IFN-λ3 stimulation but did not affect IFN-β activity (**Figure 3D**; Figure S3E in Supplementary Material). Together, HDAC inhibition enhances the ability of activated STATs to install ISG expression, indicating the involvement of epigenetic regulatory mechanisms in ISG gene expression upon IFN-λ stimulation.

Next, we interfered with the recruitment of readers of histone acetylation using the BRD3/4-specific inhibitor I-BET151. Inversely, the addition of I-BET151 suppressed IFN-λ3-dependent Mx2tRFP induction completely, whereas the ability of high concentrations of IFN-β to fully activate the Mx2 promoter was not impaired (**Figure 3E**; Figure S3A in Supplementary Material) and slightly reduced the frequency of Mx2-driven tRFP and luciferase reporter gene expression at low doses of IFN-β (20 U/ml IFN-β) (**Figure 3E**; Figure S3A in Supplementary Material). Thus, the threshold levels for IFN-λ-stimulated ISG expression depend on histone acetylation. Notably, VPA does not override the effect of



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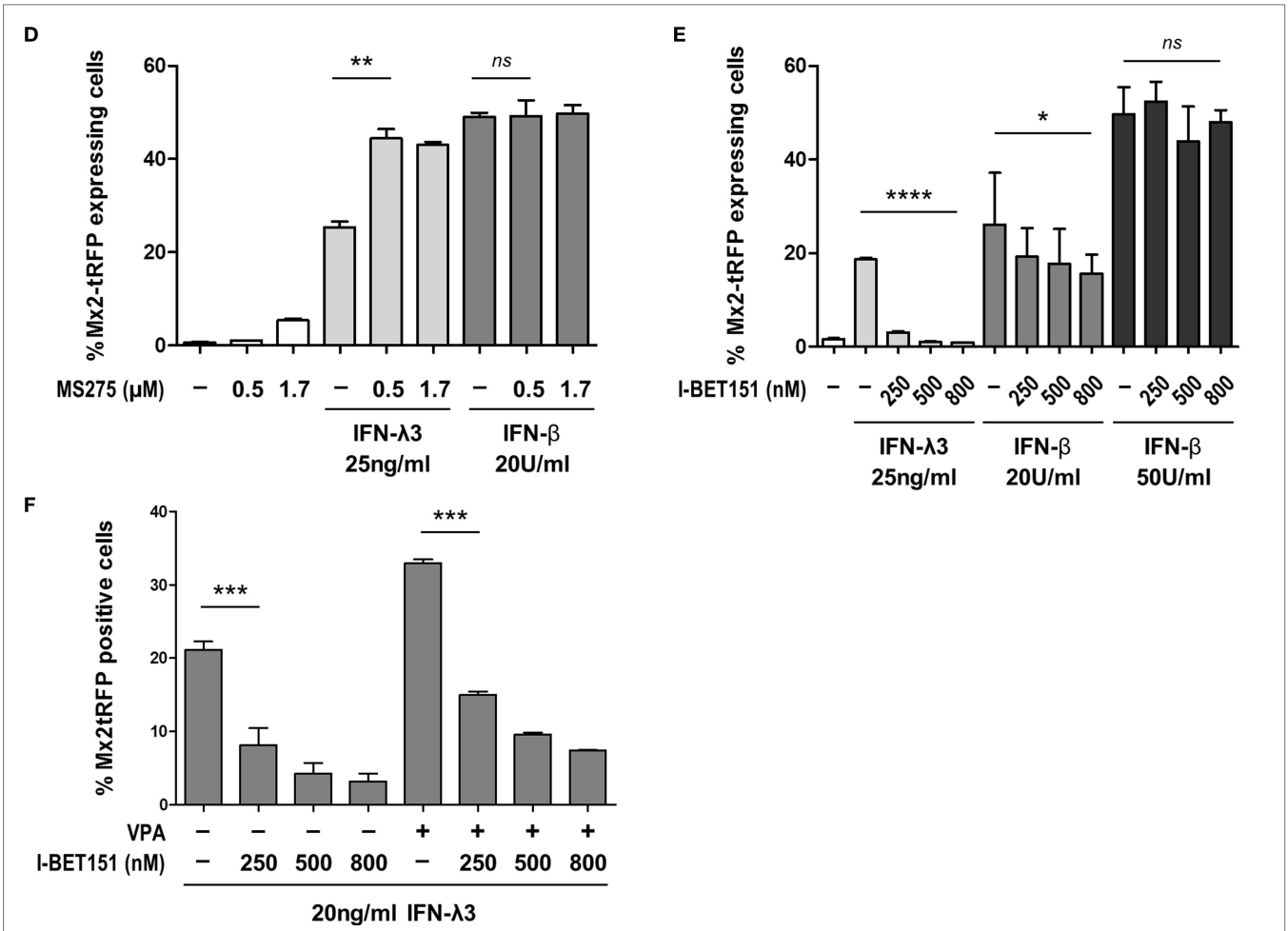

FIGURE 3 | Differential sensitivity of type I and type III interferons (IFNs) to inhibition of histone deacetylase and BRD3/4. **(A)** Intestinal epithelial cell lines (IECs) harboring Mx2tRFP were stimulated with increasing concentrations of IFN-λ3 in the absence or presence of 750 μM valproic acid (VPA). Frequency of Mx2tRFP expression was determined by flow cytometry after 24 h (n = 3–6, mean ± SEM). P values were calculated by paired t-test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). **(B)** IECs were either untreated or treated with 10 ng/ml IFN-λ3 in the absence or presence of 750 μM VPA for 16 h. RNA was isolated, and qRT-PCR was used to determine the expression of Mx2, Usp18, and Rsad2. IFN-stimulated gene expression was normalized to β-Actin (n = 3, mean ± SEM). *$P \leq 0.05$ by Mann–Whitney U test. **(C)** IECs were stimulated with 20 ng/ml IFN-λ3 in the absence or presence of 750 μM VPA for 20 h (lower panel). Control cells were not treated with IFN-λ (upper panel). Cells were infected with vesicular stomatitis virus-GFP (MOI 1) for 1 h and analyzed for eGFP expression by flow cytometry 8 h post-infection. Representative FACS dot plots show the percentage of eGFP-expressing cells. Graph represents mean eGFP frequency for each condition (n = 3, mean ± SEM). **(D)** IECs harboring Mx2tRFP were stimulated with 20 U/ml IFN-β or 25 ng/ml IFN-λ3 in the absence or presence of the indicated concentrations of MS275. Frequency of Mx2tRFP expression was determined by flow cytometry after 24 h (n = 3, mean ± SEM). **$P \leq 0.01$ by paired t-test. **(E)** IECs were stimulated with IFN in the absence or presence of the indicated concentrations of I-BET151. Percentages of Mx2tRFP-positive cells are given (n = 3–9, mean ± SEM). P values were calculated by one-way ANOVA (*$P \leq 0.05$, ****$P \leq 0.0001$). **(F)** IECs harboring Mx2tRFP were stimulated with 20 ng/ml IFN-λ3 in the absence or presence of 750 μm VPA. As indicated, different concentrations of I-BET151 were added during stimulation, and flow cytometry was performed after 24 h (n = 3, mean ± SEM). P values were calculated by one-way ANOVA, followed by Tukey's Multiple Comparison Test (***$P \leq 0.001$).

I-BET151 (**Figure 3F**), confirming the dependence of BRD3/4 action on histone acetylation for ISG induction. We asked whether the effect of HDAC inhibition would be manifested on ISG promoters. Thus, IECs were stimulated with IFN-λ3 and a medium IFN-β concentration that induces Mx2tRFP expression in less than 50% of cells in the absence or presence of VPA. ChIP experiments were performed, and proximal promoter regions of three prototypical ISGs were assayed for histone H3K9 acetylation that is known to be sensitive toward treatment with VPA (26, 27). All tested ISG promoter regions showed increased levels of H3K9 acetylation upon IFN-λ3 stimulation (Figure S3F in Supplementary Material). Interestingly, histone H3K9 acetylation was slightly reduced by the addition of VPA, indicating that these sites are not the target of the HDAC inhibition effect. Despite the fact that a medium concentration of IFN-β (50 U/ml) indeed induced Mx2RFP expression to slightly higher frequencies compared to that by IFN-λ3, the level of H3K9 acetylation was not increased 5 h after stimulation.

Together, these observations suggest that IFN-λ-dependent gene induction in IECs depends mainly on histone acetylation

events and that HDAC activity is a critical factor to control the threshold of promoter induction. In contrast, IFN-β-induced promoter activation is insensitive toward inhibition of histone acetylation. Thus, both pathways differ in the extent of the influence of chromatin modifications on gene induction.

Polarization of IECs Reinforces IFN-λ Responsiveness

All experiments with the immortalized epithelial cell line described above were performed on flat-bottom plastic culture dishes. Under these conditions, IFN-β responsiveness was robust, whereas only a moderate response was observed following IFN-λ stimulation. These results are in accordance with previous studies and further demonstrate that type I and type III IFN signaling realizes gene induction with different kinetics (8, 25). Importantly, a hallmark of the mature epithelium *in vivo* is an apical–basolateral cell polarization, an intrinsic feature of epithelial surface barrier formation. This phenotype can also be achieved *in vitro* by long-term cultivation on semipermeable transwell filter inserts (28). Under these conditions, intestinal epithelial cells polarize into apical and basolateral membrane domains with tight cell junctions and 100% confluency (21). To analyze the influence of epithelial polarization on IFN signaling, IECs were routinely grown for at least 21 days on transwell inserts. TEER was measured to confirm cellular polarization and confluency (Figure S4A in Supplementary Material). For comparison, cells were cultured for only 3 days on transwell inserts resulting in incomplete polarization. Cells were stimulated for 20 h with either type I or type III IFN, and Mx2tRFP expression was analyzed by fluorescence microscopy. Stimulation of fully polarized confluent enterocytes with IFN-λ3 in contrast to conventionally cultured (**Figure 1**) or short-term transwell-cultured cells reached a similar signal strength as that with IFN-β with respect to both the number of activated cells and the intensity of cellular Mx2tRFP expression (**Figure 4A**). Further, the HDAC inhibitors VPA and MS275 as well as the addition of I-BET151 did not alter the IFN-λ-induced Mx2tRFP expression as observed under conventional culture conditions (**Figure 4A**). To quantify the results obtained by microscopic analysis of Mx2tRFP induction, we determined IFN-λ3-induced Mx2-Luciferase activity in the cell line IEC-Mx2Luc-10. Luciferase measurements indicated that IFN-β-mediated induction of the Mx2 promoter did not change upon polarization (**Figure 4B**). However, IFN-λ activity strongly increased after 21 days of cultivation on Transwell inserts, and HDAC inhibition did not further stimulate Mx2-driven Luciferase expression (**Figure 4B**). Thus, these quantitative data confirm the results obtained from Mx2tRFP expression. Of note, the IFN-λ-mediated induction of other ISGs such as IRF7, IFI44, Rsad2, and USP18 in fully polarized and confluent cells grown on transwell inserts reached levels similar to those with IFN-β exposure (**Figure 4C**).

IFN-stimulated gene and Mx2 reporter gene induction and antiviral activity depend on STAT1 activation. Western blot analysis revealed that IFN-λ3 induced a much weaker STAT1 phosphorylation at tyrosine residue 701 under conventional culture conditions compared to that by IFN-β (**Figure 4D**; Figures S4B,C in Supplementary Material). In contrast, IFN-λ3 stimulation of transwell-grown polarized cells resulted in an enhanced Y701-phosphorylation of STAT1 reaching levels comparable to levels obtained after IFN-β treatment (**Figure 4E**). In order to understand the source of the increased type III IFN responsiveness in polarized IECs, we compared gene expression of the IFN-λ receptor between IECs cultivated under non-polarizing versus polarizing conditions. Interestingly, IECs displayed elevated levels of IL28R mRNA when cellular polarization was established, whereas the expression of the type I receptor chain IFNAR2 was not affected (**Figure 4F**).

Next, we tested whether IEC polarization also exerts a significant influence on the described delay in ISG expression following IFN-λ3 stimulation (compare Figure S1A in Supplementary Material). IECs harboring Mx2tRFP were grown for 21 days on transwell inserts and stimulated with both types of IFNs. Time-lapse microscopy revealed that IFN-λ3 stimulation of fully polarized cells indeed resulted in a rapid induction of Mx2tRFP expression with onset times of 5–7 h similar to what was observed after IFN-β exposure (**Figures 5A,B**). In contrast and consistent with our previous results, IFN-λ-induced Mx2tRFP expression under standard 2D culture conditions exhibited delayed onset time points after IFN-λ3 stimulation varying between 6 and 14 h after stimulation (**Figure 5C**). Here, IFN-λ3-induced fluorescence intensities did not reach levels observed after administration of high concentrations of IFN-β. Of note, VPA co-treatment did not alter the kinetics of Mx2tRFP gene induction upon IFN-λ stimulation under standard 2D culture conditions (Figure S5A in Supplementary Material). Together, these findings indicate that epithelial polarization abolishes the differences between type I and III IFN signaling and specifically enhances IFN-λ sensitivity.

Efficient Response to IFN-λ in Small Intestinal Organoids

Recent studies had indicated that stem cell-derived small intestinal epithelial organoid cultures recapitulate the polarization and differentiation observed in the adult intestine *in vivo* (22, 29). These organoid structures are characterized by a crypt-villus organization, epithelial polarization, and a functional lumen. To determine the characteristics of gene induction in response to type I and type III IFNs in gut organoid cultures, we made use of a transgenic mouse line harboring the Mx2tRFP reporter. Intestinal organoids established from Mx2tRFP transgenic reporter mice were treated with IFN-λ3 or IFN-β, and the kinetics of Mx2tRFP induction were determined by time-lapse confocal microscopy (**Figure 6A**). Upon stimulation with high dose of IFN-λ3 (20 ng/ml) or IFN-β (500 U/ml), we found no marked differences regarding the onset time points for tRFP expression (**Figure 6B**). Analysis of the mRNA induction of the ISGs IFI44 and USP18 by quantitative real-time PCR indicated an equal activity of both types of IFNs in the stem cell organoid cultures (**Figure 6C**). Interestingly, a scattered heterogeneous responsiveness of the epithelial cells within the analyzed organoids was observed upon administration of low concentrations of both types of IFNs (**Figure 6D**). Here, a high cell-to-cell variability in gene induction with distinct Mx2 expressing and non-expressing

cells was detected. This heterogeneity could be reduced by addition of the HDAC inhibitors VPA and MS275, enhancing the fraction of IFN-λ-reactive cells as well as the overall reporter gene expression level (**Figure 6E**). In accordance with the results obtained in the 2D and transwell cultivation system, HDAC inhibition did not alter the variability of Mx2tRFP expression toward low concentrations of IFN-β (**Figure 6E**). Quantitative mRNA analysis of the prototypical ISGs IFIT1 and USP18 confirmed that IFN-λ activity but not that of IFN-β is enhanced under conditions of diminished HDAC activity (**Figure 6F**).

Overall, the presented data of the stem cell organoid system indicated that intestinal epithelial cells *in situ* are fully

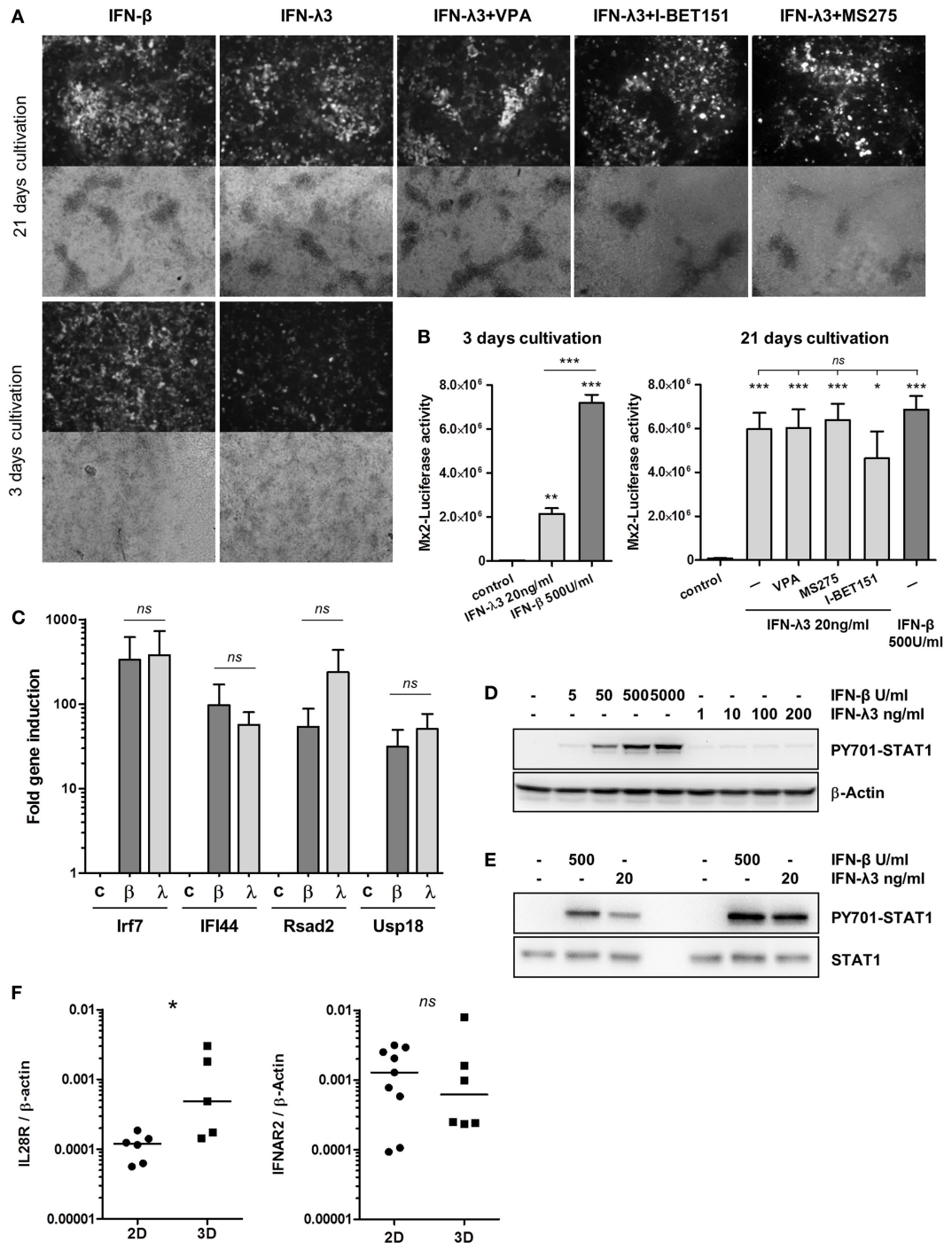

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FIGURE 4 | Continued
Cell polarization increases the responsiveness to interferon (IFN)-λ. **(A)** intestinal epithelial cell lines (IECs) harboring Mx2tRFP were cultured on transwell inserts for 3 and 21 days. Cells were treated with 500 U/ml IFN-β or 20 ng/ml IFN-λ3. As indicated, IFN-λ3 stimulation was done together with 750 μM valproic acid (VPA), 500 nM MS275, and 500 nM I-BET151. Mx2tRFP expression was determined 24 h after stimulation by fluorescence microscopy. Representative images are shown. Data are representative of three independent experiments. **(B)** IEC-Mx2Luc-10 cells containing the Mx2-Luciferase reporter were cultured on transwell inserts for 3 and 21 days as indicated. Cells were treated with 500 U/ml IFN-β or 20 ng/ml IFN-λ3. IFN-λ3 stimulation was done together with 750 μM VPA, 500 nM MS275, and 500 nM I-BET151 as indicated. Luciferase activity was determined 20 h after stimulation ($n = 3$, mean ± SEM). *P* values were calculated by one-way ANOVA, followed by Tukey's Multiple Comparison Test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). *P* values are given for differences among stimulated groups and control group (directly above columns) and between IFN-stimulated groups. **(C)** IECs cultured on transwell inserts for 21 days were treated with 500 U/ml IFN-β (β) and 20 ng/ml IFN-λ3 (λ) for 16 h. RNA was isolated, and qRT-PCR was used to determine the expression of ISGs. Fold induction after normalization to β-Actin is depicted ($n = 3$, mean ± SEM). *P* values were calculated by Mann–Whitney U test (*ns*, not significant). **(D)** IECs cultured on standard cultures dishes were treated with increasing concentrations of IFN-β and IFN-λ3 for 1 h. Western blot analysis was performed using antibodies directed against P-Y701 STAT1 and β-Actin. **(E)** IECs cultured on transwell inserts were treated with 500 U/ml IFN-β and 20 ng/ml IFN-λ3 for 1 h. Cells were lysed, and protein extracts were analyzed by Western blotting using antibodies directed against P-Y701 STAT1 and total STAT1 protein. Results of two independent experiments are shown. **(F)** IECs were cultured on standard plastic dishes (2D) or on transwell inserts (3D) for 21 days. RNA was isolated, and qRT-PCR was used to determine the expression of IL28R and IFNAR2. Receptor expression was normalized to β-Actin. *P* values were calculated by Mann–Whitney *U* test (*$P \leq 0.05$).

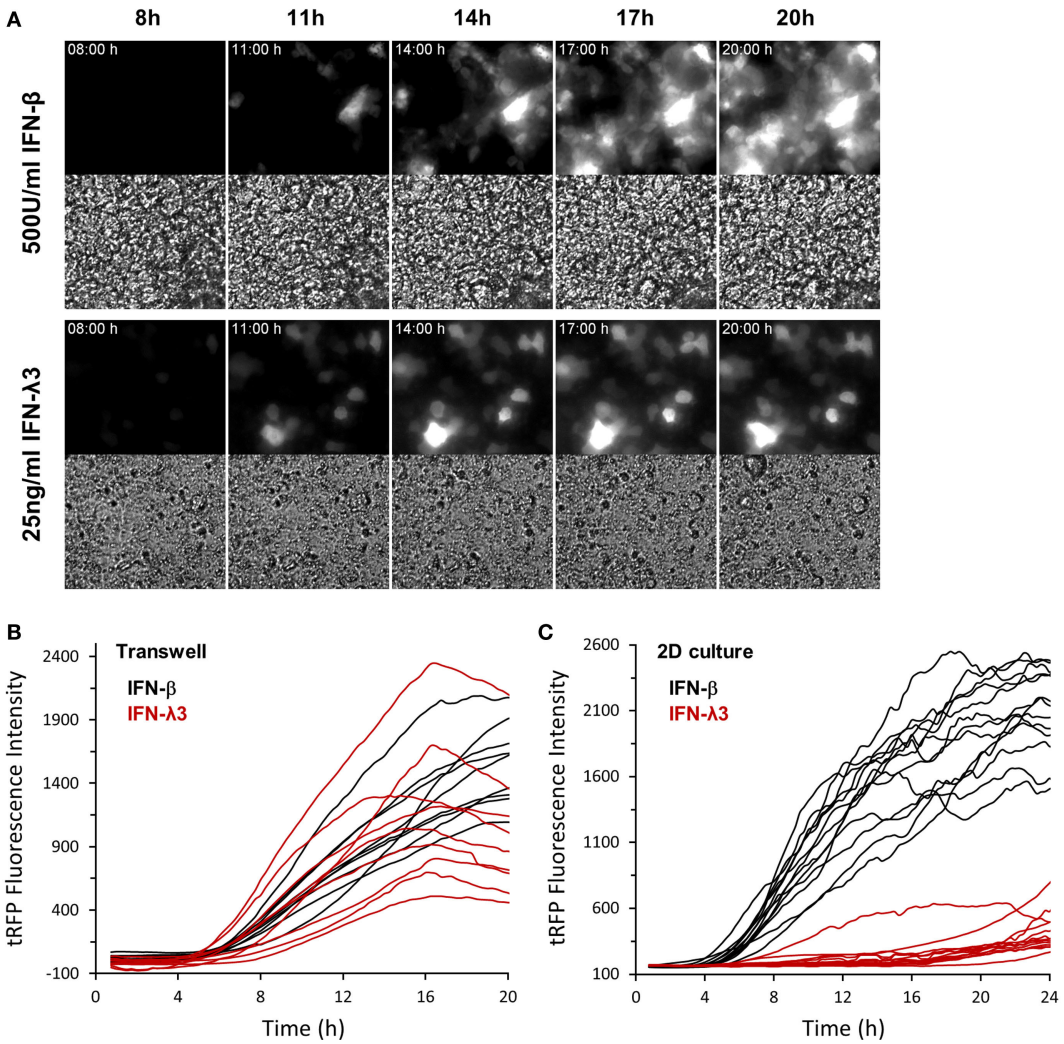
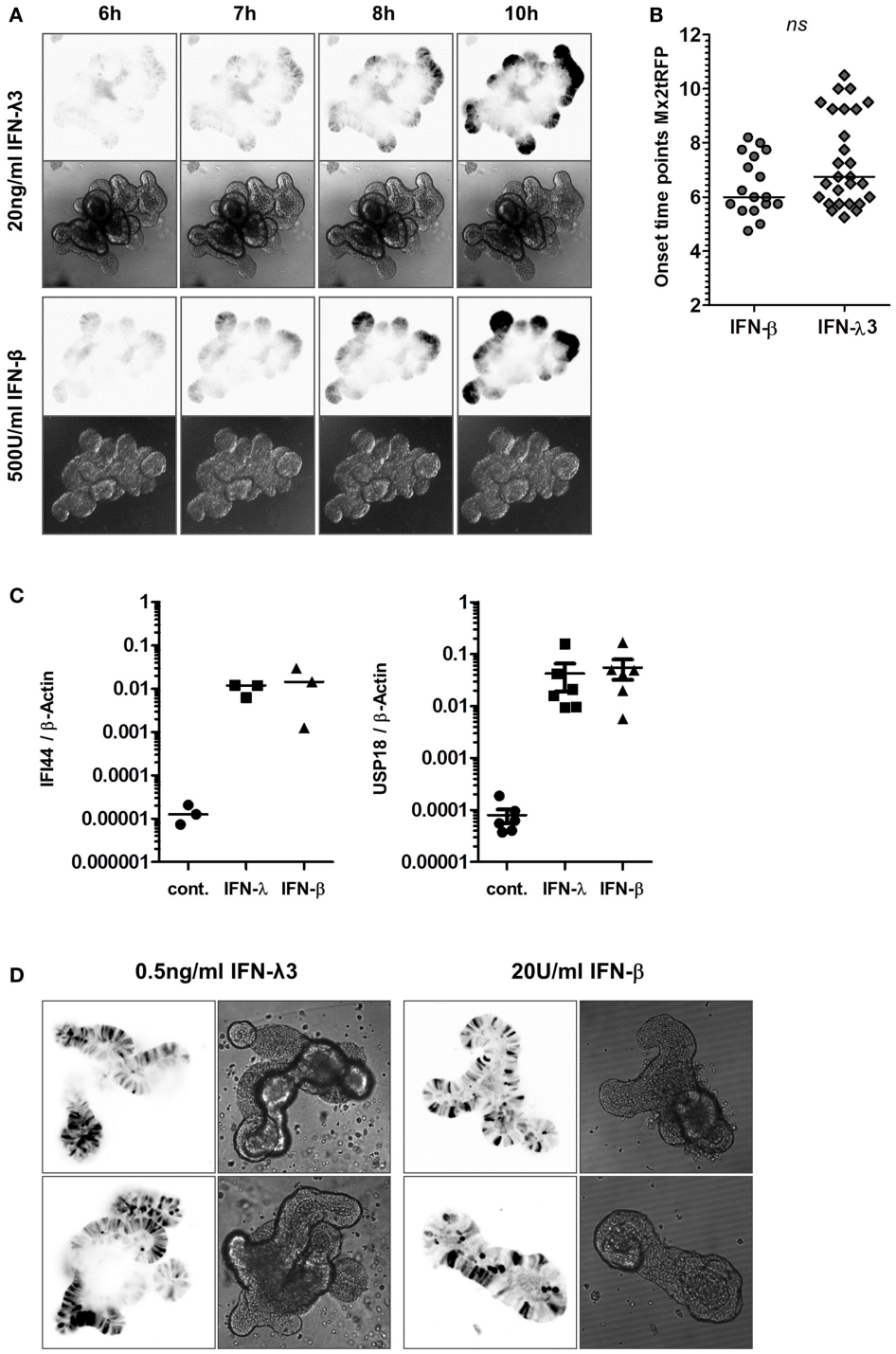


FIGURE 5 | Cell polarization abrogates differential expression kinetics of type I and type III interferons (IFNs). Intestinal epithelial cell lines (IECs) harboring Mx2tRFP were cultured on transwell inserts for 21 days. Cells were treated with 500 U/ml IFN-β and 25 ng/ml IFN-λ3. Time-lapse fluorescence microscopy was used to follow the induction of Mx2tRFP in live cells. **(A)** Representative fluorescence and corresponding bright field images at selected time points are shown. **(B)** Mx2tRFP fluorescence intensities were quantified using ImageJ software. **(C)** IECs grown on standard culture dishes (2D) were treated with 500 U/ml IFN-β and 25 ng/ml IFN-λ3 and subjected to time-lapse fluorescence microscopy. Mx2tRFP fluorescence intensities were quantified over time using ImageJ software.

responsive to IFN-λ and that this response is comparable to the response of immortalized epithelial cells cultured under fully polarizing conditions. Moreover, the physiological relevant stem cell organoid system underlined the divergent role of HDAC activity as a restriction factor for type III but not type I IFN signaling.

DISCUSSION

In this report, we took advantage of the fact that an immortalized IEC and intestinal stem cell organoids react to types I and III IFNs. These cellular models were used in combination with genetic luciferase and fluorescent reporter constructs that could



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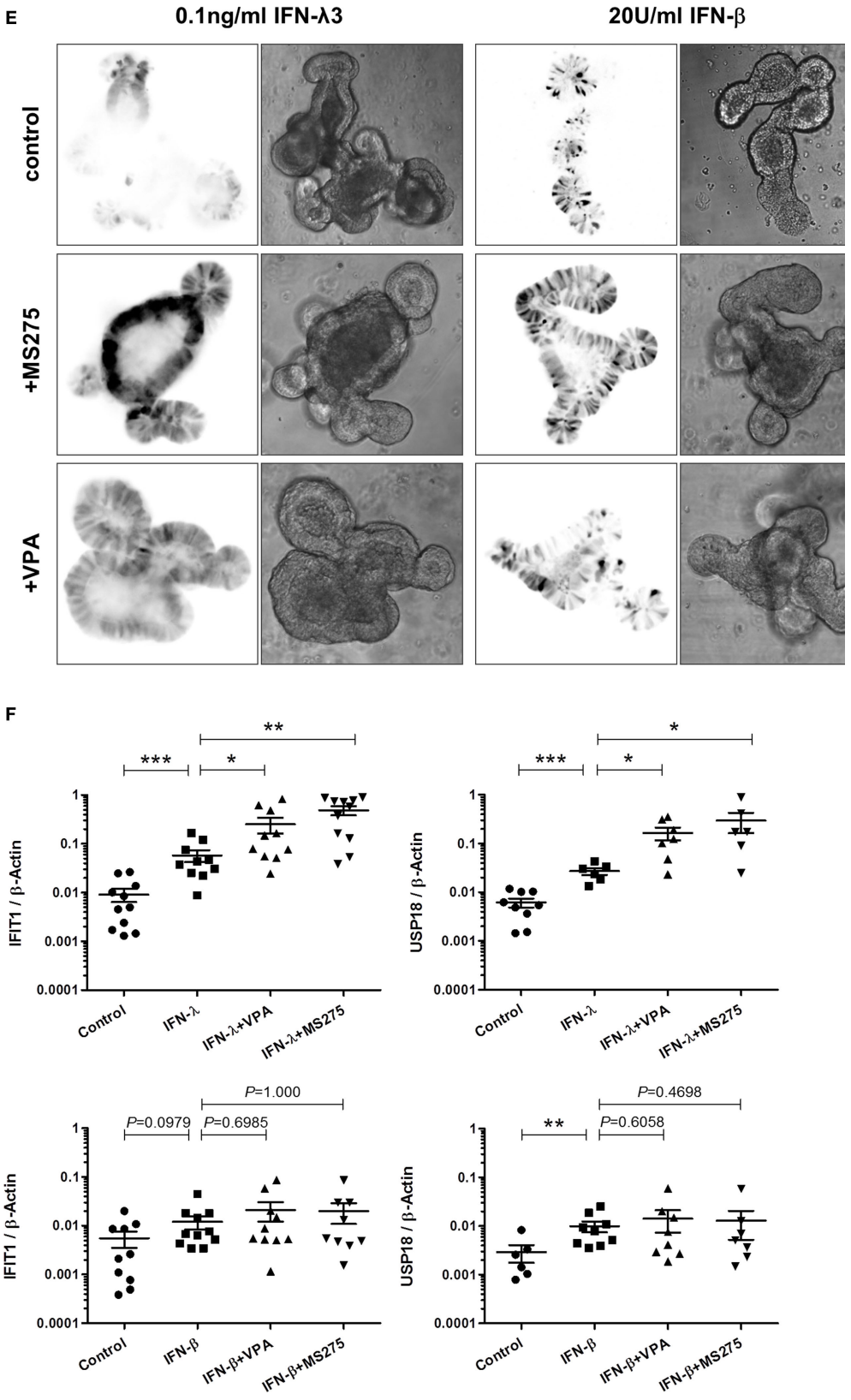

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FIGURE 6 | Continued
High responsiveness to interferon (IFN)-λ in intestinal organoids. Murine small intestinal crypts were isolated from Mx2tRFP transgenic mice. Mature organoids were obtained after incubating small intestinal crypts for 9–10 days in Matrigel. **(A)** Mx2tRFP organoids were treated with 20 ng/ml IFN-λ3 or 500 U/ml IFN-β and subjected to time-lapse confocal microscopy. Optical sections were acquired using identical acquisition settings for both types of IFNs. Mx2tRFP expression is shown at selected time points. Fluorescent images were inverted using ImageJ software. **(B)** Onset time points of Mx2tRFP expression were determined from time series after IFN-β and IFN-λ3 stimulation. *P* value was calculated by Mann–Whitney *U* test; *ns*, not significant. **(C)** Mx2tRFP organoids were treated with 20 ng/ml IFN-λ2 and 500 U/ml IFN-β for 9 h. RNA was isolated, and qRT-PCR was used to determine the expression of IFI44 and USP18. IFN-stimulated gene expression was normalized to β-Actin. **(D)** Mx2tRFP organoids were treated with 0.5 ng/ml IFN-λ3 and 20 U/ml IFN-β for 20 h and subjected to confocal fluorescence microscopy. For both IFN treatments, two representative single plane images show Mx2tRFP expression from intact organoids. Fluorescent images were inverted using ImageJ software. **(E)** Mx2tRFP organoids were treated for 16 h with 0.1 ng/ml IFN-λ3 or 20 U/ml IFN-β in the absence or presence of 750 μM valproic acid (VPA) and 0.51 μM MS275. Confocal fluorescence microscopy was used to collect single plane images from intact organoids 20 h after stimulation. Optical sections were acquired using identical acquisition settings for each type of IFN. Gain and offset were adjusted to use the entire dynamic range of the detector and to avoid saturation of the tRFP signal. Fluorescent images have been inverted using ImageJ software, and representative images showing Mx2tRFP expression are presented. **(F)** Mx2tRFP organoids were treated with 0.1 ng/ml IFN-λ3 and 20 U/ml IFN-β in the absence or presence of 750 μM VPA and 0.51 μM MS275. mRNA levels for IFIT1 and USP18 were determined by quantitative real-time PCR and normalized to β-Actin. *P* values were calculated by Mann–Whitney U test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

reflect the global ISG response (30) and the resulting antiviral status (**Figure 2A**). This approach allowed us to analyze the quantitative response over time both for the cell population and on the single cell level. Our analysis for the first time revealed that the difference in the quantitative response to both IFNs is largely based on bimodal decisions of cells, i.e., a yes/no decision of each individual cell upon stimulus exposure. This finding confirms earlier published results obtained from a different cell model (19). It could also be observed in stem cell organoids and may thus represent the behavior of intestinal epithelial cells *in vivo*. Organoids better resemble intact mature intestinal epithelial layers in their cellular composition and function but still allow to examine individual living cells under defined conditions. However, the *in vivo* situation cannot be directly compared with cell culture systems since constitutive type I and III IFN expression (31, 32) and activities from other cell types might prime the cellular response toward IFN or mask the effects of the stochastic cellular response. In addition to the bimodality, we demonstrate that IFN stimuli modulate the strength of reporter gene expression within the responding cells as represented by the MFI values in tRFP reporter cells (**Figure 1**). This effect, however, appears to be inferior to the bimodality of the response within a cell population. Moreover, it is mainly restricted to the action of type I IFNs, thus representing one of the differences between the cellular responses to both IFN types.

The discovery that type I and type III IFNs triggered the same Jak-STAT signaling pathway supported the idea that both types of IFNs would have identical functions. Indeed, both cytokines were reported to induce comparable patterns of gene expression and similar biological effects (2, 7, 8). The differential tissue distribution of the respective receptor molecules led to the concept that type III IFNs act on specific cell types, whereas type I IFNs affect all nucleated cells in the body. Our results challenge this view since we could find significant differences in the kinetics of ISG expression, the heterogeneity of the responding cell population, and cellular as well as epigenetic requirements for type I and III IFN-mediated antiviral activities.

Our results define several parameters that ultimately determine the cellular responsiveness to IFNs: IFN concentration, epigenetic modulation, and polarization status of the cells. First, the concentration of both IFNs plays a key role. Type I IFN at high concentrations is able to achieve a nearly completely homogenously reacting cell population. In contrast, a low responsiveness in combination with a delayed kinetic characterize the IFN-λ response in several cell models (8, 25, 33–35). Our results using epithelial cells cultured under conventional non-polarizing conditions confirm these observations and extend them by showing that only a fraction of cells is responding, even at high concentrations of IFN-λ. This is associated with a weak phosphorylation of STAT1.

In stark contrast, cultivation under polarizing conditions results in high IFN-λ responsiveness associated with a high fraction of responding cells within a population and strong STAT1 phosphorylation. Thus, under polarization conditions, the responsiveness to IFN-λ is largely comparable to that of IFN-β. A straightforward explanation is given by the fact that the expression of the IFN-λ receptor is, in contrast to the type I IFN receptor, dependent on the polarization status of the cells (**Figure 4F**). Polarization leads to a higher expression of the IFN-λ receptor and a higher extent of STAT phosphorylation. This reflects IFN-β activity in polarized and non-polarized cells. The role of polarization is confirmed in the organoid culture system where a closely related read-out for IFN-β and IFN-λ was found (**Figures 6A,C**). However, administration of low concentrations of both types of IFNs resulted in high cell-to-cell variability in Mx2tRFP induction (**Figure 6D**).

Another difference between the responses to the two types of IFNs concerns the modulation of the epigenetic status with HDAC blockers. While type I IFN activity in all conditions of IEC cultivation and in organoids is completely insensitive to HDAC inhibition, IFN-λ responsiveness is strongly increased. This is true for the intestinal epithelial cells in the non-polarized status and in organoids. Interestingly, the increase in Mx2 expression in IECs depends exclusively on the number of responding cells and not on the expression strength per cell. Since the HDAC inhibitor-mediated enhancement of the IFN-λ response is reduced by I-BET151 administration, histones seem to be the functionally relevant target of the HDAC inhibitors. Several reports indicate that the acetylation level of H3K9 increases across the genome following VPA treatment (26, 27). However, the global change in H3K9 acetylation and other histone modifications induced by HDAC inhibition are not recapitulated at

all individual promoter sites as measured by ChIP (36, 37). We show here that IFN-λ3 stimulation results in elevated levels of H3K9ac at proximal promoter regions when compared to those in untreated cells. However, the increased responsiveness of IECs by VPA co-treatment is not straightforward since HDAC inhibition is not reflected in a further increase in H3K9 acetylation at the analyzed proximal promoter regions (but rather limits its elevation) (Figure S3F in Supplementary Material). We propose that HDAC inhibition targets remote promoter elements or control regions that are necessary to induce expression of a larger cluster of ISGs. However, we assume that IFN-λ stimulation of cells with reduced HDAC activity mediates high level of histone acetylation at such sites providing a chromatin context that allows the expression of ISGs in an otherwise unresponsive cell population. This distinguishes this scenario from the epigenetic reactions induced by IFN-β. Further, the complexity of epigenetic regulation during IFN-mediated ISG induction and the temporal influence of HDAC inhibition on different histone modifications still have to be determined.

In contrast to IFN-λ stimulation, ChIP analysis indicated that H3K9 acetylation was not altered during IFN-β-mediated ISG promoter activation (Figure S3F in Supplementary Material; 50 U/ml IFN-β), suggesting that H3K9 acetylation at these sites is not important for gene induction. This is in line with the observation that VPA-mediated reduction of HDAC activity does not affect submaximal Mx2tRFP and ISG induction upon IFN-β stimulation. Further, addition of the BRD3/4 inhibitor I-BET151 did not reduce gene expression at IFN-β concentrations higher than 50 U/ml (**Figure 3E**; Figure S3A in Supplementary Material). Thus, we suggest that type I and type III IFN signaling induces different spectra of activating histone modifications at target genes in epithelial cells. In this scenario, other histone modifications than H3K9 acetylation possess an overriding importance for IFN-β. Indeed, active histone marks such as H3K4 and H3K79 trimethylation in the promoter regions of ISGs were found to be induced upon type I IFN treatment (38, 39). However, at low doses of IFN-β, the recruitment of readers of histone acetylation is more relevant, since I-BET151 addition leads to a distinct reduction in Mx2-Luciferase activity (**Figure 3E**; Figure S3A in Supplementary Material, 20 U/ml IFN-β). Since ISGs are targets for both types of IFNs and the HDAC-modified chromatin context is only relevant for IFN-λ, a higher complexity of signaling for IFN-β under non-polarized conditions has to be assumed. Currently, biochemical evidence for such a difference is not yet available.

An important aspect concerns the fact that the differential responsiveness toward IFN-λ is reflected by the percentage of responding cells. This suggests that the chromatin status (histone code) defines the probability for responsiveness. The responsiveness toward IFN-β is also bimodal and concentration-dependent, but is modulated neither by HDAC inhibition nor by polarization. Assuming that this effect is also based on the chromatin status, other types of histone modifications have to be considered. Binary responses have been shown to be evoked by positive feedback loops based on autocatalytic switches (40, 41). It will be of interest to see if such mechanisms apply for the bimodal response to IFNs and which molecular basis is underlying the probabilistic gene induction for both types of IFNs.

The biological function of the reported bimodality in contrast to graded induction of other genes is unknown. We speculate that it may be of advantage to maintain individual unprotected cells in an organism upon exposure to low levels of IFNs. This could allow limited virus propagation and thereby stimulation and priming of the adaptive immune system to provide subsequent protection. Alternatively, IFN-responding cells might alter their physiology in a way that hinders critical physiological functions of the gut epithelium *in vivo*. Therefore, it might be of advantage to maintain the full functionality of at least a fraction of enterocytes.

Our results ascribe a special role to IFN-λ in comparison to type I IFNs that goes beyond cell type specificity. Its transcriptional activity is strongly influenced by cell polarization and underlies a bimodal decision process and epigenetic modifications further expanding our knowledge on the complex regulation of the intestinal epithelial response to type I and type III IFNs.

AUTHOR CONTRIBUTIONS

SB, MW, HH, and MK designed the study. SB and MW performed most of the experiments. TS, LK, and MK performed some experiments. SB, MW, LK, and MK analyzed the data. DW provided essential tools. HH, MH, and MK wrote the article. SB and MW contributed equally to this work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00671/full#supplementary-material.

VIDEO S1 | Kinetics of Mx2tRFP induction in intestinal epithelial cell lines (IECs) grown under standard culture conditions. Time-lapse imaging of IECs Mx2tRFP stimulated with 500 U/ml interferon (IFN)-β (above) and 25 ng/ml IFN-λ3 (below).

VIDEO S2 | Kinetics of Mx2tRFP induction in polarized intestinal epithelial cell lines (IECs). IECs harboring Mx2tRFP were cultured on transwell inserts for 21 days. Time-lapse imaging of cells stimulated with 500 U/ml interferon (IFN)-β (above) and 20 ng/ml IFN-λ3 (below).

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MINI REVIEW
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Interferon-Lambda: A Potent Regulator of Intestinal Viral Infections

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Interferon-lambda (IFN-λ) is a recently described cytokine found to be of critical importance in innate immune regulation of intestinal viruses. Endogenous IFN-λ has potent antiviral effects and has been shown to control multiple intestinal viruses and may represent a factor that contributes to human variability in response to infection. Importantly, recombinant IFN-λ has therapeutic potential against enteric viral infections, many of which lack other effective treatments. In this mini-review, we describe recent advances regarding IFN-λ-mediated regulation of enteric viruses with important clinical relevance including rotavirus, reovirus, and norovirus. We also briefly discuss IFN-λ interactions with other cytokines important in the intestine, and how IFN-λ may play a role in regulation of intestinal viruses by the commensal microbiome. Finally, we indicate currently outstanding questions regarding IFN-λ control of enteric infections that remain to be explored to enhance our understanding of this important immune molecule.

Keywords: interferon-lambda, enteric virus, innate immunity, transkingdom interactions, norovirus, rotavirus, commensal bacteria

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AN INTRODUCTION TO INTERFERON-LAMBDA (IFN-λ) IN THE INTESTINE

Animals can mount potent and rapid innate immune responses to invading viruses. The classic signaling pathway by which this response occurs is *via* type I interferons (IFNs), including IFN-beta (IFN-β) and multiple IFN-alphas (IFN-α) (1). When cells sense viral products, type I IFNs are produced, which stimulate transcription of antiviral molecules that act in autocrine and paracrine fashion. However, in the past decade, an important paradigm shift has occurred in how we consider the compartmentalization of viral responses into systemic versus mucosal responders, driven in large part by the discovery of type III IFNs, or IFN-λ.

First described in 2003 (2, 3), the IFN-λ family of cytokines includes up to four members in humans, dependent on genetic polymorphisms (4, 5), and two functional orthologs in mice (6, 7). The family, likely arising from a common ancestral fish IFN gene that gave rise to both type I and III IFN families, is conserved to chickens (8, 9). The type III IFNs are under positive selection, with long-term persistence of duplicate copies suggesting a critical biological role for type III IFNs independent from type I IFNs (9). Pattern-recognition receptors, including RIG-I and MDA5, detect viruses and induce type I and III IFNs *via* MAVS and IRF3/IRF7 signaling (10–12) (**Figure 1**). IRF1 plays a unique role in type III IFN induction, however, being specifically stimulated by peroxisome-associated MAVS in contrast to mitochondrial-associated MAVS, which better induces type I IFNs

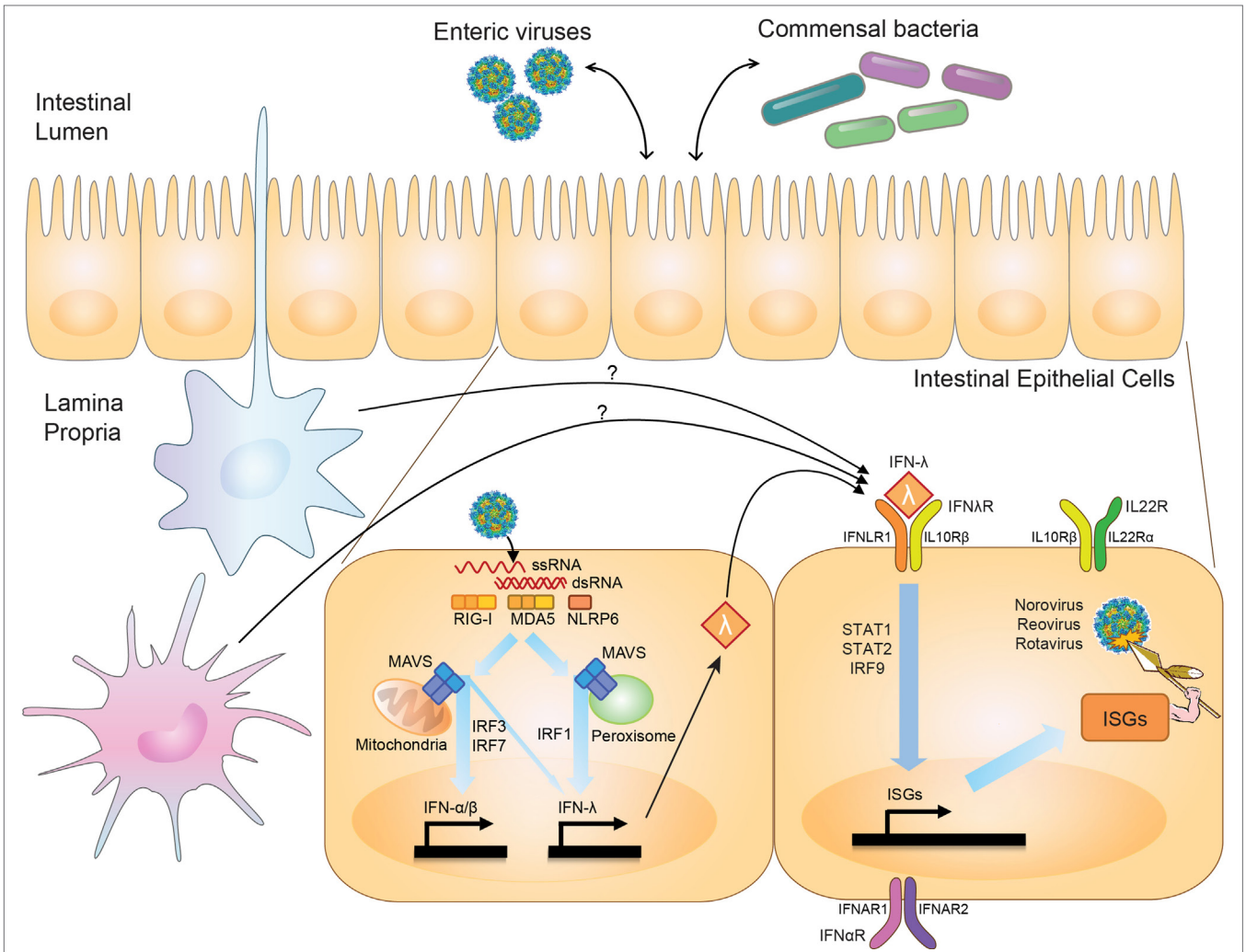

FIGURE 1 | Effects of interferon-lambda (IFN-λ) on viruses in the intestine. Upon intestinal viral infection, viral RNA is sensed by pattern-recognition receptors, RIG-I, MDA5, and NLRP6, which signal through mitochondria- or peroxisome-associated MAVS to stimulate transcription of type I and III IFN by IRF3/IRF7 and IRF1. IFN-λ is produced by intestinal epithelial cells (IECs) and possibly immune cells in the intestine. IFN-λ signaling through the IFN-λ-receptor (IFNλR) on IECs stimulates production of antiviral effectors, or interferon-stimulated genes (ISGs), *via* STAT1/STAT2/IRF9-mediated transcription. IFN-λ thus serves to regulate viral levels in the intestine. IFN-λ can interact with IL-22, whose receptor is expressed on IECs (IL22R), to coordinately regulate viral infection, and in some settings may also interact with type I IFNs, which signal through IFNαR. IFN-λ has also been shown to play a role in influencing interactions between commensal bacteria and enteric viral pathogens.

(13). Intestinal epithelial cells (IECs) produce type III IFNs with *in vivo* viral infection (14–16). However, leukocytes generate IFN-λ *in vitro* (10, 17), and intestinal eosinophils (18) and plasmacytoid dendritic cells (pDCs) (19) can produce IFN-λ *in vivo*, suggesting the possibility of additional cellular IFN-λ contributors.

While the antiviral programs induced by type I and type III IFNs exhibit substantial overlap (20–22) (**Figure 1**), a critical difference between the two is the cell types they affect secondary to receptor expression. The IFN-λ receptor consists of IFNLR1 and IL10Rβ. While the receptor for type I IFNs, IFNAR1, is expressed broadly on the majority of cell types, IFNLR1 exhibits a much more restricted pattern of expression (23). In the intestine, IFNLR1 is expressed preferentially on IECs, allowing for a compartmentalized response to viruses infecting at this mucosal surface (24, 25). While IFNLR1 expression has also been reported on NK cells, T cells, B cells, and pDCs (26–30), no role has been found for these cells in IFN-λ-mediated antiviral responses. Type I IFNs, on the other hand, are critical for preventing a virus from moving past this initial epithelial barrier into systemic tissues (24, 25, 31). The host may benefit by inducing specific and local barrier defenses at a site commonly exposed to pathogens *via* IFN-λ signaling, and thus avoid potentially detrimental systemic inflammatory responses by type I IFNs. Many autoimmune diseases, as well as the congenital interferonopathies, are secondary to excessive type I IFN activity (32, 33).

There is an ever-expanding set of roles being discovered for IFN-λ signaling, from control of viral infections in liver (34),

lung (35), and brain (36), to regulation of non-infectious diseases like inflammatory bowel disease (37) and cancer (38). Many of these intriguing advances are addressed elsewhere in this Frontiers in Immunology topic, "Interferon-λs: New Regulators of Inflammatory Processes." Here, however, we will focus exclusively on the regulation of enteric viruses by IFN-λ. We review the current literature about IFN-λ-mediated regulation of specific intestinal viruses, discuss interplay of IFN-λ with other cytokines and its regulation of viral–bacterial interactions, and highlight areas ripe for future research enterprises.

REGULATION OF SPECIFIC ENTERIC VIRUSES BY IFN-λ

Enteric viruses, including rotavirus (RV), reovirus, norovirus (NoV), and others, generally infect *via* the fecal–oral route, though other transmission routes have been described. As such, the IECs comprising the mucosal barrier of the intestine likely represent the first eukaryotic cells with which an enteric virus interacts. Here, we describe what is known about specific enteric viruses and their relationship with both the intestinal epithelium and IFN-λ (**Table 1**).

Rotavirus

Rotaviruses are double-stranded RNA viruses of the *Reoviridae* family and a major cause of severe diarrhea in children worldwide (39). RV infection exhibits a preferential tropism for IECs of the small intestinal villi in humans and mice (40). Several groups have reported antiviral effects of IFN-λ against RV in mouse models (16, 24, 41). Infection by a murine RV, EDIM-RV, induces IFN-λ in the small intestine, and endogenous IFN-λ suppresses intestinal viral replication (16, 41). RIG-I and MDA5

TABLE 1 | Interferon-lambda (IFN-λ) interactions with intestinal viruses *in vivo* and *in vitro*.

Virus	Strain	*In vivo* phenotypes	*In vitro* phenotypes	Reference
Rotavirus (RV)	EDIM	* Mice lacking *Ifnlr1* in all cells exhibit increased viral shedding, intestinal titers, and tissue damage * RV infection induces IFN-λ production in intestinal epithelial cells (IECs) * Treatment with exogenous IFN-λ prevents RV replication in the intestine		(16, 41)
	EW	* Mice lacking *Ifnlr1*, *Ifnar1*, or *Stat1* in all cells exhibit similar level of viral shedding, intestinal titers		(24)
	Rhesus strain of rotavirus	* Mice lacking *Ifnlr1, Ifnar1*, or *Stat1* in all cells exhibit increased level of viral shedding, intestinal titers		(24)
	Ito, Wa		* Human RV infection induces IFN-λ expression in human intestinal enteroids * Treatment with exogenous IFN-λ inhibits RV replication in enteroids * Blocking endogenous IFN-λ has no effect on viral production	(43)
Reovirus	Type 3 Dearing	* Adult mice lacking *Ifnlr1* in all cells exhibit higher viral shedding of reovirus * Suckling mice lacking *Ifnlr1* in all cells exhibit higher viral shedding and tissue titers of reovirus, increased tissue damage and severe mortality * Mice lacking *Ifnlr1* exhibit higher reovirus infection in IECs, while mice lacking *Ifnar1* exhibit higher infection in lamina propria cells		(14)
	Type 1 Lang	* Mice lacking *Ifnlr1* in all cells or only in IECs exhibit higher viral shedding and intestinal titers of reovirus		(50)
Norovirus	CR6	* Mice lacking *Ifnlr1* in all cells or only in IECs exhibit higher viral shedding and intestinal titers of persistent murine NoV (MNoV) * Treatment with recombinant IFN-λ prevents and cures persistent MNoV infection, dependent on IEC expression of *Ifnlr1* * NoV dependence on the commensal microbiome for infection is absent in mice lacking *Ifnlr1*		(50, 64, 93)
			* Replication of transfected human NoV RNA is sensitive to IFN-λ treatment, but does not induce IFN-λ expression	(59)
Enterovirus	EV71		* Enterovirus 71 induces IFN-λ expression in human IEC line	(72)
Parvovirus			* Canine parvovirus is more sensitive to IFN-λ than type I IFN	(73)
Coronavirus	CV777 LNCT2		* Porcine epidemic diarrhea virus is sensitive to IFN-λ when cultured in a porcine IEC line	(74)

are required for type I IFN production by IECs during RV infection (42); induction pathways for IFN-λ have not been reported. IECs produce the majority of IFN-λ, consistent with the viral IEC tropism (16). Pretreatment with exogenous IFN-λ effectively prevents EDIM-RV replication in the small intestine and colon (41). However, a recent study demonstrated that a homologous murine strain of RV, EW–RV, is largely IFN-λ-insensitive, even though EW-RV is originally derived from EDIM-RV (24). This study also showed that a heterologous rhesus strain of rotavirus (RRV) is, in contrast, highly sensitive to both IFN-α/β and IFN-λ, even though EW-RV and RRV infection both significantly induce IFN-α/β and IFN-λ production during infection (24). The reason for this discrepancy between strains is still unclear, though recently, a human RV study using human intestinal enteroids provided some hints regarding the source of this strain complexity (43). In this study, human RV infection in enteroids indeed induced IFN-λ and interferon-stimulated genes (ISGs). However, blocking IFN-λ signaling did not have any effect on viral growth. Since RV has multiple functional proteins for immune evasion (e.g., NSP1, NSP3, and VP3) (44), the effect of IFN-λ may be limited by these viral genes, and EW–RV may utilize evasion strategies to overcome IFN responses. Thus, interactions between RV and IFN-λ in the intestine are influenced by multiple host and viral factors.

Reovirus

Although reoviruses are also in the *Reoviridae* family, in contrast to RVs, they are not generally associated with serious human disease. Recently, however, they have been implicated in the pathogenesis of celiac disease, suggesting the possibility of a previously overlooked role as an environmental inflammatory trigger (45). Importantly, reoviruses have been used as a tractable experimental system for studies of viral pathogenesis in newborn mice (46). Reoviruses induce type I and III IFNs in a MAVS-dependent fashion (13, 15, 47), likely *via* RIG-I- and MDA5-mediated sensing (48, 49). Since these viruses exhibit a wide cellular tropism and a low degree of species specificity, reovirus infection of the mouse intestine is sensitive to both IFN-α/β and IFN-λ (14). In adult mice, endogenous IFN-λ inhibits reovirus strain Type 3 Dearing replication in the intestine, and reovirus replicates exclusively in IECs of *Ifnlr1*-deficient mice (14). By contrast, IFN-α/β inhibits reovirus replication in the intestine, but acts specifically on cells in the lamina propria. Another study using reovirus strain Type 1 Lang showed that endogenous IFN-λ inhibits reovirus replication in the mouse small intestine and that IFN-λ-receptor expression in IECs is critical for this antiviral activity (50). Therefore, IFN-λ in the intestine controls reovirus replication in IECs, but IFN-α/β also coordinately controls reovirus infection in non-IEC cell types in the intestine.

Norovirus

Noroviruses are positive sense non-enveloped RNA viruses in the *Caliciviridae* family (51). In humans, they are the most common cause of epidemic gastroenteritis and are a significant contributor to childhood mortality worldwide (52, 53). In addition to causing acute symptomatic infections characterized by vomiting and diarrhea, they can persist in both immunocompetent (54) and immunocompromised individuals (55), who can potentially seed future epidemics (56). Until quite recently, human NoV has been impractical to culture *in vitro* (57, 58) and lacked a robust small animal model. Secondary to these challenges, the role of IFN-λ in control of human NoV *in vivo* is unknown. *In vitro,* human NoV RNA replication and virus production, after transfection of stool-isolated RNA into mammalian cells, is sensitive to treatment with type I and III IFNs (59). However, in this system, NoV RNA replication does not induce IFNs or respond to neutralization of type I or III IFNs (59). Whether this reflects the *in vivo* effects of NoV infection remains to be seen.

The discovery of murine NoV (MNoV) (60), which is readily culturable (61) and can be studied *in vivo*, facilitated exploration of the interactions between NoV and the host immune system (62). IFNs have long been known to be important in MNoV regulation, as the virus was originally isolated from and causes severe disease and death in *Stat1*-deficient mice (60, 63). Type I and II IFNs both control acute, systemically spreading strains of MNoV [recently reviewed in Ref. (62)]. By contrast, type I and II IFNs are dispensable for intestinal regulation of persistent strains of MNoV (64), which replicate robustly in the colon and are shed at high levels in the stool (65). Instead, for persistent MNoV, IFN-λ plays a critical regulatory role. Endogenous IFN-λ controls intestinal viral replication and shedding, demonstrated by increased shedding in *Ifnlr1*-deficient mice. In addition, exogenous IFN-λ prevents and cures persistent MNoV infection in wild-type and *Rag1*-deficient mice (64). Thus, IFN-λ represents an example of sterilizing innate immunity. Because myeloid and B cells, which support MNoV replication *in vitro* (57, 61), and IECs, the target cells of IFN-λ for MNoV clearance (50), are distinct, it remains to be determined whether *in vivo* IFN-λ stimulates an antiviral program in a cell-intrinsic fashion to clear infected IECs, or instead drives production of secondary factors to target infected myeloid or B cells.

Induction of IFN-λ is also important for control of intestinal MNoV. MDA5 is critical for type I IFN responses to MNoV (66); type III IFN responses may be similarly regulated. Nod-like receptor Nlrp6 is another viral RNA sensor that regulates intestinal MNoV levels and plays a role in induction of type I and III IFNs and ISGs in response to infection (67). Activated intestinal intraepithelial lymphocytes have been shown to rapidly stimulate type I and III IFN receptor-dependent upregulation of ISGs in IECs, which correspondingly limits MNoV infection *in vivo* (68). Persistent strains of MNoV may induce lower levels of type I and III IFNs than acute systemic strains, such that avoidance of IFN upregulation may contribute to persistence of some strains (64).

Identifying viral antagonists of host pathways can highlight critical antiviral host pathways. MNoV antagonizes IFNs *via* a protein expressed from ORF4, VF1, which *in vitro* delays upregulation of innate genes including type I IFNs (69). MNoV has also been shown to diminish the host response to infection *via* its protease NS6, which specifically suppresses host ISG translation (70). However, the interactions of these genes with IFN-λ signaling in the intestine have not yet been explored. A final potential viral player of interest is MNoV NS1/2. A single amino acid difference in this gene confers the ability of the virus to persist in the intestine and stool (65). It is a tempting speculation

that intestinal viral persistence requires antagonism of IFN-λ, but further studies are needed to determine whether NoV has evolved to avoid the antiviral effects of this signaling pathway.

Other Enteric Viruses

A limited number of studies have explored the role of IFN-λ in regulation of other enteric viruses. Infection of human enteroids by echovirus 11, but not coxsackievirus B, was shown to induce expression of antiviral ISGs (71), and enterovirus 71 potently induces type I and III IFNs in a human IEC line (72). However, further studies are needed to determine the specific role of type III IFNs in control of enteroviruses. Canine parvovirus, which causes gastrointestinal disease in dogs, is more sensitive to IFN-λ than a type I IFN *in vitro* (73), but it is unknown whether this applies to human parvoviruses. Porcine epidemic diarrhea virus is an enteropathogenic coronavirus that is sensitive to both type I and III IFN treatment in a porcine IEC line (74). Finally, avian influenza virus and Newcastle disease virus induce much more robust type III than type I IFN in a primary chicken IEC culture model, suggesting a possible role for IFN-λ in prevention of intestinal infection by these viruses normally associated with respiratory infections (75). These initial findings point to the potential for a broad role for IFN-λ in control of many different enteric viruses, but additional studies are clearly needed to determine the breadth and depth of IFN-λ-mediated regulation of viral infection in the intestine.

IFN-λ INTERACTIONS WITH OTHER SIGNALING PATHWAYS

Interferon-lambda-mediated antiviral immunity in the intestine against rotavirus (EDIM-RV) and MNoV does not redundantly overlap with type I IFNs, while there is redundancy between type I and III IFNs to control influenza, SARS coronavirus, and respiratory syncytial virus in the lung, and herpes simplex virus-2 in the genital tract [reviewed in Ref. (76)]. There are two potential reasons for a non-redundant role for IFN-λ in the intestine. First, the IFN-λ receptor is highly expressed in IECs but is minimally detectable in other intestinal cell types such as lamina propria cells (50). Second, expression of IFN-α receptor subunits (i.e., IFNAR1 and IFNAR2) is less abundant in IECs than in lamina propria cells (14), and surface expression of the IFN-α receptor is polarized to the apical side (41). Interestingly, in neonatal mice, IECs are sensitive to both IFN-α/β and IFN-λ, and both IFN-α/β and IFN-λ can control RV (RRV strain) infection in suckling mice (24). It has not been explored whether this IFN-α/β-sensitivity in neonatal IECs is from altered trafficking of the IFN-α receptor to the basolateral side. Further work is needed to explore the consequences of age-related IFN-α/β sensitivity in IECs and the pathogenesis of enteric virus infection (**Figure 1**).

Another cytokine important for mucosal immunity, IL-22, has a synergistic relationship with IFN-λ. Similar to IFNLR1, the IL-22 receptor subunit, IL22Rα, associates with IL10Rβ and is expressed preferentially by IECs (77). During RV infection, IL-22 acts coordinately with IFN-λ to control virus replication and prevent tissue damage in mice (16). This antiviral activity of IL-22 is *Ifnlr1* and *Stat1* dependent but not *Stat3* dependent. IL-22 also restricts porcine enteric coronavirus infection in the intestine, for which antiviral activity is largely *Stat3* dependent (78). Since IL-22 also induces IFN-λ expression in the intestine, a *Stat3*-independent/IFN-λ-dependent role for IL-22 in control of porcine enteric coronavirus cannot be ruled out (78).

Finally, lactoferrin, a member of the transferrin family and a component of milk, potentiates IFN-λ production in a human IEC line (79), and *in vitro* lactoferrin has antiviral activity against RV (80) and MNoV (81). Thus, it would be interesting to study whether milk-derived components exhibit cross talk with IFN-λ-mediated immunity for enteric viral infections in neonatal hosts.

IFN-λ AND TRANSKINGDOM INTERACTIONS

A final critical factor for discussion of enteric viral infections and IFN-λ is the role of the commensal bacterial microbiome. For these viruses, infection occurs amidst the complex milieu of the oral and intestinal microbiome, which plays important roles in regulation of viral infectivity. Poliovirus, reovirus, and murine mammary tumor virus depend upon the presence of commensal bacteria for infection (82, 83), with direct viral binding to bacterial products like lipopolysacchide implicated as the mechanism of facilitation (84, 85). Depletion of the commensal microbiota also impairs RV infection and results in enhancement of both mucosal and systemic antibody responses against the virus (86). Human NoV binds directly to bacterial products that mimic the histo-blood group antigens (HBGAs) known to be attachment factors for NoV (87–89), and indeed culture of human NoV in B cells depends on the presence of these HBGA-expressing bacteria (57, 90). Hence, there is a common theme for enteric viruses in interacting with and depending on intestinal bacteria for infectivity, though the specific mechanisms may be virus dependent (91, 92).

The link between viral dependence on the microbiome and sensitivity to IFN-λ comes from work done with MNoV. Depletion of the commensal microbiota in wild-type mice prevents persistent intestinal MNoV infection (93), similar to what has been observed with other enteric viruses. Interestingly, in mice lacking *Ifnlr1*, *Stat1*, or *Irf3*, all important molecules for IFN-λ induction or signaling, MNoV establishes infection even in the absence of commensal microbes, implicating IFN-λ in regulation of these transkingdom viral–bacterial interactions (93) (**Figure 1**). Other enteric viruses share both a dependence on the microbiome and a sensitivity to IFN-λ; whether interplay between the microbiome and IFN-λ signaling also regulates other intestinal viruses such as RV and reovirus remains to be seen.

GUT INSTINCT ABOUT THE FUTURE OF IFN-λ

While the past decade yielded many exciting insights into regulation of enteric viruses by IFN-λ, many important questions remain. Type I and III IFNs share significant overlap in induction and signaling pathways, though there are distinctions in promoter sequences, upstream regulatory elements, and kinetics of downstream gene stimulation [reviewed in Ref. (76)]. However, most

previous studies were performed *in vitro* outside of the complex environment of the gut. How is IFN-λ production regulated in the intestine, and by what pathways is it induced *in vivo* by viral infection? Are specific ISGs induced by IFN-λ necessary for antiviral activity against enteric infections? Conversely, viruses rapidly evolve mechanisms to evade the host immune system. Are there viral factors that specifically target IFN-λ induction or signaling pathways for evasion or suppression?

In addition to important mechanistic questions for enteric viruses already known to be IFN-λ regulated, there are a number of intestinal viruses for which sensitivity to IFN-λ has not yet been explored. Astroviruses, parvoviruses, enteroviruses, and adenoviruses are among the enteric viruses for which data on IFN-λ-sensitivity in both cell culture and animal models is currently lacking. Finally, of great interest is the *in vivo* effect of IFN-λ regulation on enteric viruses in humans. Single-nucleotide polymorphisms (SNPs) in human IFN-λ genes are associated with differential responses to hepatitis B and C, human cytomegalovirus, herpes simplex virus 1, and influenza virus vaccination [reviewed in Ref. (76, 94)]. Enteric infections cause a spectrum of disease in different individuals, including variable severity and duration of infection, which may correlate with host genetic variation. Do these same SNPs correlate with differential responses to enteric viruses or to vaccination? IFN-λ is clearly an important innate immune regulator for many gut viruses, and defining the breadth of its effects and the mechanisms underlying its enteric activity represent exciting areas for future research endeavors.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this work. SL and MB conceptualized, wrote, and edited the manuscript.

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The Unresolved Role of Interferon-λ in *Asthma Bronchiale*

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Asthma bronchiale is a disease of the airways with increasing incidence, that often begins during infancy. So far, therapeutic options are mainly symptomatic and thus there is an increasing need for better treatment and/or prevention strategies. Human rhinoviruses (HRVs) are a major cause of asthma exacerbations and might cause acute wheezing associated with local production of pro-inflammatory mediators resulting in neutrophilic inflammatory response. Viral infections induce a characteristic activation of immune response, e.g., TLR3, 4, 7, 8, 9 in the endosome and their downstream targets, especially MyD88. Moreover, other cytoplasmic pattern recognition molecules (PRMs) like RIG1 and MDA5 play important roles in the activation of interferons (IFNs) of all types. Depending on the stimulation of the different PRMs, the levels of the IFNs induced might differ. Recent studies focused on Type I IFNs in samples from control and asthma patients. However, the administration of type I IFN-α was accompanied by side-effects, thus this possible therapy was abandoned. Type III IFN-λ acts more specifically, as fewer cells express the IFN-λ receptor chain 1. In addition, it has been shown that asthmatic mice treated with recombinant or adenoviral expressed IFN-λ2 (IL–28A) showed an amelioration of symptoms, indicating that treatment with IFN-λ might be beneficial for asthmatic patients.

Keywords: asthma, rhinovirus, interferon, exacerbation, epithelial cell

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ASTHMA BRONCHIALE

Currently, there are about 300 million people worldwide suffering from the chronic airway inflammatory disease *Asthma bronchiale*. There are two forms of asthma: intrinsic, or non-allergic and allergic, or atopic asthma, although both forms can co-exist in some patients. Intrinsic asthma often appears later in life and the causes are viral infections of the lower airways or irritants such as cold air, cigarette smoke, or stress whereas, allergic asthma affects prevalently children and about one half of adult asthmatic subjects. Here, the triggers for the disease are usually innocuous substances, e.g., proteins from plant or tree pollen, house dust mite, or animal dander (1–3).

The symptoms of asthma comprise airway hyper-responsiveness (AHR), mucus hyperproduction, reversible airway obstruction, airway remodeling, and in case of allergic asthma, high serum IgE levels. Together, these symptoms cause recurrent shortness of breath, wheezing, and chest tightness due to a narrowing of the airways (2, 3).

IMMUNOLOGY OF ASTHMA

The asthmatic airways host dysregulated immune reactions as a pathological response to an otherwise innocuous allergen. The dysregulated immune responses seen in asthma are mediated by both cells of the innate and adaptive immune system. In a first step, dendritic cells (DCs), located below the airway epithelium, sample allergens from the airway lumen, process these allergens into

smaller peptides, which are then presented to naïve T cells in the regional lymph nodes. Here, in the presence of interleukin 4 (IL-4), antigens activate T helper cells and facilitate their differentiation into T helper cells type 2 (Th2), which migrate into the airway mucosa, where they release high amounts of the classic Th2 cytokines like IL-4, IL-9, and IL-13 as well as the cytokines with a common beta chain involved in granulocytes development such as IL-3, IL-5, and granulocyte macrophage colony-stimulating factor (GM-CSF). These mediators have downstream effects on other immune cells, for example IL-3 differentiates mucosal mast cells which are present into the or beneath the bronchial epithelial at the site of inflammation, while GM-CSF in concert with IL-3 and IL-5 favors recruitment, maturation, and survival of eosinophils which are the predominant type of cells around the asthmatic bronchi (4). Eosinophils then release cationic proteins responsible of the cytotoxic effect on the epithelial cells observed in asthma (5). In B cells, the class-switch recombination of immunoglobulins is based to IgE production by IL-4. IgE antibodies bind to the high affinity IgE receptor expressed on, e.g., mast cells. After repeated allergen challenge, IgE cross-links the high affinity IgE receptor bound on the cell surface of mast cells and activate downstream the release of preformed broncho-constrictive, pro-inflammatory mediators such as histamine, leukotrienes, cytokines, or chemokines (2, 3).

Moreover, it has been recently demonstrated that exposure to aeroallergens results in increased differentiation and proliferation of Th17 cells producing IL-17A, resulting in neutrophils accumulation in the airways which is a signature of severe asthma (6, 7). IL 17A is also released by cells of the innate immune system like gamma delta cells, which have been shown to be important on the development of asthma (8). Furthermore IL 17A is also involved in mucosal and epithelial host defense against infections and thereby it constitutes an important cytokine mediating antiviral immune responses (9, 10).

Finally, it has been recently demonstrated that allergen can induce AHR without previous systemic sensitization *via* the upregulation of group 2 innate lymphoid cells (ILC-2) that lack antigen-specific receptor and function *via* cytokine signaling (11, 12). In the immunological response to the allergen, ILC2 are positioned downstream of infection or allergen damaged epithelial cells and after activation they produce Th2 cytokines like IL-5, IL-9, and IL-13 *via* ST2 activation and without the need of T cells. ILCs in general have also been involved in clearing infection (13–16). The mechanism involving ILC2 activation occurs *via* IL-33, a cytokine of the IL-1beta family, released by necrotic epithelial or endothelial cells after allergen challenge or virus infection (17, 18). IL-33 is the ligand of ST2, also known as IL-1RL1, and is present on the surface of both Th2, ILC2, mast cells (11). For this reason, these newly discovered cells play an important role in the asthmatic airways to resolve the inflammation and in clearing infections.

Viral infections, especially with rhinovirus (RV), play a key role in the development of asthma and asthma exacerbations, particularly in children (19–23). Therefore, the focus of this review article is the role of RV infections in asthma, the downstream interferon (IFN) immune response, with emphasis on type III IFNs, and their potential role as therapeutic agents in asthma.

RV STRUCTURE, GENOMIC ORGANIZATION, REPLICATION, AND IFN INDUCTION

Human rhinoviruses (HRVs), described first in the 1950s, are the primary causative agent of the common cold (24). HRV infections are concomitant with exacerbations of chronic pulmonary disease, severe pneumonia in elderly and immunocompromised adults, asthma development as well as serious bronchiolitis in infants and children (25, 26).

Human rhinovirus is a member of the family *Picornaviridae* and the genus *Enterovirus*. More than 150 identified HRV serotypes were divided into the three groups HRV-A, HRV-B, and HRV-C, according to their phylogenetic similarity (27–29).

Human rhinovirus is a non-enveloped positive-sense, single-stranded RNA (ssRNA) virus with a genome size of approximately 7,200 bp and a single open reading frame. The translated polyprotein can be divided into the three regions P1, P2, and P3, whereby the P1 region encodes for the viral capsid proteins VP1, VP2, VP3, and VP4 (termed the protomer). The P2 and P3 regions encode proteins involved in protein processing ($2A^{pro}$, $3C^{pro}$, $3CD^{pro}$), genome replication and assembly (2B, 2C, 3AB, $3B^{VPg}$, $3CD^{pro}$, $3D^{pol}$) (26, 30–32).

The virions consist of 60 copies each of the four capsid proteins building an icosahedral symmetric capsid of about 30 nm in diameter. Thereby VP1, VP2, and VP3 create the protein shell, by contrast VP4 is in the inner site of the virus, anchoring the RNA core to the capsid (33–35). Resolution of the structure revealed a prominent, star-shaped plateau, surrounded by a deep depression, also called canyon, which is the site of attachment to the different cell surfaces receptors (36, 37).

The replication of HRV takes place in the cytoplasm of epithelial cells of the lower and upper airways (38). In early HRV life cycle, it attaches to a cell membrane receptor. More than 90% of HRVs ("major" group), interact with the amino-terminal domain of the intercellular adhesion molecule 1 (ICAM-1; CD54) (39–41). The remaining "minor" group binds and enters the cell *via* a member of the low-density lipoprotein receptor family (42, 43), whereas some of the HRVs also use heparan sulfate as an additional receptor (44, 45). Moreover, HRV-C binds to Cadherin-related family member 3 (CDHR3) to enter the cells (38). Once HRV has attached to its cellular receptor, the virus capsid undergoes conformational changes, resulting in the release of the viral RNA into the cytoplasm directly (major group) (46), or by endosomal compartments (minor group) (47, 48). Following the translocation of the RNA into the cytoplasm, the viral genome replicates and translates to generate viral proteins, which are essential for the viral genome replication and the production of new virus particles.

During HRV infection, the virus adheres within 15 min to the cell surface receptors into the respiratory tract, thus the infection occurs very quickly. High-risk individuals for infection are children and elderly and infected individuals, which will experience

symptoms within 2 days after infection (49). Moreover, HRV-C specie may be able to cause severe infections (50).

While the virus replicates and spreads, the infected cells secrete inflammatory mediators, such as chemokines and cytokines. DsRNA produced during viral infection induces the host innate immune response. It is recognized and ligated by three pattern recognition molecules (PRMs): toll-like receptor (TLR)-3, which is localized at endosomal and plasma membranes, and cytoplasmic proteins retinoid acid-inducible gene 1 (RIG)-I, and melanoma differentiation-associated gene 5 (MDA-5), which are intracellular receptors for viral short-dsRNA and long-dsRNA, respectively (51–53).

MICROBIAL INDUCED IFN IMMUNE RESPONSES

The PRMs MDA-5 and RIG-1 are known to induce type I IFN expression by sensing viral dsRNA in the cytosol (**Figure 1**). RIG-1 has been shown to be involved in virus-induced type III IFN production. Some studies showed type III IFN expression in

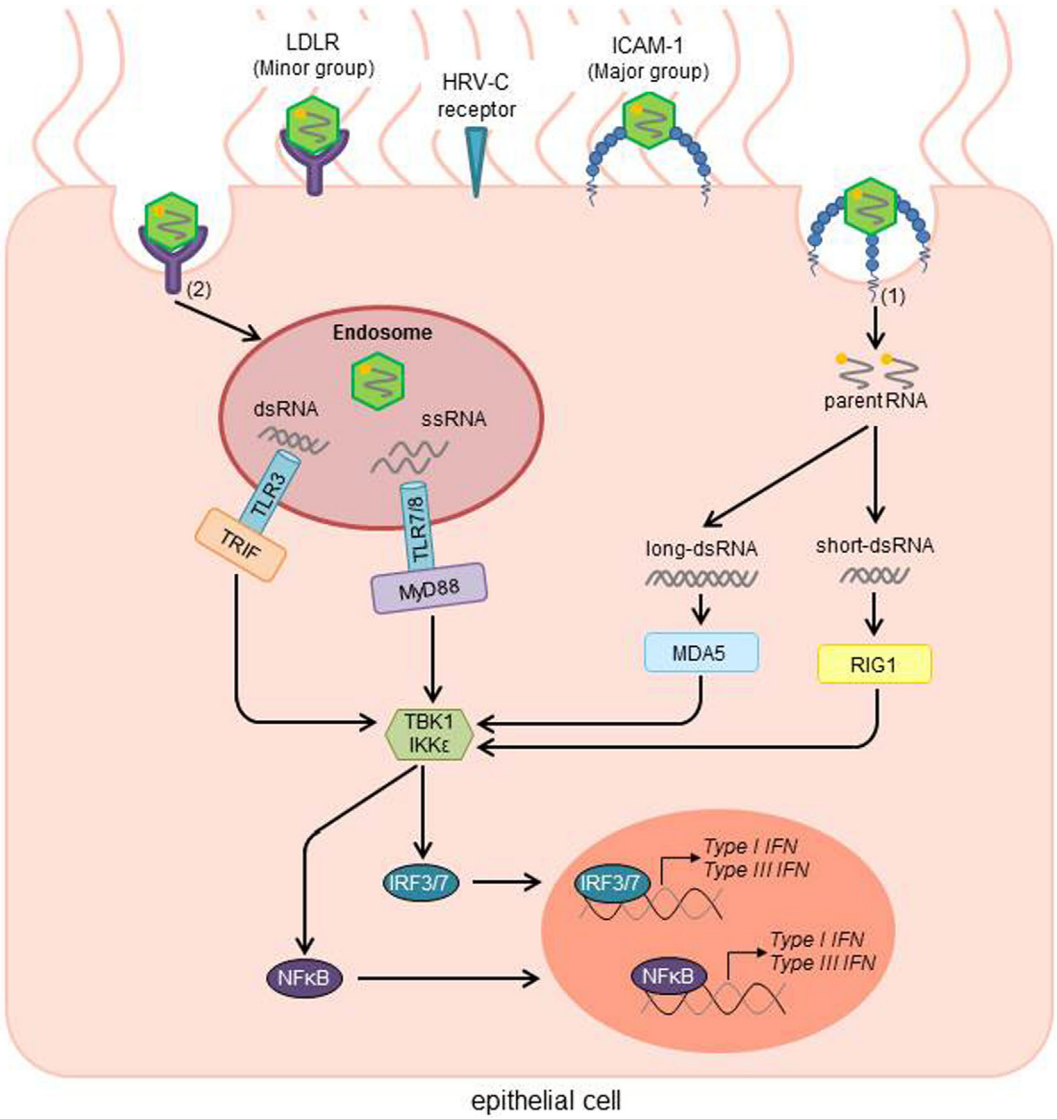

FIGURE 1 | HRV activated signal transduction pathways of the innate immune response after infection of airway epithelial cells. Depending on the receptor type, virus uptake occurs directly without a need for any cellular machinery [Major group; (1)] or *via* clathrin-dependent endocytosis [Minor group; (2)]. After uncoating, viral RNA activates cytosolic and endosomic pattern recognition molecules (PRMs). While the retinoid acid-inducible gene 1 (RIG1) recognizes short viral dsRNA, the melanoma differentiation-associated gene 5 (MDA5) binds to long dsRNA. In the endosome, viral dsRNA and ssRNA are recognized by the Toll-Like receptors TLR3 and TLR7/8, respectively. After recognition of the viral RNA, TLR7/8 then stimulates the adaptor protein myeloid differentiation factor 88 (MyD88), whereas TLR3 activates the Toll/IL-1 receptor domain-containing adaptor inducing IFNβ (TRIF). In further steps, TRIF, MyD88, MDA5, and RIG1 activate the TANK-binding kinase-1 (TBK1) and/or the inducible IκB kinase (IKK). Those factors subsequently induce the interferon regulatory factors 3 and 7 (IRF3/7), as well as NFκB as transcription factors for type I and III IFNs. HRV, human rhinovirus; ICAM-1, intercellular adhesion molecule 1; LDLR, low-density-lipoprotein receptor; RNA, ribonucleic acid; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA; IFN, interferon [adapted from Ref. (26, 55)].

DCs and monocytes after stimulation with bacterial lipopolysaccharide (LPS), transmitted by TLR4, indicating that also bacterial infections induce the production of these cytokines (54, 55). Based on the type of receptor they signal through, IFNs, cytokines named after the ability to "interfere" with viral replication, are divided into three major groups, some of which consist of subgroups, called type I, containing IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω, type II, including IFN-γ, and type III, consisting of IFN-λ (56, 57). In 2003, two independent research groups found the three highly related cytokines and while one group attributed them to the IFNs and called them IFN-λ1, IFN-λ2, and IFN-λ3, creating the type III IFNs, the other group described them as IL29, IL-28A, and IL-28B, respectively (58, 59).

Type III IFN is described to not be expressed continuously but to be co-induced with type I IFNs in different cells by various human viruses, as well as by ligands of TLR3, mimicking viral infection (synthetic dsRNA; polyI:C), TLR4 (house dust mite antigen), TLR9 (unmethylated DNA rich in CpG motifs), and TLR7 and TLR8 (guanosine or uridine-rich ssRNA or resiquimod) (55, 58, 60).

The promoters of the *IFNL* genes contain binding sites for the transcription factors NF-κB (nuclear factor κB) and AP-1 (dimerizing with other transcription factors like FOS, JUN, ATF, and MAF), and several virus response elements are the binding sites of IFN regulatory factors (IRFs). The transcription of the type I *IFN* genes is regulated by these factors as well. Furthermore, the NF-κB and IRF pathways were shown to be very relevant to the transcriptional regulation of the *IFNL* genes (60).

While the transcription of human *IFNL1* and *IFNB* genes seem to be controlled similarly by either IRF3 or IRF7, *IFN-L2/3* genes share the dependency on IRF7 with most *IFNA* genes. IRF3 is produced continuously and ubiquitously in cells and increases the expression of the *IFNB* and *IFNL1* genes on recognition of viral entry. In addition to IRF3, *IFNA* and *IFNL2/3* genes need IRF7, which is upregulated in response to IFNs and not continuously expressed in most cell types otherwise. This upregulation in cells can, in humans, be primed by both IFN-β and IFN-λ1 in response to viral induction, leading to IFN-α and IFN-λ2/3 being produced. It has been shown that, as described for type *I IFN* genes, *IFNL* genes utilize a positive feedback mechanism in their expression (60).

After entering the cell, some viruses leave the cytoplasm to enter the endocytic compartments. Depending on the compartment, different PRMs are responsible to detect the antigen, using various ways of inducing IFNs. TLR7, TLR8, and TLR9 activate the adaptor protein myeloid differentiation factor-88 (MyD88), opposed to TLR3 using the Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF). MyD88, TRIF, RIG-1, and PKR each activate the production of type I IFN by utilizing the TANK-binding kinase-1 (TBK1) and/or the inducible IκB kinase (IKK-i), which activate the transcription factors IRF3 and IRF7, inducing the transcription of type I and type III IFN (**Figure 1**) (57).

TLR7, TLR8, and TLR9 use MyD88, activating NF-κB and the mitogen-activated protein kinases (MAPKs), as well as c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK). MyD88 then utilizes the serine-threonine kinase IRAK, tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6) and a MAPK kinase kinase (MAPKKK) called transforming growth factor β-activated kinase (TAK-1), to stimulate the IKK complex. After activation IKK leads to phosphorylation and subsequent degradation of IκB, the release of NF-κB, and induction of NF-κB-dependent genes, such as the pro-inflammatory cytokines IL-1, IL-6, and TNFα. Furthermore, TAK-1 can also activate MKK3 and MKK6, two enzymes upstream of JNK, MAPK, and p38 (57).

IFN RECEPTOR SIGNALING AND RECEPTOR DISTRIBUTION

Type I IFNs, which signal through the widely distributed, heterodimeric complex consisting of the two receptor chains IFN-αR1 (IFNAR1) and IFN-αR2 (IFNAR2), whereas type III IFNs use a heterodimeric complex, which consists of the IFN-λR1 chain (IL–28RA) and the shared IL-10R2 chain. The latter receptor chain is also used by the cytokines IL-10, IL-22, and IL-26. The IFN-λR1 chain is encoded on human chromosome 1 and murine chromosome 4, the IL-10R2 chain on chromosome 21 or 16, respectively, in proximity to the IFN-αR 1 and 2 chains [reviewed in Ref. (61)].

When type III IFN binds to its unique receptor chain, IFN-λR1, this facilitates a conformational change causing the recruitment of the second part of the receptor, the IL-10R2 chain. *Via* the receptor-associated Janus kinases JAK1 and Tyk2, docking sites for different STAT proteins are created, e.g., STAT1 and STAT2. Both IFN-αR and IFN-λR signaling lead to the formation of a transcription factor complex, consisting of STAT1, STAT2, and IRF9, the so called IFN-stimulated gene factor 3 (ISGF3). This complex is able to translocate to the nucleus, where it mediates the expression of several IFN-stimulated genes (ISG), by binding to IFN-stimulated response elements (ISRE) in the promoter regions of the ISGs (**Figure 2**) [reviewed in Ref. (61)].

IFN-αR is expressed on a broad range of cells, e.g., primary fibroblasts, murine splenocytes, or human endothelial cells from the umbilical cord vein (HUVEC), while the action of IFN-λ is more limited. Here, it has been described that on epithelial cells in the respiratory, gastrointestinal and urogenital tract IFN-λR is broadly expressed, whereas the response of PBMCs and cells isolated from bone marrow show only modest responses when stimulated with IFN-λ [reviewed in Ref. (61, 62)]. In addition, it has been shown that $CD11c^+$ DCs isolated from the lung (**Figure 3**), as well as alveolar macrophages and bone marrow-derived DCs express the IFN-λR1, while inflammatory cells in the peribronchial region do not express this receptor subunit. In this study, it was also observed that $CD4^+$ T cells isolated from lung or spleen and *in vitro* differentiated Th1, Th2, and Treg cells do not significantly express IFN-λR1 and therefore are not affected by IFN-λ treatment (63). Furthermore, other groups described a lack of IFN-λR expression on leukocytes, primary fibroblasts, HUVECs, and murine splenocytes (61). Interestingly, the shared IL-10R2 chain is also expressed rather broadly, so that the limited action of IFN-λ is restricted by the selective expression of the IFN-λR chain (62).

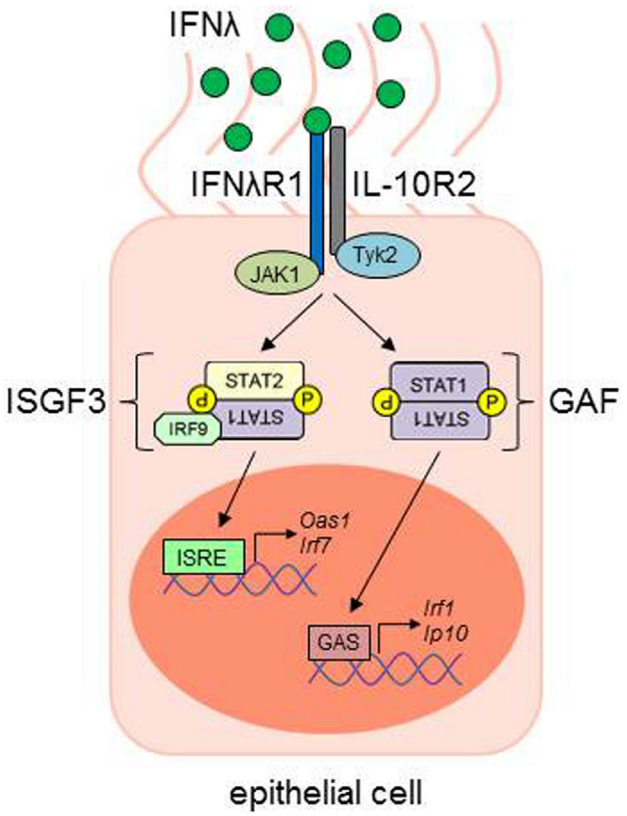

FIGURE 2 | IFN-λ signaling in epithelial cells. IFN-λ binds to its IFNλR1, which leads to a conformational change and the recruitment of the IL-10R2 chain. Receptor-associated kinases (JAK1, Janus kinase 1; Tyk2, tyrosine kinase 2) trans-phosphorylate the respective receptor chains, leading to the phosphorylation and activation of signal transducer and activator of transcription (STAT) proteins. STAT1 and STAT2, together with IFN-regulatory factor 9 (IRF9), build the IFN-stimulated gene factor 3 (ISGF3) transcription factor complex, while the STAT1 homodimer is also named gamma-IFN activated factor (GAF). ISGF3 and GAF complexes are able to translocate to the nucleus and bind to IFN-stimulated response elements (ISRE) or gamma-IFN activation sites (GAS), respectively, in the promoter regions of IFN-stimulated genes (ISG), e.g., *Oas1*, *Irf1*, *Irf7*, or *Ip10* [adapted from Ref. (61)]. ISG, interferon-stimulated genes.

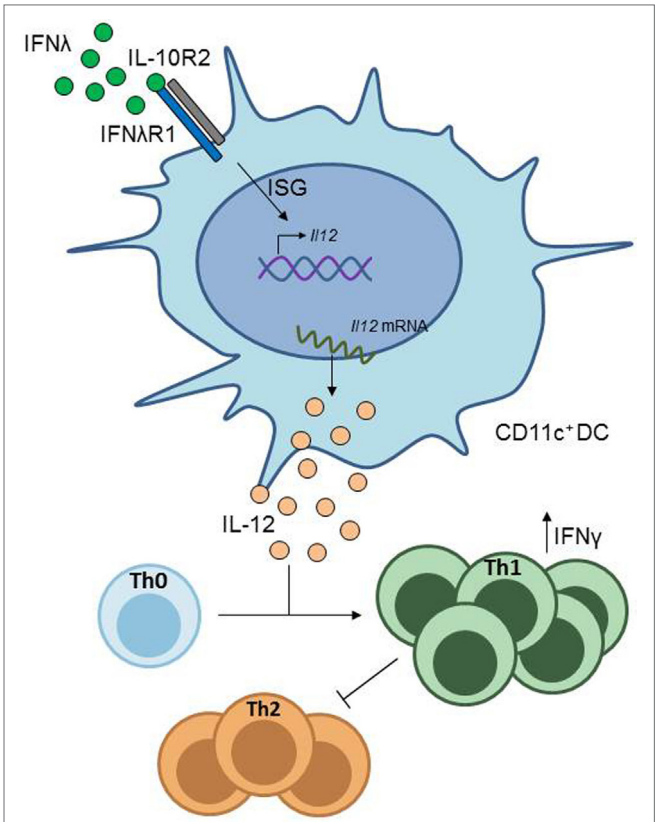

FIGURE 3 | IFN-λ signaling in CD11c⁺ dendritic cells (DCs). CD11c⁺ DCs express the IFN-λ receptor. Stimulation of CD11c⁺ DCs with IFN-λ induces IL-12 production in these cells, which drives Th1 differentiation from naïve CD4⁺ T cells, resulting in inhibition of Th2 cell differentiation.

IMMUNOLOGICAL ROLE OF TYPE III IFN IN ASTHMA

It has been previously proposed that in asthmatic patients, the IFN response to RV is impaired. *In vitro* infection of primary human bronchial epithelial cells (HBEC) isolated from asthmatic or healthy control subjects with the major group RV subtype RV16, revealed increased viral replication and delayed cell death of infected cells in cells from asthmatics. In addition, these cells released decreased amounts of the type I IFN IFN-β, while treatment of cells from asthmatics with exogenous IFN-β enhanced apoptotic cell death and reduced viral replication, especially when cells were pre-treated with IFN-β before RV infection (64). In a following study extending these findings, it was shown that in the human bronchial epithelial cell line BEAS-2B and in peripheral blood mononuclear cells (PBMC) from healthy volunteers, infection with RV16 induced Type III IFN expression both on mRNA and protein level. Furthermore, it was shown that treatment of BEAS-2B cells with exogenous type III IFN induced the expression of ISGs, e.g., CCL5 and CCL10, in resting cells, as well as after RV16 infection, in the latter case the induction of those genes was many times higher than in uninfected cells (65). As shown in the study by Wark et al., also here viral replication was increased in HBECs isolated from asthmatic subjects as compared to healthy controls, and here, it was observed that Type III-IFN expression was decreased in asthmatics. These results were then confirmed in primary bronchoalveolar lavage cells, where cells isolated from asthmatic patients and *ex vivo* treated with RV16 also secreted lower amounts of type III-IFN. To substantiate these findings, the same subjects from each cohort were experimentally infected with RV and clinical parameters were evaluated. It was shown that asthmatic patients experience a higher cold score, higher airway inflammation, and higher virus load, which inversely correlated with the decreased type III IFN secretion observed earlier in the *ex vivo* experiments, indicating a defect of patients suffering from asthma to mount effective antiviral responses against RV (65).

Another study using HBEC from healthy and asthmatic volunteers, which were then *in vitro* infected with either minor group RV1b or major group RV16, found that there was no difference in IFN-β or type III IFN secretion from both study

groups after 48 h of culture after RV1b infection. However, *IFNL1* mRNA expression was even increased in HBECs from asthmatic patients after infection with RV16 and subsequent 48 h of cell culture. The release of viral particles from RV1b or RV16 infected cells from both study groups did not differ significantly at any time-point analyzed. An explanation for the contradictory results could be the use of HBEC from patients with different asthmatic phenotypes or might be due to technical differences (66).

However, a protective role of type III IFN in asthma has also been shown by a group in a study using a murine model of allergic asthma (19). In the study, they found that in mice lacking the IL-28 receptor α chain (IL–28Rα$^{-/-}$) the asthmatic phenotype was worsened, accompanied by increased airway inflammation and mucus production, as well as higher levels of Th2- and Th17-associtated cytokine secretion. In contrast, wild type mice treated with either recombinant or adenoviral expressed IFN-λ2 (IL–28A) showed an improvement of asthmatic symptoms, concomitant with diminished Th2 and Th17 responses and an increase in IFN-γ expressing Th1 cells. This latter effect was attributed to lung CD11c^{+} DCs, which, in response to stimulation with IL-28A, downregulate the co-stimulatory molecule OX40 ligand (OX40L) and upregulate IL-12 production, a cytokine promoting Th1 cell development (**Figure 3**) (63).

In a recent study, our group has shown that in a cohort of pre-school children with and without asthma at baseline, *IFNA* mRNA levels were markedly decreased in PBMC isolated from the asthmatic group, both at mRNA level in PBMCs and at protein level in serum, when the children were sub-divided in accordance to RV detection in their upper airways. The analysis of type III IFN in serum instead revealed an increase of this cytokine in children with positive RV detection in their airways in both the control (tendency) and the asthmatic (significant) group. During the course of the study, asthmatic children were asked to come to their study center within 2 days, when they experience a respiratory infection/cold or an exacerbation of their disease (=symptomatic visit). When the baseline data of serum IFN-α were compared to those at symptomatic visits, a significant increase in IFN-α was observed in children with RV detected in their upper airways. However, at symptomatic visits, virus detection was positive in all children, so that no statement could be made about the RV negative group. In contrast, serum type III IFN levels did not significantly differ between the recruitment visit and symptomatic visit. This indicates that these responses are transient and dependent on certain stimuli (67).

Taken together, so far different results on the ability of asthmatic patients to respond to RV with IFNs have been reported. Probably, the time of analysis and the material analyzed is of importance to get significant results. Furthermore, as asthma is very heterogeneous, also the stage of disease or the underlying immunological processes might influence the detection of IFNs and thereby influence the conclusions drawn.

CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

Currently, the therapy of asthma is still mainly symptomatic with a combination of treatment with corticosteroids to inhibit the inflammatory processes in the lung and/or β2-agonists in order to relieve the bronchospasm (68). Recent therapeutic strategies focused on the neutralization of single effector molecules, such as IgE (omalizumab) and IL-9 (MEDI-528) or on receptor blockade, e.g., by a mutated form of IL-4 (pitrakinra), which binds to the IL-4Rα chain and blocks the binding of IL-4 and IL-13 [reviewed in Ref. (68)]. So far, these approaches did not bring a breakthrough in asthma therapy.

The use of IFN-α as a therapeutic agent for persistent RV infections has shown promising results, e.g., as RV RNA was cleared after subcutaneous administration to patients with hypogammaglobulinemia (69). However, in other trials treating either healthy volunteers intra nasally with IFN-α2 after experimental RV infection, or asthmatic patients with steroid-resistant disease, the symptoms were alleviated dose-dependently but side-effects such as headaches and nausea were also observed. Similar results were obtained after IFN-β administration, limiting the use of type I IFNs for asthma therapy [reviewed in Ref. (19, 70)]. As the action of type III IFNs is not as broad as that of type I IFNs, due to limited receptor distribution, their use might be advantageous in the treatment of different diseases. So far, few trials with type III IFN have been conducted in humans and many more details about the connection of different symptoms and IFN expression need to be clarified before these can be established. Yet, current data hint the potential of type III IFN administration as a therapeutic option for example for asthma, as it is able to modulate immune responses, e.g., by inhibiting Th2 and inducing Th1 responses (63, 71).

AUTHOR CONTRIBUTIONS

NS wrote the introduction and the IFN signal transduction part and generated **Figure 2**. AP and JK did the part of the virus infection and IFN-induction and generated **Figure 1**. SF supervised the all process enclosing the figures and manuscript.

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MINI REVIEW
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Interferon-λs and Plasmacytoid Dendritic Cells: A Close Relationship

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Interferon lambdas (IFNλs) are recently discovered cytokines acting not only at the first line of defense against viral infections but also at the mucosal barriers. In fact, a peculiar feature of the IFNλ system is the restricted expression of the functional IFNλR, which is known to be limited to epithelial cells and discrete leukocyte subsets, including the plasmacytoid dendritic cells (pDCs). In the latter case, current data, discussed in this minireview, indicate that IFNλs positively regulate various pDC functions, including pDC expression of interferon-dependent gene (ISG) mRNAs, production of cytokines, survival, and phenotype. Although the knowledge of the effects on pDCs by IFNλs is still incomplete, we speculate that the peculiar pDC responsiveness to IFNλs provide unique advantages for these innate immune cells, not only for viral infections but also during autoimmune disorders and/or tumors, in which pDC involvement and activation variably contribute to their pathogenesis.

Keywords: plasmacytoid dendritic cells, interferon lambda, innate immunity, IFNα, IL-3, CXCL10, TNFα

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INTRODUCTION

Human dendritic cells (DCs) in the blood typically include the myeloid DCs (mDCs), enlisting the BDCA1$^+$/CD1c$^+$ and BDCA3$^+$/CD141$^+$ DCs, as well as the plasmacytoid DCs (pDCs) (1). All peripheral DCs originate from a common DC progenitor (2) and act as antigen-presenting cells (APCs) to initiate adaptive immune responses (3). Among DCs, pDCs are distinguishable given their peculiar phenotype, tissue localization, and specialized functions (4). pDCs constitute 0.2–0.6% of the peripheral blood mononuclear cells (PBMCs) in healthy individuals (5) and are specialized in the production of type I interferon (IFN) (6–8). Human pDCs specifically express the C-type lectin BDCA2/CD303 molecule, the alpha chain of the interleukin-3 receptor (IL-3Rα/CD123), and neuropilin-1/BDCA4 (9), but not CD11c, which is instead expressed by mDCs (1, 3). Under steady state conditions, pDCs localize in the T cell areas of the lymph nodes (LNs), while they are undetectable in almost all peripheral tissues (5, 10). Migration of pDCs into LNs and inflamed tissues involves discrete adhesion molecules (CD62L, PSGL-1, β1- and β2-integrin), as well as activated chemokine receptors, including CXCR3, CXCR4, CCR2, CCR5, and CCR7 (11, 12). Once recruited into tissues, pDCs orchestrate immune responses, as well as interact with, activate, or are activated by T, B, NK cells, and other leukocytes (4, 13, 14).

Plasmacytoid dendritic cells are specialized in recognizing viral and/or self/non-self nucleic acids, for instance through TLR7 and TLR9, to ultimately produce IFNα following an intracellular signaling cascade activating interferon regulatory factor 7 (IRF7) (15). IFNα, in turn, not only induces the transcription of interferon-dependent genes (ISGs) to limit the spread of viral pathogens (16) but also amplifies immune responses by modulating selected functions of NK, myeloid, B and T cells (17, 18). TLR7/9 engagement also leads pDCs to differentiate into mature cells, thus acquiring a more DC morphology and APC capacity (5, 19, 20). Similar effects on pDCs are observed in response to IL-3, a cytokine also known to maintain pDCs alive (10). Accordingly, TLR and/or

IL-3-stimulated pDCs upregulate the expression of MHC-II and costimulatory molecules (including CD80, CD86, and CD40), as well as produce both proinflammatory cytokines (TNFα and IL-6) and chemokines (CCL4, CCL5, CXCL9, and CXCL10) (7, 11, 13, 21). Notably, endogenous TNFα concurs to pDC maturation (22), while autocrine/paracrine IFNα promotes the survival of pDCs *via* induction of antiapoptotic genes (23). Activated/mature pDCs, in turn, become able to promote the polarization of T helper lymphocytes into Th1, Th2, Th17, or also Treg cells, depending on the context (7, 8, 10, 24).

Plasmacytoid dendritic cells also produce type III IFNs/IFNλs (25), for instance in response to HSV (26–28), Sendai virus (27), Flu (27), Imiquimod/R837 (synthetic TLR7 ligands) (26–29), CpG oligodeoxyribonucleotides (26–28, 30–32), or upon cocolture with hepatitis C virus (HCV)-infected Huh7.5 (30, 31). The IFNλ family includes four members, three of them identified in 2003 (e.g., IFNλ1/IL-29, IFNλ2/IL-28A, and IFNλ3/IL-28B), the fourth one (IFNλ4), which shares only ~30% identity with other IFNλs, but signals through the same receptor complex, discovered more recently (2013) (33). IFNλs not only display potent antiviral activities (34–36) but also exert other effects involved in autoimmunity and tumor progression (37, 38). Moreover, it has become increasingly clear that IFNλs evolved to serve as a first line of defense at the mucosal barrier, particularly at the level of the respiratory and gastrointestinal tracts, which are the initial target of most invasive pathogens (36). In fact, a peculiarity of the IFNλ system is the restricted distribution of the IFNλR (39–41), which consists of a specific IFNλR1 chain (also known as IL-28R), and the ubiquitously expressed IL10R2 chain (40, 41). Epithelial cells of the intestine, lungs, skin, and liver constitutively express the IFNλR1 chain and thus represent the primary targets of IFNλs (42). In such regard, there has been a great interest in specifically characterizing the antiviral role of IFNλs during HCV and hepatitis B virus infections (43–47). In the former case, in fact, although not yet explained in the context of HCV pathogenesis, several genome-wide association studies have demonstrated a link between single-nucleotide polymorphisms near the IFNλ3 and IFNλ4 genomic loci and either the spontaneous clearance or the sustained response to IFNλ-treatment in HCV-infected patients (48–50). Moreover, IFNλ1 has been used for clinical trials in HCV patients (51) confirming an antiviral efficacy equivalent to IFNλ, but with less toxicity (51). Fibroblasts, splenocytes, bone marrow (BM)-derived macrophages, and endothelial cells do not express IFNλR1 and thus do not respond to IFNλs (42, 52, 53). Among human leukocytes, only pDCs and, less prominently, B cells, have been shown to constitutively express a complete IFNλR (26, 27). Consistently, IFNλs have been shown to trigger phosphorylation of STAT1 (27, 54, 55), STAT2 (54), STAT3, and STAT5 (55), in either freshly isolated pDCs (54) or pDCs gated among total PBMCs (27, 55), as well as various functional responses herein summarized.

PRODUCTION OF CYTOKINEs BY pDCs INCUBATED WITH IFNλs

Interferon lambdas have been described to stimulate the production of cytokines and chemokines in pDCs. We reported that human pDCs incubated for up to 42 h with 30 IU/ml IFNλ1 or IFNλ3 produce variable, but significant, levels of CXCL10, usually (but not always) followed by IFNα (54). Consistently, experiments using anti-IFNαR antibodies only partially blocked CXCL10 derived from pDCs incubated with IFNλ3 for 42 h (54). Notably, healthy donors could be categorized into two groups based on the levels of IFNα produced by their IFNλ3-treated pDCs [e.g., very modest ≤150 pg/ml/42 h: elevated ≥500 pg/ml/42 h] (54). By similar criteria, referred instead to CXCL10, healthy donors could be independently divided into three groups: one having pDCs producing modest quantities of CXCL10 (ranging from 22 ± 11 pg/ml/18 h to 163 ± 24 pg/ml/42 h); another one, having pDCs producing elevated CXCL10 levels already after 18 h (865 ± 297 pg/ml) without further increasing thereafter; and a third one, having pDCs producing maximal CXCL10 levels after 42 h of IFNλ3-treatment (1,320 ± 264 pg/ml) (54). It should be pointed out that such an extremely variable production of both IFNα and CXCL10 were shown not to depend on differences in the viability of pDCs among the donor groups. Moreover, the patterns of CXCL10 production by pDCs somewhat recalled previous data (56), likely attributable to pDCs, in which PBMCs from healthy donors were described to function either as "early" or as "late" responders to 3,500 IU/ml IFNλ1, depending, respectively, on the more rapid or more delayed kinetics of CXCL9, CXCL10, and CXCL11 transcript induction. Whatever the case is, the molecular bases underlying the variable capacity of pDCs to produce IFNα and CXCL10 by the different donor typologies, as well as their potential biologic implications, require further investigations.

In addition to CXCL10 and IFNα, we also detected low but biologically active amounts of TNFα in supernatants harvested from purified pDCs incubated with IFNλ3 (54). In fact, experiments in which supernatants from IFNλ3-treated pDCs were transferred to $CD14^+$-monocytes in the presence or absence of reagents inhibiting TNFα, namely etanercept (ETA) and adalimumab, revealed that they induced CCL4 and IκBα mRNA expression in a TNFα-dependent manner (54). It should be pointed out that, in contrast with our results, 3,500 IU/ml IFNλ1-treated PBMCs were previously found able to produce CXCL8, IL-6, and IL-10, but not TNFα or IL-1α (57), possibly because of the short stimulation period. Similarly, Flt3-generated BM-derived murine pDCs incubated with IFNλ2 were found unable to produce CXCL10 and IL-6 (58). However, whether Flt3-generated BM-derived murine pDCs express the complete IFNλR, or whether their blood counterpart behaved as human pDCs, was not reported.

Because flow cytometry experiments uncovered that both IFNλ3 and IL-3 increase the levels of surface CD123 and IFNλR1 in human pDCs (54, 59), in a subsequent study, we investigated whether IFNλ3 and IL-3 together could promote stronger pDC responses. This was found to be the case, as we could show that 30 IU/ml IFNλ3 and 20 ng/ml IL-3 induce in pDCs a synergistic production of both IFNα and TNFα (59). Moreover, endogenously produced TNFα was found to almost completely control the synergistic production of IFNα in IFNλ3 plus IL-3-treated pDCs (59). Under the same experimental conditions, or in pDCs incubated with IFNλ3 only, endogenously produced IFNα did not drive ISG mRNA expression, unlike its effect in IL-3-treated pDCs. On the

other hand, endogenous TNFα was found to drive ISG mRNA expression in both IFNλ3- and IL-3-stimulated pDCs (59).

EXPRESSION of ISG mRNAs AND PHOSPHORYLATION of STATs IN IFNλ-TREATED pDCs

Plasmacytoid dendritic cells have been shown to *de novo* express a variety of ISG mRNAs in response to IFNλs, which further support the protective role of the IFNλ/pDC system in viral infections. For example, 2'-5'-oligoadenylate synthetase 1 (OAS1) and IRF7 mRNAs were found as induced in murine pDCs incubated with 100 ng/ml IFNλ2 (52). In humans, we and others have reported that both IFNλ1 and IFNλ3 induce the mRNA expression of MX dynamin like GTPase 1 (MX1) (59, 60), protein kinase R (PKR), interferon induced protein with tetratricopeptide repeats 1 (IFIT1), ISG ubiquitin-like modifier (ISG15), and C-X-C motif chemokine ligand 10 (CXCL10) (54, 55, 59). Our unpublished observations prove that also CXCL9, TLR7, IFIT2, and TNF-related apoptosis inducing ligand (TRAIL) are induced by IFNλ3 in human pDCs. All these mRNAs were shown to reach maximal levels after 18 h of incubation of pDCs treated with 30 IU/ml IFNλ1 or IFNλ3 (54). Experiments conducted in pDCs preincubated in the presence of anti-IFNαR antibodies, and then cultured with IFNλ3 plus IL-3, which, at the 18 h-time point, express and release much higher levels of, respectively, ISG mRNAs and IFNα, than pDCs incubated with IFNλ3 alone (59), revealed that endogenous IFNα is minimally involved in autocrinally activating ISG mRNA expression (59). Consistently, and even though IFNα is typically considered more potent than IFNλ in inducing ISG gene expression, we observed that equivalent concentrations of IFNλ3 and IFNα (e.g., 30 IU/ml) induce, in human pDCs, comparable levels of STAT1 and STAT2 phosphorylation and ISG15, IFIT1, and MX1 transcripts (our unpublished observations). However, we also noticed that kinetics of both STAT phosphorylation and ISG mRNA induction were more accelerated in response to IFNα than IFNλ3, consistent

TABLE 1 | Biological effects of interferon lambdas in human plasmacytoid dendritic cells (pDCs).

IFNλ type	Dose	Investigated response in pDCs	Outcome	Modality of detection	Reference
IFNλ3	30–100 IU/ml	Modulation of IFNλR expression	Increase of mRNA and surface IFNλR1	Real-time qPCR and flow cytometry	[(59) and our unpublished observations]
IFNλ1, IFNλ2, IFNλ3	35–350 IU/ml	Activation of signaling pathways	Induction of STAT-1, -3, -4, and -5 phosphorylation[a]	Flow cytometry	(27, 55)
IFNλ3	30 IU/ml		Induction of STAT-1 and -2 phosphorylation	Immunoblotting	(54)
IFNλ1	35–350 IU/ml	Modulation of maturation markers	Upregulation of CD80, ICOS-L, CD62L, CD83, MHC-I[a]	Flow cytometry	(26, 27)
IFNλ1, IFNλ3	30–100 IU/ml		Upregulation of HLA-DR, CD123, CD83, CD86, CD303, CD62L	Flow cytometry	(54, 59)
IFNλ1	35–350 IU/ml	Survival	Counteraction of the proapoptotic effect exerted by Dexamethasone[a]	Annexin V/propidium iodide staining and intracellular detection of active caspase-3	(27)
IFNλ1, IFNλ3	30–100 IU/ml		Prosurvival effect	Vybrant DyeCycle Violet stain	(54, 59)
IFNλ1	350 IU/ml	Influence on T cell functions	Inhibition of IL-10, IL-13, and IFNγ production by PMA and ionomycin-activated allogenic T cells	ELISA	(26)
IFNλ1, IFNλ3	30–350 IU/ml	ISG mRNA expression	Induction of MX1, protein kinase R, IFIT1, ISG15, and CXCL10 transcripts	Real-time qPCR	(54, 55, 59, 60)
IFNλ3	30 IU/ml		Induction of IFIT2, TLR7, TRAIL, TNFα, IFNα transcripts	Real-time qPCR	[(54, 59) and our unpublished observations]
IFNλ2	100 ng/ml		Induction of oligoadenylate synthetase 1 and interferon regulatory factor 7 transcripts (mouse pDCs)	Real-time qPCR	(52)
IFNλ1	25 ng/ml	Cytokine production	Enhancement of IFNα production in response to hepatitis C virus-infected hepatoma cells or CpG-A	ELISA	(30)
IFNλ1	35 IU/ml		Priming effect and enhancement of IFNα and IFNλ1/3-positive pDCs in response to HSV[a]	Flow cytometry	(27)
IFNλ1, IFNλ3	30–100 IU/ml		Induction of time-dependent production of CXCL10, IFNα and TNFα	ELISA	(54)
IFNλ3	30 IU/ml		Enhancement of IL-3-induced IFNα and TNFα production	ELISA	(59)

[a]*In these papers, pDCs have been identified as BDCA2$^+$/CD123$^+$ or Lin$^-$/CD123$^+$ cells, by flow cytometry, within peripheral blood mononuclear cells previously labeled with a combination of specific antibodies.*

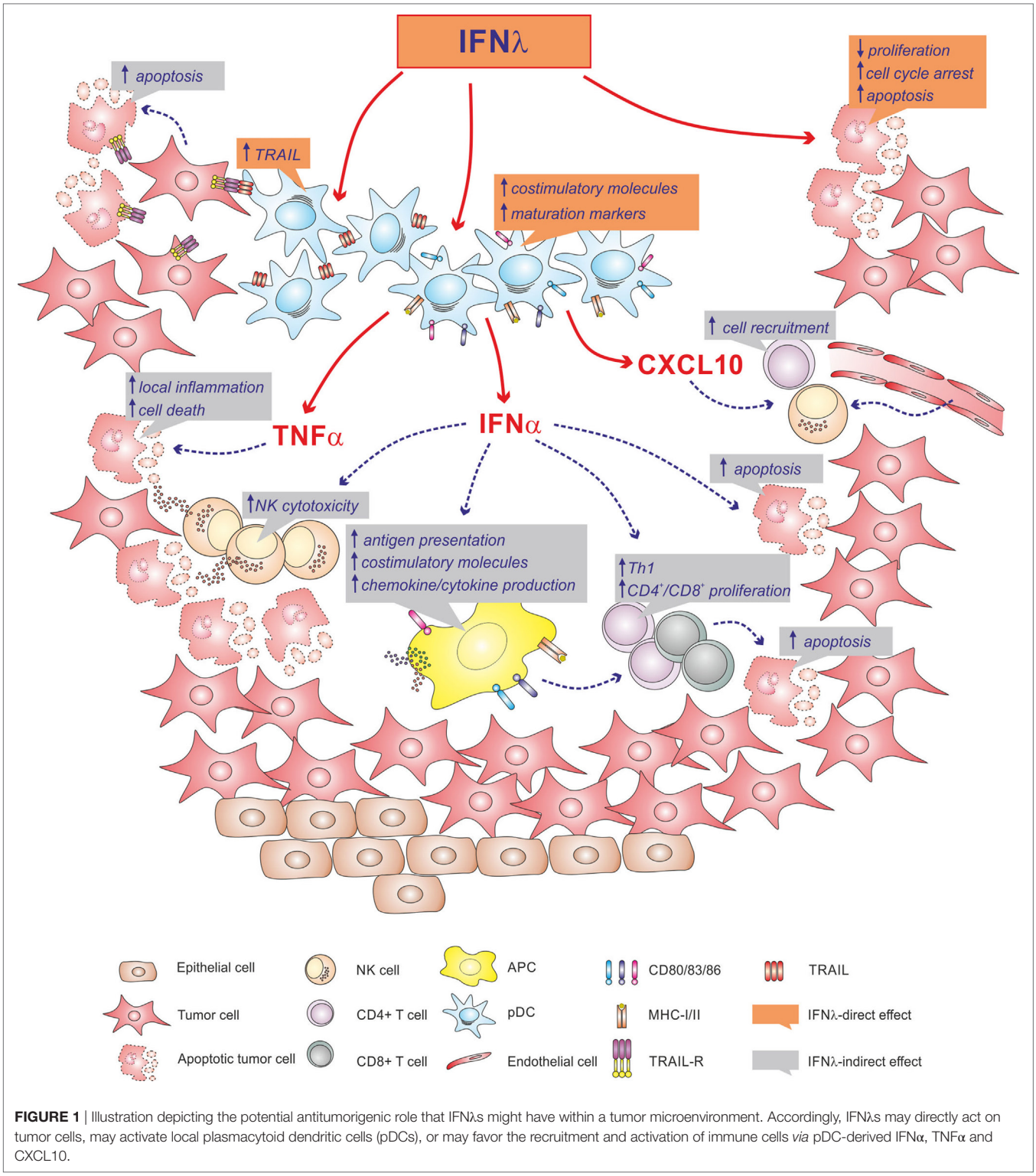

FIGURE 1 | Illustration depicting the potential antitumorigenic role that IFNλs might have within a tumor microenvironment. Accordingly, IFNλs may directly act on tumor cells, may activate local plasmacytoid dendritic cells (pDCs), or may favor the recruitment and activation of immune cells *via* pDC-derived IFNα, TNFα and CXCL10.

with studies in other cells (61–63). It should be also pointed out that, in a previous study, the levels of MX1 mRNA induced by IFNα in purified pDCs were found to be higher than those induced by IFNλ3 (60), but IFNα was used at concentrations approximately 10-fold higher than IFNλ3 (1,000 vs 100 IU/ml, respectively). Under similar experimental conditions, only IFNα, but not IFNλs, was shown to activate STAT6 phosphorylation in purified pDCs (55), independently from the concentrations used.

Recent evidence suggests that, under specific experimental settings, IFNα/β and IFNλ control gene expression, as well

as contribute to the antiviral state, by using different and non-redundant mechanisms. For instance, unlike IFNβ (64), IFNλ1 and IFNλ2 were shown to activate an alternative signaling pathway involving Jak2 in UMUC-3 and Huh7.5 cell lines (64, 65). Similarly, the antiviral activity induced in T84 cell lines by IFNλs, but not IFNα, was found to be strongly dependent on the mitogen-activated protein kinases (MAPKs) activation (66). However, whether IFNλ activates Jak2 and/or MAPK in pDCs is currently unknown.

IFNλs PROMOTE THE SURVIVAL OF pDCs

Plasmacytoid dendritic cells are known to spontaneously undergo apoptosis when cultured *in vitro* (10, 22). In this context, one of the remarkable effects that IFNλs exert in pDCs freshly purified from the blood is to prolong their survival for up to 42 h (54), similarly to IL-3 (54). While equivalent concentrations of IFNλ1 or IFNλ3 (30 and 100 IU) were found to exert comparable prosurvival activities in pDCs, no further enhancement was observed when IFNλ3 was used in combination with IL-3, indicating that each cytokine produces already the maximal prosurvival effect by itself (59). In additional experiments, we found that both endogenous TNFα and IFNα partially sustain the survival of pDCs cultured in the presence of IFNλ3. Similarly, anti-IFNαR antibodies were found to decrease survival of pDCs incubated with IL-3 alone (our unpublished observations) or CpG-C plus glucocorticoids (23), while TNFα blockers had no or only a slight effect under the same conditions (22, 23). However, no modulation of survival was found by inhibiting both TNFα and IFNα in pDCs cultured with IFNλ3 plus IL-3. Conceptually, our data not only confirm, but further support, previous observations showing that 35–350 IU/ml IFNλ1 counteracts the proapoptotic effects that dexamethasone (DEX) exerts in pDCs present within PBMCs (27). The molecular mechanisms whereby IFNλs promote pDC viability are unknown and should be characterized.

IFNλs MODULATE THE EXPRESSION OF VARIOUS SURFACE MARKERS IN pDCs

In addition to inducing cytokine production and ISG mRNA expression, or promoting survival, IFNλs have been shown to trigger the maturation of pDCs, according to phenotypic changes. For instance, incubation of PBMCs with 35–350 IU/ml IFNλ1 for 7 or 20 h has been shown to weakly increase the surface expression of CD80, ICOS-L, CD62L, CD83, CCR7, and MHC-I, but not of CD86, in $CD123^+/CD303^+$-gated-pDCs (26, 27). By using freshly isolated pDCs, we could confirm that 30–100 IU/ml IFNλ3 potently and persistently (e.g., for up to 42 h) modulates the expression of CD86, HLA-DR, CD123, and CD303, in addition to CD62L and CD83. However, in contrast with the data by Megjugorac et al. (26), we found an upregulation of CD86 upon treatment of pDCs with IFNλ3 for 42 h. Although IFNλ3-mediated effects substantially resembled those induced by IL-3 (54, 59), IFNλ3 appeared significantly less potent in upregulating HLA-DR or CD86 expression, or in downmodulating CD303 and CD62L, consistent with a weaker maturational effect on pDCs. Functionally, only one study (26) has specifically analyzed whether 350 IU/ml IFNλ1-treated pDCs could activate $CD4^+$ T cells. Accordingly, it has been reported that cocultures of IFNλ1-treated pDCs with allogenic T cells, activated by PMA/ionomycin, produce reduced levels of IL-10, IL-13, and IFNγ than in the absence of IFNλ1 (26). Whether IFNλ-treated pDCs promote Th1, Th2 or Treg polarization has not been specifically investigated yet.

CONCLUSION

As synthetically outlined in this minireview, current data suggest that IFNλ is able to regulate pDC functions at various levels (as summarized in **Table 1**), including the production of IFNα, CXCL10, and TNFα. Because IFNα has been shown to increase the production of IFNλ by $CD141^+$ DCs in response to HCV-infected hepatoma cells or poly-I:C (30), data testify for potential cross talk between pDCs and $CD141^+$ DCs *via* the two IFN systems. A strict cross talk between pDCs and B cells has been also described, as B cells are known to enhance IFNα, and possibly IFNλs, production by pDCs, *via* cell–cell contact-dependent mechanisms or soluble factors (14). Conversely, TNFα and CXCL10 secreted by IFNλ-activated pDCs might contribute to, respectively, amplify local inflammatory responses and recruit activated T lymphocytes. On the same line, modulation of pDC membrane markers by IFNλ might influence T cell polarization, either promoting or impairing T cell responses, depending on the context. Thus, *in vitro* experiments suggest that IFNλs could orchestrate complex immune cell interactions by amplifying pDC responses, both directly and indirectly. Since *in vitro* pDCs increase the expression of IFNλR1 in response to IL-3 (59), IFNλ3 (59), or R837 (our unpublished observations), it is likely that this phenomenon also happens at the site of infection in response to viral particles or other stimuli. However, whether IFNλR1 modulation positively or negatively affects pDC response to IFNλ, and, in turn, pDC cross talk with other immune cell subpopulations, is not known. Similarly, even though there are three splice variants of the human IFNλR1 gene, encoding either the full length functional IFNλR1, a soluble IFNλR1, or an IFNλR1 variant lacking a membrane-proximal region of the intracellular domain and expected to be signal-incapable (67), no information is present on how they are regulated in pDCs.

As mentioned, given the peculiar expression of IFNλR1 in hepatocytes, clinical trials of IFNλ1 therapy for HCV infection have confirmed that this cytokine has antiviral effects equivalent to IFNα without the same level of associated toxicity (51). Studies of IFNλ treatment of influenza A virus-infected mice have shown similar results (58). In this context, it would be interesting to determine if, and how, circulating and/or tissue resident pDCs are affected by the IFNλ-treatment. Such knowledge might eventually help clarifying the *in vivo* biologic implication(s) of the variable capacity of pDCs to produce IFNα and CXCL10 by the various donor typologies that we described (54). Regardless, treatment with IFNλs might be also useful in patients with autoimmune disorders. A tissue infiltration by pDCs, as well as a type I IFN signature, has been in fact described in SLE, Sjogren's syndrome, systemic sclerosis, and psoriasis patients (4). In these

diseases, pDCs are chronically activated and contribute to their pathogenesis (4). Moreover, high amounts of IFNλ1 or IFNλ2/3 have been detected, respectively, in skin lesions from psoriasis patients (68) and in serum of SLE patients (69, 70), thus pointing for some roles of IFNλs in these diseases (37, 70). In a mouse model of autoimmune arthritis, treatment with IFNλ reduced neutrophil infiltration in the joints and improved disease outcome (71). Similarly, a protective role for IFNλ in allergic asthma has also been proposed (72). Altogether, data suggest that pDCs and IFNλs can have variable contributions to the pathogenesis of autoimmune disorders and could be used as a therapeutic target by either improving or blocking their activity (73).

Neoplastic cells frequently express IFNλR1 and, after treatment with IFNλs, stop the cell cycle and/or undergo apoptosis (38, 73). In other cases, tumor cells exposed to IFNλs have been shown to become protumorigenic (73). In tumors, infiltration by pDCs is often associated with a poor prognosis, as pDCs tend to be tolerogenic and/or impaired in their functions (4, 74). However, if properly stimulated, pDCs can also promote antitumoral response, for instance, by directly killing tumor cells through TRAIL expression (75), or indirectly *via* IFNα, which mediates NK cell activation. Thus, based on our unpublished observations indicating that IFNλs, in addition to triggering IFNα production, also induce TRAIL mRNA expression in human pDCs, it would be plausible speculating a potential use of IFNλs as adjuvants to chemotherapy regimens (76). Accordingly, IFNλs may induce antitumor activities either by directly acting on tumor cells and intratumor pDCs, or by indirectly favoring the recruitment and activation of immune cells, to ultimately kill tumor cells (**Figure 1**).

AUTHOR CONTRIBUTIONS

GF, NT, and MC have contributed by writing the manuscript.

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Interferon-λs: Front-Line Guardians of Immunity and Homeostasis in the Respiratory Tract

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Type III interferons (IFNs), also termed lambda IFNs (IFNλs) or interleukins-28/29, constitute a new addition to the IFN family. They are induced upon infection and are particularly abundant at barrier surfaces, such as the respiratory and gastrointestinal tracts. Although they signal through a unique heterodimeric receptor complex comprising IFNLR1 and IL10RB, they activate a downstream signaling pathway remarkably similar to that of type I IFNs and share many functions with them. Yet, they also have important differences which are only now starting to unfold. Here, we review the current literature implicating type III IFNs in the regulation of immunity and homeostasis in the respiratory tract. We survey the common and unique characteristics of type III IFNs in terms of expression patterns, cellular targets, and biological activities and discuss their emerging role in first line defenses against respiratory viral infections. We further explore their immune modulatory functions and their involvement in the regulation of inflammatory responses during chronic respiratory diseases, such as asthma and chronic obstructive pulmonary disease. Type III IFNs are, therefore, arising as front-line guardians of immune defenses in the respiratory tract, fine tuning inflammation, and as potential novel therapeutics for the treatment of diverse respiratory diseases, including influenza virus infection and asthma.

Keywords: interferons, respiratory tract diseases, infection, asthma, cytokines, innate immunity

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INTRODUCTION

Interferons (IFNs) have a long history. Type I IFNs were first discovered in 1957 as factors that "interfere" with viral replication (1). Type II IFN was identified a few years later, in 1965, as a molecule secreted by activated lymphocytes in response to antigenic stimulation (2). Yet, it was not until 2003 that a third type of IFNs also capable of "interfering" with viral infection termed type III IFNs, lambda IFNs (IFNλs) or interleukins-28/29 was described (3, 4). This raised new questions as to why nature needs three IFN systems and new challenges as to which specific roles each type of IFN fulfils.

Type III IFNs comprise four members in humans, IFNλ1/IL-29, IFNλ2/IL-28A, IFNλ3/IL-28B, IFNλ4, and two (IFNλ2/IL-28A, IFNλ3/IL-28B) in mice (3–5). By comparison, type I IFNs in humans and most mammals are encoded by about thirteen different IFNα genes, several more distantly related genes and pseudogenes, and a single IFNβ gene (6), while type II IFNs consist of only one gene, IFNγ (7). Type III IFNs signal through a unique heterodimeric receptor complex comprising IFNLR1 (IFNLRA), conferring ligand specificity, and IL10RB (IL-10R2), also shared with IL-10 family members and required for signaling. Type I IFNs signal through IFNAR1/IFNAR2

and IFNγ though IFNGR1/IFNGR2. Notably, all IFNs share the unique ability to activate large sets of genes, collectively known as interferon-stimulated genes (ISGs) that inhibit viral replication, degrade viral nucleic acids, and induce viral resistance to neighboring cells (8). As many ISGs are known to inhibit bacterial and parasitic infection as well (9, 10), this places IFNs at the center stage of antimicrobial immunity in mammals.

Among the various IFNs, type I IFNs have long been considered to constitute the primary antiviral and antibacterial defense mechanism in the body as they can be produced by almost any cell type upon infection and can signal to almost any cell type to confer protection (11). In contrast, IFNγ does not share this ubiquitous pattern of expression. Rather, its expression is restricted to NK cells and T cells, engaged later on during the antimicrobial immune response following the production of type I IFNs, IL-12, and other innate inflammatory cues, and involved in strengthening type I IFN-mediated defenses and regulating adaptive immunity (7). However, the discovery of type III IFNs that exhibit analogous activities and expression patterns with type I IFNs has complicated this paradigm, leading to the suggestion that type III IFNs may be more important in first line defenses at barrier surfaces such as the respiratory, gastrointestinal, and urogenital tracts (12–14). Here, we review the current literature implicating type III IFNs, referred throughout as IFNλs, in the regulation of immunity and homeostasis in the respiratory tract. We highlight unique antiviral and immune modulatory functions of IFNλs not shared with type I IFNs, and discuss why two apparently similar IFN systems are needed for optimal host protection.

IFNλs EXPRESSION PATTERNS AND FUNCTIONS, AND COMPARISON TO TYPE I IFNs

IFNλs are induced in response to diverse pathogens including DNA and RNA viruses (3, 4, 15) as well as intracellular and extracellular bacteria (16, 17). In the respiratory tract, these comprise influenza viruses, rhinoviruses, respiratory syncytial viruses, *S. pneumonia*, *H. influenza*, *S. aureus*, and *M. tuberculosis*, all of which trigger high levels of IFNλs. Multiple pattern recognition receptors (PPRs) are involved in this process including endosomal toll-like receptors (TLR), such as TLR3, TLR7/8, and TLR9, and cytosolic sensors, such as RIG-I and MDA-5, recognizing double-stranded or single-stranded RNA, unmenthylated DNA, and other microbial structures (18).

Pattern recognition receptors are abundant in the respiratory epithelium and immune cells lying beneath the epithelial layer, sampling the airway lumen or residing in the lung parenchyma such as conventional and plasmacytoid dendritic cells (DCs), alveolar and interstitial macrophages, and monocytes. Interestingly, although these cells broadly respond to PRR engagement, expression of IFNλs is selective to specific cell types, most prominently epithelial cells and DCs (19–22), suggesting the involvement of additional epigenetic, transcriptional, and posttranscriptional regulation, which determines the ability of cells to make IFNλs. Indeed, RIG-I-like receptor signaling *via* mitochrondrial antiviral signaling protein (16) in peroxisomes or presence of transcriptional repressors, such as ZEB1 and BLIMP-1 (23), may provide such signals controlling IFNλ expression.

A surprising observation since the early days of their discovery was the ability of IFNλs to activate a remarkably similar downstream signaling cascade to that of type I IFNs. Despite the utilization of distinct receptor complexes, both IFNλs and type I IFNs trigger the JAK/STAT pathway, leading to the phosphorylation and nuclear translocation of STATs, the activation of interferon-regulatory factors, and the formation of the transcription complex IFN-stimulated gene factor 3 which is critically involved in the induction of ISGs (24, 25). Even on direct side-by-side comparisons in cultured cells, it has been difficult to distinguish type I from type III IFN responses (26–28). It has, therefore, been proposed that these cytokines share their antiviral activity (28–30), and indeed in numerous *in vitro* and *in vivo* studies IFNλ was shown to be as effective as type I IFNs in treating viral or bacterial infections (13, 14).

In an effort to explain why the organism employs two functional IFN systems with similar activities to confront infection, the idea of "ligand availability" was proposed (25). This was based on the notion that each unique infection induces a specific set of IFNs which accordingly determine the response. Although important, this "ligand-centric" view did not fit with many situations where both type I and type III IFNs are induced. The concept of "compartmentalization" was, therefore, put forward. This suggested that type III IFNs may be more important at barrier surfaces, such as the gastrointestinal epithelial layer, while type I IFNs may predominate once barrier surfaces are breached at the underlying tissues and the circulation. In support of that, IFNLR1 exhibits a very restricted pattern of expression compared to type I IFN receptors whose presence is ubiquitous, and is primarily found at epithelial origin cells although some leukocytes such as neutrophils can also express them (20, 21, 31, 32). Evidence for "compartmentalization" has come from recent work with intestinal pathogens indicating that IFNλs suffice to clear murine rotavirus, reovirus, or norovirus infection at the intestinal epithelium while type I IFNs are more important for preventing viral spread to the lamina propria and/or systemic dissemination (33–36). Still, compartmentalization alone may not suffice to explain the utility of two IFN systems. One report, in particular, has suggested a dispensable role for both type I and type III IFNs in murine rotavirus infection in the gastrointestinal tract, and only a temporal requirement of type III IFNs for protection against simian rotavirus infection (37). Moreover, in the respiratory track such clear-cut compartmentalization does not exist. Rather, it appears that IFNλs and type I IFNs exhibit distinct functions and activities that are only now starting to emerge.

IFNλs FUNCTIONS IN ANTIVIRAL IMMUNITY IN THE RESPIRATORY TRACT

The respiratory tract is among the sites of the body where type III IFNs are most abundantly expressed. The primary target of respiratory pathogens, such as influenza viruses and rhinoviruses, is the nose and tracheal epithelium of the upper respiratory tract but the lower airway epithelium and lung parenchyma can also

be reached. Accordingly, primary nose and airway epithelial cells, and bronchial and alveolar epithelial cell lines, can all express high levels of IFNλs following infection in culture (31, 38–40). However, smooth muscle cells, fibroblasts, and immune cells such as conventional and plasmacytoid DCs can also express IFNλs (20, 22, 41, 42), suggesting that when the epithelial barrier is breached, additional sources of IFNλ production exist.

Type I IFNs are also induced by respiratory pathogens (11, 43). Respiratory epithelial cells express IFNβ while IFNα subtypes are primarily produced by immune cells. Smooth muscle cells and fibroblasts can also make them (43). Numerous studies over the years have demonstrated the key importance of type I IFNs in providing antiviral protection against influenza and parainfluenza viruses, rhinoviruses, respiratory syncytial viruses, adenoviruses, and others. *Ifnar*$^{-/-}$ animals, in particular, have been shown to be particularly susceptible to such infections while recombinant type I IFN treatment has been shown to prevent infection (11, 44).

IFNλs have, therefore, been considered to be of secondary importance till recently. Although initial studies in mice have shown that IFNλs are the predominant IFNs produced in response to infection (45) and that *Ifnlr1*$^{-/-}$*Ifnar1*$^{-/-}$ animals are more susceptible to influenza virus infection compared to *Ifnar1*$^{-/-}$ animals, specific non-redundant functions of IFNλs in *Ifnlr1*$^{-/-}$ mice could not be described (20, 28, 46–48). IFNλs induce ISGs but so do type I IFNs. IFNλs can also activate NK cells when overexpressed (49), and endogenous IFNλ production seems to be required for optimal NK cell activity but these effects are indirect as NK cells do not express IFNLR1 (50). In addition, type I IFNs are direct and more potent activators of NK cells (51). Yet, recent more refined studies have started to uncover unique roles of IFNλs which cannot be substituted by type I IFNs. These have shown that IFNλs are the primary and earlier IFNs induced following viral infection, conferring viral resistance to the respiratory mucosa and limiting initial viral spread (32). When viral load is low, this suffices to confront infection. However, when viral load is high in the first place or escapes IFNλ control, type I IFNs are triggered in order to enhance the organism's antiviral defenses. Accordingly, *Ifnlr1*$^{-/-}$ animals exhibit markedly enhanced viral burden following infection with low viral load and upregulated type I IFN levels, highlighting the essential role IFNλs play in these processes (**Figure 1**). Central to IFNλ-mediated antiviral protection is the respiratory epithelium. This is the site where IFNλs are first induced and primarily act, limiting initial viral spread. However, neutrophils are also important as they express high levels of IFNLR1 and respond to IFNλ signaling to deal with their uptaken viral load, preventing the virus from infecting neighboring epithelial cells (32).

Beyond the "timing" component, these studies have also uncovered a fundamental functional difference between type I and IFNλs. They demonstrated that although type I IFNs trigger robust pro-inflammatory responses characterized by the upregulation of diverse cytokines and chemokines, including TNF, IL-1b, and IL-6 (32, 52), IFNλs lack this function. They only induce the expression of ISGs without affecting the production of inflammatory mediators (32). Accordingly, recombinant IFNλ2 administration in experimental animals suppressed the immunoinflammatory cascade triggered by respiratory viral infection, whereas IFNα exerted the opposite effect (32, 53). Interestingly, the expression of ISGs triggered by IFNλs follows slower and more prolonged kinetics compared to type I IFNs which induce faster but only transient expression of ISGs (26, 32, 54, 55). Central to the antiviral and/or pro-inflammatory activities of type I IFNs and IFNλs are neutrophils, which constitute the predominant leukocytes mediating initial antimicrobial immunity (56), and secreting cytokines and chemokines early during infection (57, 58).

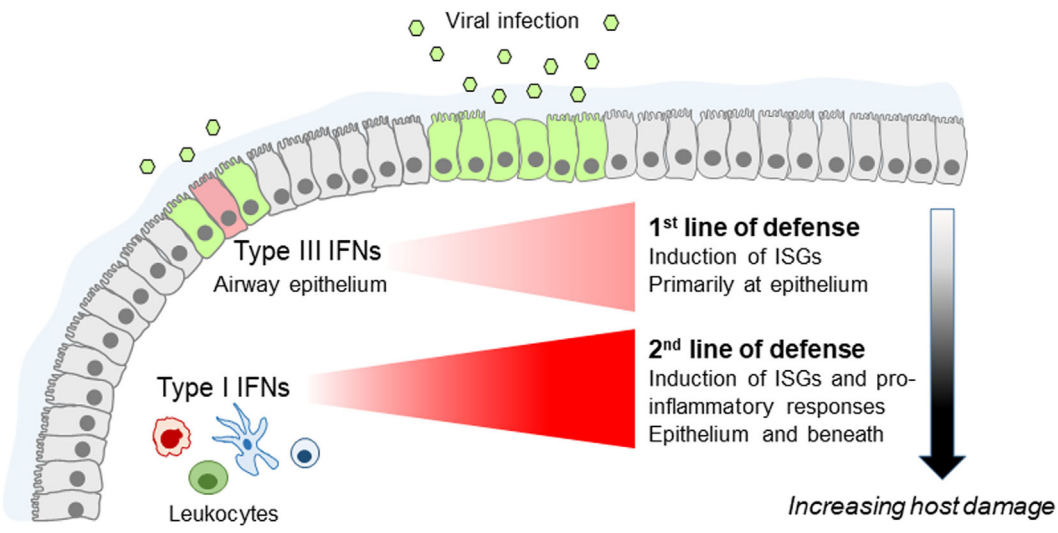

FIGURE 1 | Fine tuning of the innate antiviral immune response by type I and type III interferons (IFNs) in the lung. Type III IFNs are produced first, upon infection of airway epithelial cells, and act as the first line of defense to limit virus spread at the epithelial barrier without triggering inflammation. If infection escapes type III IFN control, type I IFNs are induced that provide the second line of defense, enhancing viral resistance beyond the respiratory epithelium and activating pro-inflammatory responses essential for providing protection but also causing immunopathology.

Although neutrophils respond to both IFNs to augment antiviral defenses, they exhibit pro-inflammatory activation only in response to type I IFNs (32), a finding that awaits confirmation in humans. Also, IFNλs directly affect neutrophil pro-inflammatory function, in both mice and humans, by suppressing reactive oxygen species production and degranulation of neutrophils, thereby limiting their tissue damaging functions and preserving barrier integrity (59).

Teleologically, this makes sense. Increased pro-inflammatory responses are needed for optimal protection against viral infection. However, they can also cause increased tissue damage, impaired respiratory function, and disease symptoms, and should not, therefore, be triggered unnecessarily. This is, in line with the emerging paradigm (schematically shown in **Figure 1**) placing type I IFNs as a second line of defense that only deal with respiratory infections that escape IFNλ control, at the expense though of host fitness.

IFNλs FUNCTIONS IN CHRONIC RESPIRATORY DISEASES

Research on IFNλs has mostly focused on their role in infections as these constitute the primary triggers of their expression *in vitro* and *in vivo*. Yet, it has been demonstrated that in settings of chronic inflammation IFNλs can also be induced independently of infectious insults, possibly through the action of cytokines and other inflammatory or environmental cues. Thus, during the development of allergic airway inflammation in mice significant levels of IFNλs have been detected in the bronchoalveolar lavage of these animals and have been shown to be required for reducing the inflammatory burden in the lung and keep allergic airway disease (AAD) under control (60). Accordingly, *Ifnlr1*$^{-/-}$ mice exhibit markedly worsened AAD while wild-type animals treated intranasally with recombinant IFNλ2 demonstrate significantly reduced type 2 inflammation and ameliorated disease. Although the molecular details of the mechanisms involved remain incompletely understood, these involve IFNλ signaling on lung conventional DCs, suppression of Th2 response, and induction of IFNγ (60). Interestingly, increased IFNλ mRNA levels have been detected in the sputum of asthmatic patients compared to healthy individuals, in the absence of evidence of viral infection, and have been shown to correlate in steroid-naïve patients with milder asthma symptoms, suggesting that IFNλs may also exhibit similar protective activities in human disease as well (61). Steady-state production of IFNλs appears, therefore, to be the key to keeping inflammation in asthma under control and reducing disease symptoms (**Figure 2**).

The effect of IFNλs to Th2 responses is not limited to the setting of AAD but may be of wider importance. IFNλs can suppress the development of primary immune responses *in vivo* as well (60). Also, IFNλs can inhibit Th2 responses *in vitro* in human cells through the reduction of GATA3 and IL-13, and possibly through the increase of IFNγ (62, 63). What remains to be clarified though is how exactly IFNλs are mediating these effects. There is a consensus that T cells do not directly respond to IFNλs to induce ISGs, the signature tag of type III IFN signaling (20, 59, 60). On the contrary, conventional DCs (60, 64, 65) and plasmacytoid DCs (20, 66–68) of either human or mouse origin, have been shown in several studies to upregulate ISGs and alter their function upon IFNλ stimulation. However, even in this case the situation is not crystal clear as there have also been reports

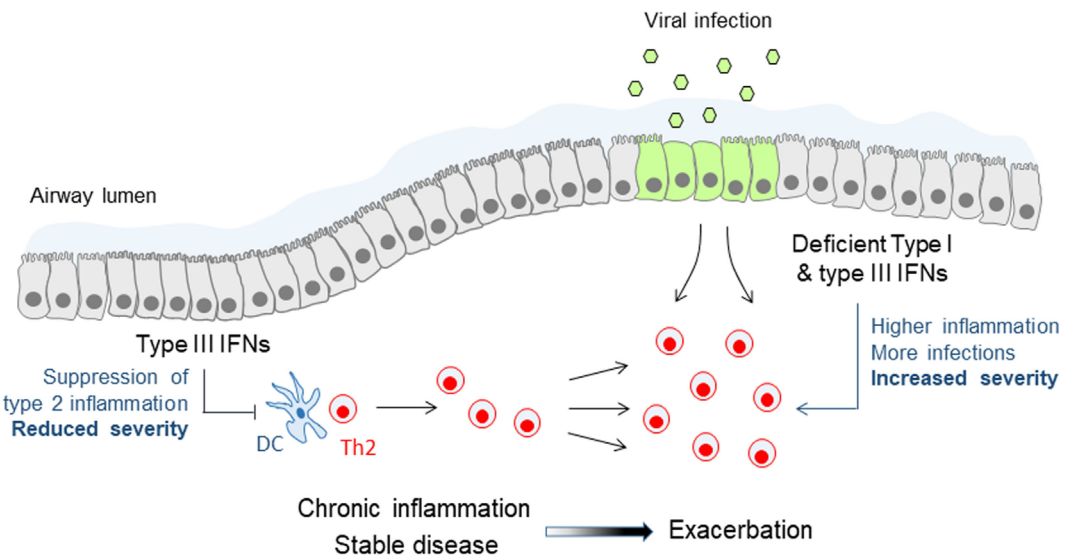

FIGURE 2 | Immune modulatory and antiviral functions of type III interferons (IFNs) in asthma. Steady-state production of type III IFNs during stable asthma suppresses effector Th2 cell responses and keeps chronic inflammation and disease symptoms under control. Deficient or lower type III IFN production leads to reduced control of Th2 cell responses and chronic inflammation, and renders patients more susceptible to viral infections, both leading to more frequent and more severe asthma exacerbations. A similar mechanism of deficient type III IFN production may also account for chronic obstructive pulmonary disease exacerbations.

that conventional (20, 59, 68) and plasmacytoid DCs (59) do not respond to IFNλs, possibly reflecting differences in their origin (e.g., spleen vs bone marrow or blood), culture or differentiation protocol, and cytokine environment (e.g., presence of IL-3, IL-4, GM-CSF, or other). More comprehensive studies addressing the responsiveness of various DC populations and subpopulations to IFNλ are, therefore, urgently needed. Noteworthy, it has been shown that IFNλs can induce the proliferation of $Foxp3^+$ regulatory T cells *in vitro* (64, 65) but confirmation of these findings *in vivo* is still awaited.

IFNλs are also particularly important during asthma exacerbations. The induction of type I and type III IFNs following viral infection is deficient in allergic asthmatic patients with poorly controlled asthma, either because of the strongly Th2-polarized environment at the respiratory mucosa and the use of corticosteroids that generically suppress IFN production and function (e.g., through the induction of SOCS1) or because of epigenetic changes that prevent optimal IFNλ gene expression and translation (31, 69, 70). In either case, this renders allergic asthmatic patients distinctly susceptible to viral exacerbations of asthma, the main cause of hospitalizations and life-threatening situations in this disease (71). These exacerbations are characterized by sudden upregulation of epithelial-derived cytokines, such as IL-25 and IL-33, and rapid aggravation of type 2 responses in the airways, which can all be regulated by type I and type III IFNs (**Figure 2**). Indeed, a Phase II clinical study, administering inhalable IFNβ in a range of asthmatic patients with moderate to severe asthma, demonstrated significant improvement in the "difficult to treat" group of patients, highlighting the potential benefit of this approach (72). Although the treatment was overall well tolerated, the long-known adverse effects of type I IFNs, such as fever, diarrhea, and flu-like disease, are still an issue of concern. IFNλs are, therefore, currently being considered as a better alternative to type I IFNs for treating asthma exacerbations as they exhibit reduced adverse effects and a safer pharmacological profile.

Deficient IFN production of the respiratory epithelium has also been observed in chronic obstructive pulmonary disease (COPD), another disease characterized by frequent virally induced exacerbations. Bronchial epithelial cells from COPD patients are not capable of mounting a full IFN response upon viral infection (73). This is possibly due to cigarette smoke exposure as bronchial epithelial cells from smokers had significantly reduced IFNβ and IFNλ levels compared to non-smokers (74). Administration of recombinant IFNλs may, therefore, be beneficial for the treatment of COPD exacerbations as well. Whether IFNλs are also important at "steady state" during stable disease and whether they can be involved in other chronic respiratory diseases remains to be investigated.

CONCLUSION AND FUTURE DIRECTIONS

Over the last years, major progress in our understanding of the unique functions of IFNλs, not shared with type I IFNs, has taken place. This has revealed the importance of IFNλs in front-line antiviral defenses in the body, especially the respiratory and gastrointestinal tracts, acting in synergy with type I IFNs to fine tune immunity for optimal protection and minimal host damage. This has also uncovered the significance of IFNλs in keeping inflammation under control and preventing exacerbations in asthma, supporting their potential use for the treatment of diverse respiratory diseases. Despite that, key gaps of knowledge exist. Thus, it remains largely unexplored whether IFNλs are also important in immunity against bacterial or fungal infections of the respiratory tract, or barrier surfaces in general and how these are positioned by comparison to type I IFNs. It also remains unclear whether IFNλs are important in adaptive immune responses against infections, such as antibody and cytotoxic T cell responses, including immunological memory, which are well known to be affected by type I IFNs. Moreover, it remains to be established whether IFNλs are important in other chronic respiratory disorders beyond asthma and COPD, and how they can affect the course of the disease process. Further studies toward these directions are, therefore, urgently needed before these highly promising therapeutic candidates can be effectively exploited in the clinic.

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Identification of a Predominantly Interferon-λ-Induced Transcriptional Profile in Murine Intestinal Epithelial Cells

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Type I (α and β) and type III (λ) interferons (IFNs) induce the expression of a large set of antiviral effector molecules *via* their respective surface membrane receptors. Whereas most cell types respond to type I IFN, type III IFN preferentially acts on epithelial cells and protects mucosal organs such as the lung and gastrointestinal tract. Despite the engagement of different receptor molecules, the type I and type III IFN-induced signaling cascade and upregulated gene profile is thought to be largely identical. Here, we comparatively analyzed the response of gut epithelial cells to IFN-β and IFN-λ_2 and identified a set of genes predominantly induced by IFN-λ_2. We confirm the influence of epithelial cell polarization for enhanced type III receptor expression and demonstrate the induction of predominantly IFN-λ_2-induced genes in the gut epithelium *in vivo*. Our results suggest that IFN-λ_2 targets the epithelium and induces genes to adjust the antiviral host response to the requirements at mucosal body sites.

Keywords: interferon-lambda, intestinal epithelium, interleukin 28 receptor, transcription, gastrointestinal tract

INTRODUCTION

The interferon (IFN) family of cytokines acts to confer protection against various pathogens. They are categorized into three different types. Whereas the type II IFN, IFN-γ, plays a key role in the host response to intracellular bacteria and parasites, members of the type I IFNs-α and β and the more recently discovered type III IFNs-λ mediate antiviral protection (1–3). Type I and type III IFNs are secreted by a wide range of different cell types upon innate immune stimulation. Differences exist with respect to their transcriptional regulation due to a distinct transcription factor requirement explaining discrepancies in their expression kinetics (4–7). Type I and III IFNs share low amino acid similarity (15–20%) and bind to structurally very different heterodimeric receptor complexes comprised of the IFN-α receptor (IFNAR) 1 and 2 chain as well as the IFN-λ receptor (IFN-λR) 1 and the IL-10 receptor (IL-10R)β chain, respectively (2, 3). The type I IFN receptor is ubiquitously expressed by all nucleated cells although differences in the expression level and functional sensitivity have been reported (8, 9). By contrast, the type III IFN receptor is restricted to epithelial cells

at mucosal body sites and distinct immune cell subpopulations such as for example polymorphonuclear cells (PMNs) (10–16). Consistently, epithelial cells of the gastrointestinal, respiratory, and reproductive tract were identified as primary targets for type III IFNs *in vivo* (8–11, 17–21). The type III IFN mediated effect on the epithelium of respiratory and gastrointestinal body surfaces thereby allow an early antiviral response in the absence of the systemic side effects and overt tissue inflammation (22).

Despite differences in their receptor utilization, both type I and type III IFNs engage the Jak/STAT signaling pathway leading to the formation of the IFN-stimulated gene factor (ISGF) 3 complex consisting of STAT1/2 heterodimers together with the interferon regulatory factor 9. ISGF3 translocates to the nucleus and binds to IFN-stimulated response elements in the promoter of so-called IFN-stimulated genes (ISGs) that ultimately generate the antiviral state. In addition to this canonical signaling, IFNAR and IFN-λR stimulation activates the mitogen-activated protein kinase pathways, i.e., the extracellular signal-regulated kinase (ERK)-1/2, the stress-activated protein kinase/c-Jun N-terminal kinase, and the p38 kinase as well as the phosphatidylinositol 3-kinase pathway *via* phosphorylation of Akt (12, 23). The functional contribution of these alternative signaling pathways *in vivo* has remained less well defined.

In accordance with the similarity of the induced signal transduction pathways, the spectrum of genes induced by the two types of IFNs is generally considered to be identical or very similar (12, 20, 24–30). This finding is consistent with the reported redundant or synergistic action of both types of IFN *in vivo* (17, 18, 20) and raises the question on the evolutionary benefit of the two distinct sets of antiviral IFNs and their respective receptors. One possible explanation is a quantitative difference in the cellular response and indeed *in vitro* studies suggested that the kinetics and magnitude of ISG induction differ between type I and type III IFN stimulation with type I IFN triggering a significantly faster and more potent transcriptional response (2, 3, 28, 29, 31, 32). However, IFN-λ was able to induce ISG expression and efficiently protect from viral infection of the intestinal and respiratory tract *in vivo* (8, 9, 17, 19, 21, 33). Another explanation might be previously undetected differences in the gene expression profile that shapes the IFN-λ response to better match the specific requirements of the mucosal antiviral host response. For example, IFN-λ may contribute to healing following mucosal tissue damage (34).

Comparative analyses of the transcriptional profile induced by type I versus type III IFN have so far been performed on hepatocytes, respiratory epithelial cells, lymphocytes, and total intestinal tissue and failed to identify IFN-λ-specific targets (12, 20, 24–30). The most discriminatory response between type I and type III IFN has, however, so far been reported at the intestinal epithelium which represents the entry port for many pathogenic viruses (9). We therefore took advantage of the recently established Mx2-luciferase transgenic gut epithelial IEC10 cells that exhibit many typical features of the intestinal epithelium and respond robustly to both type I and type III IFN (32). Comparative transcriptomic profiling of polarized intestinal epithelial cells identified a predominantly IFN-λ_2-induced set of genes. Selected target genes were confirmed *in vivo* by an analysis of intestinal epithelial cells prepared from IFN-λ_2 treated IFNAR$^{-/-}$ mice, and the critical involvement of enterocyte polarization for IL-28R expression was demonstrated.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were performed in compliance with the German animal protection law (TierSchG) and approved by the local animal welfare committee Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit Oldenburg, Germany. Mice were housed under specific pathogen-free conditions and handled in accordance with regulations defined by FELASA and the national animal welfare body GV-SOLAS.[1]

Animals

B6.A2G-Mx1-IFNAR1$^{-/-}$ mice lacking functional type I IFN receptors (IFNAR1$^{-/-}$), B6.A2G-Mx1-IL28Rα$^{-/-}$ mice carrying intact *Mx1* alleles, and lacking a functional type III IFN receptor (IL28Rα$^{-/-}$) were bred at the Central Mouse Facility of the Helmholtz Centre for Infection Research, Braunschweig and described elsewhere (17).

In Vitro Cell Culture

The intestinal epithelial cell line (IEC) Mx2Luc was generated from a transgenic mouse containing the firefly luciferase gene under control of the Mx2 promoter region as described earlier (32). IECs were cultured at 37°C, 5% CO_2, 95% RH and maintained in IEC medium (32). For cell culture under non-polarized conditions (2D), IECs were seeded in 12-well or 24-well plates at a seeding density of 2×10^5 or 2×10^4 cells, respectively, and grown to confluence. For cell culture under polarized conditions (3D), IECs were seeded at a cell density of 2×10^5 cells/mL on 0.4 μm pore size transwell cell-culture inserts (Costar). Cells were allowed to grow for 21 days to attain polarization. The cell-culture medium was changed every 3 days, and transepithelial resistance was measured (EVOM, World Precision instruments) to determine the establishment of epithelial barrier integrity. IECs were stimulated with 500 U/mL IFN-β (19) or 20 ng/mL IFN-λ_2 (Peprotech) in cell-culture medium.

Isolation and Culture of Primary Cells

For isolation of primary intestinal epithelial cells, small intestinal tissue was harvested and cut into 3–4 cm pieces. The tissue-associated fat tissue was removed using forceps, and the intestine was turned inside out. The inverted tissue was mounted on an inoculation loop, incubated for 10 min in 30 mM EDTA at 37°C and subjected to centrifugal force with a biovortexer (Sigma) using 10–12 pulses with 1–2 s duration. Epithelial cell fragments were separated from contaminating lymphoid and myeloid single cells by threefold sedimentation at $1 \times g$ for 20 min at 4°C leading to a final purity of E-cadherin positive epithelial cells of 85–90% (35). Bone marrow-derived dendritic cells (BMDCs) were obtained from female C57BL/6 WT mice by flushing the

[1] www.gv-solas.de.

bone marrow from the cavities of femurs and tibiae. Erythrocytes were depleted with ACK lysis buffer (Thermo Fisher Scientific), and the cells were plated in 12-well cell-culture plates at a seeding density of 1×10^6 cells/mL in the presence of Flt3L at 100 ng/mL (PeproTech, Rocky Hill, NJ, USA) in complete medium (RPMI supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, and 50 µg/mL gentamicin). Cultures were replenished with fresh medium every other day and stimulated at day 7. Primary alveolar epithelial cells were isolated using a modified protocol previously established (36). Briefly, the trachea of the anesthetized and exsanguinated mice was exposed and the lungs were perfused with 10–20 mL sterile PBS buffer until they were free of blood. 1 mL of dispase (BD Biosciences) was flushed into the lungs *via* the trachea. The lungs were removed and placed in a cell-culture dish containing an additional 1 mL of dispase and were cut into small pieces. They were then transferred to a 15 mL Falcon and incubated for 45 min at 37°C with gentle shaking. The crude cell suspension was passed through a sterile 70 µm strainer, and the resulting cell suspension was centrifuged at 1,500 rpm for 5 min. The pellet obtained was incubated in 5 mL of ACK buffer for erythrocyte depletion for 5 min and subsequently subjected to another round of centrifugation at 1,500 rpm for 5 min. Cells were stained with Epcam-PE (eBioscience) and magnetically sorted (MACS anti-PE Microbeads, Miltenyi Biotec GmbH) to obtain a highly enriched population of epithelial cells. Cells were plated in 12-well cell-culture plates at a seeding density of 1×10^6 cells/mL and stimulated after 5 days in culture.

Gene Expression Analysis

RNA from cell-culture experiments was isolated using the RNeasy mini kit (Qiagen) based on silica membrane containing centrifugation columns following the manufacturer's instructions. Total RNA from primary epithelial cells was isolated by guanidinium thiocyanate-phenol-chloroform extraction using Trizol LS reagent (Life Technologies) according to the manufacturer's instructions. 1–2 µg RNA was reversely transcribed into cDNA using the RevertAid RT Kit (Thermo Fisher Scientific). Newly synthesized cDNA was subjected to quantitative real-time PCR analysis in a total volume of 20 µl, using the SYBR Green PCR Kit (BioRad) in combination with a LightCycler 480 II (Roche). The expression level of the house-keeping gene *β-actin* in IEC10 cells was unaffected by IFN-β or IFN-λ_2 stimulation (Figure S2A in Supplementary Material). Changes in gene expression were calculated relative to the endogenous control *β-actin* using the formula $2^{-\Delta Ct}$. Experiments demonstrated no influence of IFN stimulation on the *β-actin* mRNA expression level (data not shown). The values obtained for individual genes after stimulation with IFN-β or IFN-λ_2 were subsequently divided by the mean values found in untreated cells (PBS). Gene expression values are presented as fold induction over the unstimulated control. Statistical analysis was performed using a (non-parametric) one-way analysis of variance (ANOVA) test with Tukey's post test, and the data are presented as mean ± SEM. The values obtained for *in vivo* gene expression were normalized to the endogenous control β-actin, statistically analyzed by the Mann–Whitney *U* test and are represented as mean ± SEM from two to three independent experiments. Murine PCR primers for *β-actin* (forward primer, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and reverse primer, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′), *Usp18* (forward primer, 5′-CAT CCT CCA GGG TTT TCA GA-3′ and reverse primer, 5′-AAG GAC CAG ATC ACG GAC AC-3′), *Ifi44* (forward primer, 5′-AAC TGA CTG CTC GCA ATA ATG T-3′ and reverse primer, 5′-GTA ACA CAG CAA TGC CTC TTG T-3′), *Ifit1* (forward primer, 5′-TGT TGA AGC AGA AGC ACA CA-3′ and reverse primer, 5′-TCT ACG CGA TGT TTC CTA CG-3′), *Mmp7* (forward primer, 5′-TAG GCG GAG ATG CTC ACT TT-3′ and reverse primer, 5′-TTC TGA ATG CCT GCA ATG TC-3′), *Serpinb1a* (forward primer, 5′-GCT GCT ACA GGA GGC ATT GC-3′ and reverse primer, 5′-CGG ATG GTC CAC TGT GAA TTC-3′), *Csprs* (forward primer, 5′-AGA GAG GCA GAG GGA CTG AG-3′ and reverse primer, 5′-GGC TTG GCT CCT GAA CAC TT-3′), *IL28R* (forward primer, 5′-CCC TGT TTC CTG ACA CTC CC-3′ and reverse primer, 5′-TCA GAA AAG TCC AGT GCC CG-3′), *IL10R* (forward primer, 5′-TCT CTT CCA CAG CAC C-3′ and reverse primer, 5′-GAA CAC CTC GCC CTC C-3′), *Ifnar1* (forward primer, 5′-CTG GTC TGT GAG CTG TAC TT-3′ and reverse primer, 5′-TCC CCG CAG TAT TGA TGA GT-3′), *Ifnar2* (forward primer, 5′-CTA TCG TAA TGC TGA AAC GG-3′ and reverse primer, 5′-CGT AAT TCC ACA GTC TCT TCT-3′).

Microarray Analysis

Microarray analysis was performed in triplicates on 3D-grown unstimulated or IFN-stimulated IECs. RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Microarray data used or referred to in this publication were generated by the Research Core Unit Transcriptomics of Hannover Medical School. Synthesis of Cy3-labeled cRNA was performed with the Quick Amp Labeling kit, one color (Agilent Technologies) according to the manufacturer's recommendations. cRNA fragmentation, hybridization, and washing steps were also carried out exactly as recommended: "One-Color Microarray-Based Gene Expression Analysis Protocol V5.7." Microarray analysis was performed using Whole Mouse Genome Oligo Microarray GPL11202 (Agilent Technologies). Slides were scanned on the Agilent Micro Array Scanner G2565CA (pixel resolution 5 µm, bit depth 20). Data extraction was performed with the "Feature Extraction Software V10.7.3.1" by using the recommended default extraction protocol file: "GE1_107_Sep09.xml." Measurements of on-chip replicates were averaged using the geometric mean of processed intensity values of the green channel, "gProcessedSignal" (gPS) to retrieve one resulting value per unique non-control probe. Single features were excluded from averaging, if they (i) were manually flagged, (ii) were identified as outliers by the feature extraction software, (iii) lie outside the interval of "1.42 × interquartile range" regarding the normalized gPS distribution of the respective on-chip replicate population, or, (iv) showed a coefficient of variation of pixel intensities per Feature that exceeded 0.5. Averaged gPS values were normalized by global linear scaling. For this approach, all gPS values of one sample were multiplied by an array-specific scaling factor. This factor was calculated by dividing a "reference 75th Percentile value" (set as 1,500 for

the whole series) by the 75th Percentile value of the particular Microarray to be scaled ("Array I" in the formula shown below). Accordingly, normalized gPS values for all samples (microarray data sets) were calculated by the following formula: normalized gPSArray i = gPSArray i × (1,500/75th PercentileArray i). A lower intensity threshold (surrogate value) was defined based on intensity distribution of negative control features. This value was fixed at 15 normalized gPS units. All measurements that fell below this intensity cutoff were substituted by the respective surrogate value of 15. The hierarchical clustering heatmap was generated using Qlucore Omics explorer (multigroup analysis: p-value = 0.003; q-value = 0.05; two-group analysis: p-value = 0.001; q-value = 0.05, fold change cutoff = 2). The group definitions for the IFN-induced genes (**Figure 1C**) were as follows: "predominantly IFN-λ_2-induced gene": fold increase by IFN-λ_2 over control/fold increase by IFN-β over control >4.5 and fold increase by IFN-β over control <2; "strong IFN-λ_2-induced gene": fold increase by IFN-λ_2 over control/fold increase by IFN-β over control >2 and fold increase by IFN-β over control >2: "Classical ISGs" were defined by their designation in the literature. Cluster of orthologous group analysis was performed using the PANTHER software.[2] Expression array data are available through GEO Series accession number GSE91382.

Statistical Analysis

The one-way ANOVA test (with Tukey's posttest) and the Mann–Whitney U test were employed for statistical analysis of quantitative RT-PCR results. The GraphPad Prism Software 7.00 was used for statistical evaluation.

RESULTS

IFN-λ_2 Induces a Unique Transcriptional Profile in Polarized IECs

The recently described intestinal epithelial IEC10 cells exhibit many properties of the natural epithelium. They respond to both type I and type III IFNs and generate a robust antiviral state making them an ideal model to study IFN-induced gene expression (32). IEC10 cells were grown to confluency on transwell cell-culture inserts and left untreated or stimulated with IFN-β (500 U/mL) or IFN-λ_2 (20 ng/mL) for 9 h. The selected IFN concentrations induced a submaximal stimulatory response (approximately 90% of the maximal Mx2 gene induction) in IEC10 cells for both cytokines as recently reported (32). Similar IFN concentrations have also been used in other comparative studies (9, 12, 20, 24–30). The stimulation time (9 h) was selected based on the kinetic of ISG (Mx2) induction following IFN-β and/or IFN-λ_2 exposure and allowed a stable gene induction for both cytokines (Figure S1B in Supplementary Material). Total RNA was isolated and subjected to transcriptome analysis. Normalization and multigroup analysis (ANOVA) revealed a total of 2,465 significantly differentially regulated genes (q-value = 0.05, p-value = 0.003). **Figure 1A** illustrates the genes significantly induced by IFN-β and/or IFN-λ. In a second approach, we subjected the genes through a two-group analysis (filtering criteria: q-value = 0.0499, p-value = 0.001, fold change cutoff = 2) and observed that 349 genes were highly expressed after stimulation with IFN-λ_2 but not IFN-β (Figure S2A in Supplementary Material). In a third approach, the top 100 (fold over control) IFN-λ_2-induced genes were selected (**Table 1**) and examined in a correlation analysis for their induction by IFN-β versus IFN-λ_2 revealing a majority of classical ISGs including the prototypical antiviral genes *Ifi44* and *Ifit1* as presented in **Figures 1B,C** (left panel). Among these genes, also a group of genes predominantly induced by IFN-λ_2 and a group of genes strongly induced by IFN-λ_2 was identified (**Figure 1B** labeled in red and blue, respectively, and **Figure 1C** middle and right panel). These genes were found to be mainly involved in cellular and metabolic processes and cellular responses to stimuli such as innate host defense, substrate transport and ion homeostasis (Figures S2B,C in Supplementary Material). Two predominantly IFN-λ_2-induced genes, *Mmp7* and *Serpinb1a*, one strongly IFN-λ_2-induced gene, *Csprs*, as well as the classical antiviral ISGs *Usp18*, *Ifi44*, and *Ifit1* were randomly selected and their transcriptional profile upon stimulation with type I or type III IFN for 9 h was confirmed by quantitative RT-PCR (**Figures 1D,E**). IFN-β was unable to induce expression of the predominantly IFN-λ_2-induced genes *Mmp7* and *Serpinb1a* also at other time points (**Figure 1F**; Figure S3 in Supplementary Material).

Expression of Predominantly IFN-λ_2-Induced Genes Requires Epithelial Cell Polarization

Apical–basolateral polarization represents a key feature of intestinal epithelial cells and is intimately linked to their physiological function such as barrier formation and nutrient absorption. To investigate the influence of cell polarization on IFN-induced gene expression, IEC10 cells were grown on conventional flat bottom culture dishes (2D) and stimulated with IFN-β (500 U/mL) or IFN-λ_2 (20 ng/mL) for 9 h. RT-PCR confirmed the ability of IFN-β and IFN-λ_2 to enhance the expression of the prototypical ISGs *Usp18*, *Ifi44*, and *Ifit1* (**Figure 2A**). The Usp18, Ifi44, and Ifit1 mRNA levels reached in response to IFN-λ_2 were less pronounced as compared with under polarized conditions. Due to the lower gene expression levels of unstimulated controls, however, the fold induction was unchanged or even increased (**Figure 1D**). Notably, IFN-λ_2 failed to enhance the expression of *Mmp7*, *Serpinb1a*, and *Csprs* under non-polarizing conditions (**Figure 2B**). Epithelial polarization might therefore critically influence the qualitative IFN-λ response. In an attempt to understand the underlying mechanism, IEC10 cells grown on flat bottom culture dishes (2D) or transwell inserts (3D) were comparatively examined for the expression levels of the IFN receptor molecules under homeostatic conditions. Epithelial cells displayed significantly increased levels of the IL-28Rα and IL-10Rβ chain expression when they attained polarization as compared with their non-polarized state (**Figure 2C**) whereas no influence of polarization was noted for the type I IFN receptor IFNAR1 and 2 (**Figure 2D**) consistent with a recent report (37).

[2] http://www.pantherdb.org/.

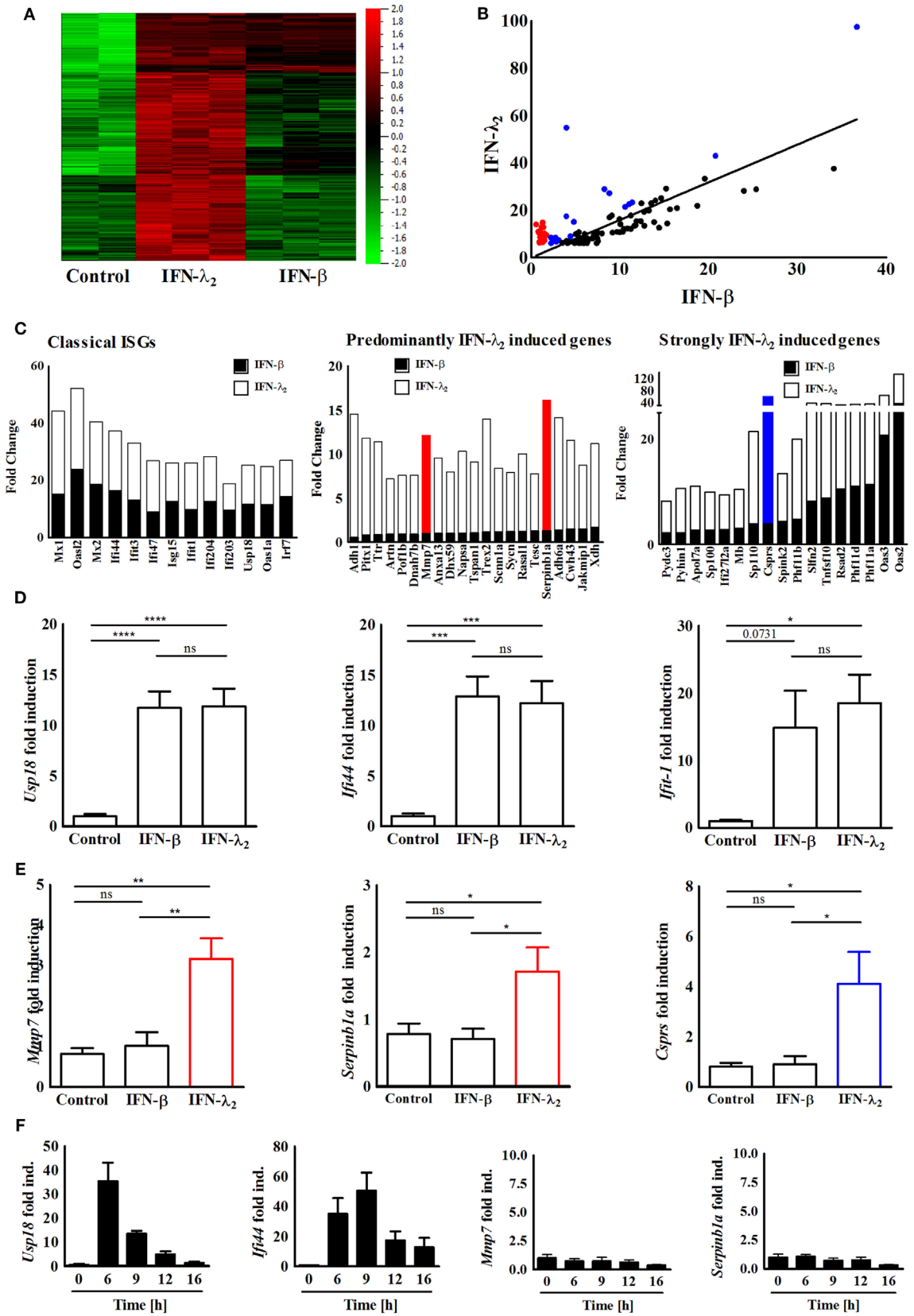

FIGURE 1 | Continued

FIGURE 1 | Continued
Identification and confirmation of a predominantly interferon (IFN)-λ_2-induced gene expression profile. **(A)** Heatmap of the genes expressed by IEC10 cells cultured on transwell filter inserts and left untreated (PBS) or exposed to IFN-λ_2 (20 ng/mL) or IFN-β (500 U/mL) for 9 h. Data were obtained using a global gene expression array. Multigroup comparison was carried out at $p = 0.003$, $q = 0.05$. **(B)** Selective analysis of the top 100 genes induced by IFN-λ_2 as identified by fold increase over unstimulated control. Correlation graph showing the fold change of these 100 genes in respect to their induction by IFN-λ_2 (20 ng/mL) versus IFN-β (500 U/mL) 9 h after stimulation. Red labeled dots illustrate a subgroup of genes that is predominantly induced by IFN-λ2; blue dots illustrate a subgroup of genes strongly induced by IFN-λ2 (for definition see Section "Materials and Methods"). **(C)** Graphical representation showing the fold change analysis of different gene subgroups ["classical antiviral IFN-stimulated genes (ISGs)," "predominantly IFN-λ_2-induced genes," "strongly IFN-λ_2-induced genes"]. **(D,E)** Quantitative RT-PCR for **(D)** the prototypical ISGs *Usp18*, *Ifi44*, and *Ifit1*, **(E)** the predominantly IFN-λ_2-induced ISGs *Mmp7*, *Serpinb1a*, and the strongly IFN-λ_2-induced gene *Csprs* performed on total RNA isolated from IEC10 cells grown on transwell inserts and stimulated for 9 h with IFN-λ_2 (20 ng/mL) or IFN-β (500 U/mL). The results are represented as mean ± SEM values from two to three independent experiments and are normalized to the values obtained for the housekeeping gene *β-actin*. Statistical significance was calculated using the one-way analysis of variance (with Tukey's posttest). **(F)** Quantitative RT-PCR for the prototypical ISGs *Usp18* and *Ifi44* as well as the predominantly IFN-λ_2-induced ISGs *Mmp7* and *Serpinb1a* performed on total RNA isolated from IEC10 cells grown on transwell filter inserts and stimulated with IFN-β (500 U/mL) for the indicated time period. The results represent the mean ± SEM values from two to three independent experiments and are normalized to the values obtained for the housekeeping gene *β-actin*.

TABLE 1 | Top 100 genes induced by IFN-λ2.

Accession ID	Description	Gene name	IFN-λ_2 fold change	IFN-β fold change	IFN-λ2/ IFN-β
NM_145227	*Mus musculus* 2′-5′ oligoadenylate synthetase 2 (Oas2), mRNA [NM_145227]	Oas2	97.48	36.70	2.66
NM_033616	*M. musculus* component of Sp100-rs (Csprs), mRNA [NM_033616]	Csprs	54.87	3.97	13.82
NM_145226	*M. musculus* 2′-5′ oligoadenylate synthetase 3 (Oas3), mRNA [NM_145226]	Oas3	43.04	20.77	2.07
NM_001139519	*M. musculus* Z-DNA binding protein 1 (Zbp1), transcript variant 2, mRNA [NM_001139519]	Zbp1	37.57	34.11	1.10
NM_030150	*M. musculus* DEXH (Asp–Glu–X-His) box polypeptide 58 (Dhx58), mRNA [NM_030150]	Dhx58	33.28	19.56	1.70
NM_010846	*M. musculus* myxovirus (influenza virus) resistance 1 (Mx1), mRNA [NM_010846]	Mx1	29.16	15.19	1.92
NM_011408	*M. musculus* schlafen 2 (Slfn2), mRNA [NM_011408]	Slfn2	28.93	8.24	3.51
NM_001289492	*M. musculus* guanylate binding protein 3 (Gbp3), transcript variant 1, mRNA [NM_001289492]	Gbp3	28.85	25.35	1.14
NM_011854	*M. musculus* 2′-5′ oligoadenylate synthetase-like 2 (Oasl2), mRNA [NM_011854]	Oasl2	28.19	23.97	1.18
NM_009425	*M. musculus* tumor necrosis factor (ligand) superfamily, member 10 (Tnfsf10), mRNA [NM_009425]	Tnfsf10	27.23	8.80	3.09
NM_001168660	*M. musculus* apolipoprotein L 9b (Apol9b), transcript variant 1, mRNA [NM_001168660]	Apol9b	25.14	14.63	1.72
NM_173786	*M. musculus* apolipoprotein L 9a (Apol9a), transcript variant 1, mRNA [NM_173786]	Apol9a	24.11	13.99	1.72
NM_172603	*M. musculus* PHD finger protein 11A (Phf11a), mRNA [NM_172603]	Phf11a	23.35	11.38	2.05
NM_010821	*M. musculus* macrophage expressed gene 1 (Mpeg1), mRNA [NM_010821]	Mpeg1	22.94	13.51	1.70
NM_001146275	*M. musculus* interferon-inducible GTPase 1 (Iigp1), transcript variant 2, mRNA [NM_001146275]	Iigp1	22.93	12.41	1.85
NM_199015	*M. musculus* PHD finger protein 11D (Phf11d), mRNA [NM_199015]	Phf11d	22.59	11.04	2.05
NM_013606	*M. musculus* myxovirus (influenza virus) resistance 2 (Mx2), transcript variant 1, mRNA [NM_013606]	Mx2	21.84	18.72	1.17
NM_021384	*M. musculus* radical *S*-adenosyl methionine domain containing 2 (Rsad2), mRNA [NM_021384]	Rsad2	21.38	10.58	2.02
NM_133871	*M. musculus* interferon (IFN)-induced protein 44 (Ifi44), mRNA [NM_133871]	Ifi44	20.93	16.42	1.27
NM_009099	*M. musculus* tripartite motif-containing 30A (Trim30a), mRNA [NM_009099]	Trim30a	20.39	15.62	1.31
NM_010501	*M. musculus* IFN-induced protein with tetratricopeptide repeats 3 (Ifit3), mRNA [NM_010501]	Ifit3	19.90	13.26	1.50
NM_199146	*M. musculus* tripartite motif-containing 30D (Trim30d), transcript variant 1, mRNA [NM_199146]	Trim30d	19.29	12.74	1.52
NM_001145164	*M. musculus* T cell-specific GTPase 2 (Tgtp2), mRNA [NM_001145164]	Tgtp2	17.80	11.22	1.59
NM_001271676	*M. musculus* IFN-λ-inducible protein 47 (Ifi47), transcript variant 2, mRNA [NM_001271676]	Ifi47	17.74	9.09	1.95
NM_175397	*M. musculus* Sp110 nuclear body protein (Sp110), transcript variant 1, mRNA [NM_175397]	Sp110	17.45	3.95	4.42
NM_011579	*M. musculus* T cell-specific GTPase 1 (Tgtp1), mRNA [NM_011579]	Tgtp1	16.98	8.83	1.92
NM_008331	*M. musculus* IFN-induced protein with tetratricopeptide repeats 1 (Ifit1), mRNA [NM_008331]	Ifit1	16.27	9.94	1.64
ENSMUST00000102642	Ubiquitin-conjugating enzyme E2L 6 [source:MGI Symbol;Acc:MGI: 1914500] [ENSMUST00000102642]	Ube2l6	15.38	12.34	1.25
NM_001037713	*M. musculus* XIAP-associated factor 1 (Xaf1), transcript variant 1, mRNA [NM_001037713]	Xaf1	15.35	11.86	1.29

(*Continued*)

TABLE 1 | Continued

Accession ID	Description	Gene name	IFN-λ_2 fold change	IFN-β fold change	IFN-λ2/ IFN-β
NM_001164327	*M. musculus* PHD finger protein 11B (Phf11b), mRNA [NM_001164327]	Phf11b	15.13	4.81	3.15
XM_006497295	PREDICTED: *M. musculus* IFN-activated gene 204 (Ifi204), transcript variant X1, mRNA [XM_006497295]	Ifi204	14.98	13.86	1.08
NM_025429	*M. musculus* serine (or cysteine) peptidase inhibitor, clade B, member 1a (Serpinb1a), mRNA [NM_025429]	Serpinb1a	14.83	1.29	11.51
NM_001045481	*M. musculus* IFN-activated gene 203 (Ifi203), transcript variant 1, mRNA [NM_001045481]	Ifi203	14.38	15.34	0.94
NM_020557	*M. musculus* cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial (Cmpk2), mRNA [NM_020557]	Cmpk2	14.15	10.05	1.41
NM_007409	*M. musculus* alcohol dehydrogenase 1 (class I) (Adh1), mRNA [NM_007409]	Adh1	13.97	0.55	25.50
NM_011909	*M. musculus* ubiquitin-specific peptidase 18 (Usp18), mRNA [NM_011909]	Usp18	13.66	11.75	1.16
NM_015783	*M. musculus* ISG15 ubiquitin-like modifier (Isg15), mRNA [NM_015783]	Isg15	13.41	12.75	1.05
NM_145211	*M. musculus* 2′-5′ oligoadenylate synthetase 1A (Oas1a), mRNA [NM_145211]	Oas1a	13.37	11.59	1.15
NM_011907	*M. musculus* three prime repair exonuclease 2 (Trex2), mRNA [NM_011907]	Trex2	12.85	1.15	11.16
NM_026945	*M. musculus* alcohol dehydrogenase 6A (class V) (Adh6a), mRNA [NM_026945]	Adh6a	12.79	1.37	9.37
NM_016850	*M. musculus* interferon regulatory factor 7 (Irf7), transcript variant 1, mRNA [NM_016850]	Irf7	12.58	14.41	0.87
NM_001039530	*M. musculus* poly (ADP-ribose) polymerase family, member 14 (Parp14), mRNA [NM_001039530]	Parp14	12.29	10.84	1.13
NM_001033450	*M. musculus* myeloid cell nuclear differentiation antigen (Mnda), mRNA [NM_001033450]	Mnda	12.14	11.22	1.08
NM_145211	*M. musculus* 2′-5′ Oas1a, mRNA [NM_145211]	Oas1a	12.10	10.42	1.16
NM_010810	*M. musculus* matrix metallopeptidase 7 (Mmp7), mRNA [NM_010810]	Mmp7	11.17	0.97	11.57
NM_011097	*M. musculus* paired-like homeodomain transcription factor 1 (Pitx1), mRNA [NM_011097]	Pitx1	11.00	0.80	13.70
NM_023386	*M. musculus* receptor transporter protein 4 (Rtp4), mRNA [NM_023386]	Rtp4	10.93	10.34	1.06
NM_010260	*M. musculus* guanylate binding protein 2 (Gbp2), mRNA [NM_010260]	Gbp2	10.82	9.66	1.12
NM_007986	*M. musculus* fibroblast activation protein (Fap), mRNA [NM_007986]	Fap	10.68	5.36	1.99
NM 028967	*M. musculus* basic leucine zipper transcription factor, ATF-like 2 (Batf2), mRNA [NM_028967]	Batf2	10.66	9.97	1.07
NM_013697	*M. musculus* transthyretin (Ttr), mRNA [NM_013697]	Ttr	10.58	0.86	12.33
NM_145153	*M. musculus* 2′-5′ oligoadenylate synthetase 1F (Oas1f), mRNA [NM_145153]	Oas1f	10.56	9.16	1.15
NM_001146007	*M. musculus* tripartite motif-containing 12C (Trim12c), transcript variant 1, mRNA [NM_001146007]	Trim12c	10.50	7.49	1.40
NM_019440	*M. musculus* immunity-related GTPase family M member 2 (Irgm2), mRNA [NM_019440]	Irgm2	10.22	7.98	1.28
NM_181323	*M. musculus* cell wall biogenesis 43 C-terminal homolog (*S. cerevisiae*) (Cwh43), mRNA [NM_181323]	Cwh43	10.08	1.49	6.76
NM_194336	*M. musculus* guanylate binding protein 6 (Gbp6), mRNA [NM_194336]	Gbp6	9.99	6.01	1.66
NM_001170853	*M. musculus* myeloid nuclear differentiation antigen like (Mndal), mRNA [NM_001170853]	Mndal	9.94	7.07	1.41
NM_001256005	*M. musculus* guanylate binding protein 4 (Gbp4), transcript variant 1, mRNA [NM_001256005]	Gbp4	9.93	5.02	1.98
NM_011723	*M. musculus* xanthine dehydrogenase (Xdh), mRNA [NM_011723]	Xdh	9.49	1.71	5.54
NM_008437	*M. musculus* napsin A aspartic peptidase (Napsa), mRNA [NM_008437]	Napsa	9.31	1.02	9.13
NM_183284	*M. musculus* serine peptidase inhibitor, Kazal type 2 (Spink2), transcript variant 2, mRNA [NM_183284]	Spink2	9.00	4.41	2.04
NM_013832	*M. musculus* RAS protein activator like 1 (GAP1 like) (Rasal1), transcript variant 1, mRNA [NM_013832]	Rasal1	8.79	1.23	7.15
NM_027211	*M. musculus* annexin A13 (Anxa13), mRNA [NM_027211]	Anxa13	8.58	1.00	8.58
NM_145209	*M. musculus* 2′-5′ oligoadenylate synthetase-like 1 (Oasl1), mRNA [NM_145209]	Oasl1	8.48	5.36	1.58
NM_008505	*M. musculus* LIM domain only 2 (Lmo2), transcript variant 1, mRNA [NM_008505]	Lmo2	8.40	6.16	1.36
NM_175026	*M. musculus* pyrin and HIN domain family, member 1 (Pyhin1), mRNA [NM_175026]	Pyhin1	8.38	2.22	3.77
NM_029419	*M. musculus* apolipoprotein L 7a (Apol7a), transcript variant 1, mRNA [NM_029419]	Apol7a	8.36	2.73	3.06
NM_023141	*M. musculus* torsin family 3, member A (Tor3a), mRNA [NM_023141]	Tor3a	8.32	7.42	1.12

(*Continued*)

TABLE 1 | Continued

Accession ID	Description	Gene name	IFN-λ_2 fold change	IFN-β fold change	IFN-λ2/ IFN-β
NM_008326	*M. musculus* immunity-related GTPase family M member 1 (Irgm1), mRNA [NM_008326]	Irgm1	8.16	6.84	1.19
NM_011852	*M. musculus* 2′-5′ oligoadenylate synthetase 1G (Oas1g), mRNA [NM_011852]	Oas1g	8.11	5.56	1.46
NM_145545	*M. musculus* guanylate binding protein 7 (Gbp7), transcript variant 1, mRNA [NM_145545]	Gbp7	8.11	6.28	1.29
NM_010708	*M. musculus* lectin, galactose binding, soluble 9 (Lgals9), transcript variant 1, mRNA [NM_010708]	Lgals9	8.06	6.89	1.17
NM_133681	*M. musculus* tetraspanin 1 (Tspan1), mRNA [NM_133681]	Tspan1	8.05	1.07	7.56
NM_010426	*M. musculus* forkhead box F1 (Foxf1), mRNA [NM_010426]	Foxf1	7.67	6.22	1.23
NM_013593	*M. musculus* myoglobin (Mb), transcript variant 2, mRNA [NM_013593]	Mb	7.33	3.09	2.37
NM_178394	*M. musculus* janus kinase and microtubule interacting protein 1 (Jakmip1), mRNA [NM_178394]	Jakmip1	7.24	1.49	4.85
NM_013673	*M. musculus* nuclear antigen Sp100 (Sp100), mRNA [NM_013673]	Sp100	7.23	2.74	2.64
NM_011324	*M. musculus* sodium channel, non-voltage-gated 1 alpha (Scnn1a), mRNA [NM_011324]	Scnn1a	7.22	1.18	6.10
NM_145226	*M. musculus* 2′-5′ oligoadenylate synthetase 3 (Oas4), mRNA [NM_145226]	Oas4	7.04	3.98	1.77
NM_001139519	*M. musculus* Z-DNA binding protein 2 (Zbp2), transcript variant 2, mRNA [NM_001139519]	Zbp2	6.99	6.35	1.10
NM_030150	*M. musculus* DEXH (Asp–Glu–X-His) box polypeptide 58 (Dhx58), mRNA [NM_030150]	Dhx59	6.98	1.01	6.93
NM_025378	*M. musculus* IFN-induced transmembrane protein 3 (Ifitm3), mRNA [NM_025378]	Ifitm3	6.94	7.55	0.92
NM_197944	*M. musculus* hematopoietic SH2 domain containing (Hsh2d), mRNA [NM_197944]	Hsh2d	6.72	4.40	1.53
NM_001160386	*M. musculus* dynein, axonemal, heavy chain 7B (Dnah7b), mRNA [NM_001160386]	Dnah7b	6.72	0.92	7.29
NM_026716	*M. musculus* syncollin (Sycn), mRNA [NM_026716]	Sycn	6.71	1.21	5.53
NM_023835	*M. musculus* tripartite motif-containing 12A (Trim12a), mRNA [NM_023835]	Trim12a	6.70	5.83	1.15
NM_181579	*M. musculus* premature ovarian failure 1B (Pof1b), mRNA [NM_181579]	Pof1b	6.68	0.92	7.26
NM_029803	*M. musculus* IFN, alpha-inducible protein 27-like 2A (Ifi27l2a), transcript variant 1, mRNA [NM_029803]	Ifi27l2a	6.65	2.82	2.36
NM_021344	*M. musculus* tescalcin (Tesc), mRNA [NM_021344]	Tesc	6.51	1.28	5.09
NM_181728	*M. musculus* ADP-ribosyltransferase 3 (Art3), mRNA [NM_181728]	Art3	6.45	5.17	1.25
NM_001284192	*M. musculus* artemin (Artn), transcript variant 2, mRNA [NM_001284192]	Artn	6.29	0.91	6.91
NM_029000	*M. musculus* GTPase, very large IFN-inducible 1 (Gvin1), transcript variant 1, mRNA [NM_029000]	Gvin1	6.16	5.74	1.07
NM_013585	*M. musculus* proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2) (Psmb9), mRNA [NM_013585]	Psmb9	6.16	3.51	1.76
NM_001146007	*M. musculus* tripartite motif-containing 12C (Trim12c), transcript variant 1, mRNA [NM_001146007]	Trim12c	6.11	5.41	1.13
NM_023141	*M. musculus* torsin family 3, member A (Tor3a), mRNA [NM_023141]	Tor3a	6.11	5.96	1.03
NR_030671	*M. musculus* expressed sequence AW011738 (AW011738), long non-coding RNA [NR_030671]	AW01173 8	6.11	7.36	0.83
NM_021274	*M. musculus* chemokine (C–X–C motif) ligand 10 (Cxcl10), mRNA [NM_021274]	Cxcl10	6.10	4.89	1.25
NM_001025208	*M. musculus* MHC class I family member (LOC547349), mRNA [NM_001025208]	LOC5473 49	6.10	4.08	1.49
NM_030253	*M. musculus* poly (ADP-ribose) polymerase family, member 9 (Parp9), mRNA [NM_030253]	Parp9	6.04	6.28	0.96
NM_001162938	*M. musculus* pyrin domain containing 3 (Pydc3), mRNA [NM_001162938]	Pydc3	6.03	2.21	2.72

Cell-Type Specificity of the IFN-λ-Induced Transcriptional Profile

Dendritic cells were reported to respond to type III IFN (14, 38). Therefore, BMDCs were examined following stimulation with IFN-β (500 U/mL) or IFN-λ_2 (20 ng/mL) for 9 h by RT-PCR. Expression of the prototypical ISGs *Usp18*, *Ifi44*, and *Ifit1* was increased following exposure to IFN-β. By contrast, no influence of IFN-λ_2 on the expression level of *Usp18*, *Ifi44*, and *Ifit1* (**Figure 3A**) or the expression level of *Mmp7*, *Serpinb1a*, and *Csprs* was observed (**Figure 3B**). To determine if the predominantly IFN-λ_2-induced gene signature was restricted to the epithelial cells of the intestine, we next analyzed epithelial cells of another important mucosal organ, the lung. Lung epithelial cells have previously been reported to express receptors for both, type I and type III IFNs. Primary lung epithelial cells cultured for 5 days before stimulation were analyzed. Stimulation with IFN-β (500 U/

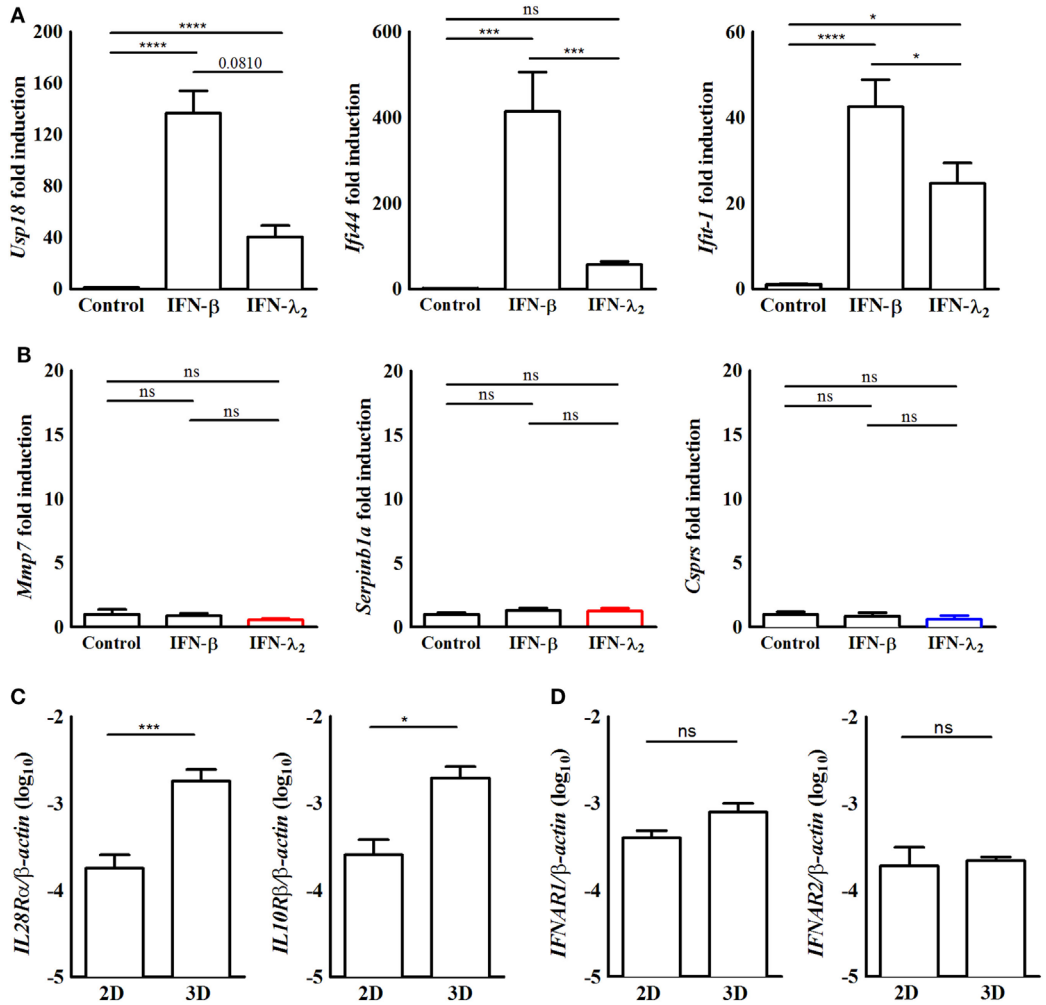

FIGURE 2 | The induction of predominantly interferon (IFN)-λ_2-induced genes requires epithelial polarization. **(A,B)** Quantitative RT-PCR for **(A)** the prototypical IFN-stimulated genes (ISGs) *Usp18*, *Ifi44*, and *Ifit1* or **(B)** the predominantly IFN-λ-induced ISGs *Mmp7*, *Serpinb1a*, and *Csprs* performed on total RNA isolated from IEC10 cells grown in conventional flat bottom 12-well tissue culture plates under 2D conditions and stimulated for 9 h with IFN-λ_2 (20 ng/mL) or IFN-β (500 U/mL). The results represent the mean ± SEM values from two independent experiments and are normalized to the values obtained for the housekeeping gene *β-actin*. Statistical significance was calculated using a one-way analysis of variance (with Tukey's posttest). **(C,D)** Quantitative RT-PCR for **(C)** IL-28Rα and IL-10 receptor (IL-10R)β and **(D)** IFNAR1 and IFNAR2 performed on total RNA isolated from unstimulated IEC10 cells grown either on conventional flat bottom tissue culture plates (2D) or transwell inserts (3D). The results are normalized to the values obtained for β-actin and are represented as mean ± SEM values from two independent experiments performed in quadruplicates. Statistical significance was calculated using the Mann–Whitney *U* test.

mL) or IFN-λ_2 (20 ng/mL) for 9 h induced a significant increase of the prototypical ISGs *Usp18*, *Ifi44*, and *Ifit1* (**Figure 3C**) but failed to enhance the expression level of *Mmp7* and *Serpinb1a* (**Figure 3D**). *Csprs* expression was significantly enhanced by IFN-β but not IFN-λ_2 indicating a more pronounced effect of IFN-λ_2 on intestinal as compared with lung epithelial cells.

In Vivo Induction of the IFN-λ_2 Stimulated Gene Signature

To confirm expression of the predominantly IFN-λ-induced genes *in vivo*, 8-week-old IFNAR1-deficient female mice were intraperitoneally stimulated with 1 µg murine IFN-λ_2. 9 h after administration, intestinal epithelial cells were prepared and analyzed by RT-PCR. IFN-λ_2 administration significantly enhanced expression of the prototypic ISG *Ifit1* (**Figure 4A**). It also significantly enhanced the expression level of the predominantly IFN-λ-induced genes *Mmp7* (**Figure 4B**) and *Serpinb1a* (**Figure 4C**). By contrast, intraperitoneal administration of 500 U IFN-β to IL-28R deficient failed to induce the prototypic ISG *Ifit1* (**Figure 4D**). Also, neither an increase of *Mmp7* (**Figure 4E**) nor *Serpinb1a* expression was observed (**Figure 4F**). Immunostaining subsequently confirmed induction of the prototypic ISG IFIT1 in the intestinal villus epithelium of IFN-λ_2 treated IFNAR1-deficient animals (**Figure 4G**). Finally, also enhanced expression of the predominantly IFN-λ_2-induced target MMP7 was noted in crypt based Paneth cells of IFNAR1-deficient animals following IFN-λ_2 administration (**Figure 4H**).

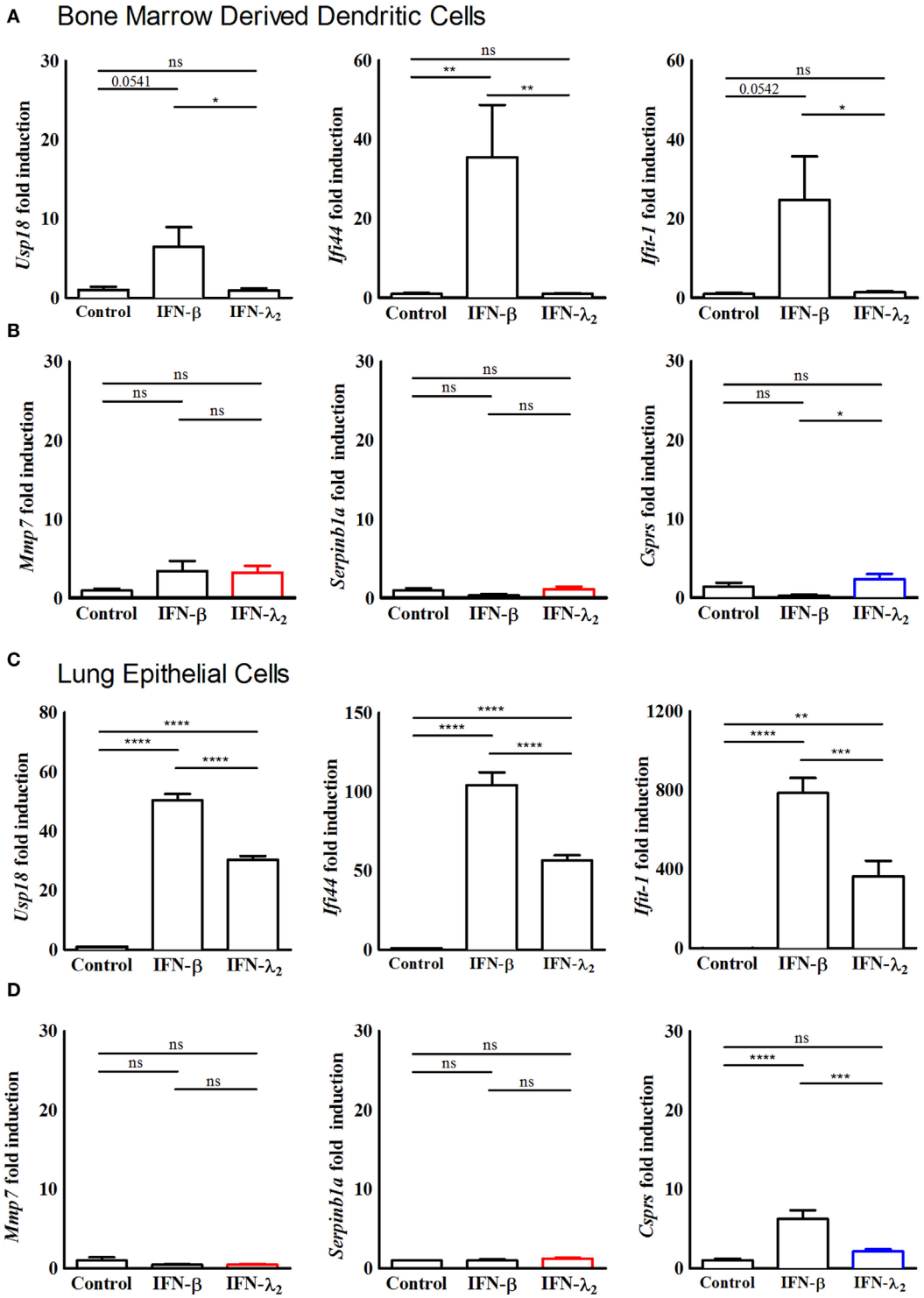

FIGURE 3 | Predominantly interferon (IFN)-λ-induced genes are not induced in bone marrow-derived dendritic cells and primary lung epithelial cells. **(A,B)** Quantitative RT-PCR for **(A)** the prototypical IFN-stimulated genes (ISGs) *Usp18*, *Ifi44*, and *Ifit1* and **(B)** the predominantly IFN-λ-induced ISGs *Mmp7*, *Serpinb1a*, and *Csprs* performed on total RNA isolated from bone marrow-derived dendritic cells isolated from 8-week-old female wild-type mice and cultured *in vitro* for 7 days. The dendritic cells were stimulated with Flt3 ligand to initiate maturation, following which they were stimulated with IFN-λ_2 (20 ng/mL) or IFN-β (500 U/mL) for 9 h. **(C,D)** Quantitative RT-PCR for **(C)** the prototypical ISGs *Usp18*, *Ifi44*, and *Ifit1* and **(D)** the predominantly IFN-λ-induced ISGs *Mmp7* and *Serpinb1a* and *Csprs* performed on total RNA isolated from primary lung epithelial cells isolated from 8-week-old female wild-type mice and cultured *in vitro* for 5 days before stimulation for 9 h with IFN-λ_2 (20 ng/mL) or IFN-β (500 U/mL). The results are normalized to β-actin and are represented as mean ± SEM values from two independent experiments performed in quadruplicates. Statistical significance was calculated using a one-way analysis of variance (with Tukey's posttest).

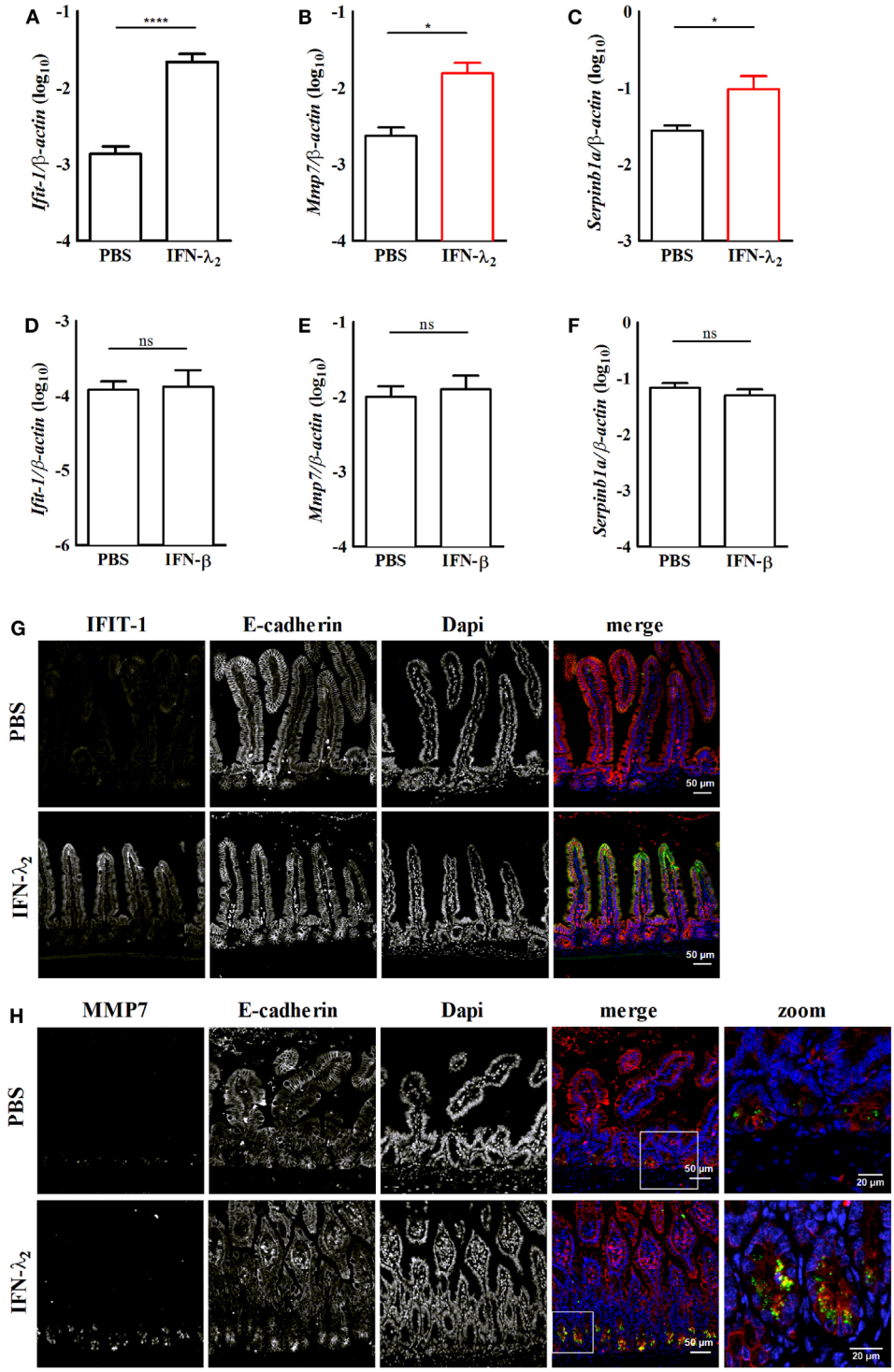

FIGURE 4 | *In vivo* confirmation of the expression of predominantly interferon (IFN)-λ2-induced genes in the intestinal epithelium. Primary intestinal epithelial cells were isolated from IFNAR1$^{-/-}$ **(A–C)** or IL28R$^{-/-}$ **(D–F)** female 8-week-old adult mice 9 h after intraperitoneal injection of IFN-λ2 [1 µg, **(A)**] or IFN-β [500 U, **(D)**], respectively. Control animals in each group received PBS. Quantitative RT-PCR for the prototypical IFN-stimulated gene (ISG) *Ifit1* [**(A)**, $n = 12$ animals per group; **(D)**, $n = 8$ animals per group], the predominantly IFN-λ2-induced ISGs *Mmp7* [**(B)**, $n = 8$ animals per group; **(E)**, 8 animals per group] and *Serpinb1a* [**(C)**, $n = 12$ animals per group; **(F)**, 8 animals per group] performed on total RNA prepared from primary intestinal epithelial cells. Epithelial cells were isolated from PBS or IFN-λ2 treated IFNAR1$^{-/-}$ mice or PBS or IFN-β treated IL28R$^{-/-}$ animals. The results are normalized to β-actin and are represented as mean ± SEM from two to three independent experiments. Statistical analysis was performed using the Mann–Whitney *U* test. **(G,H)** Paraffin-embedded samples from IFNAR1$^{-/-}$ mice treated with PBS or IFN-λ2 as indicated were subjected to simultaneous staining for **(G)** IFIT1 (green) and E-cadherin (red) or for **(H)** MMP7 (green) and E-cadherin (red). Counterstaining was performed with DAPI (blue). White squares depict the zoomed area of the merged images in panel **(H)**. Scale 50 and 20 µm in the zoomed areas.

DISCUSSION

The major biological difference between type I and type III IFN was shown to reside in their organ and cell-type tropism. Whereas most nucleated cells respond to type I IFN, type III IFN appears to play a non-redundant role in the protection of epithelial cells at mucosal body sites such as the gastrointestinal and respiratory tract *in vivo* (8, 9, 18). This renders type III IFNs critical components of the epithelial antiviral host response and raises the question of the evolutionary benefit of an additional epithelium-specific IFN system. First, an epithelium-specific antiviral host response acts early during the infectious challenge and may be able to cope with the microbial challenge in the absence of the well-known side effects of a systemic IFN response (39, 40). Indeed, a recent study demonstrated that the early protective IFN-λ effect occurs in the absence of significant tissue inflammation, which might be particularly important in respect to the function of the respiratory and gastrointestinal tract (22). Consistently, IFN-λ has been shown to also exert an immunomodulatory effect on PMNs (15, 16). The use of IFN-λ as an alternative therapeutic option to type I IFN has been therefore suggested for human viral hepatitis in an attempt to reduce the systemic side effects (41). Second, type III IFN may be able to simultaneously induce gene products that tailor the response to fit the needs of an anti-infectious host response at colonized mucosal surfaces.

Previous studies did not identify a type III IFN-specific gene profile (12, 20, 24–30). Notably, however, these studies employed hepatocytes or immortalized liver cell lines as well as lung epithelial cells possibly missing out on genes involved to maintain host–microbial homeostasis at the most densely colonized body surface, the intestinal tract. The striking species-specific activity of type III IFN on human but not mouse hepatocytes underlines the exceptional phenotype of hepatocytes (42). Also, our results revealed no expression of the predominantly IFN-λ-induced genes in lung epithelial cells. Intestinal epithelial cells might therefore represent the most promising cell type to investigate an IFN-λ_2-specific cell response. Indeed, differences in the IFN receptor signal cascade have previously been observed between different cell types (12, 23, 43).

In this study, we employed a recently described immortalized intestinal epithelial cell line that exhibits a potent response to both type I and III IFN (32). These cells express a number of typical intestinal epithelial cell marker proteins and exhibit a polarized growth with increase in the transepithelial electrical resistance when cultured on porous transwell culture surfaces. Most importantly, stimulation of ISGs in IEC10 cells was induced by both, type I and III IFN in a dose-dependent manner. This cell-culture model therefore represents an ideal tool to investigate the differential response to type I versus type III IFN at the intestinal epithelial lining. In addition, we employed IL-28R and IFNAR-deficient animals in combination with protocols to isolate highly enriched primary gut epithelial cells to confirm the induction of a predominantly IFN-λ-induced gene, *Mmp7*, *in vivo* (9).

Comparative analysis of IFN-β versus IFN-λ_2 stimulated IEC10 cells resulted in the identification of a predominantly IFN-λ_2-induced gene expression profile. The identified genes do not belong to the previously defined group of classical ISGs associated with viral inhibition but their function demonstrates a clear association with the gut epithelial barrier function. MMP7 plays a critical role in tissue remodeling, encodes an immunomodulatory activity and activates Paneth cell-derived antimicrobial peptides (44). Other gene products such as the vitamin A transporter transthyretin, the $Na^+HCO_3^-$-cotransporter NBCn1 (Slc4a7), the surface membrane protein annexin A13 or the Na channel α-ENaC (encoded by Scnn1A) may contribute to metabolism, transcellular transport and ion homeostasis at the epithelium (45, 46). The dynein protein Dnah7b (dynein axonemal heavy chain 7B) the mucin-synthesis core 2 1,6-*N*-acetylglucosaminyltransferase enzyme (C2GnT-M encoded by the GCNT3 gene) and the desmosome protein premature ovarian failure 1B (Pof1b) may reinforce epithelial barrier formation (47–49). Other proteins such as the HIF1-associated regulator paired-like homeodomain pituitary transcription factor Pitx1 or the Ca dependent GTPase RAS protein activator (Rasal1) may be involved to tailor cellular functions and epithelial gene expression (50, 51). Thus, enhanced expression of predominantly IFN-λ_2-induced gene products may help to control the inflammatory reaction at impaired mucosal body sites and reconstitute the epithelial barrier integrity and host–microbial homeostasis following viral clearance.

This hypothesis is also consistent with the fact that type III IFNs belong to the IL-10 cytokine family, a large group of cytokines that also includes IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26. Members of this family play a critical role in the maintenance and repair of the epithelial barrier function during infectious and inflammatory challenges (52). They exhibit a strong immunomodulatory activity illustrating the adverse effect of uncontrolled mucosal inflammation and the need to maintain the integrity of body surfaces and host–microbial homeostasis. This is nicely illustrated by IL-10 that is able to repress pro-inflammatory responses playing a critical role to maintain mucosal homeostasis in the colon (53). Also, IL-22 strengthens the mucosal barrier and induces antibacterial effector molecules in the absence of an inflammatory response. Of note, IL-22 and type III IFN were recently shown to synergize to restrict viral replication at the intestinal epithelium (54).

Cell polarization appears to play a critical role for the expression of the predominantly IFN-λ_2-induced gene profile. Apical–basolateral polarization represents a key feature of intestinal epithelial cells and has previously been functionally associated with the response of gut epithelial cells to IFN (9). Strikingly, epithelial cell polarization significantly enhanced the expression level of the IL-28Rα chain but not of the IFNAR receptor complex confirming a previous report (37). It is therefore tempting to speculate on a possible functional link between the level of expression of the type III IFN receptor and the ability to induce additional cellular signal transduction pathways ultimately inducing a predominantly IFN-λ_2-induced gene profile. Alternatively, the cell polarization itself may influence downstream events of the IL-28 receptor complex. Future investigations will be needed to identify and dissect the involved signaling pathways.

In conclusion, we here report on the first evidence for the existence of a predominantly IFN-λ_2-induced gene expression profile in

polarized intestinal epithelial cells *in vitro* and *in vivo*. Expression of predominantly IFN-λ_2-induced genes was restricted to gut epithelial cells and required apical–basolateral cell polarization. The existence of a predominantly IFN-λ-induced gene set at the intestinal epithelium might significant extend the biological role of IFN-λ and shed light on the particular situation at microbially colonized mucosal surfaces during infectious challenges.

ETHICS STATEMENT

All animal experiments were performed in compliance with the German animal protection law (TierSchG) and approved by the local animal welfare committee Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit Oldenburg, Germany. Mice were housed under specific pathogen-free conditions and handled in accordance with regulations defined by FELASA and the national animal welfare body GV-SOLAS (www.gv-solas.de/index.html).

AUTHOR CONTRIBUTIONS

TS, SB, MK, and MH performed experiments. TS, SB, MK, DW, HH, and MH planned the experiments and evaluated the results. HH, MK, and UK provided critical reagents. TS, MK, DW, HH, UK, and MH wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01302/full#supplementary-material.

FIGURE S1 | Interferon (IFN) effect on house-keeping gene expression and time kinetic of IFN-β and IFN-λ_2-induced IFN-stimulated gene induction. **(A)** mRNA expression (intensity) of the house-keeping gene *β-actin* in unstimulated IEC10 cells (control) and IEC10 cells stimulated with IFN-β (500 U/mL) and IFN-λ_2 (20 ng/mL) for 9 h. The results represent the mean ± SEM values from one experiment performed in triplicates. **(B)** IEC10 cells carrying an Mx2-luciferase reporter were stimulated with IFN-β (500 U/mL) and IFN-λ_2 (20 ng/mL) for the indicated time periods and the luciferase production was determined. The data are presented as mean ± SD from one experiment performed in triplicates.

FIGURE S2 | Gene induction by interferon (IFN)-λ_2 in IEC10 cells. **(A)** Heatmap of stimulated genes in IEC10 cells cultured on transwell filter inserts and left untreated (PBS) or exposed to IFN-λ_2 (20 ng/mL) or IFN-β (500 U/mL) for 9 h. Data were obtained using a global gene expression array. Two-group analysis for the induction of IFN-λ_2-induced genes were carried out at $p = 0.001$, $q = 0.05$, FC = 2 represented as a hierarchical cluster of 349 genes upregulated by IFN-λ_2. **(B–D)** Clusters of orthologous group analysis of the **(B)** "predominantly IFN-λ_2-induced genes," **(C)** "strongly IFN-λ_2-induced genes," and **(D)** "classical antiviral IFN-stimulated genes," shown in **Figure 1C**.

FIGURE S3 | Early time kinetic of interferon (IFN)-β and IFN-λ_2-induced IFN-stimulated gene (ISG) induction. **(A,B)** Quantitative RT-PCR for the prototypical ISGs *Usp18* and *Ifi44* as well as the predominantly IFN-λ_2-induced ISGs *Mmp7* and *Serpinb1a* performed on total RNA isolated from IEC10 cells grown on transwell filter inserts and stimulated with **(A)** IFN-β (500 U/mL) and **(B)** IFN-λ_2 (20 ng/mL) for the indicated time periods. The results represent the mean ± SEM values from one experiment performed in triplicates and are normalized to the values obtained for the housekeeping gene β-actin.

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REVIEW
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Interferon (IFN)-λ Takes the Helm: Immunomodulatory Roles of Type III IFNs

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Type III interferons (IFNs) (or IFN-λ) are the latest addition to the IFN family. Even though they share little protein homology with type I IFN, both exhibit remarkable functional similarities: each can be induced in response to viral infections, and both lead to Janus kinases (JAK) and signal transducer and activator of transcription (STAT) activation. The JAK/STAT pathway induces antiviral responses and IFN-stimulated gene transcription. However, despite the similarities in their effector functions with type I IFNs, IFN-λ also has a non-redundant role in protecting barrier organs: epithelial cells preferentially produce IFN-λ rather than type I IFNs; and interferon lambda receptor 1 (IFNLR1), the specific receptor for IFN-λ, is highly expressed on cells of epithelial lineage. Thus far, IFN-λ has been considered mainly as an epithelial cytokine, which restricts viral replication in epithelial cells and constitutes an added layer of protection at mucosal sites. However, it is now increasingly recognized that IFNLR1 is expressed broadly, and that immune cells such as neutrophils and dendritic cells also respond to IFN-λ. Moreover, in many *in vivo* models, IFN-λ modulates immune cell functions and thereby configures itself less as a cytokine that is only specific to the epithelium, and more as a cytokine that directly controls the inflammatory response at mucosal sites. Here, we critically review the recent literature on immune modulatory roles for IFN-λ, and distinguish between the direct and indirect effects of this IFN on immune cell functions in different inflammatory settings.

Keywords: interferon lambda, dendritic cells, neutrophils, natural killer cells, type III interferon, viral infection, bacterial infections, fungal infection

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INTRODUCTION

First described more than 60 years ago (1) interferons (IFNs) were the first family of cytokines to be discovered. Since then, IFNs have been extensively studied, and their presence is correlated with a number of immunological and biological processes, such as cell proliferation, regulation of cell survival, and modulation of immune functions. IFNs can be divided into three major subfamilies: type I IFNs (comprising mainly IFN-β and over 20 subtypes of IFN-α, -ε, and -ω), type II IFNs (IFN-γ), and the recently identified type III IFNs (IFN-λ) (2, 3) that comprise four members in human (IFN-λ1/IL-29, IFN-λ2, IFN-λ3/IL-28A-B, and IFN-λ4) and two in mice (IFN-λ2/IL-28A and IFN-λ3/IL-28B, while IFN-λ1 is a pseudogene interrupted by a stop codon). IFN-λ2 and IFN-λ3 are highly related and have 96% sequence identity, while IFN-λ1 shares 81% sequence identity with IFN-λ2 and IFN-λ3 (4).

The gene and protein structure of IFN-λ2 and -λ3 share little homology to those of type I IFNs (15%) (4); but they exert remarkably overlapping functions. The heterodimeric receptor for IFN-λ is named IFNLR (or IL-28R), and comprises the specific subunit interferon lambda receptor 1 (IFNLR1, also known as IL-28R1) plus the IL-10R2 subunit that is common to many type II cytokines (such as IL-10, IL-22, IL-24, and IL-26). Once IFNLR is engaged, IFN-λ activate an antiviral response that is very similar to the one triggered by type I IFNs (5). In fact, both engage a similar JAK–STAT pathway, with the only difference that IFN-λ can also use the adaptor JAK2 (6). Both cytokine families also induce IFN-stimulated gene (ISG) transcription, and both confer protection against viral infections (5). This overlap in functions raises the question of why two distinct but similar IFN systems have been maintained throughout evolution, considering that these two systems separated as far back in evolution as did amphibians, reptiles, and birds (7).

The main distinction between the two IFN systems has to do with the tropism between expression of the cytokine and its specific receptors. Myeloid cells at mucosal sites express both type I IFNs and IFN-λ in response to viral as well as bacterial ligands (8–11). However, type I IFN and IFN-λ production are regulated differently. Stimulation of plasma membrane toll-like receptor (TLR) (such as TLR2 and TLR5), both in myeloid and epithelial cells, selectively induces IFN-λ, and not type I IFN, mRNA expression. Moreover, activation of TLR5 has recently been proved to be essential for the induction of IFN-λ upon *Salmonella* encounter (9). Also, cells of epithelial lineage, both in the gut (12) and in the liver (13), preferentially produce IFN-λ over type I IFNs in response to viral ligands. In particular, while both IFNs are induced downstream of pattern recognition receptor and mitochondrial antiviral signaling protein (MAVS), the production of IFN-λ is favored subsequent to activation of the MAVS that reside in peroxisomes (6, 14). The abundance of peroxisomes in cells of epithelial lineage could explain the tropism of IFN-λ production (13).

Other than the tropism of IFN-λ production, the selective expression of the receptor governs the tropism of IFN-λ response. The receptor for type I IFNs (which comprises receptor subunits IFNAR1 and IFNAR2) is expressed in virtually every cell type, while expression of the IFNLR1 receptor is much more specific, and is believed to be most abundant in cells of epithelial origin that are present at barrier surfaces (15). This pattern of expression, along with the recently documented non-redundant role of IFN-λ in protecting against virus infection at mucosal sites [e.g., at the intestinal barrier (12, 16–18) and in the lung (19)], suggest a model in which IFN-λ represents an epithelial cytokine that protect mucosal surfaces without activating widespread and possibly nocuous immune responses, while type I IFNs represent a more general and potent system that is activated once the mucosal barrier is broken. However, recent findings challenge the view that IFN-λ is primarily an epithelial cytokine, describe IFN-λ's ability to directly and indirectly modulate immune cell functions and document the expression of IFNLR1 on immune cells; they also document that among immune cells, neutrophils express IFNLR1 and directly respond to IFN-λ, in the setting of viral infections (19) as well as other forms of acute inflammation (20–22). IFN-λ reportedly also interferes with the function of NK cells (23, 24), and favors the skewing of T cell activation toward type I (rather than type II) responses, by modulating DC functions (25). While the study of immunomodulatory effects of IFN-λ is still in its infancy—in part due to a lack of specific tools such as good antibodies against IFNLR1—a new role for IFN-λ in shaping the mucosal immune response is emerging. In this review, we critically examine recent literature on the role of IFN-λ in immune cells, differentiating between a direct IFN-λ effect on specific cell types and possible indirect phenomena; we also evaluate what is known about how IFN-λ participates in the control of mucosal immune responses.

MODULATION OF IMMUNE CELL FUNCTIONS BY IFN-λ

Neutrophils

Neutrophils are the first line of defense of the immune system: following pathogen invasion or tissue injury, these cells are quickly and massively recruited to barrier sites, where they protect the host by killing invading pathogens *via* a very rapid release of toxic mediators, independent of *de novo* protein synthesis (26). At later stages, neutrophils regulate the inflammatory response, either passively by undergoing apoptosis and turning off their toxic potential, or actively by secreting anti-inflammatory cytokines and lipidic mediators (27). The ability of these cells to potently kill bacteria is also accompanied by the necessary evil of tissue damage, since many of the toxic mediator released, such as reactive oxygen species (ROS) and proteases, are unable to discriminate between host and pathogen cells. Given the tropism of IFN-λ production to mucosal sites and the complex crosstalk between epithelial cells and neutrophils at mucosal surfaces (28, 29), it is remarkable that among murine immune cells, neutrophils express IFNLR1 at the highest level (19–21). Murine neutrophils express IFNLR1 at very high levels (19–21, 30) that are comparable to those in colonic epithelial cells (20) and in epithelial cells from the lung (19). Human neutrophils have also been found to express IFNLR1 at higher levels as compared to lymphocytes (30) and upregulate its expression following treatment with pro-inflammatory agents such as LPS (20), or after encounter with *Aspergillus fumigatus* (30). In addition to the high levels of receptor expression, mouse and human neutrophils also respond to IFN-λ stimulation (19–21, 30), and activate the canonical JAK–STAT pathway, that leads to phosphorylation of STAT1, STAT2, and STAT3 (21, 30) and induces upregulation of ISGs at levels similar to those induced by type I IFNs (19, 20). Surprisingly, in addition to the canonical ISG response induced downstream of the JAK–STAT pathway, IFN-λ also down-modulates tissue-damaging, transcription-independent responses such as production of ROS, granule mobilization (20), release of neutrophil extracellular traps (NETs) (22), and cellular migration (21); while cytokine production in response to inflammatory stimuli, phagocytosis, and apoptosis is not affected by IFN-λ (20).

Irina Udalova and colleagues were the first to report that neutrophils respond to IFN-λ (21), and that treatment of neutrophils

with IFN-λ *in vitro* leads to activation of the JAK–STAT pathway and STAT1 phosphorylation; they also first described the ability of IFN-λ to regulate pro-inflammatory neutrophil functions. In arthritic mice treated with recombinant IFN-λ, they observed a defect in neutrophil migration to the inflamed joint; this defect was attributed to the capacity of IFN-λ to directly inhibit neutrophil migration. Also in an air pouch model of acute inflammation, and when neutrophil migration toward leukotrien B4 was assessed *in vitro*, the cells exhibited a defect in migration: fewer neutrophils were recovered in the air pouch in the presence of IFN-λ, and a shorter Euclidean distance was traveled by neutrophils treated with IFN-λ *in vitro* (21).

More recently, we showed that IFN-λs (but not type I IFNs) are able to regulate a non-translational signaling pathway that diminishes ROS production by neutrophils as well as degranulation following activation of the cells with pro-inflammatory stimuli, but that it does not alter cytokine production induced by inflammatory stimuli or phagocytosis (20). We additionally demonstrated that IFN-λ inhibits degranulation and decreases ROS production even when *de novo* protein synthesis is inhibited with cycloheximide, or when STAT1 or STAT3 are genetically ablated or pharmacologically inhibited. Inhibition of all JAK kinases, or specific inhibition of JAK2, which is involved only in IFN-λ signaling (and not in type I IFN responses) (6, 14) impairs the ability of IFN-λ to inhibit ROS production and degranulation (20). Neutrophils treated with IFN-λ are nevertheless able to phagocytose both opsonized and non-opsonized *E. coli,* and to produce cytokines in response to LPS. Human neutrophils appear to have similar regulating mechanisms: treatment with IFN-λ reduces the ability of these cells to produce ROS (20), and also impairs their ability to generate NETs in an *in vitro* model of thromboinflammation, wherein neutrophils are incubated with activated platelets in the presence of IFN-λ (22). IFN-λ treatment also inhibits NET generation in response to platelet-derived inorganic polyphosphate (polyP) and interferes with the ability of polyP to inhibit mTOR activation and induce the autophagy marker LC3, which is a requisite for NET release (31). IFN-λ, thus, profoundly influences neutrophil non-transcriptional functions and engages a pathway that is independent of the canonical JAK–STAT pathway and does not rely on *de novo* protein synthesis. In contrast to the transcriptional responses, these characteristics are not shared with type I IFNs and seem to specifically target the potent cytotoxic responses that can threaten mucosal integrity.

As previously described for epithelial cells (2, 3, 5, 32), IFN-λ induces a transcriptional response remarkably similar to that of type I IFNs. So far, no genes have been identified that are selectively upregulated by IFN-λ (and not by type I IFNs), and the upregulation of antiviral ISGs is largely overlapping; however, IFN-λ (as opposed to IFN-α) is unable to directly induce upregulation of pro-inflammatory cytokines, such as TNF, IL-1β, and IL-6, or chemokines, such as CCL2 and CXCL1. The influence of IFN-λ on neutrophils appears, thus, to be anti-inflammatory. Indeed, IFN-λ is able to down-modulate nocuous neutrophil functions—such as the production of toxic mediators or the production of NETs—without interfering with the capacity of these cells to engulf pathogens, or to orchestrate the inflammatory response *via* cytokine secretion (**Figure 1**). The importance of such regulation of neutrophil functions has been documented *in vivo* following viral infections and also in inflammatory pathologies. In fact, when IFNLR1 is depleted specifically in neutrophils, mice are more susceptible to a sublethal dose of influenza virus infection and present a higher viral load, higher number of leukocytes in the BAL, and higher levels of expression of inflammatory cytokines (19). Notably, when low doses of virus are used for infection, IFNLR expression is required both in epithelial cells and in neutrophils to confer maximum protection. In fact, mice with a conditional ablation of IFNLR1 in pulmonary epithelial cells or in neutrophils only partially recapitulate the total knock-out phenotype (19).

Interferon-λ also influences neutrophil functions during acute inflammation in the gut mucosa. We and others have described a protective role for IFN-λ in a mouse model of DSS-induced colitis (20, 33, 34). In fact, IFNLR1$^{-/-}$ mice are more susceptible to the induction of colitis than are wild-type mice and present a more severe disease phenotype, which is characterized by shorter

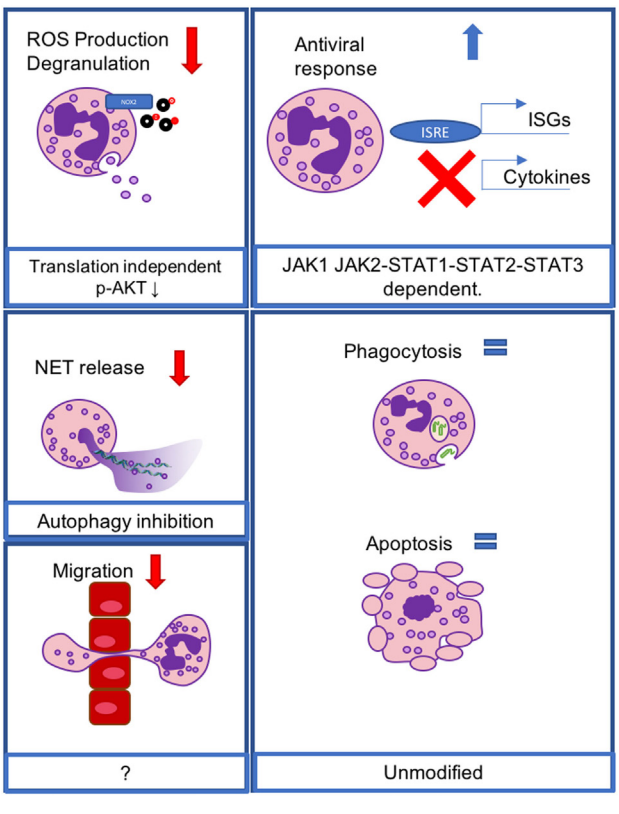

FIGURE 1 | IFN-λ modulates neutrophil functions at the transcriptional and non-transcriptional levels. Reactive oxygen species production and degranulation are regulated at a non-translational level, involving AKT inhibition (upper left), neutrophil extracellular trap release is inhibited *via* inhibition of autophagy (middle left), and neutrophil migration is inhibited *via* an unknown mechanism (lower left). Transcriptional antiviral responses lead to the induction of IFN-stimulated genes, but do not mediate cytokine production, and act through a JAK1- and JAK2-dependent, STAT1, -2, -3-dependent mechanism (upper right). Phagocytosis and apoptosis are not affected (lower right).

colons, greater weight loss, more severe histological damage, and augmented oxidative stress (20). This effect is entirely dependent on the action of IFN-λ on immune cells, because chimeras in which only radio-resistant cells are IFNLR1$^{-/-}$, and mice that harbor a deletion of IFNLR1 specific to epithelial cells are equally sensitive to DSS administration as are their wild-type counterparts (20).

By contrast, bone marrow chimeras in which IFNLR1 is depleted only in cells of hematopoietic origin, and mice with conditional depletion of IFNLR1 expression restricted to neutrophils, recapitulate the aggravated phenotype of IFNLR1$^{-/-}$ mice. Notably, both chimeras deleted in the hematopoietic compartment, neutrophils specific IFNLR1$^{-/-}$ mice and total IFNLR1$^{-/-}$ mice have a more severe oxidative stress transcriptional signature in the colon epithelium, when compared to their wild-type counterparts. These data strongly suggest that the control exerted by IFN-λ on neutrophil ROS production is pivotal to protect the intestinal mucosa during acute inflammation (20). In the absence of an active viral or bacterial infection, the source of tonic IFN-λ signaling is represented by the commensal virome. In fact, while depletion of intestinal viruses aggravates colitis in wild-type mice (20, 35) as well as in mice that are deficient in type I IFN signaling (20), IFNLR1$^{-/-}$ mice phenocopy WT mice that are depleted of intestinal viruses in that they are insensitive to treatment with antiviral drugs. In particular, alteration of the intestinal virome in humans that are similar to the alteration obtained in mice treated with antiviral drugs is associated with ulcerative colitis and Crohn's disease (36, 37).

It was recently shown that IFN-λ action on neutrophils can also protect the host during fungal infections (30). In a model of invasive aspergillosis, both IFNLR1$^{-/-}$ and mice bearing neutrophil-specific depletion of IFNLR1 succumb faster after pulmonary infection with *A. fumigatus* and present an aggravated disease, with higher CFUs recovered from the lungs and more severe invasion as measured by histology. Curiously, neutrophils deficient for IFNLR1 had reduced intracellular ROS levels when stained *ex vivo*. This phenotype was recapitulated in neutrophils deficient for STAT1 suggesting that, during fungal infections, IFN-λ-dependent STAT1 activation mediates a transcriptional program that protects the host. While early, translation independent, regulation of neutrophil function by IFN-λ suppresses ROS production and degranulation in response to inflammatory stimuli, during fungal infections, STAT1-dependent action is critical for the activation of neutrophil functions *in vivo*. The apparent contrast between the two mechanisms can be explained by the differential regulation of neutrophil biology in response to different stimuli. Moreover, while immediate responses, such as ROS production and degranulation, are not typically transcriptionally regulated, the optimal expression of NADPH enzymes during neutrophil development could contribute to the protective effect of IFN-λ against fungi. Indeed, in our hands, when neutrophils were stimulated *in vitro* with *C. albicans* hyphae, ROS were produced both in the absence and in the presence of recombinant IFN-λ (our *unpublished data*). Altogether, these data suggest that IFNLR1-stimulation is not necessary to induce ROS production by neutrophils upon fungal encounter *in vitro* but that, *in vivo*, IFN-λ can contribute to prime neutrophils during a stage of differentiation that could not be recapitulated *in vitro*.

Finally, the inhibitory activity of IFN-λ on neutrophils can also be exploited therapeutically: in fact, IFN-λ administration is protective in pulmonary infections with influenza virus (19), during DSS colitis (20) and in an inflammatory setting such as rheumatoid arthritis (21) or a mouse model of vascular injury (22), where IFN-λ is not produced naturally.

Dendritic Cells (DCs)

Conventional mouse DCs and human plasmacytoid DCs (pDCs) express low levels of IFNLR1 yet respond to IFN-λ stimulation. In mice, DCs that are derived from the lung express low levels of IFNLR1 (25). Despite these low levels of expression, the central role of DCs at the crossroads between adaptive and innate immunity makes their responses to IFN-λ highly significant. Koltsida and colleagues report that DCs stimulated with IFN-λ, despite responding poorly in terms of ISG induction, are nonetheless able to upregulate T-bet and produce higher levels of IL-12 following LPS stimulation. In the same conditions, they also fail to upregulate OX40L and assume a Th1-polarizing phenotype (25). Indeed, when DCs sorted from the lungs of mice infected with a replication-defective adenovirus expressing IFN-λ under the CMV promoter—or from mice that are treated with recombinant IFN-λ—are used to stimulate T cell polarization *in vitro*, they favor Th1 skewing. This ability of IFN-λ to induce the skewing of T cell responses is particularly relevant in a model of allergic airway disease (25). In fact, IFNLR1$^{-/-}$ mice present a more severe disease phenotype, with elevated production of type II cytokines, a higher histopathological score, and increased eosinophilic infiltration in the BAL. Moreover, when IFN-λ is administered—either directly or *via* an IFN-λ-producing adenovirus—mice are protected from allergic airway disease (25). Also, adoptive transfer of DCs purified from mice treated with IFN-λ-producing adenovirus confers protection. Early reports also suggest that when DCs are stimulated with IFN-λ, they acquire a regulatory phenotype and promote FOXP3^{+} Treg proliferation (38), and that T cell responses can, thus, be skewed toward a Th1 phenotype *in vitro* (39). These data strongly support a role of IFN-λ-stimulated DCs in skewing T cell responses *in vivo,* and underscore the need to further investigate how IFN-λ affects DCs (25).

As mentioned above and recently reviewed (40), human pDCs serve an important role in IFN-λ biology. Human pDCs express IFNLR1 and are able to produce as well as respond to IFN-λ (40–42). When stimulated with IFN-λ, they induce the canonical JAK–STAT pathway (43, 44) and upregulate low levels of ISG transcription (43–45). IFN-λ also influences pDC-specific functions: in particular, it can stimulate pDCs to produce type I IFNs and induce the expression of low levels of TNF (44). Moreover, IFN-λ acts synergistically with IL-3 to hyperactivate pDCs and induce higher levels of inflammatory cytokines (45). Treatment of pDCs with IFN-λ also influences the activation status of pDCs, inducing an upregulation of CD80 and CD86. The functional significance of these regulations remains to be determined: while some researchers claim that IFN-λ inhibits the ability of pDCs to activate T cells (42), the enhancement of pDC activation suggests

that IFN-λ stimulates pDCs and enhances their capacity to combat viral infections.

While the ability of IFN-λ to influence the activity of DCs is intriguing and could have a substantial effect on how DCs govern innate and adaptive responses, more work is needed to clarify the specific response of DCs to IFN-λ. The discovery of new non-transcriptional pathways induced by IFN-λ should elucidate whether non-transcriptional responses are active in DCs and help reveal additional specific effects of IFN-λ on DCs. But while scattered reports in the literature link IFN-λ to the skewing of T cells toward a Th1 phenotype (46), the expression of IFNLR in T cells and the responsivity of T cells to IFN-λ has not been formally established; this suggests that the influence of this IFN on T cell functions *in vivo* represents indirect effects that require activation of DCs.

NK Cells

Emerging evidence documents that IFN-λ affects NK cell activity *in vivo* (23, 24). NK cells are believed to be essential for IFN-λ-mediated protection against influenza virus (24), against tumor growth (23), and in a model of LPS-induced or cecal-ligation puncture (CLP)-induced septic shock (23). However, whether IFN-λ can act directly on NK cells is debated (47–49). Smyth and colleagues (50) report low levels of IFNLR1 expression on mouse NK cells, and to date, there is no evidence of a direct response of NK cells to IFN-λ; in fact, treatment of NK cells with IFN-λ does not activate STAT1 phosphorylation, nor does activate ISG expression (23). However, despite the lack of receptor expression on NK cells and the lack of responsiveness of these cells to IFN-λ *in vitro*, a model of acute endotoxemia shows that NK cells derived from IFNLR1$^{-/-}$ spleens have defective IFN-γ production, and IFNLR1$^{-/-}$ mice are partially protected from lethal doses of LPS or in a CLP model of sepsis, in a IFN-γ-dependent manner. Together, these observations point to an indirect effect of IFN-λ on NK cells. While NK cells transferred from INFLR1$^{-/-}$ mice into Rag$^{-/-}$ γc$^{-/-}$ mice are also defective in the production of IFN-γ after LPS treatment (23), this does not exclude the possibility that IFNLR1$^{-/-}$ NK cells have defects in differentiation/development. Observations on a recent model of influenza virus infection support this notion: administration of IFN-λ (by continuous overexpression *via* hydrodynamic gene delivery) protected mice from the viral infection, and influenced NK cell differentiation; indeed, NK cells in these mice exhibited a more mature phenotype and proliferated at a higher rate. However, these authors also claimed that NK cells express extremely low levels of IFNLR1, and they attributed the observed phenotype to the expression of IFNLR1 on myeloid cells. Notably, depletion of phagocytes by administering clodronate liposomes abolishes the protective effect of IFN-λ (24).

While the above findings unequivocally establish that NK cell functions are modified by IFN-λ *in vivo*, they also strongly suggest that NK cells can be instructed by other cell types that directly respond to IFN-λ stimulation. DCs and neutrophils—the two cell types that do express IFNLR1 and respond to IFN-λ—can influence NK cell functionality *in vivo*. In fact, DCs activate NK cells by secreting cytokines, such as IL-2, IL-18, and IL-12; and DCs also present IL-15 to NK cells in an IFN-β-dependent manner (51–56). It will be important to test in the future the hypothesis that, similarly to type I IFNs (19), IFN-λ could also directly induce low levels of IL-15 that are presented to NK cells. In the same model of airway allergic inflammation that revealed IFN-λs ability to influence DC-mediated skewing of the immune response, it was shown that NK cells preferentially produced IFN-γ and that they were protective against airway inflammation (57). While a direct activity of IFN-λ on NK cells for the observed protection cannot be excluded, the striking similarity of the two models implicates DCs in both skewing NK cell activation and inducing IFN-γ production.

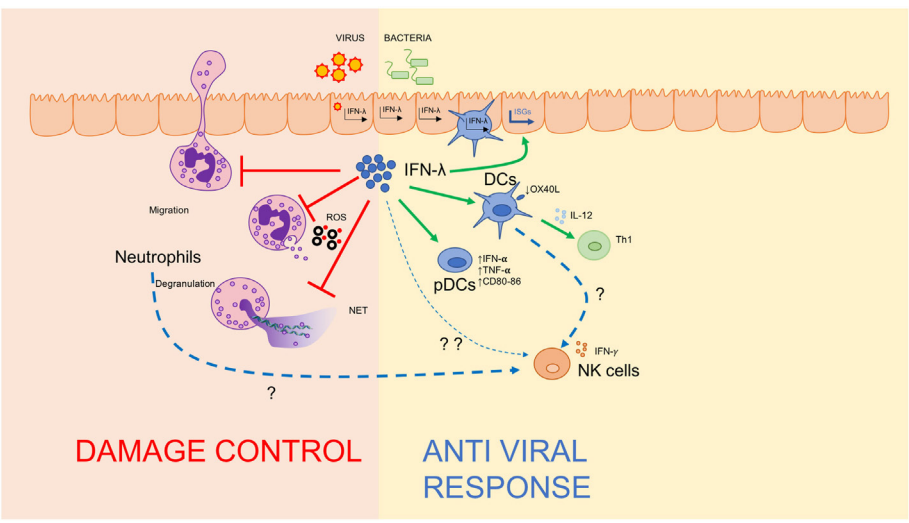

FIGURE 2 | IFN-λ regulates the mucosal inflammatory process. Schematic depiction of IFN-λ's ability to regulate immunity at mucosal sites by amplifying the antiviral response *via* directly stimulating dendritic cells and plasmacytoid DCs (right), and dampening damage-inducing neutrophil functions to maintain mucosal integrity (left).

Neutrophils also profoundly influence the functions of NK cells. Consistent with the model in which IFN-λ regulates NK cell maturation, defects in NK cell terminal differentiation and survival were observed in congenitally neutropenic mice and in mice depleted of neutrophils, as well as in patients with neutropenia (57). Also, ROS produced by human neutrophils inhibit NK cell functions *in vitro* (58). The ability of IFN-λ to suppress ROS production and to counteract this inhibition feedback can potentially explain the increased activation of NK cells in the presence of IFN-λs. Some early *in vivo* studies support the hypothesis of a crosstalk between neutrophils and NK cells that governs the antitumoral activity of IFN-λ. In fact, when IFN-λ is administered *via* retroviral transduction into a mouse fibrosarcoma cell line, it is effective in controlling tumor growth, but this protective effect is lost when either NK cells or neutrophils are depleted (59). While IFN-λ undeniably influences NK cell functions *in vivo*, the phenotypes observed might be ascribed to unexplored modulation of NK cell functions by neutrophils or DCs. However, the emergence of non-transcriptional roles for IFN-λ on neutrophils opens up the possibility that similar overlooked non-transcriptional pathways are active in NK cells.

Other Cell Types

Reports of other cell types expressing IFNLR1 and responding to IFN-λ stimulation exist in the literature. In particular, human B cells have been shown to express IFNLR1 (43, 60, 61) and respond to IFN-λ by upregulating ISGs (61). While the functional role of IFN-λ in B cells is still open for investigations, early pieces of evidence suggest that, similar to type I IFNs, IFN-λ augments TLR-mediated activation of B cells.

Scattered reports describing a role of IFN-λ in human macrophage activation also exist. In particular, IFN-λ can protect human monocyte-derived macrophages from HIV infection (62, 63) and treatment of human monocyte-derived macrophages with IFN-λ augments the production of pro-inflammatory cytokines following stimulation with LPS or R848 (64).

CONCLUSION

Historically, IFN-λ has been recognized as an epithelium-specific cytokine that affects antiviral responses in epithelial cells; however, a growing body of literature supports a critical role for these IFNs in influencing the modulation of immune responses. The action of IFN-λ on immune cells is now configured in a model wherein this cytokine represents the first line of defense of mucosal surfaces. In fact, IFN-λ has non-redundant functions in conditions such as low viral loads (19), or when the epithelial layer is preferentially affected (12): under these conditions, IFN-λ acts directly on epithelial cells to exert local antiviral activity and on DCs to skew the T cell response toward an antiviral Th1 response; IFN-λ also acts directly or indirectly on NK cells to potentiate their activation and protect against viruses. At the same time, IFN-λ also serves important functions in neutrophils, inhibiting tissue-damaging events, such as ROS production, degranulation, and NET formation, without impairing cytokine production or pathogen engulfment. Indeed, IFN-λ activity on neutrophils does not impair, but enhances, responses to pathogenic fungi (30). This modulation of neutrophil activities is pivotal for protecting the mucosae from excessive damage and for maintaining the integrity and barrier functions of epithelia at mucosal sites. IFN-λ is, thus, deemed to be a mucosal cytokine whose evolutionary role is to precede activation of type I IFN, eliminate invading pathogens at mucosal sites without compromising their barrier functions, and limit dissemination of the pathogen (**Figure 2**). If the pathogen spreads and reaches the underlying tissues, a more potent inflammatory response orchestrated by type I IFNs is needed, but comes at the cost of extensive tissue damage. Such protective activity is also relevant in the absence of a viral infection: tonic IFN-λ production induced by commensal viruses protects the colon mucosa during experimental colitis by dampening neutrophil responses, and administration of IFN-λ is protective in a number of inflammatory settings such as allergic airway diseases, or arthritis. Such evidence of immunomodulatory roles for IFN-λ *in vivo* highlights that these cytokines have additional, as yet unexplored roles in the stimulation of immune cells.

However, support for a direct role for IFN-λ in the modulation of immune functions is fragmented. This is in part due to the lack of biological tools such as specific antibodies against IFNLR1 and the existence of a splicing variant of IFNLR1 in humans that gives rise to a secreted protein with decoy functions (65), which further complicate the correlation of IFNLR1 expression and IFN-λ responsiveness. The translation of findings based on mouse models to human biology is further complicated by the apparent different pattern of expression of the IFNLR1. Indeed, while pDCs and B cells express IFNLR1 and respond to IFN-λ stimulation in humans, the same cell types are not responsive to IFN-λ in mice. Also, while both murine and human neutrophil express the IFNLR1, it is still a matter of discussion if and how inflammatory stimuli and differentiation status of these cells can influence IFNLR1 expression. Despite these confounds, recent reports have uncovered the immune-modulating properties of IFN-λ, as well as new specific non-translational pathways that further differentiate its action from that of type I IFNs. These new insights will pave the way toward an in-depth understanding of the physiological role of these cytokines and will help in exploring the unappreciated functions of IFN-λ in the context of immune cells.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Interferon Lambda Genetics and Biology in Regulation of Viral Control

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Type III interferons, also known as interferon lambdas (IFNλs), are the most recent addition to the IFN family following their discovery in 2003. Initially, IFNλ was demonstrated to induce expression of interferon-stimulated genes and exert antiviral properties in a similar manner to type I IFNs. However, while IFNλ has been described to have largely overlapping expression and function with type I IFNs, it has become increasingly clear that type III IFNs also have distinct functions from type I IFNs. In contrast to type I IFNs, whose receptor is ubiquitously expressed, type III IFNs signal and function largely at barrier epithelial surfaces, such as the respiratory and gastrointestinal tracts, as well as the blood–brain barrier. In further support of unique functions for type III IFNs, single nucleotide polymorphisms in *IFNL* genes in humans are strongly associated with outcomes to viral infection. These biological linkages have also been more directly supported by studies in mice highlighting roles of IFNλ in promoting antiviral immune responses. In this review, we discuss the current understanding of type III IFNs, and how their functions are similar to, and different from, type I IFN in various immune cell subtypes and viral infections.

Keywords: interferon lambda, interferon, immunity, immune cells, infectious disease, virus

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EVOLUTION OF TYPE III IFN GENES

Type I IFN is produced and secreted rapidly following viral infection (1, 2). It subsequently signals to surrounding cells to initiate an antiviral state as a critical host defense mechanism. In humans, there are 13 subtypes of IFNα as well as IFNβ, IFNε, IFNκ, and IFNω [reviewed in Ref. (3)]. Type I IFNs are intronless genes clustered on chromosome 9 in humans and chromosome 4 in mice. In mammals, birds, reptiles, and amphibians, type I IFN genes lack introns, which suggests their origin may have been from retrotransposed genetic elements [reviewed in Ref. (4)]. However, type I IFNs in fish harbor introns and are thought to have arisen through a common ancestor of IL-10 family [reviewed in Ref. (5)]. Amphibians have been recently described to have both intron-containing and intron-less type I IFN genes (6). The current understanding of interferon evolution has not distinguished whether an independent or retrotransposition event led to the generation of intron-less type I IFN genes that may have been the ancestor of the intron-less type I IFN locus in reptiles, birds, and fish.

IFN lambda family members were initially named as interleukin-28 (IL-28) and IL-29 and classified into the IL-10 family genes as they signal through the common IL-10 receptor subunit 2 (IL-10R2) (7, 8). Humans have four *IFNL* genes, *IFNL1* (*IL29*), *IFNL2* (*IL28A*), *IFNL3* (*IL28B*), and *IFNL4*. *IFNL* genes are present in tetrapods, but in contrast to the evolutionary diversity seen in type I IFNs, throughout vertebrates the type III IFN locus comprised of two to four family

members, each containing introns (9). While IFN lambdas are most functionally similar to type I IFNs, they are structurally similar to members of the IL-10 family. Type III IFNs have a phase 0 intron–exon structure and utilize a component of IL-10R2 as a part of their receptor heterodimer complex for signaling (10). Sequence identities of type III IFNs when compared with type I IFNs (15–19% aa) or IL-10 (11–13% aa) are low (8). Among type III genes, *IFNL1* and *IFNL2* share 81% amino acid identity, whereas *IFNL2* and *IFNL3* share 96% amino acid identities. *IFNL4* shares only ~28% amino acid identity with other *IFNL* genes, leading to speculation *IFNL4* may have been introduced *via* a separate duplication event. While the evolutionary history of type III IFNs is still incomplete, a number of groups are working to understand the evolutionary constraints on type III IFNs [reviewed in Ref. (4, 11, 12)]. Utilizing an evolutionary genetics approach, Manry et al. demonstrated that type I and type III IFNs, and even individual genes within each of these types, have been subjected to distinct evolutionary pressures (11). This work suggests both redundant and specific, unique roles for these IFN families in pathogen defense.

In contrast to humans, in mice only *Ifnl2* and *Ifnl3* are functional; *Ifnl1* and *Ifnl4* are pseudogenes (13). Despite differences in human and murine *Ifnl* gene composition, murine studies have provided critical insights into the antiviral and immune modulatory functions that have relevant correlates to human infection. For example, in a murine asthma model, interferon lambda (IFNλ) treatment was demonstrated to lead to a Th1-biased immune response (14). In humans, IFNλ leads to enhanced Th1 responses during influenza virus vaccination (15). In addition, respiratory viral pathogens have evolved mechanisms to suppress IFNλ function or downstream signaling, highlighting the critical importance of IFNλ to respiratory immunity in particular, but also the contribution of IFNλ to infection at mucosal barriers in general (16, 17).

EXPRESSION IFN LAMBDA GENES DURING VIRAL INFECTION

IFNs are expressed following detection of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) [reviewed in Ref. (18–20)]. Sensing of PAMPs by the RIG-I-like receptors results in the recruitment of mitochondrial antiviral signaling protein (MAVS) to mitochondrial associated membranes or peroxisomes, leading to activation of the transcription factors NF-κB and interferon regulatory factors (IRFs), which induce expression of both type I IFN and IFNλ (21). Multiple toll-like receptors induce expression of type I and III IFNs (22, 23). While the signals and pathways that induce type I and type III IFNs largely overlap, one notable exception does exist in the DNA sensing pathway. In HEK293 and THP-1 cells, binding of DNA to the cytosolic sensor Ku70 induces production of IFλ1 and IFNλ2/3 but not type I IFN (24). Following transfection of DNA or herpes simplex virus-2 infection, DNA binding to Ku70 leads to recruitment of STING and subsequent activation of IRF3 in addition to IRF1 and IRF7 (24, 25). Whether this novel, IFNλ-specific IFN induction exists in other cell types following DNA sensing is an interesting possibility that has not yet been investigated.

Although type I and III IFNs are all induced following infection, the transcription of these genes is temporally regulated. Type I IFNs are induced and resolved rapidly, followed by a delayed but sustained induction of *IFNL* genes (19, 26, 27). The mechanisms responsible for a distinct temporal induction pattern of type I and type III IFNs is currently unknown, but this could be due to utilization of different signaling molecules or transcription factors. The *IFNL1* and *IFNL3* promoters harbor binding sites for IRF1, IRF3, IRF7, and NF-κB (28). However, in contrast to type I IFNs, studies have suggested that transcription of *IFNL* is primarily dependent on NF-κB, and activation of both IRF and NF-κB signals is required for a robust induction of *IFNL* (29). The differential requirement for IRFs and NF-κB in the induction type I and type III IFNs following PAMP engagement by the PRRs could potentially contribute to the temporal difference in their transcriptional regulation of type III IFNs compared with type I IFNs.

Both type I and type III IFNs are produced following rotavirus infection in an adult murine model, but intestinal epithelial cells (IECs) respond preferentially to type III IFN (27, 30), suggesting a predominant role for IFNλ in antiviral defense in the intestine. In addition, type III IFNs are produced more abundantly at mucosal sites by epithelial and myeloid cells in response to viral infection (31). The mechanism for this preferential induction of type III IFN by IECs remains to be fully elucidated, but it might be due in part to the preferential induction of IFNλ upon MAVS localization to peroxisomes, which are highly abundant in epithelial cells, following PAMP sensing (21). Another possible mechanism is that undefined tissue-specific factors present at the epithelial barrier surfaces may promote IFNλ over type I IFN, similar to the IFNλ response in hepatocytes during hepatitis B and hepatitis C virus (HCV) infection (32). Further, IFNλ can be induced by type I IFN similar to an interferon-stimulated gene (ISG) in a feed-forward fashion (33). This type I IFN enhancement of *IFNL* is at least partially due to the ability of type I IFN to increase TLR expression; however, the functional consequences of this co-regulation remain to be tested.

Overall, a lack of IFNλ-specific mouse models and antibody detection reagents for ligands and receptors has slowed progress in determining the contribution of IFNλ to immunity. While whole body knockout mice lacking IFNλR exist, dissection of the role IFNλ signaling in various tissues and cell types *in vivo* will be advanced by studies in mice utilizing a recently reported floxed IFNλR model (34). In addition, an IFNλ2 cytokine reporter mouse has recently been developed (35). These new models will likely lead to a rapid advancement in understanding the unique functions of IFNλ *in vivo*.

IFN LAMBDA RECEPTOR EXPRESSION AND SIGNALING

The general induction and signaling cascades of type I and type III IFNs are summarized in **Figure 1**. Type I and III IFNs each signal through distinct receptor heterodimer complexes [reviewed in Ref. (3, 17, 19, 36)]. Type I IFN binds to a receptor complex comprised of IFNAR1 and IFNAR2, which is broadly expressed

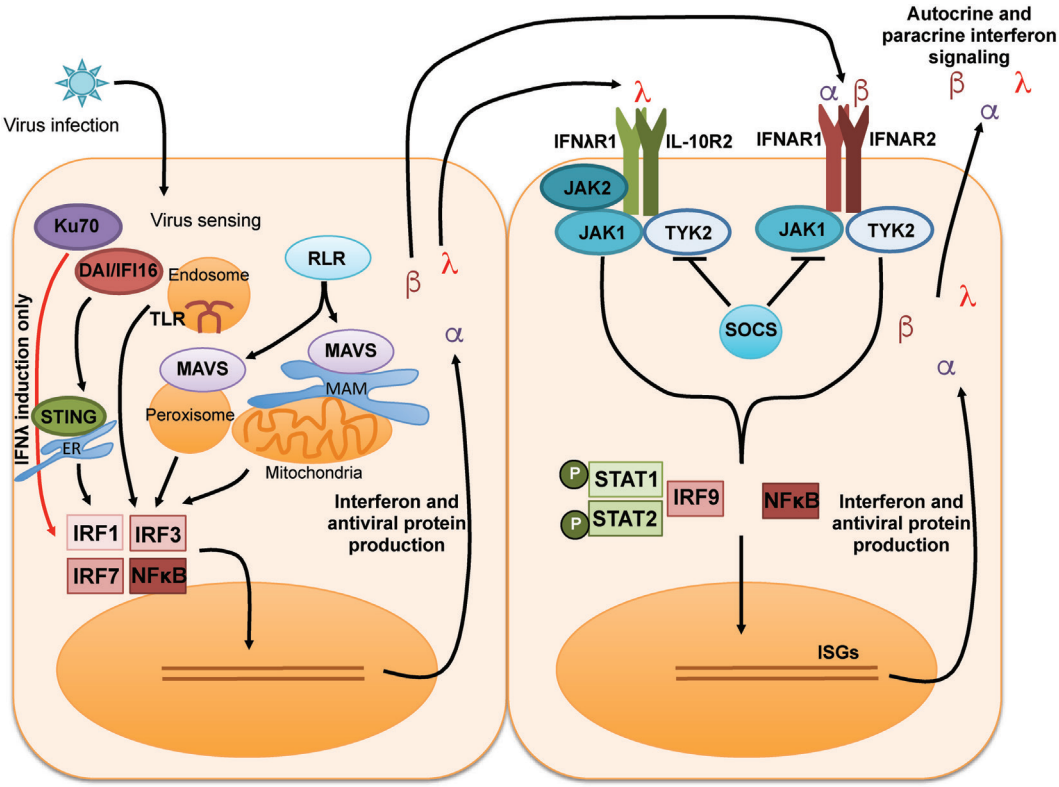

FIGURE 1 | General induction and receptor signaling pathways of type I and type III IFNs. Recognition of virus by multiple pattern recognition receptor pathways leads to the activation of the transcription factors IRF1, IRF3, IRF7, and NF-κB to induce transcription, translation, and secretion of type I IFN (IFNα and IFNβ) and type III IFN [interferon lambda (IFNλ)]. Type I and type III IFNs signal to surrounding cells *via* distinct receptors to induce activation of the JAK–STAT pathway leading to the production of IFN-stimulated genes (ISGs) that can amplify the IFN signal and induce an antiviral state in infected cells/tissues.

on most cells [reviewed in Ref. (1, 2)]. IFNλ signals through a heterodimeric receptor comprised of IFNλR1 and IL-10R2 (7, 8); IL-10R2 is a receptor subunit that is broadly expressed and shared for signaling by members of the IL-10 cytokine family [reviewed in Ref. (37)]. By contrast, the expression of IFNλR1 is much more restricted to epithelial cells, subsets of myeloid cells, and neuronal cells. This limited expression likely explains the importance of IFNλ at mucosal sites and the blood/brain barrier [reviewed in Ref. (17, 38)]. Engagement of all IFNs with their receptors initiates downstream signaling events, namely, activation of the JAK–STAT signaling cascade. JAK1, TYK2, and potentially JAK2 are phosphorylated and activated, leading to subsequent phosphorylation and activation of STAT1 and STAT2, which then associate with IRF9. Together, the complex of STAT1, STAT2, and IRF9 is referred to as the interferon-stimulated gene factor 3 (ISGF3) transcriptional complex. Activated ISGF3 translocates to the nucleus and binds to the interferon-sensitive response element, initiating the transcription of a wide array of ISGs. SOCS1 can provide negative regulation of this JAK–STAT signaling pathway downstream of IFN *in vitro* and *in vivo* (39–41).

In addition to activation of the JAK–STAT pathway, IFNs also activate PI3K and MAPK signaling cascades (1, 2). Perhaps the shared utilization of these signaling pathways between IFN and many other cytokines may help to explain the varied role of IFN in modulating antiviral and immune responses in various contexts and locations. Different affinities for their respective receptors exist among IFN subtypes, which may alter the signal strength upon receptor engagement, thus potentially adding another layer of regulation in control of immune responses by IFNs. Mendoza et al. developed a high-affinity IFNλ3 to discern the structure of the cytokine. When used in *in vitro* experiments this high-affinity IFNλ3 was found to have enhanced HCV and hepatitis B virus (HBV) antiviral activity (42). These results support the idea that enhancing the strength of the interaction of IFN with its receptor can modulate downstream functions. While this particular study investigated antiviral and anti-proliferative responses, it would be interesting to discern whether engineering of high-affinity IFNλ molecules can alter other facets of immunity. The recently solved IFNλ3/IFNλR1/IL-10R2 signaling complex structure could aid in answering these questions and in the development of IFNλ therapeutic agonists that have differential affinities for the receptor complex and downstream signaling strengths (42). Other mechanisms to regulate the response to IFNλ at the level of the IFNλR are conceivable. For example, in addition to the restricted nature of the IFNλR1 subunit, a soluble, secreted IFNλR1 has been described that could potentially sequester IFNλ as a regulatory mechanism (43). In summary, further studies are needed to dissect the intricate interplay of how IFN

signaling pathways function in concert with stimulation by other cytokines that may activate similar or overlapping intracellular signaling pathways.

ANTIVIRAL EFFECTS OF TYPE III IFNs

Interferon lambda is important in a wide variety of viral infections that including HCV, HBV, influenza virus, rhinovirus, respiratory syncytial virus (RSV), lymphocytic choriomeningitis virus (LCMV), rotavirus, reovirus, norovirus, and West Nile virus (WNV) [reviewed in Ref. (17, 19, 44–47)]. Many of these studies of IFNλ antiviral responses have been focused on viruses that infect the liver, the respiratory, and gastrointestinal mucosa, and, more recently, those that cross the blood–brain barrier (BBB) to cause a neuroinvasive viral infection. Experimental *in vivo* approaches using IFNλR knockout mice have highlighted the importance of IFNλ signaling in control of influenza A virus (IAV), SARS coronavirus, RSV, and human metapneumovirus levels in the lung as well as norovirus, reovirus, and rotavirus levels in the gastrointestinal tract (30, 48–50). It is also of note that type I and type III IFNs also have roles in cancer, parasitic infections, fungal infections, and several bacterial infections that include potential respiratory pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*, as well as *Listeria monocytogenes* and *Salmonella typhimurium* in addition to IFNs regulation of viral infections [reviewed in Ref. (47, 51)]. As the contribution of type I and type III IFNs in these other settings has been recently reviewed, we will not elaborate further herein.

Multiple reports have suggested redundant roles for IFNα/β and IFNλ in response to infection (23, 28, 52). However, distinct contributions for IFNα/β and IFNλ to infection have begun to be appreciated. **Table 1** summarizes viral infections where IFNλ has been demonstrated to contribute in comparison with the known role of IFNα/β in these infections *in vitro* and *in vivo*. While the differences between IFNλ and IFNα/β are still being investigated, studies have demonstrated the ISG response induced by IFNλ is reduced compared with IFNα/β, while *in vivo* IFNλ is much less inflammatory than IFNα/β (53–55). Interestingly, IFNλ retains many antiviral properties despite the less inflammatory response compared with type I IFNs. This has spurred development of IFNλ for clinical use as an alternative treatment to IFNα for HCV infection has been of recent interest (53). Enthusiasm within the HCV field for IFNλ as a therapeutic treatment has waned as a result of the availability of direct-acting antiviral drugs capable of clearing HCV infection (56). However, harnessing the potential antiviral and less inflammatory functions of IFNλ as a therapeutic may be useful in treatment of other hepatic viral infections.

More recent and broad hypotheses posit that IFNλ treatment could also be utilized to control respiratory viral infections. In several experimental studies, prophylactic and therapeutic treatment of mice with IFNλ2 or IFNλ3 was shown to control IAV pulmonary titers similarly to IFNα or IFNβ treatment (54, 55). Importantly, IFNλ treatment avoided excessive pulmonary inflammation associated with IFNα treatment (54). The authors of this study speculated treatment with either cytokine overcame the known IAV NS1 mediated block on the induction of both type I and type III IFNs. The IFNλ treatment used in this study also altered responses in pulmonary monocytes and antigen presenting cells; however, the potential direct effects of IFNλ on these specific cell populations have not been characterized in an antiviral therapeutic setting.

GENETIC ASSOCIATION OF IFN LAMBDA LOCUS TO VIRAL SUSCEPTIBILITY

The function of *IFNL* genes and their ability to regulate immunity is further impacted by a number of single nucleotide polymorphisms (SNPs) that have been identified in genome-wide association studies and correlate strongly to infectious disease outcome. These have been described in great detail elsewhere [reviewed in Ref. (47)]. Here, we will briefly discuss more recent findings related to these SNPs where the mechanism of their function and direct outcome on immune responses has been described. There has been considerable progress in understanding the direct impact of these SNPs on immunity to infection and disease outside of correlative phenotypes.

Multiple SNPs in *IFNL3* are associated with response to interferon-based therapeutics and natural clearance of the HCV (68–72), although until recently the mechanism of regulation provided by these SNPs had not been understood. Our group has recently described the mechanism of one *IFNL3* SNP (rs4803217) where presence of the G allele correlates with HCV clearance, whereas the unfavorable T allele correlates with HCV persistence (103). Specifically, HCV was found to regulate expression of two microRNAs (miR-208b and miR-499a-5p) that target the 3′ untranslated region (UTR) of *IFNL3* leading to its degradation, allowing for viral persistence. The T allele leads to changes in the 3′ UTR allowing for enhanced binding of these HCV-induced microRNAs and AU-rich element-mediated decay of *IFNL3*, impacting expression of the cytokine and the outcome of HCV infection. Intriguingly, these same microRNAs also dampen type I IFN signaling in HCV-infected hepatocytes by downregulating expression of IFNAR1, a mechanism distinct from miR-208b and miR-499a-5p regulation of type III IFN (104).

Mechanistic studies have also defined the immunological consequence of another SNP impacting the production of IFNλ4. Approximately, 40% of Caucasians have an intact open reading frame for *IFNL4* gene (105). However, a frame-shift mutation (TT>dG at ss469415590) in *IFNL4* renders it a pseudogene. Intriguingly, the G gene variant encoding full-length *IFNL4* is strongly correlated with persistence of HCV. It was hypothesized, but not demonstrated, that *IFNL4* may have an intracellular role for dampening the antiviral response. However, it is speculated that this effect could be at least in part an indirect one as the dG *IFNL4* allele is linked with the less favorable *IFNL3* genotype at rs12979860 and rs4803217 (106). This work confirmed IFNλ4 has similar antiviral function to IFNλ3. However, the functional full-length *IFNL4* is induced at lower levels compared with *IFNL3* and is poorly translated due to intron-retention splice isoforms and weak polyadenylation (polyA) signal. Interestingly, non-human primates do not contain the dG>TT frame-shift mutation, but still limit IFNλ4 translation by production of intron-retention splice isoforms and a weak

TABLE 1 | Interferon lambda (IFNλ) and IFNα/β functions in viral infection.

Virus infection	Role of IFNλ	Role of IFNα/β
Negative-sense RNA viruses		
Human metapneumovirus (−ssRNA Pneumoviridae)	• IFNλ treatment reduces titer in murine model (57) • Increased titers in mice lacking IFNλR and IFNAR (49)	• Increased titers in mice lacking IFNλR and IFNAR (49) • Increased titers and reduced CD8 T cell response in mice lacking IFNAR (58)
Influenza virus (−ssRNA Orthomyxoviridae)	• Increased virus titers in human cells and murine models in the absence of IFNλR (48, 49) • IFNλ reduced influenza A virus (IAV) titers with minimal-associated pulmonary damage in murine *in vivo* models (35, 48, 54, 55) • Increased IFNλ [human single nucleotide polymorphism (SNP) rs8099917] correlates with increased Th1 skewing of CD4 T cell response and reduced sero-conversion following vaccination (15)	• Mice lacking IFNAR1 and IFNλR in the stromal compartment are more susceptible to IAV infection (52) • Therapeutic treatment of IAV-infected mice with IFNα leads to reduced IAV titers, but pulmonary damage (54)
Lymphocytic choriomeningitis virus (−ssRNA Arenaviridae)	• IFNλ2 and IFNλ3 inhibit infection of human lung epithelial cells (59) • IFNλR−/− mice have no change in virus titer, but increased CD8 T cell response to acute infection and reduced CD8 T cell response to chronic infection (60, 61)	• Blockade of type I IFN controls persistent infection (62, 63)
Respiratory syncytial virus (−ssRNA Paramyxoviridae)	• Increased titers in mice lacking IFNλR and IFNAR (49)	• Increased titers in mice lacking IFNλR and IFNAR (49)
Positive-sense RNA viruses		
Dengue (+ssRNA Flaviviridae)	• IFNλ1 induces expression of CCR7 and *in vitro* dendritic cell (DC) migration (64) • IFNλ1 and IFNλ2 inhibit virus in a human epithelial cell line (65)	• Mice lacking IFNAR are more susceptible to infection (66) • Mice lacking IFNAR on CD11c+ or LysM+ cells have increased disease during infection, but still mount protective CD8 T cell responses against the virus (67)
Hepatitis C virus (HCV) (+ssRNA Flaviviridae)	• SNPs rs4803217, rs8099917, rs12979860, and rs368234815 correlate with response to IFN therapeutic and spontaneous virus clearance (68–72)	• IFNα therapeutic effective in control of HCV, but highly inflammatory (source)
Human immunodeficiency virus (+ssRNA Retroviridae)	• IFNλ1, 2, 3 treatment of human monocyte-derived macrophages inhibits infection *via* JAK–STAT (73, 74) • Pretreatment of human primary CD4 T cells with IFNλ1 or IFNλ2 reduced HIV integration and posttranscriptional events, but IFNλ1 was not negatively correlated with HIV levels *in vivo* (75)	• Type I IFN can inhibit HIV *in vivo* in a humanized murine mouse model of infection (76) • High, sustained type I IFN associated with pathogenicity during SIV infection of rhesus macaques (77) • Serum IFNα inversely correlates with CD4 T cell counts in human patients with HIV-1 (78)
Norovirus (+ssRNA Caliciviridae)	• Recombinant IFNλ clears persistent norovirus infection in a murine model, dependent upon IFNλR signaling in intestinal epithelial cells (IECs) (34, 50, 79) • Mice lacking IFNλR have increased titers and virus shedding (50)	• Persistence of norovirus in mice lacking IFNAR specifically on CD11c+ cells (80)
Rhinovirus (+ssRNA Picornaviridae)	• IFNλ levels inversely correlate with rhinovirus replication in a human bronchial epithelial cell line (81)	• Type I IFN response contributes to control of rhinovirus in murine airway cells at 37° (82)
SARS coronavirus (+ssRNA Coronaviridae)	• IFNλR−/− mice have increased viral titers and shedding (49)	• Type I IFN signaling in hematopoietic cells drives SARS-CoV pathogenesis in a murine model (83)
West Nile virus (+ssRNA Flavi)	• Treatment with IFNλ protects mice from lethal infection • IFNλR−/− mice have increased permeability of the blood–brain barrier and neuroinvastion of virus (84)	• Mice lacking IFNAR have enhanced viral loads, increased tropism, and complete mortality (85)
Zika virus (+ssRNA Flaviviridae)	• Knock down of IFNλR in HBMECs leads to increase in ZIKV dsRNA (86)	• Mice lacking IFNAR susceptible to Zika virus infection (87) • Zika virus antagonizes type I IFN response in human DCs (88)
Double stranded RNA viruses		
Reovirus (dsRNA Reoviridae)	• Fatal disease in neonatal mice lacking IFNλR • Mice lacking IFNλR fully or specifically in IECs have increased virus shedding and growth in IECs (34, 89)	• No enhanced disease or systemic spread in IFNAR−/− mice infected intracranially (90)
Rotavirus (dsRNA Reoviridae)	• IFNλ treatment (synergistically with IL-22) reduces rotavirus titer (91) • Mice lacking IFNλR have increased virus titer (30)	• Minimal role for IFNAR signaling in control of viral disease in mice (89)
DNA viruses		
Cytomegalovirus (dsDNA Herpesviridae)	• IFNλ reduces replication and CD4 T cell proliferation in human PBMCs (92)	• Type I IFN released by DCs inhibits replication (93) • CMV directly inhibits type I IFN (94)
Hepatitis B virus (dsDNA Hepadnaviridae)	• Restricts virus in murine cell line (32) • Pegylated IFNλ augmented antiviral reduction in hepatitis B virus (HBV) levels of infected patients (95)	• Type I IFN restricts HBV in hepatocytes (96) • HBV inhibits type I IFN induction (97, 98)
Herpes simplex virus (HSV) (dsDNA Herpesviridae)	• IFNλ inhibits HSV-1 and HSV-2 in human epithelial cells (99, 100) • SNP rs12979860 correlates with HSV-1 severity upon reactivation (101)	• INFAR−/− adult mice are susceptible to infection of the choroid plexus and HSV encephalitis, similar to newborn WT (102)

polyA signal, suggesting the functional IFNλ4 isoform has been selected against before the arise of the pseudogene frame-shift mutation in humans (106). It is still currently unclear as to why IFNL4 is suppressed, and perhaps undergoing pseudogenization. Perhaps *IFNL4* arose more recently through genetic duplication of *IFNL3* but did not develop a specific function distinct from IFNλ3, similar to what has occurred for other IFNs. Future studies without the confounding factor of linkage of the unfavorable *IFNL3* genotypes may reveal the function of bioactive IFNλ4 to antiviral immunity. In addition, more studies parsing out the mechanisms of *IFNL* SNPs regulation of disease could provide important insights for the development and functionality of IFNλ therapeutics.

TYPE I VS III IFNs IN AUTOIMMUNITY

The contribution of type I IFNs to development and manifestation of autoimmunity is well established [reviewed in Ref. (1, 107, 108)]. Type I IFNs are commonly upregulated in systemic autoimmune diseases such as systemic lupus erythematosus (SLE), Aicardi–Goutieres syndrome, Sjogren's syndrome, type I diabetes, and psoriasis. More than half of adult patients, and 90% of pediatric patients, with SLE have elevated peripheral IFNα (109). Mechanistic studies have identified plasmacytoid dendritic cells (pDC), which are a major source of type I IFN, to be enriched in SLE lesions in humans and mice (110–112). Interestingly, type III IFNs do not seem to be linked to exacerbation of autoimmune diseases. In fact, type III IFN has been demonstrated to remediate symptoms in a mouse model of arthritis (113). Further, in a murine model of colitis, a disease that can be autoimmune in humans, IFNλ signaling specifically in neutrophils leads to a reduction on the release of reactive oxygen species and prevention of intestinal pathology (114). In addition, mice lacking the IFNλR1 have exacerbated disease in a model of asthma (14). This potentially protective role of IFNλ in asthma has also begun to be explored in humans (81). One recent paper has identified a correlation between systemic sclerosis and elevated IFNλ1 levels (115), but mechanistic studies clearly identifying a role for IFNλ in autoimmune disease are lacking. Interestingly, pDC have been shown to express the IFNλR and respond directly to IFNλ (116, 117). Whether IFNλ signaling is altered in pDC in the context of immunity is an interesting question that could have implications for immune-mediated treatment.

IFNλ IMMUNE MODULATORY EFFECTS

The optimal induction of interferon to control infection while simultaneously avoiding host immunopathology is critical for an effective immune response against pathogens. Although IFNλ is generally considered to be less inflammatory than type I IFN, a full understanding of IFNλ's regulation of immune responses outside of direct antiviral action has remained largely unknown (**Figure 2**). Recent studies, predominantly in the context of viral infections, have begun to elucidate the contribution of IFNλ to the regulation of the broader innate and adaptive immune responses (see **Table 1**). While the role of IFNα/β and IFNλ is similar in many viral infections, some notable differences exist. For example, therapeutic treatment of influenza virus infected mice with IFNα leads to enhanced pulmonary inflammation and mortality, while IFNλ is protective (54, 55). In addition, IFNλ is critical for protection against intestinal viral pathogens such as reovirus and rotavirus (89, 90). This is likely due to the fact that IECs respond robustly to IFNλ, but not type I IFN, *in vivo* (30). However, there are also other important roles for IFNλ in other immune cell types in the intestine, such as murine neutrophils, that have only begun to be investigated (114). As part of the ongoing efforts to understand specific function of IFNs in host defense, more studies designed to examine specific effects on different tissues and cell types that contribute to innate and adaptive immunity will be informative.

Effects of IFN on Innate Immune Cells

IFN has been well described to have a direct antiviral effect in epithelial cells. Further, type I IFNs function on innate immune cells, such as DCs and macrophages. However, the functions of IFNλ on immune cells still remain largely unknown. For example, type I IFN signaling is well described to promote activation, survival, and cytotoxic function of NK cells during infection (118–120). By contrast, whether NK cells express IFNλR and respond directly to IFNλ to modulate NK cell function remains unclear (119, 121, 122). In this section, we will review the literature on the effect of IFNλs on innate immune cells.

IFN in Monocytic Cell Populations

Type I IFN promotes the polarization of macrophages to an inflammatory "M1" phenotype and increases the production of nitric oxide (73, 123–125). Type I IFN also enhances DC function by promoting their generation from monocytic precursors and leads to upregulation of MHC and costimulatory molecules in addition to increasing IL-12 production and enhancing DC migration (2, 126, 127). Conversely, the role of IFNλ in macrophages and DCs remains unresolved as studies have both supported and refuted the ability of these cells to directly respond to IFNλ (73, 128–130). Limited studies have indicated a role for IFNλ in the modulation of DC function. For example, it was demonstrated that human DC migration was enhanced as a direct response to IFNλ1 in the context of Dengue virus infection (64). In a separate report, IFNλ2 treatment increased IL-12 production and alteration of expression of the costimulatory molecule OX40L in CD11c+ cells (14). These changes, which may result in enhanced T cell immunity (131), indicate that IFNλ2 may also have a functional role at the interface of the innate and adaptive immune responses. While data implicating IFNλ regulation of DC functions are intriguing, further studies are needed to determine whether the defect in DCs in the absence of IFNλR signaling is intrinsic to these cells or influenced by the IFNλ response in epithelial cells at infection sites.

A possible explanation of the mixed reports regarding the contribution of IFN to DC function is that this could be due to differential responsiveness of various DC subsets to IFNλ and/or type I IFN. For example, pDC have been described to respond to both type I and type III IFNs to enhance their upregulation of ISGs, maturation, and antigen presentation function. We will not

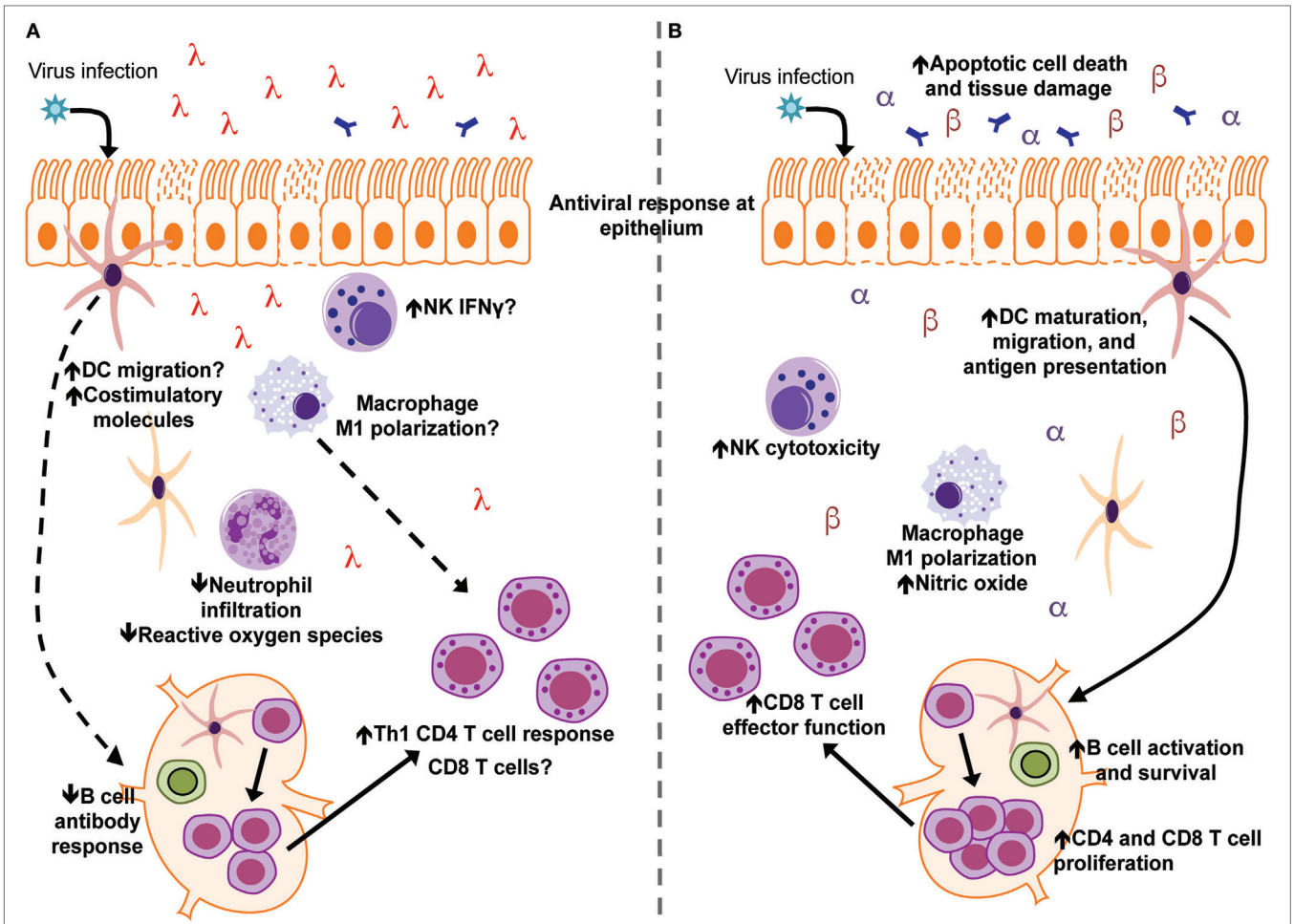

FIGURE 2 | Interferon lambda (IFNλ) and IFNα/β differentially modulate immune responses during acute viral infection and tissue inflammation. **(A)** Following viral infection/tissue inflammation, IFNλ modulates functions of dendritic cells (DCs) neutrophils, CD4 T cells, and the B-cell antibody response. IFNλ signaling may also regulate macrophage, NK cell, and CD8 T cell function during infection/tissue inflammation **(B)** type I IFN (IFNα and IFNβ) have been the subject of a greater number of studies and have more defined roles during virus infection and tissue inflammation. Type I IFN enhances functions of DCs, macrophages, NK cells, B cells, CD4 T cells, and CD8 T cells toward an inflammatory/antiviral state.

elaborate herein on the functions of IFN in pDC, as they have been well described in other recent reviews (132, 133). Given that there is a specific response of pDC to IFNλ that is not observed in the bulk heterogeneous DC population, it is possible other DC subsets may respond to IFNλ. During influenza and other viral infections in mice, CD103+ DCs are integral in delivery of antigen from the infected tissue to lung-draining lymph nodes where they can activate T cells (134–136). CD103+ DCs are less responsive to type I IFN, allowing for viral replication within these cells, and potentially leading to enhanced antigen presentation (137). Whether this difference could be due to preferential usage of IFNλR signaling to enhance antigen presentation has not been addressed. Interestingly, however, the ImmGen database indicates murine CD103+ DCs have higher levels of IFNλR compared with other DC subsets (138). However, as of this writing, the responsiveness of various DC and macrophage subsets to IFNλ signaling remains unclear. As T cells do not respond directly to IFNλ, it is likely that differential IFNα/β signaling compared with IFNλ in DCs could be modulating T cell responses (43, 61). Indeed, during Dengue virus infection, IFNλ leads to enhanced migration of DCs *in vitro* and increases CCR7 required for migratory function on DCs (64). Perhaps IFNλ signaling in DCs allows for optimal maturation and antigen presentation to T cells without excessive inflammation associated with IFNα/β signaling. It is also possible that at mucosal and barrier epithelial sites, epithelial cells themselves are regulating the alteration in DC response. Future studies in mice conditionally lacking IFNλR1 or IFNAR1 in DCs or epithelial cells specifically will delineate the role of IFNλ in these cell population.

IFN in Neutrophils

While few studies that have interrogated the direct effect of IFNα/β signaling on neutrophils, type I IFNs have been demonstrated to play a role in activation of neutrophil function (139). Murine neutrophils have recently been shown to express high levels of *Ifnlr1* and respond directly to stimulation with IFNλ

(114). Treatment of mice with arthritic symptoms with IFNλ2 was shown to prevent neutrophil infiltration into arthritic joints (113). While the potential therapeutic application of IFNλ to limit neutrophil-mediated pathology is interesting in this arthritis model, whether this paradigm is true should continue to be examined in the context of other inflammatory events. Neutrophils are known to significantly exacerbate disease severity during respiratory viral and bacterial infections and directly contribute to lung pathology [reviewed in Ref. (140)]. It is intriguing that IFNλ could potentially reduce or prevent neutrophil-mediated detrimental lung inflammation during respiratory infection *via* a similar mechanism. IFNλ has recently been demonstrated to act on neutrophils to control both influenza virus infection and DSS-induced colitis in murine models, indicating IFNλ directly alters neutrophil function in addition to recruitment as previously described (35, 114, 141). Interestingly, this IFNλ-specific dampening of neutrophil function is mediated in a non-transcriptional/translational fashion *via* Akt's regulation of the release of reactive oxygen species (114). Importantly, this study represents the first reported such function of IFNλ and opens the intriguing possibility for IFNλ to yield changes in immune cells in a mechanism distinct from canonical JAK–STAT signaling. While these studies are intriguing, they have thus far only been validated in murine neutrophils. Future studies will be needed to determine whether human neutrophils respond to IFNλ in a similar fashion.

Effects of IFNs on Adaptive Immune Cells

Adaptive immunity is critical in controlling and providing long-term protection against infection. IFNs act at the interface of innate and adaptive immunity, by directly regulating innate as well as adaptive immune cells. For example, type I IFN promotes B-cell activation and class switching during acute viral infection [reviewed in Ref. (2)]. While there is currently no evidence demonstrating IFNλ has direct effects on the function of B cells, humans receiving influenza virus vaccination who had lower levels of circulating IFNλ correlated with increased sero-conversion (15). In addition, IFNλ has been reported to augment TLR-mediated activation and function of human B cells, but IFNλ could not directly and independently impact B-cell activation (142). Conversely, in a murine model of WNV infection, IFNLR1−/− mice had no effect on antibody responses compared with wild-type control mice (84). However, evidence supporting a role for IFNλ regulation of B cell functions is currently lacking but this still an area of active investigation.

IFN in T Cells

While T cells do not respond directly to IFNλ (43, 61), it is clear that IFNλ regulates function of T cells. IFNλ enhances T cell proliferation and Th1/Th17 cytokine production following treatment of peripheral blood mononuclear cells with IFNλ and in the context of asthma and influenza virus vaccination (14, 15). IFNλ has been shown to polarize the response toward a Th1 phenotype while suppressing Th2 and associated B cell responses. This is supported by studies in humans evaluating a SNP (rs8099917) in the *IFNL* locus, where individuals with the SNP that correlate to high IFNλ3 levels have lower sero-conversion rates following influenza virus vaccination, but a greater induction of Th1 CD4 T cells (15). Therefore, IFNλ-mediated effects on the T cell response might be indirect mediated by another cell subset known to express IFNλR. It is likely that IFNλ signaling in DCs is responsible for this alteration of the T cell response; however, the direct action of DCs in regulating Th1/Th2 responses is still unknown. In addition, whether IFNλ alters DCs to regulate CD8 T cell responses, which are critical for clearance of virus during many infections, is still unknown. Intriguingly, a report investigating acute and chronic LCMV responses in a murine model suggest IFNλ signaling negatively regulates virus-specific T cell responses during acute infection, but is required for the persistence of the T cell response during chronic infection (61). While the mechanism remains unclear, a study in macaques demonstrated that IFNλ3 drives cytotoxic ability of CD8 T cells, overall providing further evidence of the potential of IFNλ to function as an immune adjuvant or therapeutic agent to promote antiviral T cell responses (143).

In contrast to the absence of direct effects by IFNλ on CD4 and CD8 T cell activation, proliferation, and cytokine production, type I IFN directly regulates these T cell functions [reviewed in Ref. (144)]. Type I IFN signaling can regulate T cell responses *via* indirect effects on DCs or macrophages in addition to direct signaling effects on T cells themselves. This difference in mechanisms of T cell regulation is a major distinction between type I and type III IFNs that has not yet been fully evaluated. The potentially distinct, indirect mechanism of IFNλ regulation of T cell responses could yield interesting insights into the ability of IFNλ to be utilized as a therapeutic or vaccine adjuvant to augment the immune response against viral infections.

ROLE OF TYPE III IFNs AT THE BBB

In addition to impacts on immune cells, IFNλ has also been described to regulate the BBB during WNV infection (84). Mice lacking the IFNλR1 show increased viral titers in central nervous system tissues and increased BBB permeability following WNV infection. Interestingly, IFNλ-mediated restriction of the endothelial tight junctions in an *in vitro* BBB model is independent of STAT1 or protein synthesis. These findings suggest there may be an undescribed, novel IFNλ signaling pathway that regulates endothelial cells. As endothelial cells are significant regulators of inflammatory responses, IFNλ could exert important effects on these cells types that would also be applicable to infection at the site of pulmonary and gastrointestinal barriers.

OUTSTANDING QUESTIONS

While the functions of IFNλ and IFNα/β overlap in many infections and cell types, a growing number of notable differences are allowing for a better understanding of specialized roles of IFNs in regulation of immunity. In addition, the difference in IFNAR and IFNλR1 expression levels on various cell subsets

and tissues could contribute to specific action of IFNλ vs type I IFN. This regulation along with SNPs in *IFNL* genes highlight that IFNλ has a unique role in antiviral immunity independent of type I IFN. While nearly 15 years of research has led to many insights in the function of IFNλ and its contribution to immunity, many questions remain to be answered. What are the distinct and redundant functions of each *IFNL* gene? Are there spatiotemporal effects that provide distinctions of IFNλ subtypes? Are there other signaling pathways that are active downstream of IFNλR differentially activate immune and epithelial cell subsets to modulate innate and adaptive immune response? Answering these questions with the help of newly available murine models will be critical to gain insights into the function of IFNλ, and to continue to develop IFNλ for use as a therapeutic against viral infections in the liver and at barrier surfaces.

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