STRUCTURES, SIGNALING MECHANISMS, AND FUNCTIONS OF TYPES I AND III INTERFERONS

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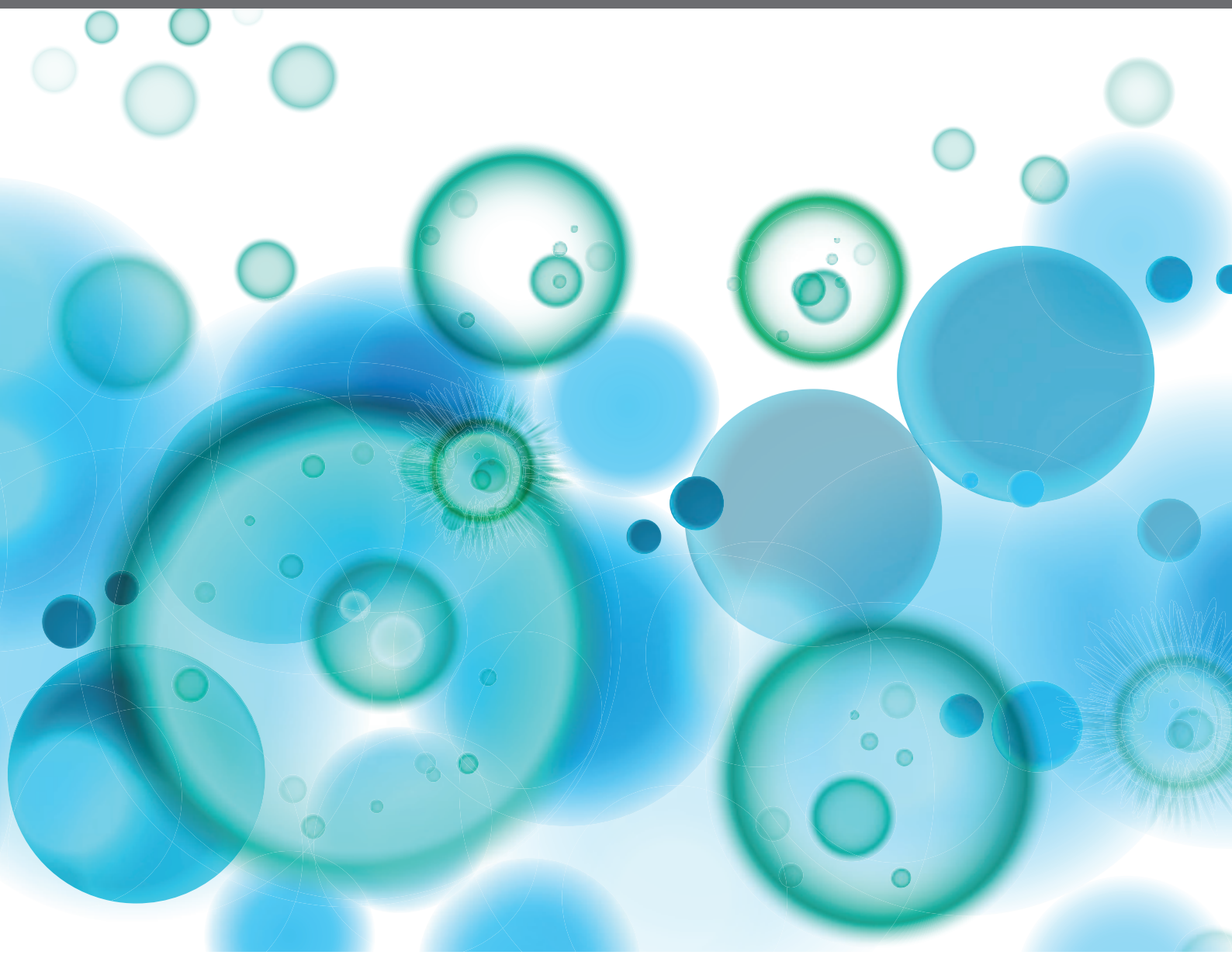

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STRUCTURES, SIGNALING MECHANISMS, AND FUNCTIONS OF TYPES I AND III INTERFERONS

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EDITORIAL
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Editorial: Structures, Signaling Mechanisms, and Functions of Types I and III Interferons

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Editorial on the Research Topic

Structures, Signaling Mechanisms, and Functions of Types I and III Interferons

Never has understanding the fundamental roles of the interferons (IFNs) been more important than in the year 2021. It is well-recognized that IFNs play critical roles inducing an antiviral state in cells. However, their influence on innate and adaptive immunity continues to expand. As a result, IFNs play critical roles in protecting the host from pathogens, controlling cellular transformation, and when dysregulated, promoting autoimmunity. Since the seminal discovery of Type I IFNs over 60 years ago (1), the Type I and Type III IFN family has grown to include 20 distinct members consisting of 16 Type I IFNs (12 IFNαs, IFNβ, IFNε, IFNκ, and IFNω) and four Type III IFNs (IFNλ1-IFNλ4) (2) that signal through common type-I (IFNAR1/IFNAR2) and type-III IFNλR1/IL10R2 receptor complexes. The paucity of studies that define the role of the IFN subtypes (Type I/Type III) in cellular function and disease was a major driver of this research topic.

"Structures, Signaling Mechanisms, and Functions of Types I and III Interferons" is a collection of eight review articles that are intended to summarize current knowledge on fundamental aspects of interferon signaling and biology. Three articles address IFN receptor biology and signaling. Walter compares the structures of types I, II, and III IFNs and their receptor complexes, providing insights into how subtle structural differences in the IFNs may modulate downstream signaling. Notably, the study highlights murine IFNβ does not share equivalent structural and biophysical properties with its human counterpart, highlighting the difficulties in direct comparisons of type-I IFN signaling between species. Subsequently, Zanin et al. reviews type I IFN receptor trafficking. These authors comprehensively discuss post-translational modification of the type I IFNAR receptors and the role of clathrin-mediated endocytosis on signaling, recycling, or degradation of receptor components. Mazewski et al. review canonical and non-canonical signaling mechanisms, and discuss their roles in infectious and autoimmune diseases, and in cancer. Ultimately, the outcome of IFN signaling is the production of various levels of IFN stimulated genes (ISGs). Thus, Yang and Li provide a detailed review of the complex anti-viral defense mechanisms used by a small set of ISGs to inhibit viral RNA replication.

Three additional articles review our current understanding of the unique and overlapping roles of the type-I and type-III IFNs. Stanifer et al. discuss redundant and non-redundant expression patterns, signaling, and functional outcomes between types I and III IFN at respiratory and intestinal barriers. Notably, this work highlights the role of epithelial cell heterogeneity and cell polarity in explaining the non-redundant activities of type III IFNs at barrier surfaces. Two articles focus on functional differences among different type I IFN subtypes. Fox et al. provide an extensive review highlighting differences among IFNα and IFNβ subtypes in murine infectious disease models, with potential insights for human disease. Wittling et al. review human type I IFNs

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to propose that regulatory elements, expression patterns, and primate evolution can serve as a guide toward revealing unique roles for the IFNα subtypes. These reviews, as well as Mazewski et al. discuss various aspects of using type I IFNs to treat patients with chronic autoimmune and infectious diseases. As expected, the potential of type I IFN as a therapy for SARS-CoV-2 is discussed in several reviews. In particular, the review by Schreiber highlights the roles for type I IFNs in viral defense and as a therapeutic agent to ameliorate the ongoing pandemic caused by the virus.

We wish to extend our extreme gratitude toward our colleagues who contributed to this review series as well as to those who took the time to review these manuscripts under the challenges of a pandemic. We hope this research topic summarizes the field for experienced biologists, serves as a foundation for neophytes entering the field, and stimulates novel research directions to harness the power of the IFNs to treat human disease.

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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REVIEW
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The Role of Structure in the Biology of Interferon Signaling

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Interferons (IFNs) are a family of cytokines with the unique ability to induce cell intrinsic programs that enhance resistance to viral infection. Induction of an antiviral state at the cell, tissue, organ, and organismal level is performed by three distinct IFN families, designated as Type-I, Type-II, and Type-III IFNs. Overall, there are 21 human IFNs, (16 type-I, 12 IFNαs, IFNβ, IFNϵ, IFNκ, and IFNω; 1 type-II, IFNγ; and 4 type-III, IFNλ1, IFNλ2, IFNλ3, and IFNλ4), that induce pleotropic cellular activities essential for innate and adaptive immune responses against virus and other pathogens. IFN signaling is initiated by binding to distinct heterodimeric receptor complexes. The three-dimensional structures of the type-I (IFNα/IFNAR1/IFNAR2), type-II (IFNγ/IFNGR1/IFNGR2), and type-III (IFNλ3/IFNλR1/IL10R2) signaling complexes have been determined. Here, we highlight similar and unique features of the IFNs, their cell surface complexes and discuss their role in inducing downstream IFN signaling responses.

Keywords: interferon, IFN, type-I, type-II, type-III, receptor complex, IFN signaling, structure

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INTRODUCTION

IFNs were discovered more than 60 years ago (1957) as substances that protect cells from viral infection (1, 2). Based on their sensitivity to pH, IFNs were designated as either type-I (pH stable) or type-II (pH sensitive) (2, 3). Characterization of their distinct amino acid sequences and crystal structures (4, 5) (6–8) further validated the classification of IFNα/β and IFNγ as type-I and type-II IFNs, respectively. The type-I family expanded (9) to include 12 IFNαs (10–13) encoded by 13 genes (IFNα1/13 encode the same protein), IFNβ, IFNϵ (14), IFNκ (15), and IFNω (16). Genome analysis in 2003 identified a new type-III IFN family (IFNλs) (17, 18), which by sequence and subsequent structure analysis (19) were similar to IL10 family cytokines (12, 20–22), in particular IL-22 (23, 24). With the discovery of IFNλ4 in 2013 (25), a total of 21 IFNs (**Table 1**) exhibit not only antiviral activity, but anti-tumor actions, and the ability to modulate the adaptive immune response.

The pleotropic biological activities of the three IFN families are initiated by binding and subsequent assembly of heterodimeric receptor complexes on the cell membrane (**Table 1**). The 16 type-I IFNs bind and signal through the IFNAR1 and IFNAR2 receptor complex, type-II IFNγ binds to IFNGR1 and IFNGR2 chains, and the type-III IFNs signal through IFNλR1 and IL-10R2 receptor chains. Each receptor heterodimer consists of a high affinity receptor chain (e.g., IFNAR2, IFNGR1, IFNλR1) and a low IFN affinity receptor chain (IFNAR1, IFNGR2, IL10R2). The high and low affinity receptors exhibit nM and μM/mM affinity, respectively, for their cognate IFNs (26–30). Despite variable affinities, the high and low affinity type-I and type-II receptors are specific for their cognate IFN family members. In contrast, IFNλR1 is specific for type-III IFNλ family members, but

TABLE 1 | IFN families and their receptor complexes.

	High Affinity Receptor	Low Affinity Receptor	IFNs						
Type-I IFNs	**IFNAR2**	**IFNAR1**	IFNα1/13*, IFNα10, IFNκ,	IFNα2, IFNα14, IFNω	IFNα4, IFNα16,	IFNα5, IFNα17,	IFNα6, IFNα21,	IFNα7, IFNβ,	IFNα8, IFNε,
	JAK1	TYK2							
Type-II IFNs	**IFNGR1**	**IFNGR2**	IFNγ						
	JAK1	JAK2							
Type-III IFNs	**IFNLR1**		IFNλ1,	IFNλ2,	IFNλ3,	IFNλ4			
		IL10R2	IFNλ1,	IFNλ2,	IFNλ3,	IFNλ4,	IL10,	IL22,	IL26
	JAK1	TYK2							

**IFNα1/13 encode the same amino acid sequence [(see 9)].*

the low affinity IL-10R2 chain is a shared receptor that also participates in IL10, IL22, and IL26 signaling complexes (12, 31–33).

IFN receptor complex formation activates Janus kinases (JAKs) that initiate IFN-mediated intracellular signaling cascades (34–38). The JAKs constitutively associate with the intracellular domains (ICDs) of the IFN receptors through non-covalent interactions (**Table 1**). Type-I and type-III IFN receptors use the same JAKs for signal transduction. The high affinity IFNAR2 and IFNλR1 receptors associate with JAK1, while low affinity IFNAR1 and IL10R2 associate with TYK2. In contrast, type-II IFNGR1 and IFNGR2 associate with JAK1 and JAK2, respectively (39, 40). The ICDs of the low affinity receptors are 69–100 amino acids long and their main purpose appears to be to bind their respective kinases for activation upon receptor complex formation. The high affinity receptor ICDs range from 223 to 271 amino acids in length and contain multiple tyrosine residues that upon phosphorylation by the JAKs, recruit STATs that become phosphorylated themselves, and translocate to the nucleus where they activate interferon-stimulated genes (ISGs) (40, 41). In addition to using the same JAKs, type-I and type-III IFNs induce the same STAT1/STAT2/IRF9, ISGF3 transcription complex (40–42). IFNγ activates phospho-STAT1 homodimers, but not ISGF3, which is reflected in the ~1,000-fold lower anti-viral activity of IFNγ compared to the type-I and type-III IFNs (43, 44). In addition to activating distinct intracellular signaling pathways, type-I/III IFNs are produced in cells upon viral infection, or infection by other pathogens, through pattern recognition receptor pathways, including RIGI, MDA7, PKR, TLR3, TLR7, TLR9, and STING (40, 45–48). In contrast, type-II IFNγ is produced predominantly by antigen-activated T lymphocytes (39). Thus, type-I/III IFNs are products of innate immune system, designed to establish direct and immediate antiviral states in cells, yet can also modulate adaptive immune responses. Type-II IFNγ is itself a product of adaptive immunity that acts on cells of innate immunity, notably macrophages. As a potent macrophage activator, IFNγ is essential for combating mycobacteria and other intracellular pathogens (49, 50). IFNGR1 deficiencies in individuals are associated with mycobacterial infections, while individuals with IFNAR2, or IFNAR1, deficiencies have had life threatening illness following vaccination with mumps, measles, and rubella (MMR) vaccines (51, 52). Together, these data highlight the distinct roles of these IFNs in controlling different pathogens.

While there is only one IFNγ, it is remarkable that humans encode 16 different type-I and 4 type-III IFNs that induce the same fundamental ISGF3-mediated anti-viral program in cells (17, 18, 53, 54). The necessity of this remarkable arsenal of IFNs to combat virus, and other pathogens (55–58), remains an area of intense investigation. Given the complexity of IFN signaling, this review describes the fundamental structural organization of each IFN receptor complex in generating IFN signaling responses. The main emphasis is to define how structure impacts IFN-IFN receptor affinity, specificity, and the role of the overall architecture of the complex to position receptor ICDs for intracellular JAK/STAT activation and subsequent cellular activity.

Structures of the Type-I, Type-II, and Type-III IFNs

All IFNs adopt α-helical structures with unique up-up-down-down topology (21), relative to other α-helix bundle proteins (**Figure 1**). Each IFN consists of six secondary structural elements, denoted A-F, of which helices A, C, D, and F form an anti-parallel four helix bundle. Loop elements B and E exhibit more variable secondary structures, ranging from additional helices to extended segments that pack against the edge of the four-helix bundle (helices A, C, D, and F). The α-helices of the Type-I IFNs are long, straight, and essentially parallel to one another (**Figure 1A**). Despite considerable sequence diversity (35%–95%), all 16 IFNs adopt the same α-helical structure (4, 5, 59–63). In contrast to type-I IFNs, type-III IFNs are comprised of shorter helices that contain several kinks, which form a more compact bundle (**Figure 1B**). As a result, type-III IFNs adopt structures that are more similar to the IL-10 family cytokine IL-22 than to type-I IFNs (12, 19, 23, 24, 64). This is interesting from a functional perspective since IL-22 induces anti-bacterial activity in the gut and skin through a tissue-restricted receptor complex of IL22R1 and IL10R2 (22, 32, 65–70). Thus, IFNλs and IL-22 control viral and bacterial challenges, respectively, at barrier surfaces (22, 64, 71). As a "mucosal IFN", IFNλs have been promoted as an optimal drug to treat respiratory viruses, such as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which causes COVID-19 (72). However, IFNλ signaling in mice prevents lung epithelial repair, leading to bacterial superinfections (73, 74). Other studies suggest type-I IFNs, not IFNλs, might be most efficacious and safe in treating SARS-CoV-2 (75). Overall, these studies highlight the

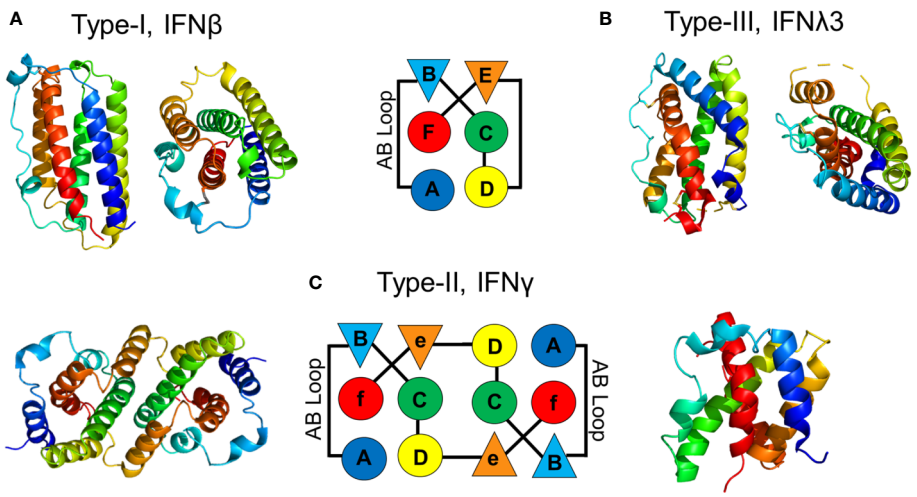

FIGURE 1 | Structures of IFN family members. Schematic, and ribbon diagrams, show the six secondary structural elements of the type-I **(A)**, pdbid = 1AU, type-III **(B)**, pdbid = 3HHC, and type-II **(C)**, pdbid = 6E3K IFNs. IFN structures are rainbow colored from the N-terminus helix A (blue) to the C-terminal helix F (red).

complexity of IFN signaling at barrier surfaces and differences in IFN signaling outcomes in mice vs. humans.

In contrast to the monomeric type- I and type-III IFNs, IFNγ adopts an intercalated dimer structure, where helices E and F from one chain are "swapped" with the other subunit of the dimer (**Figure 1C**). Like the IFNλs, the structure of IFNγ is most similar to IL10, which is the founding member of the IL-10 cytokine family (12, 21, 32, 76–78). These data confirm that each IFN family adopts a distinct α-helical scaffold, which must "handle" various amounts of sequence variation to regulate engagement of their cellular receptors. For example, there is one highly conserved type-II IFNγ dimer, whereas there are 16 monomeric type-I IFNs (35%–95% sequence identity) and 4 type-III IFNs (28%–96% sequence identity) that exhibit variable amino acid sequence identities. This highlights the distinct mechanisms used by each IFN family to regulate biological activity. Receptor homodimerization by IFNγ, versus variable IFN/IFN-receptor contacts by monomeric type-I and type-III IFNs. These mechanisms will be reviewed in more detail below.

The Type-III IFNλ/IFNλR1/IL10R2 Complex

The type-III IFNλ receptor complex (79) exhibits the simplest architecture of the three IFN families. Monomeric IFNλs assemble 1:1:1 signaling complexes with high affinity IFNλR1 and low affinity IL10R2 receptors (**Figure 2A**). IFNλR1 and IL10R2 both consist of two β-sandwich domains (D1, D2), where the D2 domains are positioned closest to the membrane. IFNλR1 binds to the IFNλs using five receptor loops (L2-L6) that are located at the junction of the D1 and D2 domains. The IFNλR1 binding loops contact IFNλ residues located on helix A, the AB loop, and helix F. Although differing in detail, the high affinity IFNλ/IFNλR1 site-1 binding site is conserved with type-I and

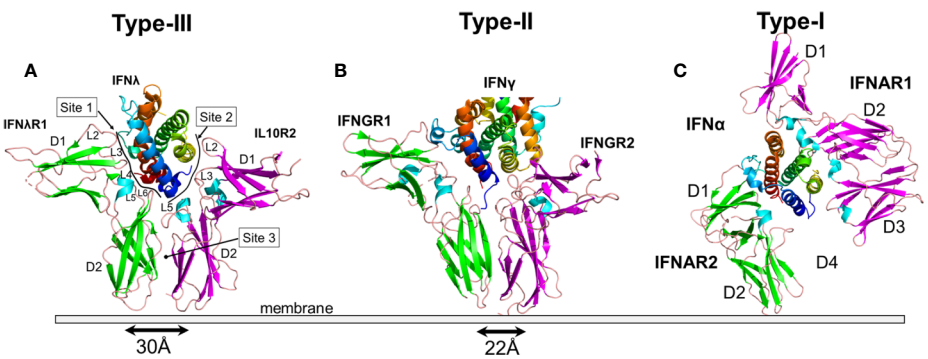

FIGURE 2 | Structures of the IFN Receptor Complexes. Ribbon diagrams of the type-III **(A)**, pdbid = 5T5W, type-II **(B)**, pdbid = 6E3K, and type-I **(C)**, pdbid = 3SE4, receptor complexes. IFNs are rainbow colored as described in Figure 1. The β-strands of the high affinity receptor chains are colored green and low affinity chains are colored magenta. For the type-II IFNγ receptor complex, only one IFNγ subunit is shown to emphasize the similarity of "half" of the complex with the type-III IFN receptor complex. The separation of the C-termini of the type-III IFNλR1/IL-10R2 and type-II IFNGR1/IFNGR2 receptor chains, where they enter the membrane are 30Å and 22Å, respectively. A D2-D4 interaction was not observed in structures of the IFN/IFNAR1/IFNAR2 complex.

type-II high affinity receptor complexes (**Figure 2**). The low affinity IL10R2 binding site-2 consists of N-terminal IFNλ residues, prior to the start of helix A (e.g., the pre-A region (80), also see **Figure 3A**), residues on helix C, and on the segment of helix D that runs parallel to the pre-A region. IL10R2 uses a subset of the same loops used by IFNλR1 (loops L2, L3, and L5) to contact IFNλ. Thus, the IFNλ-IL10R2 site-2 interface is discontinuous, making a smaller L2/helix D contact (site-2a) and a larger interaction between L3/L5 and IFNλ pre-A and helix D (Site 2b).

In addition to IFNλ-IL10R2 site-2 contacts, IL10R2 forms an additional D2-D2 site-3 interface with IFNλR1. Thus, the complete IL10R2 binding site is only formed once IFNλ binds to IFNλR1. This structural organization ensures IFNλ receptor complex formation is cooperative, where the IFNλ/IFNλR1 complex forms first, followed by binding of IL10R2 to site-2 and site-3. Once formed, the assembled IFNλ complex positions the C-terminal ends of IFNλR1 and IL10R2 30Å apart from one another, prior to entering the membrane. The combined site-2 and site-3 interfaces bury over 1,500Å (2) of surface area, which is more than twice the surface area buried in the high affinity IFNλ3/IFNλR1 site-1 interaction. However, despite this extensive interface, there are few energetically critical interactions. Thus, the affinity of IL-10R2 for the IFNλ3/IFNλR1 complex (e.g., site-2 + site-3) is 15 μM (79), which is ~15× lower than the affinity of IFNAR1 for most IFN subtypes (26, 27). While IFNλ3/IFNλR1 represents the "high affinity" interaction in the complex, the measured *K*D of 850nM (79) is ~1 log lower than the affinity of the weakest type-I IFN for IFNAR2 (e.g., IFNα1, *K*D ~100nM).

Due to the low affinity of the IFNλs for their receptors, the IFNλs are sensitive to the expression levels of their receptors on cells. In fact, a major distinction between type-I and type-III IFNs is the unique distribution of their receptors on different cell types (81, 82). Type-I IFNAR1 and IFNAR2 receptors are present on all nucleated cells, while IFNλR1 expression is predominantly limited to epithelial cells, as mentioned for IL22R1 earlier (22, 70). Thus, IFNλ signaling appears to be specialized for combating viral infections at epithelial barrier surfaces such as the lung, gut, and liver (83). This has most impressively been shown by demonstrating IFNλ, but not type-I IFN, is essential for controlling norovirus infection (84). Although gut epithelial cells in this study express type-I IFNARs, their expression is limited to the apical surface of the cells, and no IFNAR expression is observed on the basolateral surface. Thus, the selective signaling of IFNλ in gut epithelial cells was only fully appreciated within the organization of the intact gut in animals. While IFNλ activity appears "weak" in many cell-based assays, *in vivo* data suggests potent IFNλ signaling in the context of tissues and organs. It should be noted that type-I IFNs, IFNϵ and IFNκ, protect the female reproductive track (85–87) and skin (15, 88), respectively. Notably, like the IFNλs, IFNϵ and IFNκ exhibit "low" affinity for the type-I receptors, relative to most type-I IFNs (89).

Insights From IFNλ1/IFNλR1 and IFNλ3/IFNλR1 Binary Structures

Both IFNλ1/IFNλR1 and IFNλ3/IFNλR1 binary complex structures have been solved (79, 90). IFNλ1 and IFNλ3 adopt very similar structures, with a root-mean-square deviation (r.m.s.d.) of 0.6Å. Similarly, IFNλR1 binding to either IFNλ1 or IFNλ3 exhibits an r.m.s.d. of 0.68Å. Finally, the structure of unbound IL10R2 (91) and IL10R2 bound to IFNλ3 exhibit an r.m.s.d. of 1.3Å. The larger r.m.s.d. is due to changes in the conformation of the IL10R2 L5 binding loop upon contacting IFNλ3. Despite this difference, the overall structures of bound and unbound IL10R2 are the same. These structural comparisons suggest all IFNλs assemble a signaling complex with the same overall architecture. Thus, IFNλ biological potency is not regulated by the structure of the ternary complex, but by the affinity of each IFNλ for the IFNλR1 and IL10R2 chains, and ultimately the stability of the complex.

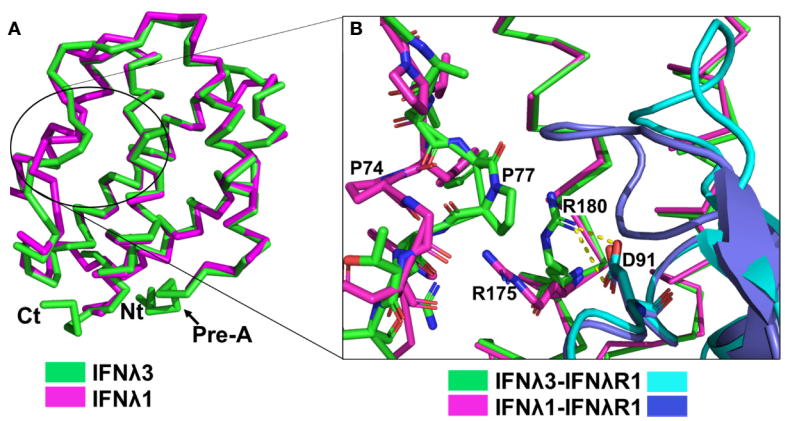

FIGURE 3 | Subtle structural changes between IFNλ1/IFNλ3 alter IFNλR1 Contacts. **(A)** Alpha carbon diagram of the superposition of IFNλ1 and IFNλ3. The location of structural differences in the B loop regions of IFNλ1 and IFNλ3, as discussed in the text, are circled. **(B)** Enlargement of the B loop "proline flip" observed in IFNλ1 and IFNλ3 structures and its influence on the conformation of Arg-180$^{IFN\lambda3}$ (green), where it makes a salt bridge with IFNλR1 Asp-91. In contrast, IFNλ1 Arg-175 (magenta) extends away from IFNλR1 Asp-91 towards the B loop.

In vitro cell-based assays demonstrate IFNλ3 exhibits twofold greater antiviral potency than IFNλ1 (92). Although a detailed analysis of IFNλ receptor binding affinity has not been completed, we expect the IFNλ3/IFNλR1 complex should exhibit differences from the IFNλ1/IFNλR1 complex, consistent with a higher affinity interaction. Comparison of IFNλ1 and IFNλ3 structures (**Figure 3A**) reveals the B loop regions of IFNλ1 and IFNλ3 exhibit different conformations, particularly Pro-74$^{IFN\lambda1}$/Pro-77$^{IFN\lambda3}$ (**Figure 3B**). In IFNλ3, Pro-77 moves in toward helix F, while in IFNλ1 Pro-74 moves away from helix F. This "proline flip" alters the position of the conserved Arg-175$^{IFN\lambda1}$/Arg-180$^{IFN\lambda3}$, located on helix F (**Figure 3B**). In IFNλ3, the guanidino group of Arg-180 packs against Pro-77, which positions it for a bivalent salt bridge with IFNλR1 residue Asp-91. A series of IFNλ3 alanine mutants were tested for antiviral activity and identified Phe-179 as the most important IFNλ3 residue for inducing antiviral activity (19). Since IFNλ3 Phe-179 is adjacent to Arg-180, it is likely that mutation of Phe-179 to an alanine disrupts the Arg-180$^{IFN\lambda3}$/Asp-91$^{IFN\lambda R1}$ salt bridge, which reduces IFNλR1 binding affinity and antiviral activity.

The "proline flip" observed between IFNλ1 and IFNλ3 (**Figure 3B**) may also provide mechanistic insight into the reduced biological activity of the IFNλ4 single nucleotide polymorphism (SNP), rs117648444. Rs11768444 corresponds to IFNλ4-Pro70Ser, which exhibits reduced antiviral activity, relative to wildtype IFNλ4 (25, 93). Understanding IFNλ4 SNPs is important since several groups have mapped the major genetic determinant of hepatitis C virus (HCV) clearance, in response to treatment with IFN-α plus ribavirin, to the type-III IFN loci (94–96). Ultimately, IFNλ4 activity has been implicated as the causative agent of HCV clearance failure in patients that encode "active" IFNλ4 protein, as opposed to inactive IFNλ4 protein (25). Despite sharing ~28% sequence identity with IFNλ3, IFNλ4 adopts the same α-helical fold as other IFNλs and binds to IFNλR1 and IL10R2 (97). Amino acid sequence alignments show IFNλ4 Pro-70 is identical to IFNλ3 Pro-77, suggesting the IFNλ4 Pro70Ser mutation impacts IFNλ4-IFNλR1 interactions by altering the structure of IFNλ4 Arg-163, as described for Arg-180 in IFNλ3 (**Figure 3B**).

IFNλ2 has not been studied to the same extent as the other IFNλs, presumably because it was shown to exhibit ~5–10× lower antiviral activity (53, 98). The IFNλ2 amino acid sequence differs from IFNλ3 by only 6 amino acids. Modeling the structure of IFNλ2 based on the structure of IFNλ3 suggests, R28H occurs in a non-structured region at the N-terminus of the molecule, where it is not predicted to alter receptor binding. K70R and R72H are located in the AB loop of IFNλ2, but do not contact IFNλR1. Furthermore, an IFNλ3 R72A mutant reduced IFNλ3 anti-viral activity by only 30%, suggesting these residue changes cannot explain the lower activity of IFNλ2. Residues V92M and H156Y are located on exposed surfaces of IFNλ2 helices C and E, respectively, which are located opposite the IFNλR1 and IL10R2 binding sites. Thus, if these amino acids were responsible for the lower activity of IFNλ2, this would support the hypothesis of some groups that IFNλ may bind to another, unidentified, receptor chain (83). Finally, L133F is located on helix D, where the sidechain is buried in the hydrophobic core of IFNλ2. The L-to-F amino acid change cannot be incorporated into the hydrophobic core of the IFNλ3 structure without distorting helices A, D, or F. This suggests L133F may be the main residue responsible for the reduced biological activity of IFNλ2, relative to IFNλ3.

The Type-II IFNγ/IFNGR1/IFNGR2 Complex

The type-II IFNγ receptor complex provides an important structure to further understand the type-I and type-III complexes (99). The unique intercalated dimer structure (6) of IFNγ distinguishes it from the disulfide-linked monomeric type-I and type-III IFNs (4, 19, 100). The IFNγ dimer assembles a symmetric 1:2:2 IFNGR1/IFNGR2 heterodimeric complex (99, 101) (**Figure 4**), compared to the 1:1:1 heterodimeric complexes of the type-I and type-III IFNs (**Figure 2**). In the dimeric complex, the twofold-related C-termini of the IFNGR1/IFNGR2 heterodimers are positioned 85Å apart from one another. As suggested from the analysis of the structurally related IL10 dimer (102), the dimeric IFNγ positions IFNGR1 and IFNGR2 (**Figure 4**), and their respective ICDs, in an optimal dimeric arrangement to recruit inactive STAT1 dimers (103) for subsequent phosphorylation and activation of STAT1 homodimers (104). Disruption of the dimeric IFNγ receptor complex architecture, using engineered monomeric IFNγs, which assembles ½ of the dimeric IFNγ/IFNG1/IFNGR2 (see **Figure 2** vs. **Figure 4**), drastically reduced some IFNγ-induced biological activities (7, 8, 99, 102, 105). Additional IFNγ mutants confirmed the dimeric arrangement of IFNGR1, not IFNGR2, was essential for full STAT1 phosphorylation (99). In contrast to

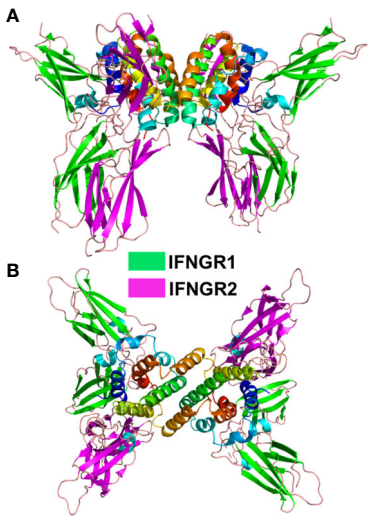

FIGURE 4 | Dimeric IFNγ/IFNGR1/IFNGR2 Complex. Ribbon diagram of the 1:2:2 IFNγ dimer/IFNGR1/IFNGR2 complex (pdbid = 6E3K). Two views of the complex are shown. The first is approximately perpendicular to the IFNγ twofold axis **(A)** and the second is parallel to the twofold axis **(B)**.

STAT1, many additional pathways activated by IFNγ, including MAP kinase, PI3K, and CaMKII (106), appear not to be equally sensitive to IFNγ-mediated IFNGR1/IFNGR2 dimerization. Thus, at least on some cells, engineered IFNγ monomers can induce the same levels of cell surface HLA-A as the WT IFNγ dimer (99). Interestingly, it should be noted that neurons appear to naturally manipulate IFNγ signaling outcomes by maintaining low STAT1 levels, which results in potent IFNγ-mediated activation of ERK1/2 (107). Overall, the dimeric architecture of the IFNγ/IFNGR1/IFNGR2 complex is critical for inducing the full spectrum of IFNγ-mediated pleotropic activities (108), which includes macrophage activation (109, 110), tumor surveillance (111, 112), and protection from intracellular pathogens, including mycobacteria (50, 113).

Despite the larger dimeric assembly, within one IFNγ subunit, IFNGR1 and IFNGR2 form similar site-1, site-2, and D2-D2 site-3 interfaces, as previously described for the IFNλ/IFNλR1/IL10R2 complex (**Figure 2B**). Compared to IFNλ/IFNλR1, the IFNγ site-1 interface is more extensive with major contacts between the AB loop and helix F of IFNγ and IFNGR1 L2-L6 loops. The site-2 IFNγ/IFNGR2 interface is comprised almost exclusively of contacts with IFNγ helix D and no contacts with helix A, the main contact region in the IFNλ complex. Despite these differences, IFNGR2 still forms a D2-D2 site 3 interface with the IFNGR1, which positions the C-termini of the receptors 22Å apart at the cell surface prior to their entry into the membrane. Thus, assembly of the IFNγ signaling complex is cooperative, requiring the formation of the IFNγ/IFNGR1 binary complex first, followed by IFNGR2 binding to induce cell signaling.

The Type-I IFN/IFNAR1/IFNAR2 Complex

The type-I IFN receptor complex is distinct from both the type-II and type-III receptor complexes (**Figure 2**). The high affinity IFNAR2 chain adopts a two-domain D1/D2 receptor structure, as observed for IFNλR1 and IFNGR1 chains (**Figure 2**) (114). NMR and X-ray structures confirm IFNAR2 binds to an IFN site-1 epitope that is comprised of residues on helix A, the AB loop, and helix F, similar to the type-II and type-III IFNs (100, 115, 116). IFNAR2 makes extensive interactions with Arg-33 (IFNα2 numbering) in the AB loop of the IFNs. Arg-33, and the structurally adjacent Leu-30, account for approximately two thirds of the IFNα2/IFNAR2 binding energy (29, 100, 117). Additional critical contacts occur with the IFNAR2 L3 and L4 binding loops, which contact helix F residues Met-148 and Arg-149 (IFNα2 numbers) (117). Although we know that all 16 IFNs exhibit a variety of affinities for IFNAR2 (26–28, 89), the mechanisms that control IFNAR2 affinity for each IFN subtype remains incomplete. In general, it appears that subtle changes to residues around these energetically critical residues modulate IFN-subtype IFNAR2 affinity.

The type-I IFN low affinity receptor chain, IFNAR1, is completely unique relative to the other IFN and IL10 family cytokine receptors (**Figure 2**). IFNAR1 consists of four β-sandwich domains (D1-D4), similar to tandem D1/D2 receptors, where the D4 domain is the membrane proximal domain. The D2 and D3 domains of the receptor form an extensive interface with one another, while the D1 domain can undergo rigid body movements. Overall, IFNAR1 D1-D3 domains form an IFN-binding module, while the D4 domain is attached to D3 by a flexible linker that allows the D4 domain to adopt multiple conformations, even when bound to IFN (100, 118). Despite a unique structure, IFNAR1 loops at the ends of D1, D2 and D3 domains contact IFN helices C, D, and E, with the D1 domain "closing down" on helix E, like a hand grabbing a glass.

Based on the features described above, the binding of type-I IFNs by IFNAR1 represents a novel protein recognition paradigm. First, the IFNAR1-IFN contact surface, consisting of IFN helices C, D, and E, is larger than for the other IFN complexes. Second, the membrane proximal D4 domain of IFNAR1 does not form a site 3 interface, at least not a stable interface, with the D2 domain of IFNAR2. This suggests that by increasing the size of the IFNAR1-IFN site-2 interface (see **Figure 2C**), using novel D1/helix E interactions, the type-I IFN complex no longer requires a site-3 interface. Thus, for the type-I IFN complex, there is no structure-based cooperativity enforced by a D2-D4 site-3 interaction. Rather, receptor complex assembly and stability is controlled completely by IFN-IFNAR2 and IFN-IFNAR1 affinities. While it is possible that free IFNs, and IFNs bound to IFNAR2, could exhibit different affinities for IFNAR1, resulting in an affinity-based cooperative binding mechanism, this has not been demonstrated experimentally.

The mechanistic role of the IFNAR1 D4 domain in type-I IFN receptor activation remains unclear since the D4 domain was not observed in crystal structures of the IFN/IFNAR1/IFNAR2 complex (**Figure 5A**). To identify possible location/s of the IFNAR1 D4 domain, the IFNλ3/IFNλR1/IL10R2 complex was superimposed onto the IFNω/IFNAR1/IFNAR2 complex (**Figure 5B**). In this model, the D1 domain of IL10R2 overlaps with the IFNAR1 D3 domain and the putative location of the IFNAR1 D4 domain, represented by the IL10R2 D2 domain, is adjacent to the IFNAR2 D2 domain creating a D2-D4 site-3 interface, as observed in type-II and type-III complexes (**Figure 2**). A second possible position of the D4 domain is provided by the structure of the murine IFNβ/IFNAR1 binary complex (119), where all four domains of IFNAR1 were observed. Superposition of the murine IFNβ/IFNAR1 complex on the IFN/IFNAR1/IFNAR2 human complex places the C-terminal ends of IFNAR2 D2 and IFNAR1 D4 51Å apart (**Figure 5C**), in contrast to 30Å and 22Å for the IFNλ and IFNγ complexes, respectively. These models lead to two possible conclusions. First, type-I IFNs assemble a novel "open" complex with the C-terminal ends of IFNAR1 and IFNAR2 separated by ~50Å. Second, the "open" conformation is an inactive complex, which must "close" to form a D2/D4 site-3 interface to induce IFN activity. Our analysis suggests that IFN binding to IFNAR2 and IFNAR1 promotes transient IFNAR2-D2/IFNAR1-D4 interactions. Thus, the stability of the IFN/IFNAR1/IFNAR2 interaction would control the number of transient "open"/"closed" D2-D4 site-3 binding events, which could influence signaling strength. Thus, the stability of the IFN/IFNAR2 and

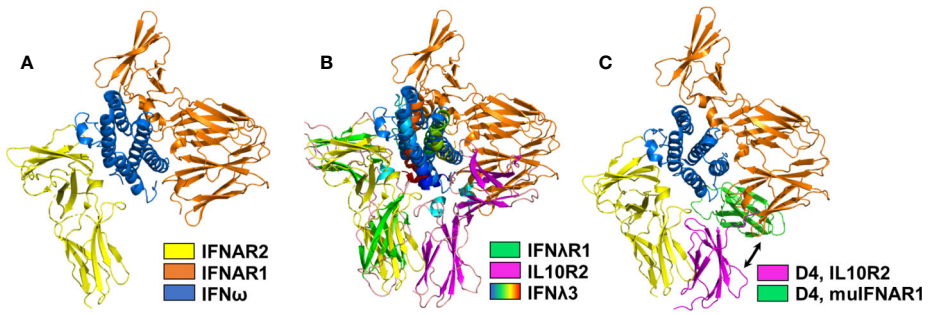

FIGURE 5 | Structural Models of the IFNAR1 D4 Domain. **(A)** Ribbon diagram of the type-I IFN (IFNω, blue)/IFNAR1 (orange)/IFNAR2 (yellow) complex structure (pdbid = 3SE4), which lacks the IFNAR1 D4 domain. **(B)** Superposition of the IFNλ3 (rainbow)/IFNλR1 (green)/IL10R2 (magenta) ternary complex on the IFN/IFNAR1/IFNAR2 structure positions the IL10R2 D2 domain (magenta), such that it could represent the transient location of the IFNAR1 D4 domain forming an IFNAR2 D2-IFNAR1 D4 stem interaction. **(C)** A second possible location of the human IFNAR1 D4 domain is shown by superimposing the murine IFNβ/IFNAR1 complex (pdbid = 3WCY) on the IFN/IFNAR1/IFNAR2 complex. The position of the modeled D4 domain (green), derived from the murine IFNβ/IFNAR1 structure is shown in green, and the location of the IFNAR1 D4 domain obtained from superimposing the IFNλ receptor complex is shown in magenta. Since the human IFNAR1 D4 domain does not form a stable D2-D4 interaction with IFNAR2, D4 may transition between green and magenta conformations to induce biological activity. The exact role of the D4 domain in IFN signal transduction remains unknown.

IFN/IFNAR1 interactions would regulate signaling, as has been previously described (120).

Despite structures that reveal extracellular IFN-receptor recognition and assembly mechanisms, there remain questions about IFN-mediated signal transducing events that initiate and sustain cellular activation. For example, it remains unclear how all 16 IFNs, that exhibit a spectrum of affinities for the IFNARs (weak/strong), can all activate a subset of genes associated with antiviral activity on all cells, while additional cellular functions of the IFNs, one such readout being anti-proliferative activity, correlates with IFN-IFNAR affinity (121). These two distinct cellular readouts, labeled as robust and tunable activation (121), might be explained by an IFNAR1/2 pre-association model (122) and an IFN-mediated IFNAR1/2 heterodimerization model (123), respectively. The IFNAR pre-association could account for rapid IFN-mediated activation of antiviral gene expression, while IFN-mediated IFNAR dimerization could account for tunable gene expression. The implication of the pre-association model is that the IFNs induce a structural change in the IFNARs that activate JAK1/TYK2 and induce rapid anti-viral gene expression, while the dimerization model relies solely on IFN-mediated dimerization of the IFNARs to activate JAK1/TYK2 and subsequently induce IFN-mediated gene expression. Technical issues, specifically analysis of artificially high IFNAR expression levels, have been suggested to be responsible for the observation of pre-associated IFNARs (123). Unfortunately, the investigators criticizing the pre-association model did not confirm that overexpression of the IFNARs leads to IFNAR1/2 interactions. Nonetheless, the cortical actin cellular meshwork and/or lipid rafts could provide a suitable mechanism to "concentrate" IFNARs for rapid induction of robust antiviral genes by all IFNs, while still allowing tunable activities that are dependent on IFN-IFNAR affinities (124). Overall, the data suggest that the major mechanism regulating IFN activation is IFN-mediated IFNAR1/2 heterodimerization, although some recent data suggests IFN-induced IFNAR conformational changes may also regulate IFN activity (125).

The Murine Type-I IFN Family Is Distinct From Human Type-I IFNs

The murine IFNβ/IFNAR1 binary complex structure provides an important datapoint in the proposed model of human type-I IFN signaling. However, my lab and others have previously noted the "uniqueness" of type-I IFN families in different animals (10, 126–129). For example, the murine IFN system consists of 14 IFNαs (note that murine and human IFNα subtype designations have no bearing on their interspecies sequence and/or functional similarities), as well as IFNβ, IFNϵ, IFNκ, limitin (130), but do not encode an IFNω (126). Thus, it is necessary to ask if the murine IFNs and receptor proteins, as well as their biological outcomes, can be extrapolated to humans. From a structural biology perspective, the overall folds of murine (62) and human (5) IFNβ, which share 47% sequence identity, are almost identical (**Figure 6A**). The extracellular regions of human and murine IFNAR1 share 49% amino acid sequence identity and the structures of D1-D3 domains of murine and human IFNAR1s are also almost identical (119). These findings suggest the overall model proposed for the missing D4 domain in the human IFN/IFNAR2/IFNAR1 complex is plausible (**Figure 5**).

Despite similar overall receptor complex structures, the receptor binding properties of murine and human IFNβ are distinct. Human IFNβ binds to IFNAR1 and IFNAR2 with ~30nM and ~0.1nM *K*D values, respectively (28). However, in the mouse, IFNβ receptor affinities are "flipped" such that the IFNβ-IFNAR1 forms the high affinity interaction (*K*D ~10nM) and the IFNβ-IFNAR2 forms the low affinity interaction (*K*D ~1.7μM) (86). Structural comparisons of human and murine IFNβ reveal the AB loop of murine IFNβ, which forms a major part of the IFNAR2 site-1 binding site, exhibits a distinct structure compared to human IFNβ (**Figure 6**). In human

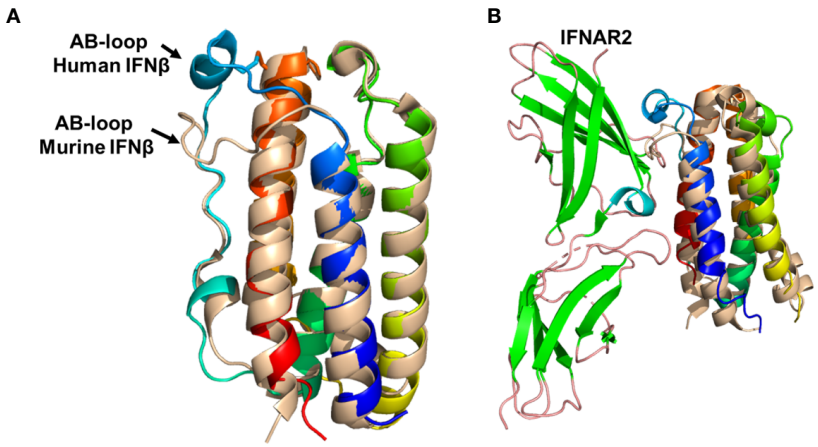

FIGURE 6 | Structural Comparison of human and murine IFNβ. **(A)** Structural superposition of human IFNβ (colored as in **Figure 1**, pdbid = 1AU1) and murine IFNβ (wheat, pdbid = 1WU3), highlighting their distinct AB loop structures. **(B)** Structural superposition of murine and human IFNβ onto IFNα2 from the human IFNα2/IFNAR2 crystal structure. The resulting structural model results in steric clashes between the murine IFNβ AB loop and IFNAR2 binding loops, but not for the human IFNβ/IFNAR2 model. This structural analysis provides an explanation for the low affinity of the murine IFNβ/IFNAR2 interaction, compared to the high affinity human IFNβ/IFNAR2 interaction.

IFNβ, the AB-loop arches toward the N-terminal end of helix-F, "over" helix F itself, where the loop connects to helix F by a disulfide bond. In contrast, the murine IFNβ AB-loop wraps "across" helix F where it would disrupt high affinity IFNAR2 interactions, as observed in the human IFNα/IFNAR2 crystal structure (**Figure 6B**). Interestingly, sequence alignments reveal the murine IFNAR2 receptor binding loops that contact the AB loop region of murine IFNβ are the same length as human IFNAR2. In addition, murine IFNαs bind with high affinity (*KD* ~1nM) to murine IFNAR2 (86). Thus, it is likely murine IFNAR2 receptor binding loops do not change their lengths, or grossly change their conformations, to accommodate the distinct murine IFNβ AB loop structure. Together, these structural observations provide an explanation for the low affinity of the murine IFNβ/IFNAR2 interaction, compared to the human IFNβ-IFNAR2 interaction. While this structural analysis is satisfying with respect to murine and human IFNβ, it highlights the many distinct properties of the murine IFNs, from structure to mechanism to *in vivo* outcomes, remain uncharacterized.

Moving Forward

This review has focused on fundamental structural features of the three human IFN families, highlighting similar and unique features of each receptor complex. The ultimate goal of structural studies is to define mechanisms that can be used to discover optimal IFN therapeutics that harness the antiviral activity of the IFNs to improve human health (131). The importance of this goal is highlighted by the SARS-CoV-2 pandemic that is ravaging our society (72, 132–134). Based on the critical role that IFN – IFN receptor affinity plays in varying IFN activity (26, 120, 135), type-I and type-III IFNs with increased receptor affinity have been designed, yet they have not advanced into the clinic (79, 136, 137). Presumably because we still do not know the optimal design principles to create an optimal IFN therapeutic. Given that humans produce 20 different type-I/III IFNs in response to pathogens, the design may not be simple and might require the synergistic actions of both type-I and type-III IFNs. For example, type-I IFNβ and type-III IFNλ3 induced distinct anti-viral gene expression profiles with distinct kinetics on human hepatocytes (138). Specifically, high affinity IFNβ induced potent antiviral protection almost immediately (~2 h) after addition to cells that waned after ~48 h. In contrast, IFNλ3 antiviral activity was not observed until ~12 h after treatment, but was sustained for at least 72 h post-treatment (138). These data highlight the interplay of distinct receptor affinities and negative feedback mechanisms (139, 140), which synergistically control IFN-mediated antiviral signaling. Notably, type-III IFN signaling has been shown to be resistant to USP18-mediated negative feedback regulation, which potently regulates type-I IFN signaling (141). USP18 is induced by type-I and type-III IFNs, but specifically binds to the ICD of IFNAR2 and disrupts IFNα-mediated IFNAR1/IFNAR2 complex formation. These studies demonstrate that the anti-viral signaling cascade induced by type-I and type-III IFNs is very similar, yet multiple mechanisms can tailor the response for optimal functional outcomes, which include eliminating the virus and protecting the host. These studies, and more like them, are providing new design principles to further our quest for safe and efficacious IFNs with broad-spectrum antiviral activity.

AUTHOR CONTRIBUTIONS

MRW performed literature searches, made figures, and wrote the manuscript.

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REVIEW
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Interferon Receptor Trafficking and Signaling: Journey to the Cross Roads

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Like most plasma membrane proteins, type I interferon (IFN) receptor (IFNAR) traffics from the outer surface to the inner compartments of the cell. Long considered as a passive means to simply control subunits availability at the plasma membrane, an array of new evidence establishes IFNAR endocytosis as an active contributor to the regulation of signal transduction triggered by IFN binding to IFNAR. During its complex journey initiated at the plasma membrane, the internalized IFNAR complex, i.e. IFNAR1 and IFNAR2 subunits, will experience post-translational modifications and recruit specific effectors. These finely tuned interactions will determine not only IFNAR subunits destiny (lysosomal degradation vs. plasma membrane recycling) but also the control of IFN-induced signal transduction. Finally, the IFNAR system perfectly illustrates the paradigm of the crosstalk between membrane trafficking and intracellular signaling. Investigating the complexity of IFN receptor intracellular routes is therefore necessary to reveal new insight into the role of IFNAR membrane dynamics in type I IFNs signaling selectivity and biological activity.

Keywords: transmembrane receptor, interferon, endocytosis, intracellular signaling, traffic, JAK - STAT signaling pathway

INTRODUCTION

The IFNAR signaling pathway plays a central role in the defenses of the organism by supporting one of the major anti-viral and anti-proliferative cellular responses. Its dysregulation can also lead to deleterious auto-inflammation in humans (1). Nowadays, it is accepted that endocytosis holds an essential role in the activity of a large number of receptors including receptor tyrosine kinases (RTK) and G protein-coupled receptors (GPCR) families [reviewed in (2, 3)]. The role of endocytosis in the modulation of type I interferons receptor (IFNAR) has however lagged behind. Endocytosis is an essential mechanism by which a cell can efficiently achieve the uptake of transmembrane proteins, lipids, nutrients, extracellular molecules, and more generally cell surface cargos. The identification and characterization of clathrin-coated pits shed light upon endocytosis as being an active and highly regulated process mediated by clathrin and dynamin (4–6). Over the last decades, a strong body of work has contributed to the complexification of the mechanisms involved in the regulation of endocytosis. Early on, endocytosis was categorized as being mediated either by

clathrin-dependent or clathrin-independent means. This simplistic binary classification did not resist further investigations and today several molecular machineries including caveolin, endophilinA2, RhoA, Cdc42, Arf6, flotillin or endophilinA3/galectin8 selectively control distinct endocytic pathways (7–9). Despite the existence of specific molecular machineries, these different endocytic pathways share the common property that is to modulate the cell surface density of a multitude of receptors, a process that is essential for the cell homeostasis and the transduction of receptor signaling (10, 11). Whereas the IFNAR signaling cascade has long been thought to be linear and exclusively controlled at the plasma membrane (PM) [reviewed in (12)], these studies allow to revisit its regulation in the context of membrane trafficking. In this review, we will describe recent studies on IFNAR journey from the cell surface to the different endosomal compartments and how it is connected with the regulation of signaling outputs.

MECHANISMS THAT CONTROL STEADY STATE TYPE I INTERFERON RECEPTOR LEVELS

The type I IFN receptor is composed of the IFNAR1 and IFNAR2 subunits. IFNAR1 exists only as one isoform whereas differential splicing of the *IFNAR2* gene generates three isoforms. The firstly discovered IFNAR2c is the longer isoform with the full intracellular domain (13). IFNAR2b is a shorter transmembrane isoform lacking the intracellular domain while IFNAR2a is a soluble truncated form. The two latter isoforms can still bind type I IFNs and interact with IFNAR1 but are unable to transduce signal (14), suggesting they would be negative regulators of JAK/STAT signaling (15). We will focus here only on the full-length IFNAR2c which will be referred to as IFNAR2.

IFNAR1 and IFNAR2 are ubiquitously expressed (16) albeit with highly variable levels. The first quantifications of PM levels relied on standard Scatchard analysis, which is based on the saturation of IFNAR binding with iodinated IFNs and allow to precisely determine ligand affinity and number of binding sites (17). Published results showed large variations among cell types with a number of binding sites ranging from 200 to up to 250,000 (18, 19). Today, Scatchard analysis has been replaced by more acute and sophisticated measurements. For instance, single-molecule imaging of fluorescently labelled IFN-α2 by total internal reflection fluorescence (TIRF) microscopy could measure a density of around 0.55 IFN bound per μm^2 in HeLa cells, corresponding to 500–1000 binding sites per cell (20). The same technique measured 0.58 IFNAR1 and 0.72 IFNAR2 per μm^2 of PM in human retinal pigmented epithelial RPE1 cells (21). While the IFNAR cytoplasmic pool is likely to be important, few if any studies have determined the ratio of internal versus surface IFNAR. Likewise, the PM IFNAR1/IFNAR2 ratio is certainly critical for IFNAR signal transduction. Finally, the two IFNAR2a and b shorter forms can compete with the long IFNAR2c by forming a non-signaling complex (14).

Type I Interferon Receptor Intimate Cytosolic Interactors

The lack of IFNAR intrinsic tyrosine kinase activity is compensated by a non-covalent and constitutive association with Janus tyrosine Kinases (JAK). The JAK family is composed of four members: JAK1 and JAK2 (22), TYK2 (23, 24), and JAK3 (25). JAKs are large multidomain proteins with an N-terminal part dedicated to the recognition and interaction with the PM proximal region of the receptor and a C-terminal part regulating its kinase activity (**Figures 1A, B**) [for more details see reviews (26, 27)]. IFNAR1 is constitutively associated with TYK2 (28, 29) and IFNAR2 with JAK1 (13, 30, 31). IFN-induced association of the two IFNAR chains allow JAK1 and TYK2 to form a functional signaling unit (**Figure 1C**). The precise mapping of interactions between IFNAR and associated JAK are well described (32, 33). TYK2 interaction with IFNAR1 was shown to stabilize IFNAR1 at the PM (34–36) thereby controlling IFNAR1 PM pool. Moreover, both JAK1 and TYK2 play a central role in IFNAR trafficking and signaling by regulating the recruitment and/or the activity of other IFNAR interactors through JAK-dependent tyrosine phosphorylation.

The canonical type I IFN signaling pathway relies on the phosphorylation and nuclear translocation of signal transducers and activators of transcription (STAT) proteins. STATs share a similar structure with an SH2 domain that allows the interaction with cytokine receptors and regulators, and a DNA binding domain that modulates gene transcription (37). Pull-down experiments suggested that STAT2 was constitutively associated with the cytosolic domain of IFNAR2 (**Figure 1B**) (38). This interaction was further confirmed and visualized in cells expressing HaloTag-IFNAR2 spatially constrained at the PM by HaloTag ligand functionalized on a micropatterned surface (39). Whereas STAT2-EGFP showed a strong colocalization with patterned IFNAR2, a very limited colocalization of STAT1-EGFP could be detected in the same conditions. This probably reflects that STAT1 docking to IFNAR2 occurs through STAT1-STAT2 heterodimerization in agreement with the requirement of STAT2 for STAT1 phosphorylation by IFN (37, 40). These results suggest that additional sites for STAT docking may be created by IFNAR phosphorylation, thus enhancing binding of STAT1 and STAT2. The precise mapping of STAT2 interaction that is still unknown should lead to a better understanding of the IFNAR complex organization.

STAT2 mediates the recruitment of the ubiquitin specific peptidase 18 (USP18) to the IFNAR2 subunit (**Figure 1B**). USP18 is an isopeptidase that promotes the de-conjugation of the ubiquitin-like modifier interferon-stimulated gene 15 (ISG15) (41), a reaction known as deISGylation. USP18 is first recruited to STAT2 (42), and then to IFNAR2 where it competes with JAK1 binding, which results in JAK/STAT signaling inhibition (43). USP18 binding to IFNAR2 stabilizes the

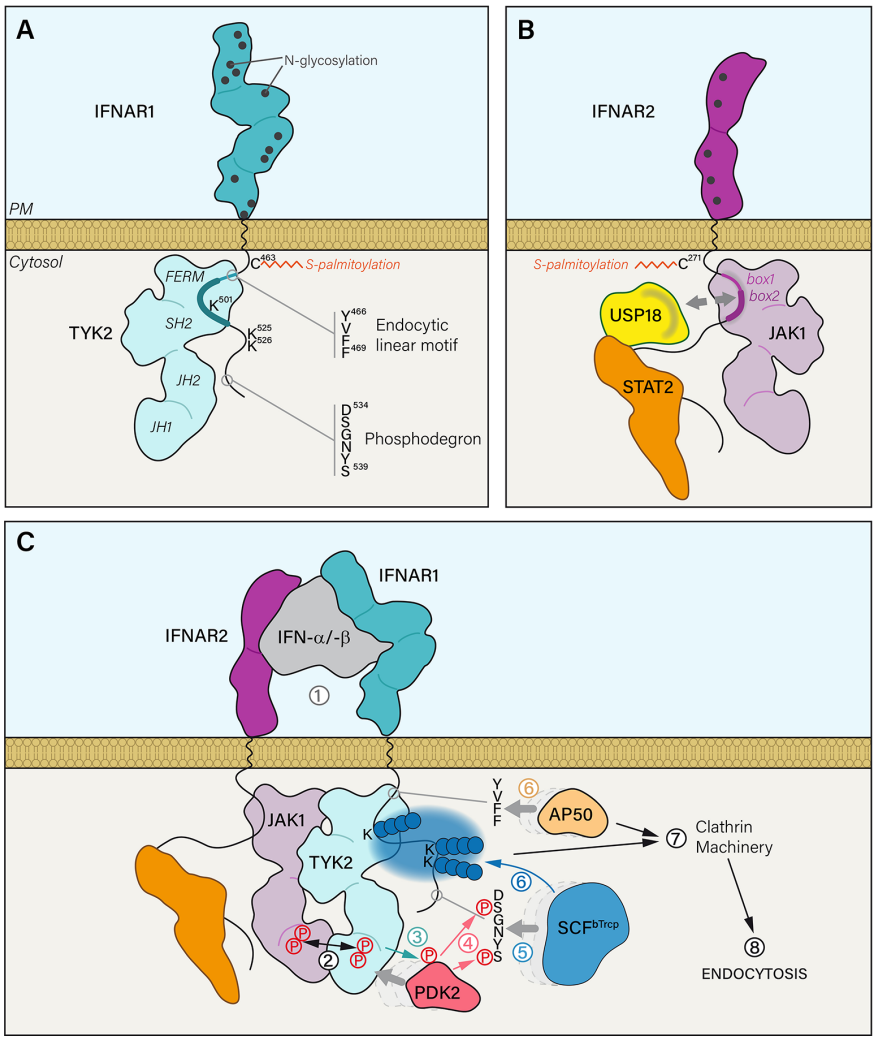

FIGURE 1 | **(A)** The extracellular part of IFNAR1 is composed of four domains and exhibits 12 residues that are potentially N-glycosylated. IFNAR1 is S-palmitoylated on the PM proximal Cys^{463} residue. IFNAR1 cytosolic tail interacts with TYK2 kinase FERM and SH2 domains through a minimal region that corresponds to 486–511 residues (thick blue line). IFNAR1 interaction domain with TYK2 can be extended to a maximal region from residues 465 to 511 (thick and thin blue line) that also covers a canonical tyrosine-based linear endocytic motif ^{466}YVFF^{469}. IFNAR1 cytosolic tail has three lysine residues that can be ubiquitinated and a phosphodegron motif. **(B)** The extracellular part of IFNAR2 is composed of two domains presenting five putative N-glycosylation sites. IFNAR2 is meant to be S-palmitoylated on two Cys residues: one near the PM (Cys^{271}) and another one less likely, closer to the C-terminal part (Cys^{395}). IFNAR2 interacts with its associated JAK1 kinase through cytosolic tail box 1 and box 2 domains. At steady state, ubiquitin specific peptidase 18 (USP18) interacts with the DNA-binding and coiled-coil domains of STAT2 but also with IFNAR2 box 1 and box 2 domains. Therefore, it can compete with JAK1 binding on IFNAR2. **(C)** (1) IFN-α/-β binding to IFNAR1 and IFNAR2 subunits triggers several mechanistic events leading to the internalization of the receptor complex. (2) IFNAR associated JAK kinases are brought in close proximity resulting in the concomitant tyrosine phosphorylation of JAK1 and TYK2 on Tyr^{1022}-Tyr^{1023} and Tyr^{1054}-Tyr^{1055} residues, respectively. (3) Activated TYK2 can then phosphorylate the serine/threonine kinase PDK2 which in turn (4) phosphorylates the two serine residues of the IFNAR1 phosphodegron ^{534}D**S**GNY**S**^{539}. It acts as a docking site (5) for the Skp1-Cullin1-F-box complex E3 ubiquitin ligase (SCF$^{\beta Trcp}$). (6) Once bound to IFNAR1, SCF$^{\beta Trcp}$ is able to polyubiquitinate (blue spheres) lysine residues 501, 525 and 526 by adding Lys^{48} and Lys^{63} linkages. In parallel, the endocytic linear motif ^{466}YVFF^{469} recruits AP50, the μ2 subunit of AP2 adaptor complex. (7) Together, AP50 binding and IFNAR1 polyubiquitination trigger the association of IFNAR receptor complex with the clathrin machinery and its endocytosis *via* clathrin-coated vesicles.

interaction of STAT2 with IFNAR2 (39) and inhibits IFN-α binding (44, 45). Thus, both STAT2 and USP18 are important for IFNAR complex trafficking and signaling.

The adaptor protein receptor for activated protein kinase C-1 (RACK-1) is also constitutively associated with IFNAR2 (46). This interaction is maintained after IFN stimulation. RACK-1 is a scaffold protein with no enzymatic activity, which recruits specific signaling elements. RACK-1 directly interacts with IFNAR2, TYK2, and JAK1, and indirectly with IFNAR1 (47). RACK1 is necessary for STAT1 and STAT2 phosphorylation. The RACK-1 binding site on IFNAR2, partially overlaps with JAK1 binding site.

Type I Interferon Receptor Proper Function Depends on Posttranslational Modifications

Receptor glycosylation is an important posttranslational modification regulating their activity through various mechanisms including proper folding in the ER, localization at the PM (*via* interactions with galectins and with gangliosides in microdomains for instance), ligand binding, intracellular trafficking and signaling (48, 49). The two IFNAR subunit extracellular domains are highly glycosylated resulting in their high molecular weight (MW) (**Figures 1A, B**). Thus, human IFNAR1 has an approximately 130 kDa MW instead of the theoretically calculated 63.5 kDa MW corresponding to the 557 amino acids (aa) of IFNAR1, and human IFNAR2 has a 100-110 kDa MW instead of a theoretical 57.8 kDa MW for its 515 aa (50).

Levels of IFNAR1 and IFNAR2 at PM are likely to be regulated independently from each other. The PEPD prolidase is required for the maturation and surface expression of IFNAR1 but not of IFNAR2 (51). The importance of these posttranslational modifications in IFN biological activity is illustrated by NS5 viral proteins that are produced by some Flaviviruses. NS5 can suppress IFNAR1 glycosylation by binding to PEPD thereby interfering with IFNAR1 delivery from the trans-Golgi network (TGN) to the PM (51, 52). Hence IFNAR1 glycosylation is likely to play an important role in signaling by regulating IFNAR1 density at the cell surface.

Both IFNAR chains are palmitoylated on cysteins (**Figures 1A, B**) (53). Palmitoylation has been involved in the proper addressing of proteins from the TGN to the PM, in protein-protein interactions, and in the association with lipid nanodomains, all steps that are important for the targeting, stability and function of receptors at the PM (54). IFNAR1 is mono-palmitoylated on Cys^{463} and the corresponding C463A IFNAR1 mutant, totally lacking palmitoylation, showed no alteration either in stability at the PM, IFN-α-induced endocytosis or intracellular distribution but showed some defects in the later steps of JAK/STAT signaling (53). Indeed, although the very first events of JAK/STAT signaling (i.e. JAK1, TYK2 and IFNAR1 tyrosine phosphorylations) were unchanged, IFNAR1 palmitoylation was found to be required for STAT1 and STAT2 downstream phosphorylation and nuclear translocation. Therefore, IFNAR1 palmitoylation may participate to target IFNAR1 to the proper PM nanodomains or the recruitment of effectors associated to the signaling receptor complex. Although IFNAR palmitoylation is not involved in IFNAR1 addressing to the PM and IFNAR1 internalization, it is worth noting that the inhibition of global protein palmitoylation by 2-bromopalmitate strongly affects IFNAR1 internalization after IFN stimulation, suggesting that yet to be identified palmitoylated effectors, associated or not with the IFNAR complex control these processes. Although little is known about the molecular mechanisms linking palmitoylation and JAK/STAT signaling, this posttranslational modification remains an interesting avenue of investigation, as it may help us to reveal new links between IFNAR subunit trafficking and its signaling.

Whether multi-chain cytokine receptors are pre-associated or not before their activation at the PM remains highly debated for some members of this family as recently illustrated by the IFN gamma receptor (55, 56). The debate seems closed for IFNAR as several studies convincingly established that IFNAR1 and IFNAR2 are not pre-associated at steady state and that receptor dimerization occurs only upon ligand binding (57–60). The two chains are nevertheless associated to other partners such as lipids or proteins, whose main function is to maintain the receptor at the PM and keep the receptor-bound JAK kinases inactive.

Receptor Partitioning in Plasma Membrane Nanodomains

Based on IFNAR density at the cell surface, the constant rate of ligand association/dissociation predicts that IFNAR1 and IFNAR2 subunits should not be able to form stable complexes if they were uniformly distributed (59). The two chains of IFNAR should therefore be clustered in the same local structures at the PM. Twenty years ago, in mouse embryonic fibroblasts (60), IFNAR1, like many other receptors at that time (61), was proposed to be associated with lipid rafts, these PM asymmetric lipid assemblies thought to be detected in detergent resistant membranes (DRM). If this technique is today outdated, more sophisticated cell imaging methods have led to a finest characterization of the nanoscale distribution of IFNAR at the PM.

Caveolae are characteristic small buds present at the PM of many cell types, that are enriched in cholesterol and glycosphingolipids, and coated with the structural protein caveolins (Cav) and assembly proteins cavins (62). The early finding that caveolae could biochemically fractionate in DRM fractions has led to refer to them as a subtype of lipid rafts (63). While this definition is not accurate any longer (64), the role of caveolae in IFNAR distribution has remained elusive. Indeed, early electron microscopy (EM) studies failed to detect human IFN-α or murine IFN-β in caveolae (65, 66). More recently, the overexpression of the chain cytokine receptor family B1 (CRFB1), the zebrafish IFNAR ortholog, resulted in its colocalization with Cav1β domains at the PM using super resolution microscopy (67). The finding that Cav1β depletion significantly decreased CRFB1 PM clustering and IFN-induced STAT1 signaling is in strong support of an important role of Cav1β in zebrafish IFN-R system and not of caveolae since the other caveolae constituents like cavins were not studied. However, the analysis of gene sequences and structures of zebrafish IFN-R complexes such as CRFB5 chain associated with either CRFB1 or CRFB2, revealed important differences with mammalian IFNAR and closer homology with IFN-λR (68, 69). IFN-φ was proposed as a new nomenclature for fish IFN to close the debate (70). The regulation of CRFB1 by Cav1β seems therefore less relevant to human IFNAR for which no strong evidence of a role of caveolae and/or caveolin was brought so far.

By using super-resolution microscopy restricted to the PM, a study revealed that overexpressed IFNAR1 and IFNAR2 were partially co-clustered in nanoscale domains (71). These clusters

were weakly co-localized with clathrin, the structural core protein of endocytic clathrin-coated pits, but frequently found in the vicinity of actin structures. On the contrary, a new study by the same team based on quantitative ligand-binding with fluorescently-labeled IFNs described the presence of continuously diffusing IFNAR in a random and non-clustered distribution at the PM (20). This apparent discrepancy could result from the high density of receptors caused by their overexpression, which could artificially induce IFNAR1/IFNAR2 co-clustering at steady state (72). Nevertheless, the confinement of the receptor chains at physiological density (5–10 chains per μm^2) in the cortical actin meshwork was confirmed by single quantum dot tracking and localization microscopy (73). In this study, the longer lifetime of the IFN-α2 induced ternary complex measured in cells in comparison with artificial membranes *in vitro* was attributed to its stabilization through an active nano-confinement in 70 nm cortical actin sub-domains organizing larger domains of 300 nm. Disrupting these domains decreased the stability of the ternary complex and JAK/STAT downstream signaling. In summary, these studies indicate that IFNAR chains are confined in actin-dependent nanodomains at the PM. It is likely that the continuous technological developments in super-resolution microscopy will reveal new features of IFNAR nanoscale partitioning and functions at the PM that could not be detected with conventional fluorescence microscopy.

THE BASIC INTERFERON—TYPE I INTERFERON RECEPTOR COMPLEX

Binding of IFN to IFNAR2 is followed by IFNAR1 association with the IFN/IFNAR2 subcomplex (**Figure 1C**). IFNAR1 binding is associated with a major conformational change involving movement within the four extracellular SD domains, resulting in the efficient capping of IFN molecule (74, 75). IFN binding has also been shown to reduce the force needed to unfold the IFNAR1 extracellular domains (76). The reduction in IFNAR1 rigidity would enable the propagation of IFN-induced conformational changes closer to or even across the PM. Interestingly, comparison of the ternary complexes formed by the two IFNAR1 and IFNAR2 receptor chains with IFN-α or IFN-β by single-particle EM analysis could not reveal any difference (74). Likewise, no difference could be detected regarding the IFNAR1 residues involved in the interaction with IFN-α or IFN-β. For instance, IFN-β maintains the same overall fold, and shares the same binding interface with IFNAR1 and IFNAR2, similarly to IFN-α2 (10). Interestingly, it was observed that among the IFN subtypes, increased binding affinities are correlated with a higher rate of IFN-IFNAR endocytosis (75). This faster entry within the endosomal system has been proposed to lead to earlier signal triggering but also to rapid termination. Thus, it was proposed that it could explain, at least partially, some of the signaling differences, such as anti-proliferative activities, observed between IFN-α, IFN-β, and IFN-ω.

Upon formation of the ternary complex, JAK1 and TYK2 are both brought in close contact, which leads to a repositioning of their respective pseudokinase domain, thereby relieving the self-inhibition by the JH1/JH2 domains (77). While the exact underlying molecular mechanism remains debated, the first activating events would involve the concomitant tyrosine phosphorylation of JAK1 and TYK2 on Tyr^{1022}-Tyr^{1023} and Tyr^{1054}-Tyr^{1055} residues, respectively (**Figure 1C**) (78).

Following JAK activation, several other post-translational modifications within the receptor complex lead to the recruitment and regulation of new partners, to the internalization of the complex and also to the priming of the IFN-induced signaling cascade. Several of these steps have been studied but there is still a lot of mechanisms of this finely tuned pathway that need further investigation.

Upon IFN stimulation, the serine/threonine kinase PKD2 is recruited to IFNAR1. PKD2 is then TYK2 phosphorylated on Tyr^{438} (79) and activated PKD2 can in turn phosphorylate IFNAR1 on Ser^{535} and Ser^{539} residues within the ^{534}DSGNYS^{539} phosphodegron—also called destruction motif (**Figure 1C**) (80). The $SCF^{\beta Trcp}$ (Skp1-Cullin1-F-box complex) E3 ubiquitin ligase binds to the destruction motif of IFNAR1 and adds polyubiquitin chains on lysine residues 501, 525, and 526 (80, 81). The ubiquitination process plays an import role in the internalization of the IFNAR complex since interfering with either Ser^{535} phosphorylation, $SCF^{\beta Trcp}$ recruitment or polyubiquitination inhibits endocytosis (82). TYK2 kinase activity is essential for IFNAR1 Ser^{535} phosphorylation, which in turn is required for IFNAR1 ubiquitination-dependent endocytosis. It is therefore expected that IFNAR1 Ser^{535} phosphorylation and ubiquitination should represent the very first steps that follow the reunion of the two chains of the receptor initiated by IFN binding.

BEGINNING OF THE JOURNEY: EN ROUTE TO THE ENDOSOME

IFNAR, like most transmembrane signaling receptors, is endocytosed by clathrin mediated endocytosis (CME). Earlier EM studies localized IFN-α (65) and IFN-β (66) in clathrin coated pits (CCP). The role of CME in IFNAR uptake was definitely established by the finding that IFNAR uptake required key elements of the clathrin-dependent endocytosis machinery (**Figure 2**) including clathrin heavy chain, the α2 adaptin protein-2 (AP2) complex, the GTPase dynamin and Eps15 (53, 82, 83).

CME is initiated at the PM by the recruitment of transmembrane receptors to the clathrin machinery thanks to the interaction of the AP2 adaptor complex with a tyrosine-based linear endocytic motif YXXΦ that is found in the receptor cytoplasmic tail (7). Although this endocytic motif is present in many transmembrane receptors, very few examples have documented a direct interaction with AP2 (5, 6, 84). It is therefore worth noting that AP50, the μ2 subunit of AP2, was shown to recognize ^{466}YVFF^{469} domain in IFNAR1 (**Figure 1A**), a canonical tyrosine-based linear endocytic motif. At steady state, the interaction between IFNAR1 and AP50 is prevented

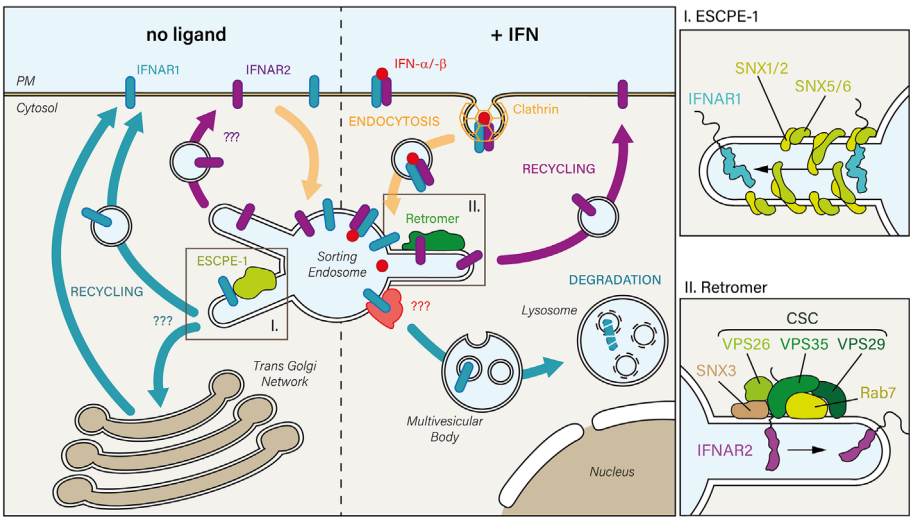

FIGURE 2 | In the absence of IFN (left), constitutively internalized IFNAR1 and IFNAR2 subunits may recycle from the sorting endosome to the PM. While the mechanism is unknown for IFNAR2, IFNAR1 is sorted back to the PM through its interaction with the endosomal sorting nexin (SNX)-BAR sorting complex for promoting exit 1 (ESCPE-1). ESCPE-1 complex (top right inset) is composed of SNX heterodimers formed by SNX1 or SNX2 that are associated with SNX5 or SNX6. Whether IFNAR1 transits through the trans-Golgi Network during its recycling to the PM has not been investigated yet. Upon IFN stimulation (right), the IFN-bound receptor complex is endocytosed *via* clathrin-coated pits. Upon arrival to the sorting endosome, IFNAR receptor complex is dissociated: 1) IFNAR1 is addressed to the lysosomal degradative pathway, first through multivesicular bodies, then in lysosomes where it is fully degraded, and 2) IFNAR2 interacts with the endosomal retromer complex which controls its recycling to the PM. The retromer (bottom right inset) is formed by the cargo-selective-complex (CSC), a trimer composed of vacuolar protein sorting-associated protein 35 (VPS35)-VPS29-VPS26 proteins, associated with the nexin SNX3 and the small GTPase Rab 7.

by the physical masking of ^{466}YVFF^{469} by TYK2 (36). The endocytic motif is in close proximity to the TYK2 minimal interaction domain (486-511) and is inserted in the TYK2 maximal interaction domain (465-511) (32). It was shown that IFNAR1 stability at the PM is reduced in the absence of TYK2, probably by allowing at steady state the interaction of the otherwise masked endocytic motif with AP50 and thus the internalization of IFNAR1 (34–36). Upon ligand binding, one can speculate that the endocytic motif would be unmasked by TYK2 as a result of the various conformational changes associated with IFNAR chains complexes and JAK rearrangement and activation.

The ^{466}YVFF^{469} motif is highly conserved within species except for the mouse (36), which could explain why the stability of murine IFNAR1 at the PM was not affected by the absence of TYK2 (85–87). This does not explain however the mechanism by which the AP2 complex would be recruited to the murine receptor. Other sequences such as di-leucine and iso-leucine can also be recognized by clathrin adaptors subunits (84). Indeed, leucine-based motifs are found in the mouse but also in the human IFNAR1. While it is likely that these sequences can also modulate the uptake of IFNAR, no study has so far documented their function. It is also possible that the endocytosis of murine IFNAR depends exclusively on IFNAR1 ubiquitination since the S526A ubiquitination deficient mutant, which is unable to interact with SCF$^{\beta Trcp}$, shows a significant reduced internalization (80). Finally, one cannot rule out a participation of several of these mechanisms in a process as finely tuned as CME.

If the importance of IFNAR1 Tyr^{466} in clathrin-dependent endocytosis is clear, the role of phosphorylated Tyr^{466} is more obscure. Phosphorylation of the tyrosine motif YXXΦ is likely to inhibit AP50 recruitment, as the negatively charged phosphate group would prevent the endocytic motif to fit into AP50 binding pocket (88, 89). The fact that IFNAR1 Tyr^{466} phosphorylation occurs as early as 5 min after IFN-α stimulation (29) questions the chronology of these events within the time range of internalization and signaling. That AP50 cannot interact with the phosphorylated (pY)XXΦ motif (90) infers that IFNAR1 Tyr^{466} should not be phosphorylated during the recruitment of the endocytic machinery. It was suggested that Tyr^{466} was phosphorylated immediately after ligand binding, and then dephosphorylated by the PTP1B phosphatase, thereby allowing the interaction with AP50 (91). This phosphorylation/dephosphorylation cycle is, however, unlikely if one considers that IFNAR1 is endocytosed very rapidly after IFN stimulation whereas IFNAR1 Tyr^{466} phosphorylation can still be detected by western blotting at longer time points (92). Although experimental evidence is yet lacking, it seems more likely that IFNAR1 Tyr^{466} phosphorylation occurs after AP50 binding. Whether it happens at the PM just after AP50 recruitment to the IFNAR1 or later after IFNAR complex endocytosis is unknown. In this context, it was shown that AP50 binding to the EGFR, that occurs only when the YXXΦ motif is not phosphorylated, was still maintained after phosphorylation of the YXXΦ motif (90).

The formation of the IFNAR complex allows the activation and recruitment of additional regulating partners such as

phosphatases (SHP1, SHP2, PTP1B, TCPTP, CD45) or SH2 domain-containing proteins (SOCS, LNK) [reviewed in (77, 78)]. Whether the recruitment of these effectors takes place at the PM or later along the endocytic route is a question that is difficult to tackle because of the intrinsic rapidity of the endocytic process. New approaches such as the functionalization of micropatterned surface with Halo ligands that retain IFN-IFNAR complex at the PM will help to better understand these processes (39).

During acute brain infection by the simian immunodeficiency virus (SIV), IFN-α signaling is drastically hampered with a down-regulation of downstream effectors such as TYK2, STAT1 and IRF7, in contrast to IFN-β signaling that remains fully active (93). A follow-up study using SIV as a model of infection in macrophages, revealed that the inhibition of IFN-α signaling in the central nervous system was triggered by CCL2, a chemokine secreted by astrocytes (94). Interestingly, confocal microscopy revealed in leukocytes that β-arrestin 2 was recruited to endomembranes positive for CCR2B, the CCL2 receptor, upon CCL2 treatment (95). β-arrestins (1 and 2) play a central role in GPCR desensitization, internalization and intracellular trafficking. They act as adapters that build a bridge between activated i.e. phosphorylated GPCRs and the two main components of the clathrin-dependent endocytic machinery, AP-2, and clathrin (96–98). In support of this, it was recently reported that silencing RNA against β-arrestin 2, but not β-arrestin 1, restored IFN-α levels of SIV infected macrophages in a CCL2/CCR2-dependent manner (99). They further showed that β-arrestin2 was required for IFNAR1 internalization, in agreement with the first implication of AP2 in IFNAR1 endocytosis (83).

Type I Interferon Receptor Endocytosis Is Mandatory for JAK/STAT Signaling

Although JAK/STAT signaling has a pivotal role in key cellular processes, the underlying molecular mechanisms controlling its activation by IFNAR and the determination of IFN signal specificity have remained poorly understood (100–104). In 2006, in line with the original studies establishing the role of endocytosis in the regulation of receptor signaling, the Lamaze group revealed for the first time that IFNAR endocytosis was required to trigger JAK/STAT signaling downstream of IFN-α stimulation (83) (**Figure 3**). Blocking IFNAR CME with the dominant negative mutant DynK44A or a siRNA against clathrin heavy chain strongly decreased the level of STAT1 and STAT2 phosphorylation induced by IFN-α but not by IFN-γ. Accordingly, it was later shown that IFN-γ receptor subunits need to be associated with specific cholesterol/sphingolipid enriched PM nanodomains for JAK/STAT activation IFN-γ, independently from receptor endocytosis (55). The essential role of CME in JAK/STAT signaling was further confirmed a year later in Drosophila with the receptor Domeless (Dome) whose internalization is also required to transduce JAK/STAT signaling, (105). Like IFNAR1 and IFNAR2, Dome presents fibronectin type-III extracellular domains (106) and a conserved di-leucine motif in its cytosolic tail (107). These data emphasize that the control of JAK/STAT signaling by receptor endocytosis is a conserved mechanism among species.

SORTING ENDOSOME: THE PLACE TO BE

Today, the central role of the endosomal network in membrane trafficking is not questioned. Endosomes have been classically divided into early and late endosomes to reflect the chronology of cargo delivery. Early endosomes represent the first intracellular station downstream of endocytosis at the PM, where receptors are sorted from ligands. The central function of early endosomes in cargo sorting led to rename them as sorting endosomes [for review see (108, 109)]. In the late 1990s, two seminal studies simultaneously revealed that the sorting endosome could serve as

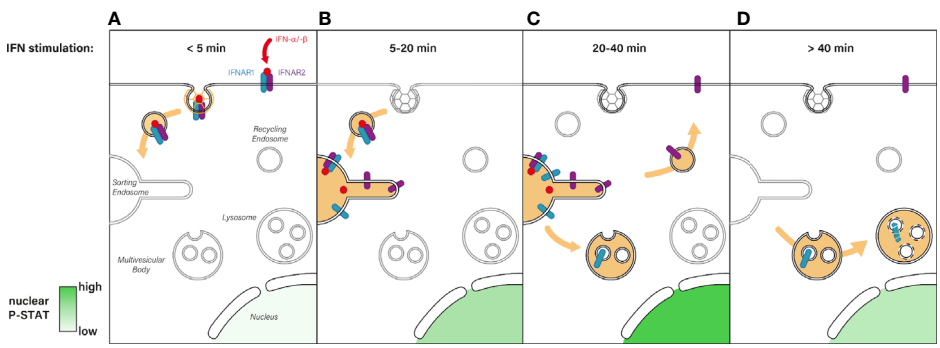

FIGURE 3 | **(A)** Immediately after type I IFN binding the IFNAR1-IFNAR2 receptor complex is actively endocytosed from the PM and addressed to the endosomal network. **(B)** Five to 20 min after IFN stimulation, the IFN-IFNAR complex arrives at the sorting endosome where JAK/STAT signaling is triggered, which results in P-STAT nuclear translocation. **(C)** For the following 20 min, IFNAR signaling is maintained at the endosome where it reaches its maximum (highest P-STAT1 level). The subsequent sorting of IFNAR1 to multivesicular bodies results in signaling termination whereas IFNAR2 is recycled to the PM. **(D)** After 40 min of IFN stimulation, IFNAR1 is degraded through the lysosomal pathway whereas IFNAR2 is ready for a new round of IFN binding at the PM. Nuclear levels of P-STAT return to pre-stimulation levels.

a relay where receptor signaling could be controlled. Thus, nerve growth factor (NGF) and its receptor $gp140^{TrkA}$ (TrkA) were rapidly endocytosed by CME into early endosomes where TrkA -bound NGF could activate TrkA resulting in TrkA tyrosine phosphorylation and in the binding to the downstream effector PLC-γ1 (110). At the same time, another study took advantage of the first molecular possibility to selectively inhibit clathrin-dependent endocytosis using a dominant negative mutant of dynamin, the GTPase required for the scission of clathrin-coated pits from the PM (111). Inhibition of EGF receptor (EGFR) endocytosis with the dynamin mutant revealed that in addition to attenuate EGFR signaling, endocytosis was also required to deliver activated EGFR in early endosomes where EGF specific signaling pathways could be either activated or terminated (112). These pioneering studies established the essential role that endosomes could play in the control of signal transduction. This groundbreaking work has been followed by numerous studies that contributed to definitely upgrade the early endosome from a sorting organelle to an active signaling hub and to establish the new concept of the *signaling endosome*. The signaling endosome has since been extensively investigated and became a major center of interest in the understanding of the cell biology of signaling receptor trafficking and signaling as described in many reviews (113–118).

The requirement for IFNAR endocytosis in JAK/STAT signaling implies that endosomal sorting machineries should be at work in this process. Recent studies have indeed uncovered the role of a major endosomal sorting machineries in IFNAR trafficking and signaling, namely the endosomal sorting complex required for transport (ESCRT). ESCRT has a central position as the major complex mediating the entry of ubiquitinated cargos into the lysosomal degradation pathway (119, 120). The ESCRT machinery consists of four protein complexes: ESCRT-0, -I, -II, and -III, and include several accessory components that are highly conserved from yeast to human. Over the past decade, structural and biochemical studies have uncovered the sequential process by which ESCRT assembly occurs [reviewed in (121–123)]. ESCRT-0 is tethered on the endosomal membrane where it recruits ubiquitinated cargos and interacts with ESCRT-I. ESCRT-0 is composed of two subunits: hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which binds to the ubiquitinated substrate, and signal-transducing adaptor molecule (STAM) (124–127). Hrs and its partner STAM, both ubiquitously expressed, play a vital role in biology and development as shown by the death of Hrs deficient mice *in utero* (128–130). Hrs is recruited to the early endosome where it binds to endosomal specific PI(3)P phosphoinositide through its FYVE-domain and initiates the formation of intraluminal vesicles (ILV) found inside multivesicular bodies (MVB) (123).

During the last decades, our understanding of the cellular functions of ESCRT have evolved from a sequential process controlling cargo entry into the lysosomal degradation pathway to more diverse biological activities such as virus budding, membrane repair, cytokinesis, regulation of gene transcription, autophagy, quality control of nuclear pore complexes (131–137).

Hrs/STAM for Type I Interferon Receptor Sorting: Insights From *Drosophila*

Following the first report of EGFR signaling control by clathrin-dependent endocytosis (112), efforts were directed at elucidating the molecular mechanisms underpinning EGFR endosomal sorting and signaling. Thus, EGFR sorting toward the lysosomal degradation pathway was found to require Hrs recognition of EGF-induced ubiquitinated EGFR (113, 138–142). Before arrival to the lysosome, ubiquitinated EGFR accumulate into ILVs of MVBs (143). This physically removes the signaling tail of EGFR from the cytosol, effectively terminating the downstream signaling cascade (144). In this context, cells depleted for the *Hrs* gene revealed that Hrs negatively controls EGFR signaling in Drosophila embryogenesis with an increase of EGFR signaling activity and an accumulation of ubiquitinated cargos in enlarged endosomes, including EGFR and other activated signaling receptors such as PDGF/VEGF receptors, or Notch (105, 145–147).

Based on the prototypical example of EGFR sorting and signaling control by Hrs, numerous studies have addressed the role of Hrs in receptor signaling especially for GPCRs. Thus, the β_2-adrenergic receptor (β_2AR) and the δ-opioid neuropeptide receptor (DOR), are two examples of GPCRs that are regulated by Hrs. Both receptors are ubiquitinated on lysine residues in the carboxyl terminal tail and are endocytosed in a clathrin- and β-arrestin-dependent manner (148–155). Similarly to EGFR, DOR sorting toward the lysosomal degradation pathway requires Hrs (156). Hrs has a different effect on β_2AR since it mediates its recycling to the PM, a process associated with β_2AR resensitization (157). Hrs-dependent recycling was shown to rely on an acidic di-leucine motif present in the C-terminal tail of the β_2AR but not on ubiquitination (158, 159). Hrs and STAM were also required for efficient fibroblast growth factor receptor (FGFR) endosomal sorting and signaling in Drosophila. This study revealed an opposite role of Hrs and STAM depending on the location of the complex since EGFR signaling was downregulated by Hrs and STAM in the embryo but fully activated during wing development (147).

The role of Hrs in IFNAR trafficking and signaling had not been investigated until recently. A first evidence came from studies in Drosophila where the disruption of Hrs in egg chambers transiently expressing Dome receptor led to an inhibition of STAT activation upon Dome stimulation (105). A recent study showed the constitutive association of STAM2, without Hrs, with IFNAR1 and TYK2 at the PM preventing TYK2 activation by IFN (160). IFN-α induced receptor endocytosis delivers the STAM-IFNAR1-TYK2 complex to early endosomes positive for PI(3)P where Hrs interaction abolishes STAM inhibitory effect and triggers IFNAR endosomal signaling. In contrast, IFN-β stimulation results in IFNAR sorting to a distinct endosomal subdomain where endosomal JAK/STAT signaling occurs independently from Hrs. This study put into question the classical dogma stating that Hrs and STAM are always constitutively associated on the endosomal membrane to form the ESCRT-0 complex. Indeed, a few studies suggest that this assumption may suffer some

exceptions. Thus, a truncated mutant of STAM lacking the coiled-coil domain of interaction with Hrs was reported to promote the relocation of Hrs in the cytoplasm (161). Hrs was reported to be targeted to early endosomes independently of STAM, and STAM was also localized at the PM in HeLa cells (162). In line with this study, a recombinant Hrs purified in the absence of STAM could be detected on membranes as hexamers (163).

The role of Hrs/STAM in cytokine signaling is not exclusive to JAK/STAT signaling by the IFNAR complex. Indeed, more than fifty cytokines can signal *via* the JAK/STAT pathway (164). Among them, IL-4 signals through both the type I receptor consisting of the IL-4Rα and the common gamma chain (γC), and the type II receptor composed of IL-4Rα and IL-13Rα1 (164). IL-2 activates JAK/STAT signaling downstream of the trimeric IL-2R composed of α, β, and γC chains (165). Hrs controls IL-2R and IL-4R signaling albeit through its classical regulatory function as it is required for IL-4Rα and IL-2Rβ endosomal sorting toward the degradation pathway, resulting in receptor cell surface downregulation and signaling termination. Thus, in contrast to IFNAR1, the binding of Hrs on IL-4Rα and IL-2Rβ is not required for endosomal signaling, (166, 167). Finally, as reported above for GPCRs, Hrs can regulate IL-2Rα recycling to the PM by binding to a hydrophobic amino acid cluster in an ubiquitin-independent manner (167). The absence of such motifs in the C-terminal tail of IFNAR1 and IFNAR2 may explain why Hrs is not involved in IFNAR recycling (see below).

ENDOSOMAL EXIT: CHOOSE YOUR DESTINY

After internalization and arrival in early endosomes, cargos are classically sorted to three possible destinations: (1) fast recycling to the cell surface *via* the endosomal recycling pathway, (2) recycling to the PM through the retromer or retriever complexes or *via* the TGN (retrograde recycling), and (3) degradation in lysosomes. Although these three main routes have been investigated for several signaling membrane receptors, mainly EGFR, IL2-R, and growth hormone receptor (GHR), the intracellular fate of the IFNAR receptor complex upon its endocytosis has long remained mysterious.

Type I Interferon Receptor Recycling to the Plasma Membrane

In contrast to IFNAR1, whose final fate is degradation in the lysosome (see below), IFNAR2, which is not ubiquitinated, takes a different path. IFNAR2 intracellular trafficking has long remained poorly characterized. A first study measured unchanged IFNAR2 levels at the PM during IFN-α stimulation while IFNAR1 was efficiently degraded (168). These data suggested that after IFNAR endocytosis, IFNAR2 was probably recycled back to the PM by unknown mechanisms.

The retromer complex plays a central role in both the retrograde transport of endosomal proteins to the TGN and in the endosomal recycling of cargos to the PM (169–171). The retromer complex is assembled by a first sub-complex called cargo-selective-complex (CSC), a trimer made of vacuolar protein sorting-associated protein 35 (VPS35)-VPS29-VPS26 proteins, that are highly conserved among species (169, 172, 173). The CSC binds PI(3)P, an early endosome specific phosphoinositide, through the Phox-homology (PX) domain present in sorting nexins (SNX), which together with the small GTPase Rab7a, assembles the second sub-complex of the retromer (174, 175). In addition to membrane tethering, SNX bend and remodel the endosomal membrane to create recycling tubules for cargo trafficking [reviewed in (176)]. Cargo selection is mediated through the FERM-like domain of SNX17 whereas SNX27 interacts with cargos *via* its PDZ domain. Recently, SNX17 has been shown to interact with another endosomal sorting complex called the retriever, a heterotrimer harboring similarities with the retromer. The retriever and SNX17 can associate with other complexes to prevent cargo lysosomal degradation and to promote cell surface recycling (177).

Upon IFN-α stimulation, IFNAR2, in contrast to IFNAR1, could not be colocalized with LAMP1, a *bona fide* marker of late endosomes and lysosomes (21). Instead, IFNAR2 was accumulated in early endosomes in the absence of Rab11A or Rab4, two GTPases involved in cargo recycling to the PM. These findings therefore suggest that IFNAR2 is not directed towards the lysosomal degradation pathway but recycled back to the PM. Indeed, mass spectrometry analysis revealed that upon IFN-α stimulation, IFNAR2 could interact with Rab11A and Rab4. IFNAR2 could also interact with Rab35 and VPS26A, VPS29 and VPS35 – the components of the retromer cargo-recognition trimer (**Figure 2**). Accordingly, under IFN stimulation, cells depleted of VPS35 accumulated IFNAR2 in early endosomes together with a decreased amount of IFNAR1 sorted for lysosomal degradation. The same phenotype was observed in the absence of IFN stimulation, indicating that IFNAR is sorted by the retromer complex under basal and stimulated conditions. In agreement with the interaction of the Rab7 GTPase with the retromer complex (175), Rab7 depletion led to the same phenotype than Vps35 depletion. These data agree with a previous study showing an accumulation of IFNAR1 in early endosomes in Rab7A depleted cells (178). Although the retromer complex has been involved in the retrograde trafficking of mannose-6-phosphate (179), sortilin-related (180) and Wnt receptors (181) from endosomes to the TGN, the possibility of IFNAR2 retrograde trafficking to the TGN by the retromer complex was ruled out (21).

A recent proteomic study identified IFNAR1 as a possible cargo recognized by SNX5 nexin through binding to ^{466}YVFFP^{470}, a consensus ΦxΩxΦ recycling motif (182). Interestingly, the SNX5-binding motif covers the endocytic motif ^{466}YVFF^{469} described before (36). The endosomal SNX-BAR sorting complex for promoting exit 1 (ESCPE-1), identified in this study, allows to couple cargo recognition with SNX-mediated biogenesis of tubulo-vesicular transport carriers that

recycle cargo to the PM or send them to the TGN (182). Whether this new recycling pathway mediates IFNAR1 basal recycling to the PM will need further investigation (**Figure 2**).

Degradation of Type I Interferon Receptor 1 in Lysosomes

Early studies performed with human radiolabelled ^{125}I-IFN-α led to the first report of the degradation of IFN-α after IFNAR endocytosis, probably in lysosomes since it was blocked by chloroquine, a lysosomotropic agent (65, 183). IFNAR1 degradation was first described after IFN-α stimulation of Daudi cells (19). The lysosomal proteolysis of IFNAR1, excluding proteosomal degradation, was later established with lysosome inhibitors (80, 184). Several IFN-induced post-translational modifications in the cytosolic tail of IFNAR1 are necessary for its targeting to lysosomes. Upon IFN stimulation, the ubiquitination of IFNAR1 proximal lysine residues adds K48 and K63 linkages which are known to sort polyubiquitinated cargos to the proteasome or lysosomes for degradation (**Figure 2**) (81, 185, 186).

IFNAR1 ubiquitination occurs probably mainly at the PM after IFN binding, but could also take place at the endosomal level as described for GHR (187). This study showed that $SCF^{\beta\text{-}Trcp}$ was active at the cell surface and in endosomes. Silencing $SCF^{\beta\text{-}Trcp}$ or deleting the GHR ubiquitin-dependent endocytosis motif forced GHR recycling from endosomes to the PM, indicating that GHR sorting to lysosomes depends on an active ubiquitin system. More recently, several studies revealed that ubiquitinated receptors can be sorted to ILVs independently from ESCRT as shown for GPCR with ALG-2-interacting Protein X (ALIX) or EGFR, PDGFR and α5β1 integrin with the histidine domain phosphotyrosine phosphatase (HD-PTP) [review in (188)]. The precise molecular mechanism mediating IFNAR1 lysosomal degradation will require further investigations. Whether ALIX and HD-PTP are part of IFNAR1 sorting machinery are still open questions.

Type I Interferon Receptor Complex Dissociation and Signaling Termination

Whether and how the retromer complex contributes to the regulation of intracellular signaling are still poorly understood. How ligand-induced receptor phosphorylation can influence decisions between recycling versus degradation and signaling was first described for β_2AR and EGFR. EGFR activation by TGFα led to sustained MAPK activation and retrieval/recycling of the receptor, whereas activation by EGF induces fast receptor degradation and a transient MAPK activation (189). The SNX27-retromer retrieval subdomain allows to terminate G protein-coupled parathyroid hormone receptor signaling, a key regulation as shown by the deleterious effect of its constitutive activation on bone formation observed in *Snx27* deficient mice (190).

By controlling the residency time of internalized IFNAR complex in the endosome, the retromer is directly implicated in the fine tuning of JAK-STAT signaling duration and downstream transcription outputs (**Figure 3**). Indeed, a significant upregulation of genes known to be dependent on IFN stimulation was observed in VPS35 depleted cells upon IFN-α/-β activation, suggesting an aberrant prolonged activation of the JAK/STAT pathway (21). Thus, this study establishes a direct link between retromer-mediated sorting and modulation of intracellular signaling and gene transcription. In this context, a recent study proposed that the long-term effects of type I IFN could be explained by the persistence of receptor bound IFN-α2 inside endosomes. Endosomal IFN could even continue to signal from this compartment for days when the IFN-IFNAR complex negative regulators ISG15 or USP18 were missing (191). The endosome is therefore a crucial sorting station where a concerted choreography between Hrs/STAM and retromer complexes sequentially control the initiation and the termination of IFN-induced JAK/STAT signaling.

DISCUSSION

Since their discovery more than 60 years ago, numerous studies have tried to unravel the mechanisms underlying the signaling activity of IFNs and their cognate IFNAR receptor. Until recently, these studies have mainly focused on the initiation of JAK/STAT signaling at the plasma membrane in a linear manner. IFNAR membrane trafficking has been much less studied and all the less so when it comes to understand the role of the intracellular journey of IFNAR and its signaling. In agreement with the dogma that has long prevailed for transmembrane receptors, IFNAR trafficking was seen as a simple way to terminate signaling by passively removing receptors from the PM away from IFNs. This simplistic picture has recently changed with the demonstration that IFNAR trafficking is tightly associated with the control of JAK/STAT signaling. Nevertheless, the characterization of IFNAR trafficking has only recently begun and further studies are clearly needed to better understand the role of each trafficking step in the final IFN signaling response.

While most studies have addressed these mechanisms using IFN-α2 as a ligand, how IFN-β can transduce distinct activities remains a challenging and unresolved question. This is also the case for the other human type I IFNs including the twelve subtypes of IFN-α, IFN-ϵ, IFN-κ, and IFN-ω. Their distinct structures and IFNAR binding affinities may be translated into a selective modulation of IFNAR trafficking characteristics that it would be interesting to relate to their specific activities. Distinct IFN affinities could determine distinct IFNAR endocytosis rate and potentially control when signal is terminated at the endosomal level. Modulations of these parameters would eventually adjust the signal duration for each IFN subtype. Therefore, the regulation of trafficking events may add another level of complexity and control of the IFN stimulation outcomes.

The importance of better understanding IFNAR trafficking is not restricted to IFNAR as it will probably establish new paradigms in the control of signaling by trafficking for cytokine and transmembrane receptors beyond the prototypical EGFR and GPCRs. The extremely dynamic nature of the endosomal network and the rapid movement of vesicles through the cytosol, make it challenging to follow receptors during their journey in live cells. In particular, it is not known whether IFN receptor complexes made

from different IFN subtypes would be found in common or separate endosomal compartments. However, the recent and continuous improvements in live cell imaging such as super resolution microscopy and AI-based segmentation approaches will enable us to make substantial progress in the near future. No, the journey does not end here.

AUTHOR CONTRIBUTIONS

NZ, CV, CL, and CB wrote the review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Type I Interferon (IFN)-Regulated Activation of Canonical and Non-Canonical Signaling Pathways

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For several decades there has been accumulating evidence implicating type I interferons (IFNs) as key elements of the immune response. Therapeutic approaches incorporating different recombinant type I IFN proteins have been successfully employed to treat a diverse group of diseases with significant and positive outcomes. The biological activities of type I IFNs are consequences of signaling events occurring in the cytoplasm and nucleus of cells. Biochemical events involving JAK/STAT proteins that control transcriptional activation of IFN-stimulated genes (ISGs) were the first to be identified and are referred to as "canonical" signaling. Subsequent identification of JAK/STAT-independent signaling pathways, critical for ISG transcription and/or mRNA translation, are denoted as "non-canonical" or "non-classical" pathways. In this review, we summarize these signaling cascades and discuss recent developments in the field, specifically as they relate to the biological and clinical implications of engagement of both canonical and non-canonical pathways.

Keywords: interferon, signaling, MAP kinase signaling, signal transducer and activator of transcription, mammalian target of rapamycin, mRNA translation, SARS-CoV-2, COVID-19

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INTRODUCTION

Established cellular signaling pathways have been referred to in the context of canonical or "classical" and non-canonical or "non-classical" signaling cascades that control distinct outcomes in the cell. A canonical pathway indicates the conventional protein signaling, typically considered the main effect or, maybe more appropriately, the first effect discovered and elucidated; non-canonical pathways are alternative pathways to the canonical, but that should not imply less importance (1). Perhaps, the most well-described signaling in terms of canonical and non-canonical pathways is Wnt signaling, specifically the canonical β-catenin pathway (2). Additionally, inflammation and immunoregulatory related pathways such as nuclear factor-κB (NF-κB) and interferon (IFN) signaling are described as canonical and non-canonical (3, 4). Recent discoveries of additional non-canonical pathways, some that interconnect with canonical signaling, add to the complexity surrounding different biological outcomes.

The IFNs are cytokines that can be divided into three groups: type I (IFNα, IFNβ, IFNδ, IFNϵ, IFNκ, IFNτ, IFNω, and IFNζ), type II (IFNγ), and type III (IFNλ) (5). Type I IFNs were first discovered in 1957, followed by type II in 1965, while much more recently, in 2003, type III IFNs were identified (6–8). Type I IFNs have the most family members. The predominant type I IFN subtypes studied are IFNα and IFNβ, partially due to IFNδ, IFNτ, and IFNζ not having human homologs, more specific cellular sources of IFNϵ and IFNκ, mainly female reproductive organs and keratinocytes, respectively, and IFNω being studied more in felines (5, 9, 10). The roles of IFNα and IFNβ in antiviral responses have been most reported, but these type I IFNs also have significant relevance in cancer and autoimmune diseases (11–13).

Production of type I IFNs is induced by pathogen-associated molecular patterns, viral RNA or DNA fragments, and is associated with activation of pattern recognition receptors (11). Once activated, the receptors initiate signal transduction that involves adapter proteins, eventually leading to activation and translocation of IFN regulatory factor 3 (IRF3) and NF-κB, which promote type I IFN production either directly or indirectly through IRF7 (11). IFNα is mainly produced by plasmacytoid dendritic cells (pDCs), whereas IFNβ is ubiquitously produced by immune cells (13).

Following transcriptional activation and mRNA translation, type I IFNs are secreted from immune cells and, on neighboring cells, bind to the two cellular receptor subunits IFNα receptor 1 (IFNAR1) and IFNAR2, which are associated with tyrosine kinases TYK2 and Janus kinase 1 (JAK1), respectively (9). Dimerization of the receptor initiates the autophosphorylation of JAK1, which phosphorylates and activates signal transducers and activators of transcription 1 (STAT1) and STAT2 proteins, which form a complex with IRF9, resulting in a well-characterized complex, IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates to the nucleus where it binds to IFN-stimulated response elements (ISREs) in the promoters of genes, leading to transcription of IFN stimulated genes (ISG) (14). Additionally, JAKs can phosphorylate and initiate the formation of phosphorylated STAT complexes of STAT1 and STAT3 homodimers, where the STAT1 homodimer is associated with a pro-inflammatory response, mediated by binding to gamma activated sequences (GAS), and the STAT3 homodimer indirectly inhibits inflammatory gene expression, restraining pro-inflammatory responses (15). These JAK/STAT IFN-signaling pathways are considered the canonical pathways. In addition, type I IFNs have also been reported to activate the formation of STAT2:STAT3 heterodimers and a STAT5:CrkL complex, invoking transcriptional activation of ISGs (16, 17).

Non-canonical type I IFN signaling pathways are similarly activated by IFNs binding to the extracellular regions of the dimeric IFNAR1 and IFNAR2 complex, leading to JAK1/TYK2 activation, but diverge from that point, specifically, not involving STAT activation by the JAKs. Evidence points to the regulation of STATs by non-canonical modifiers, with serine phosphorylation of STATs versus the tyrosine phosphorylation by JAK1/TYK2 (18). The main non-canonical IFN pathways identified thus far are the MAP kinase (MAPK) and phosphoinositide 3-kinases (PI3K)/mammalian target of rapamycin (mTOR) pathways, but there are other non-canonical modifiers such as SIRT2 and the Schlafen (SLFN) family (18, 19). MAPK and PI3K/mTOR pathways have been shown to elicit effects on ISG transcription and mRNA translation while also having some interaction with STATs in the canonical cascade (18). Further discoveries on the effectors of these pathways, such as the importance of Unc-51–like kinase (ULK1) in MAPK type I IFN-induced signaling, add to the complexity of type I IFN signaling cascades and demonstrate that the focus cannot be limited to the classical pathways (20). Other non-canonical modifiers include SLFN family members. Type I IFNs upregulate SLFN gene expression, and SLFN5 interaction with STAT1 has been demonstrated, indicating its effect downstream of JAK1 (21). SLFNs have been shown to be involved in antiviral responses, and their high expression in specific human immune cell subsets has been identified, such as elevated SLFN5 in T cells (22). These non-classical IFN-induced effectors have critical roles in ISG transcription, independent of or in conjunction with the canonical pathway, eliciting specific biological responses. A summary of the canonical and non-canonical pathways of type I interferon signaling is shown in **Figure 1**.

Below we provide an update on type I IFN canonical and non-canonical signaling, related to antiviral responses, antiproliferative effects in cancer, and immune regulation in autoimmune diseases, focusing on studies within the last few years. We address the type I IFN response to SARS-CoV-2 and the potential for therapeutic use for COVID-19.

BIOLOGICAL EFFECTS IN DISEASES

Canonical and Non-canonical IFN Signaling in Malignancies

Type I IFNs have been studied in a wide range of cancers in the last few years, as illustrated in **Table 1**. These studies have focused exclusively on the IFNα and IFNβ subtypes, demonstrating their clinical relevance over other type I IFN subtypes. The signaling analyses in the last few years have still focused more on the JAK/STAT cascades, specifically STAT1 effects in type I IFN signaling. However, some reports explored the impact of STAT3 versus STAT1, as well as the non-canonical involvement of MAPKs, SIRT2, and SLFN5.

STAT1 phosphorylation and the induced expression of various ISGs such as *OASL* and *ISG15* have commonly been used as indicators of a type I IFN response (19, 27, 28, 34, 35). In a study on cervical cancer, the importance of IFN-inducible activation of STAT1 and STAT2 was demonstrated through the use of STAT1 and STAT2 knockout human HeLa cells, yet the STAT3 knockout did not have any effect on ISGs (24). By contrast, in colorectal cancer, inhibition of p-STAT3 but not p-STAT1 decreased IFNα and IFNβ induced granzyme B expression in cytotoxic T lymphocytes (25). These differences highlight how different effectors activated by type I IFNs are dependent on cell type and disease specificity. Bazhin et al. also explored IFN-activated STAT3 effects, identifying a non-canonical

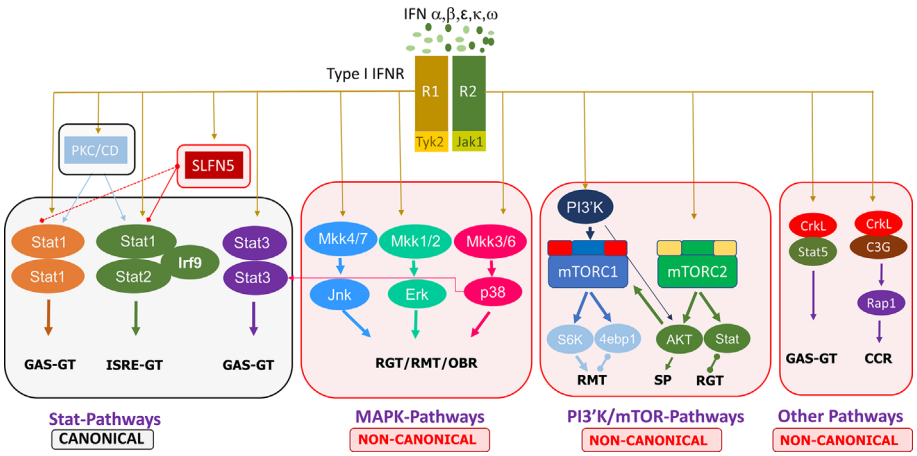

FIGURE 1 | Summary of the canonical and non-canonical pathways involved in type I interferon signaling. 4ebp1, eukaryotic translation initiation factor 4E binding protein 1; CCR, cell cycle regulation; ERK, extracellular signal-regulated kinase; GAS, gamma-activated sequence; GT, gene transcription; IFN, interferon; IFNR, interferon receptor; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; JAK, janus kinase; Jnk, c-Jun N-terminal kinase; MKK, mitogen activated protein kinase kinase; mTORC, mammalian target of rapamycin complex; PI3′K, phosphoinositide 3-kinase; PKC, protein kinase C; CD, Calmodulin-dependent kinase, R1/R2, receptor 1/2; Rap, Ras-related protein; RGT, regulation of gene transcription; RMT, regulation of mRNA translation; OBR, other biological responses; S6K, ribosomal protein S6 kinase; SLFN, Schlafen; STAT, signal transducer and activator of transcription; SP, survival pathways.

interaction with p38 MAPK on STAT3 phosphorylation in mature DCs (31). ULK1 has been identified as a regulator of p38 MAPK and ISGs, downstream of mTOR, in type I IFN signaling in myeloproliferative neoplasms (20). This demonstrates a connection between both major IFN activated non-canonical signaling pathways. Another MAPK, extracellular signal-regulated kinase (ERK), is involved in non-canonical type I IFN signaling in malignancy, where mitogen-activated protein kinase kinase kinase 8 (MAP3K8) and ERK phosphorylation were decreased upon IFNα treatment in bladder cancer cells (23). Further elucidation is needed on the STAT-dependent and -independent non-canonical functions of the many MAPK pathway proteins.

Additional effects of non-canonical type I IFN-induced signaling in various malignancies have been examined. A glioblastoma study identified SLFN5 as a regulator of STAT1 induction by type I IFNs (26). In leukemia and lymphoma cells, type I IFN induced phosphorylation of STAT1 on serine 727 is mediated by cyclin dependent kinase 9 (CDK9), and this activation is dependent on the deacetylation of CDK9 by SIRT2 (19). Additionally, quercetin, a natural compound, decreases Src Homology Phosphatase 2 (SHP2), a negative regulator of STAT1 (27).

An important issue related to the clinical use of IFNs is toxicity and adverse events. Although approved in 1986 by the FDA for the treatment of malignancies and viral disorders, with demonstrated positive disease outcomes, IFNα is currently not commonly used in cancer treatment due to adverse effects (36, 37). A pilot study looked at the potential of decreasing the dose of IFN-α2b for the treatment of melanoma over the course of an 11-month treatment period. Despite the dose reduction, p-STAT1 levels were induced at comparable levels throughout the 11 months, and the IFN was well-tolerated (28). An alternative strategy has been to stimulate the endogenous type I IFN response in immune cells. Tsuchiya et al. genetically engineered induced pluripotent stem cell (iPSC)-derived proliferation myeloid cells (iPSC-pMCs) to produce IFNα. When injected into mice, these IFN-producing iPSC-pMCs exerted immunomodulatory effects analogous to direct type I IFN administration, yet without adverse effects or hematopoietic stem cell exhaustion (37). Brown et al. studied recombinant poliovirus/rhinovirus chimera PVSRIPO effects in cancer immunosuppression and found PVSRIPO infection of DCs increased IFNβ production and a sustained type I IFN response, as indicated by p-STAT1 and ISG induction (*IFIT1*, *ISG15*) (34). In a separate study, the use of photodynamic therapy (PDT) lead to the upregulation of type I IFNs in melanoma cells and DCs co-cultured with the PDT treated cells; the authors proposed this *ex vivo* strategy of stimulating DCs with the use of PDT as a possible immunotherapy (29).

Distinct from the positive outcomes of type I IFN treatment for malignancies, a number of studies have addressed the potential link of IFN treatment with chemotherapy resistance, immunosuppression, and driving of cancer stemness. Qadir et al. found chronic CD95 activation leading to cancer stemness was driven by IFNα/β-STAT1 canonical signaling (32). They also provided evidence that radio-resistant squamous cancer cells had increased p-STAT1 and ISG expression and that type I IFN treatment of breast and squamous cancer cells increased stemness and sphere formation, which was blocked by JAK inhibition, indicative of the involvement of canonical signaling.

Several studies have evaluated the effects of type I IFN administration in combination with immunotherapy. One group showed that IFNα increased programmed

TABLE 1 | Canonical and non-canonical type I interferon signaling in malignancies.

Type of cancer	IFN pathway (canonical or non-canonical)	Type I IFN (related) used/ analyzed	Methods/models	Main results	Ref.
Bladder	Non-canonical—MAP3K8 (TPL2)/ ERK	IFNα	*In vitro* bladder - T24, 5637, HEK293A *In vivo:* T24 or 5637 cells SC into flanks BALB/c (nu/nu*):* mice – IFNα, roflumilast *Clinical:* MIBC tissue microarray chips (n=126) *Bioinformatics:* TGCA & Oncomine	-IFNα decreased COX-2, TPL2, ERK, IKK α/β, & cAMP levels but little effect on JAK/STAT -TPL2 co-IP with IFNAR2 (not IFNAR1), IFNα & TPL2i decreased pTPL2-IFNAR2 -IFNα + roflumilast synergistically suppressed tumor growth, cAMP & PGE2 sera levels in mice	(23)
Cervical	Canonical—JAK/ STAT1, 2, & 3	IFNα2 IFNβ (IFNAR1/2)	*In vitro:* HeLa human cervical cancer cells, KO clones: IFNAR1, IFNAR2, STAT1, STAT2, STAT1 + STAT2 dKO, STAT2 + IRF1 dKO, & STAT3	-KO of IFNAR1 or 2 inhibited p-STATs & ISGs -STAT1 or 2 KOs had low ISG & dKO blocked ISG -STAT3 KO had no effect on ISG, p-STAT1 or 2, or IFNβ induced negative feedback regulators	(24)
Colorectal	Canonical or non-canonical—STAT3	IFNα IFNβ (IFNAR1)	*Bioinformatics:* TCGA dataset *In vitro:* Murine colon carcinoma -MC38 *In vivo:* IFNAR1-KO, IFNAR1-TKO, WT C57BL/6 & SJL mice - MCA or MC38 SC *Clinical:* Peripheral blood - healthy donors SCBC, CRC tissues - GCC	-Tumors grew faster & larger in IFNAR1-KO mice vs WT & in IFNAR1-TKO vs WT -Inhibition of p-STAT3 (not p-STAT1) decreased a granzyme B expression increase by IFNα/β in CTLs	(25)
Glioma	Non-canonical—SLFN5-STAT1	IFNα IFNβ	*Bioinformatics:* GlioVis Database *In vitro:* GBM - LN18, LN229, LN443, U87MG, MBM - DAOY & D556, PDX derived GSC	-SLFN5 expression increased at basal levels & further induced by IFNα or IFNβ in PDX glioma stem cell & established GBM & MBM cells -SLFN5 co-IP'd with STAT1, not STAT3 or 5, in 293T cells & signal increased with IFNβ treatment	(26)
Hepato-cellular	Non-canonical—SHP2/STAT1	IFNα	*In vitro*: hepatocellular - HepG2, Huh7, human embryonic kidney- HEK293A *In silico*: SHP2 & quercetin computational docking	-Quercetin increased IFNα induced p-STAT1 & ISG expression & decreased SHP2 expression in HepG2 -SHP2 overexpression decreased IFNα (+ quercetin) ISRE reporter expression in HepG2	(27)
Leukemia, lymphoma	Non-canonical—SIRT2/CDK9	IFNα IFNβ	*In vitro*: leukemia – HEL, KT-1, lymphoma - U937, Sirt2+/+, Sirt2–/–, Sirt1+/+, Sirt1 –/–, Sirt6+/+, and Sirt6–/– MEF	-Sirt2–/– MEF had no IFNβ induced STAT1 activation or expression of ISG (Oasl2 Cxcl10 ISg15, ISg54) -SIRT2 regulated IFNβ induced CDK9-mediated p-STAT1 -SIRT2 KD leukemia cells less sensitive to IFNα–mediated antiproliferative effect	(19)
Leukemia, lymphoma, myeloma	Non-canonical—ULK1	IFNβ	*In vitro*: leukemia—KT-1, lymphoma—U937, myeloma—U266, Akt1/2+/+, Akt1/2–/–, Ulk1/2+/+ & Ulk1/2–/– MEFs	-IFNβ induced p-ULK1 Ser757 (mTORC1 phospho site) -ULK1/2 KO reduced ISRE & GAS activity & IFNβ induced ISG transcription, p38 activation, & antiproliferative effects	(20)
Melanoma	Canonical—JAK/ STAT1	IFN-α2b	*Clinical*: NCT01460875 – SC IFN-α-2b 3/week 10 MU/M^2—4 weeks, dose reduction every two weeks after first month—total 11 months	–91% of patients had stable or increased p-STAT1 levels over time of dose reduction -ISGs (OAS1 CXCL10, CD69 and SOCS1), not significantly less at end/ with lower IFN-α-2b dose -Higher p-STAT1 after initial dose had lower recurrence	(28)
Melanoma	Canonical—JAK/ STAT1	IFNα IFNβ	*In vitro:* mouse melanoma - B16-OVA; Me-ALA incubation + irr *In vivo*: C57BL/6 and IFNAR1–/– mice; dendritic cells collected from bone marrow	-PDT of melanoma cells increased IFNα/β and apoptosis -PDT increased cGAS receptor (not MDA-5, TLR3, RIG-1), p-STAT1 & ISGs (CXCL10, ISG15, MX1) -WT DCs migrated toward PDT melanoma cells more than IFNAR–/– DCs	(29)
Ovarian	Canonical—JAK/ STAT	(IFNAR1 ISG15),	*In vitro:* ID8-Defb29/Vegf-a mouse ovarian cancer cells - AZA *In vivo:* Pre-treated & ID8-VEGF-Defensin cells IP in C57BL/6 or NSG mice - AZA & anti-IFNAR1 IP	-anti-IFNAR1 inhibited AZA induced anti-tumorigenic response, survival benefit, increase in CD45+ immune cells, activation of CD8+ T and NK cells, & increase in ISG15 in immunocompetent mice but not in NSG mice	(30)
Immune focused	Non-canonical—p38/STAT3	IFNα	*Ex vivo* mDC isolated from PBMCs from human blood (MBDS*):*, DC/T cell co-culture *In vivo:* C57BL/6 mice—IFNα IP	-IFNα upregulates PD-L1 expression on myeloid immune cell & T-cell populations & on DC in mice -IFNα increased p38 and STAT3 activation & STAT3i & p38i (not PI3Ki or ERKi) decreased IFNα induced PD-L1 expression in mDC	(31)
Multiple	Canonical—JAK/ STAT1	IFNα IFNβ	*In vitro*: breast—MCF-7, Hs578T, SK-BR-3, HCC70, T47D, melanoma - MDA-MB-435, Squamous - SCC61, Nu61, MES glioma cells *In vivo*: MCF-7 pre-treated anti-APO-1 or IFNβ injected into fat pad NGS mice	-Long term CD95 stimulation induced type I IFNs, p-STAT1, & increased ISGs in cancer cells -CD95L or type I IFN increased stemness and sphere formation in MCF-7 & SCC61, blocked by JAK1/JAK2i -p-STAT1 correlates with cancer stemness & KO of STAT1 blocked CD95L or type I IFN induced stemness	(32)

(Continued)

TABLE 1 | Continued

Type of cancer	IFN pathway (canonical or non-canonical)	Type I IFN (related) used/ analyzed	Methods/models	Main results	Ref.
Multiple	Canonical—JAK/ STAT1	IFNβ (IFNAR1)	*In vitro* melanoma—B16F10 lung—TC-1, lymphoma—YAC-1, thymoma—EG7 *In vivo:* C57BL/6, Ly5.1þ & IFNAR1–/– mice, LCMV-clone 13 (Cl13),: IP or IV, anti-CD4	-Chronic Cl13 infection lead to elevated IFNβ in sera -STAT1 mRNA higher in NK & protein expression higher in NK & T cells from Cl13-infected mice -Anti-IFNAR1 increased tumor metastasis 20% in Cl13-infected mice	(33)

death-ligand 1 (PD-L1) expression on various immune cells through non-canonical p38/STAT3 signaling (31). The inference is that combining immunotherapy with IFNα treatment would limit the immunosuppressive effects of IFN treatment and permit effective growth inhibition. Similarly, another study provided evidence that IFNα-iPSC-pMC treatment increased PD-L1 mRNA, and when combined with a PD-L1 inhibitor, synergistic anti-tumor effects were reported (37). The poliovirus/rhinovirus type I IFN induced response likewise increases PD-L1 expression (34). Additionally, a bioinformatics examination of IFN gene deletions revealed that homozygous deletion of IFN was significantly associated with non-response to anti-CTLA4 treatment among melanoma patients (38). Overall, these studies suggest type I IFNs may have a critical role in immunotherapy strategies, possibly *via* a combination of type I IFN treatment with PD-L1 inhibition. Moreover, the data suggest that PD-L1 expression may be affected by IFN-induced non-canonical signaling.

Canonical and Non-canonical Signaling in Autoimmune Diseases

Accumulating evidence implicates chronic and persistent type I IFN signaling in systemic inflammation that promotes the pathogenesis of some autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis, multiple sclerosis (MS), type I diabetes (T1D) and Sjögren's syndrome, among others (13, 39). These conditions are associated with different clinical symptoms and management strategies, yet there are common features related to the underlying inflammatory signaling pathways involved and the dysregulated immune response. **Figure 2** summarizes the cell type-specific type I IFN-induced canonical and non-canonical signaling pathways recently implicated in autoimmune diseases.

IFNα has been shown to impact the onset and progression of T1D, which involves the autoimmune attack of pancreatic β cells (40). One study demonstrated that IFNα activated STAT1, STAT2, and STAT3 in pancreatic β cells through TYK2, and that STAT2 was more critical than STAT1 in mediating the inflammatory and endoplasmic reticulum (ER) stress response (41). Another study likewise reported on IFNα induction of ER stress in pancreatic β cells, leading to the downregulation of insulin production and influence on T1D onset (42). A mouse model study revealed that inhibition of IFNα, but not IFNβ, in the pre-diabetes stage prevented the onset of T1D and blocked autoreactive T cells from entering and killing β cells in the pancreatic islets (43). Notably, patients with neutralizing autoantibodies to type I IFNs, specifically IFNαs, are less likely to develop T1D (44). These studies identify the negative impact of IFNα on the development of T1D.

Sjögren's syndrome is an autoimmune disease with glandular lymphocyte infiltration leading to symptoms of dry mouth and eyes, where approximately 50% of patients have a type I IFN signature (45, 46). Given that this IFN signature is not present in all patients, one study analyzed the effects of IFN-α2b treatment of peripheral blood mononuclear cells (PBMCs) from patients with Sjögren's compared with PBMCs from healthy donors, including type I IFN signature-positive and negative patients (45). Baseline effector protein phosphorylation levels differed predominantly in T cells in Sjögren's patients compared with healthy individuals, with higher p-p38 and p-STAT1 (Y701, S727). Sjögren's patients also exhibited increased IFNα-inducible JAK phosphorylation of STAT1 (Y701). Further, IFNα-2b treatment of PBMCs upregulated p-STAT1 (Y701) in B cells and downregulated p-STAT3 on S727 in T cells in type I IFN signature-positive patients.

SLE manifestations include organ damage and skin rash (47, 48). There is an IFNα signature in sera of SLE patients. A recent study using inducible IFNα transgenic mice found that upregulation of IFNα alone was capable of inducing an SLE phenotype (47). SLE pathogenesis is characterized by inflammasome overactivation; one study demonstrated that prolonged IFNα treatment increased inflammasome activity, which was eliminated with knockdown of IRF1 in SLE monocytes (49). IFNα treatment increased p-STAT1 and p-STAT2 at tyrosine residues, indicative of a classical JAK/STAT driven response. Another group that analyzed B cells from SLE patients, found increased baseline p-STAT3 (Y705), not p-STAT1, compared to B cells from healthy individuals (50). Additionally, these investigators found that IFNα treatment polarized naïve B cell differentiation towards a lupus-like phenotype, which was reversed by a STAT3 inhibitor and was absent in STAT3-deficient donor naïve B cells. In SLE monocytes, Gkirtzimanaki et al. identified IFNα induced mTOR activity, which promoted oxidative stress, revealing non-canonical IFNα signaling in SLE (51).

Cognizant of the persistent IFNα signature in SLE patients, a phase IIb clinical trial evaluated the effects of vaccination with IFNα kinoid, which produces anti-IFNα antibodies (52). Although the trial did not see a benefit in Based Composite Lupus Assessment (BICLA), the drug did provoke anti-IFN-α2b

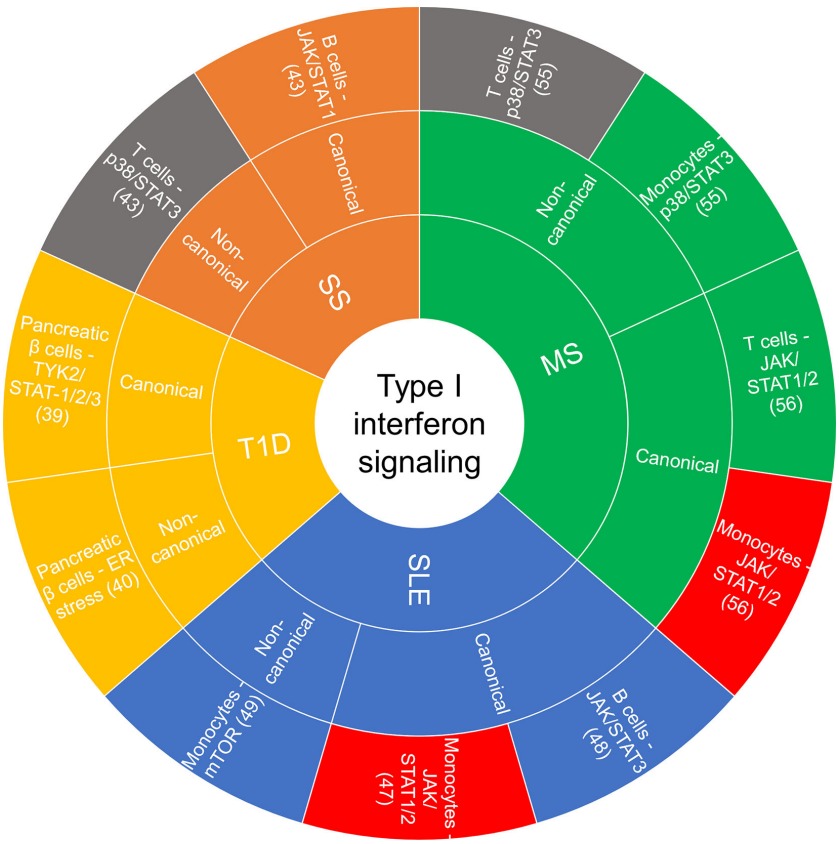

FIGURE 2 | Sunburst chart of cell-specific canonical and non-canonical signaling recently reported on in autoimmune diseases. If there was a commonality in a canonical or non-canonical signaling demonstrated in the same cell type but different disease, they were color coded the same: red—JAK/STAT1/2 in monocytes, gray—p38/STAT3 in T cells. ER, endoplasmic reticulum; JAK, janus kinase; MS, multiple sclerosis; mTOR, mammalian target of rapamycin; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; STAT, signal transducer and activator of transcription; T1D, type I diabetes.

serum antibodies and decreased the IFN gene signature in 91% of patients. An anti-IFNAR1 monoclonal antibody, anifrolumab, has been evaluated in 11 clinical trials for SLE (9), Sjögren's (1), and rheumatoid arthritis (1), with encouraging results (53). A recent phase III trial in SLE did not meet its primary endpoint of response, as per the SLE Responder Index; however, the same group conducted another phase III trial using the of British Isles Lupus Assessment Group (BILAG)-BICLA response as the primary endpoint and reported a statistically significant higher percentage of patients having a response as well as seeing a decrease in secondary endpoints, suggesting that a chronic IFNα response in SLE patients may contribute to disease pathogenesis (48).

Interestingly, while IFNα has been implicated in the pathogenesis of various autoimmune diseases, IFNβ has been used to successfully treat MS (54). Employing a mouse model of MS, studies with mice that lack the IFNβ gene revealed that in the absence of IFNβ the mice had a more severe disease with earlier onset and that the lack of IFNβ predisposed the mice to a pro-inflammatory Th17 immunophenotype (55, 56). Given the heterogeneity of the disease, and differing patient responses to IFNβ treatment, the identification of potential biomarkers of response to IFNβ therapy is receiving considerable attention. One study suggested predictors of response could be based on cell type-specific responses to type I IFN signaling, such as higher activation of STAT1, STAT3, and p38, leading to higher TRAIL expression in monocytes of IFN responders (57). Hurtado-Guerrero et al. analyzed monocytes from MS patients *ex vivo*, either left untreated (baseline) or after short-term IFNβ treatment (58). At baseline, there were no detectable differences in the levels of IFNAR1, IFNAR2, p-STAT1, and p-STAT2 among responders and non-responders, yet following IFNβ treatment, differences were observed. They found a pattern of decreased IFNAR1 and increased IFNAR2, p-STAT1, and p-STAT2 levels representing 68.4% of responder IFNβ-stimulated monocytes. Other groups have employed bioinformatics to uncover gene signatures that determine a response to IFNβ. One study used a feature selection computational method on a longitudinal microarray dataset of relapse-remitting MS (RRMS) patients treated with IFNβ-1b, and found a predictive seven gene signature (*CXCL9*, *IL2RA*, *CXCR3*, *AKT1*, *CSF2*, *IL2RB*, *GCA*) with 65.08% predictive accuracy (59). Using an alternative method of Elastic net modeling, Fukushima et al. analyzed time-course microarray datasets from PBMCs of

MS patients and identified eleven (*ZBTB16*, *ZFP37*, *HPS5*, *HOPX*, *ARFGAP3*, *CALML5*, *VPS26A*, *SLC5A4*, *MBL2*, *DLGAP4*, *CACNA1C*) and eight (*SMA4*, *MIR7114_NSMF*, *LSM8*, *FLAD1*, *RRN3P1*, *RASL10A*, *IER3IP1*, *CDH2*) genes predictive of an IFNβ response, with 81% and 78% accuracy, respectively, for each dataset (60). A different study employed the GeneRank method to identify monotonically expressed genes (MEGs) that determine a good response (*AFTPH*, *ALOX5*, *ATG7*, *MYD88*, *LILRB1*, *PRKAB1*, *PSEN1*, *VAMP3*) and a bad response (*AGFG1*, *CHM*, *IGLL1*, *PELI1*, *PTEN*) for responders, and two bad response MEGs for non-responders (*NAP1L4*, *MMS19*) in IFNβ treated RRMS patients (61). As an alternative strategy to gene analysis, a logistic regression modeling method was used to examine metabolites from the sera of a cohort of MS patients to predict the production of anti-drug antibodies (ADA) to IFNβ treatment (62). Differences in 29 metabolites were shown to be indicative of ADA production, and the top ten most significant metabolites were lipid related. Another study using a systems immunology approach evaluated ADA production differences in three IFNβ treated cohorts and showed reduced baseline NOTCH2 expression and that a pro-inflammatory phenotype in monocytes was predictive of ADA development (63). Given the preceding, there is a need for further identification and characterization of biomarkers that are reproducibly predictive of an IFNβ response in RRMS patients.

The differences between IFNα and IFNβ in the generation of effects in autoimmune diseases requires additional analysis. Although both type I IFNs bind to and initiate signaling cascades through the dimeric IFNAR, they do differ in primary amino acid sequences and in binding affinity to the receptor which may account for varying impacts of the response on cells (54, 64). Binding affinity for IFNAR1 and IFNAR2 varies among IFNα subunits, with overall higher affinity for IFNAR2 over IFNAR1, and IFNβ has tighter binding to each receptor subunit than any of the IFNα subunits (64, 65). How the induced signaling can differ after the type I IFN ligand binds is not well understood but studies have shown differences further downstream in genes and transcription factor binding sites of IFNα versus IFNβ signaling, such as enrichment of IRF8 binding sites in IFNβ response (54). As previously mentioned, cell-type and disease state lead to variance in type I IFN signaling which is further complicated by the differences invoked by IFNα and IFNβ and requires further studies, especially to understand the protein signaling cascades after binding of type I IFNs to the IFNAR.

Canonical and Non-Canonical IFN Signaling in Antiviral Responses

IFNs are critical effectors of an antiviral response in mammalian cells. Following viral infection, type I IFNs are produced by immune and non-immune cells, bind to and activate IFNAR, and signal through canonical and non-canonical pathways (66–68). An area of interest has been the involvement of the IFN system in the pathophysiology of Coronavirus Disease 19 (COVID-19).

Since the emergence of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012, therapeutic options for treatment have been limited (69). Type I IFNs are attractive therapeutic candidates because of their ability to clear virus through direct inhibition of viral replication of both DNA and RNA viruses and their effects on the activation of specific immune cell subsets to assist with viral clearance (70). Many viruses, including coronaviruses, evade an IFN antiviral response by inhibiting the production of type I and III IFNs (71–73). Scrutiny of the SARS-CoV genome identified the genes NSP1, NSP3, ORF3b, and ORF6 that are antagonists for type I IFNs, as well as the N protein (74). ORF6 not only inhibits the production of IFN but can also inhibit the expression of ISGs by inhibiting STAT1 nuclear translocation, through disruption of karyopherin-mediated transport. IRF3 is an important transcription factor necessary for IFNβ expression. The papain-like protease (PLpro), conserved in both SARS-CoV and SARS-CoV-2, inhibits the phosphorylation required for IRF3 homodimerization and nuclear translocation leading to its association with CBP/p300 and NF-κB for IFNβ expression (75–77). Comparing the gene sequences between SARS-CoV and SARS-CoV-2 for NSP3, ORF3b, and ORF6, revealed sequence differences that may contribute to the greater sensitivity of SARS-CoV-2 to type I IFNs (77). Konno et al. made the observation that ORF3b inhibits type I IFN induction more so in SARS-CoV-2 than in SARS-CoV, and a naturally arising SARS-CoV-2 variant exerts even greater antagonism of type I IFN induction by ORF3b (78). Accumulating data continue to provide further evidence of a blunted IFN response in COVID-19 cases (79–83).

Recently, data have emerged that indicate that SARS-CoV-2 is sensitive to the antiviral effects of both IFNα and IFNβ in cell culture assays, similar to the sensitivity of SARS-CoV *in vitro* (84–86). A pilot clinical study during the SARS outbreak of 2003 demonstrated that treatment with an IFNα resulted in reduced disease-associated impaired oxygen saturation and rapid resolution of lung abnormalities (87). The evidence of SARS-CoV-2 sensitivity to IFN treatment and accumulating clinical studies suggest that IFN treatment may have therapeutic benefits for COVID-19 (88). Early on in the pandemic, Zhou et al. provided evidence that treating COVID-19 patients with nebulized IFN-α2b with or without the antiviral drug, arbidol, accelerated viral clearance from the airways of infected patients and also reduced the circulating levels of the inflammatory cytokines, IL-6 and CRP (89). Following up from this exploratory study, there have been several clinical studies evaluating the therapeutic benefit of IFNα and IFNβ treatment for COVID-19 (see **Table 2**). *In vitro* studies suggested greater antiviral effectiveness of IFNβ over IFNα against SARS CoV (97). This prompted the WHO SOLIDARITY randomized controlled trial of a combination of lopinavir/ritonavir, ribavirin, and IFNβ-1b versus lopinavir/ritonavir in SARS-CoV-2 (90). The findings suggest that the triple combination treatment was more effective than lopinavir/ritonavir alone, reducing symptom severity and time to viral clearance. Given the emerging evidence that lopinavir/ritonavir treatment may be ineffective against SARS-CoV-2, the ongoing trial had been amended to compare the therapeutic effectiveness of IFNβ with remdesivir, a viral

TABLE 2 | Clinical studies involving type I interferons in SARS-CoV-2.

Type I IFN (administration or collection)	Other drugs in combination	Study type	Outcomes if applicable	Trial # (reference)
IFNα-2b (nebulized)	Umifenovir	Uncontrolled, exploratory cohort study	IFN-α2b (± arbidol) reduced time to viral clearance and circulating inflammatory cytokine (IL-6, CRP) levels	(89)
IFNβ-1b (subcutaneous)	Lopinavir/Ritonavir Ribavirin	Randomized controlled phase 2 trial	Triple combination treatment more effective than lopinavir/ritonavir alone, reducing symptom severity and time to viral clearance	NCT04276688 (90)
IFNα-2b (intramuscular)	Lopinavir/Ritonavir Chloroquine	Multicenter prospective study	Higher proportion of patients discharged from hospital in IFN-treated vs. non-IFN treated group	RPCEC00000318 —Cuban Registry (91)
IFNα-2b (nebulized)	Lopinavir/Ritonavir Umifenovir	Retrospective cohort study	Early IFN-α2b administration reduced in-hospital mortality but increased mortality and delayed recovery with late administration (>5 days post hospital admission)	(92)
IFNα	Lopinavir/Ritonavir Ribavirin	Retrospective, single-center study	Time to clearance positively correlated with length of hospital stay in patients treated with IFN-α+lopinavir/ritonavir (± ribavirin)	(93)
IFNβ-1b	Hydroxychloroquine	Single center randomized controlled phase 2 clinical trial	Completed—no results posted	NCT04343768 (94)
IFNβ-1a (subcutaneous)	Lopinavir/Ritonavir			
IFNβ-1b (subcutaneous)	Hydroxychloroquine	Prospective open-label randomized controlled phase 2 trial	Completed – no results posted	NCT04350281
IFNα-2b (nebulized)	Ganovo Ritonavir	Open controlled phase 4 trial	Completed – no results posted	NCT04291729
IFNβ-1a (subcutaneous)	Remdesivir	Adaptive randomized double-blind multicenter placebo-controlled phase 3 trial	Recruiting, Adaptive COVID-19 Treatment Trial 3	NCT04492475
IFNα IFNκ (plasma and serum),		Observational study	Autoantibodies for IFNα, IFNκ, or both found in 101 of 987 patients with life-threatening pneumonia, none in 663 patients with no or mild symptoms, 4 of 1227 healthy patients	(95)
ISGs (bronchoalveolar lavage fluid)		Observational study	COVID-19 patients had higher expression of ISGs with a proinflammatory subset, compared to healthy and pneumonia patients	(96)

polymerase inhibitor that has demonstrated limited therapeutic efficacy in COVID-19 cases. A prospective observational study was conducted to assess the therapeutic efficacy of IFN-α2b in SARS-CoV-2 patients during the first month after the COVID-19 outbreak began in Cuba. Intramuscular administration of IFN-α2b improved both the rate of recovery and case fatalities (91). However, a retrospective cohort study demonstrated that there is great importance on the timing of administration of IFN-α2b with reduction of in-hospital mortality when administered the first five days of admission but increased mortality and delayed recovery was seen if given later (92). Additionally, inborn errors of type I IFNs and presence of autoantibodies against type I IFNs can be determinants of severity of disease and effectiveness of type I IFN treatment (95, 98). Roughly 10% of COVID-19 patients with severe pneumonia in a cohort of 987 patients had neutralizing autoantibodies against IFNα, IFNω, or both, where patients with no or mild symptoms had no detectable autoantibodies (95). These findings demonstrate that administration of IFNα may not be effective in patients with severe condition and autoantibodies, but since IFNβ autoantibodies were uncommon in the same patients, IFNβ may provide a more beneficial treatment. The same group analyzed a separate cohort of patients with life-threatening pneumonia and found 3.5% had inborn errors in type I IFN related genes, specifically in loci pertaining to TLR3- and IRF7-dependent type I IFN induction (98). This showed a commonality with influenza since similar type I IFN related gene defects have been demonstrated in life-threatening influenza pneumonitis (99).

Similar to SARS-CoV, SARS-CoV-2 interacts with the angiotensin-converting enzyme 2 (ACE2) for cell entry, while MERS-CoV exploits the dipeptidyl peptidase 4 (DPP4) receptor for entry into human cells (100–102). Ziegler et al. demonstrated that nasal secretory cells (goblet cells), type II pneumocytes, and absorptive enterocytes of the ileum are positive for the two critical receptors for SARS-CoV-2 cell entry, ACE2 and the type II transmembrane serine protease, TMPRSS2 (103). Their observation that ACE2 expression is induced by type I IFNs in primary upper airway basal cells and lung tissue is hard to reconcile with IFNs inhibiting infection by SARS-CoV-2, yet recent emerging data suggesting a role for the renin-angiotensin pathway in protection from specific clinical features of COVID-19 would support a role for ACE2 in limiting COVID-19 severity. The inability of mice to uptake SARS-CoV-2 infection through the mouse ortholog of entry receptor ACE2 prompted Israelow et al. to create an adeno associated virus-mediated human ACE2 mouse model that can be utilized to analyze SARS-CoV-2 in mice, and they found increased type I IFN signaling ISGs in the lungs and limited control of SARS-CoV-2 replication by type I IFNs (104). The involvement of canonical versus non-canonical pathways in the induction of IFN-responses against SARS-CoV2 remains to be elucidated.

SARS-CoV-2 and influenza viruses are respiratory infections where disease severity results in lung hyper-inflammation and acute respiratory distress. Findings from clinical studies suggest that the early viral phase of both infections is associated with a blunted IFN response, yet progression to severe disease shows no such failed IFN response, specifically elevated levels of ISGs in PBMCs are observed (105–107). The implications are that the therapeutic benefits of IFN treatment are applicable in the early viral phases of COVID-19 and influenza, but that once the pulmonary phases of both infections progress to hyper-inflammation, IFN treatment is likely to be contra-indicated. Non-canonical effects in type I IFN signaling in influenza have been demonstrated as with p38 MAPK signaling, shown to be important in affecting type I IFN production and signaling in highly pathogenic avian influenza virus infected endothelial cells (108). Additionally, IFN-κ treatment inhibits influenza replication in lung cells, dependent on IFNAR, p38, CHD6, and Fos activation, but not STAT1 (109). Notably, IFNα induced STAT3 activation is crucial for inhibition of influenza viral replication and ISG transcription in mouse embryonic fibroblasts (110).

Although antiretroviral therapy (ART) for Human Immunodeficiency Virus (HIV) infection has transformed this infection from a fatal one to a chronic disease, viral reservoirs complicate efforts for HIV elimination, and a recent review paralleled HIV reservoir persistence to immuno-editing and immune evasion in cancer (111). The roles of type I IFNs in the pathophysiology of HIV infection are not fully understood, but IFNα has been implicated as an adverse factor in the persistence of HIV-1. When circulating levels of IFNα were measured for healthy donors, primary-infected, and chronically-infected patients, higher IFNα levels were associated with higher viral loads and higher expression of the ISG, *USP18*, which negatively regulates IFNα signaling by displacing JAK2 bound to IFNAR2 (112). Humanized mouse models have provided evidence that whereas type I IFNs suppress early HIV infection, type I IFN signaling induces T cell depletion and impaired functionality during persistent infection. When IFN signaling is blocked in HIV-infected mice or in monkeys receiving ART, this reduces the HIV reservoir, rescues anti-HIV T cells, and reduces HIV-induced inflammation (113–115). Notably, HIV-1 proteins, Vpu and Nef, inhibit ISG expression through canonical IFNα mediated JAK/STAT1 signaling, blocking any antiviral benefits from IFNα (116). Knockout of IFNAR1 in an HIV-induced brain injury mouse model provided memory benefits and neuronal injury protection while suppressing p38 activation, indicating involvement of type I IFN non-canonical signaling in HIV-1–related neurotoxicity (117). Indeed, there is accumulating evidence that sustained type I IFN signaling, surprisingly, can promote viral replication for a number of viruses, mediated by induction of certain ISGs and inhibition of IRFs (14). IFN induced 2′5′-oligoadenylate synthetase-like (OASL) limits RNA virus replication through enhancing RIG-I signaling yet inhibits cGAS and promotes viral replication for DNA viruses such as HSV.

Of late, there are emergent data that SLFN proteins, non-canonical effectors of type I IFN signaling, have a role as antivirals. IFN induced SLFN11 expression controls protein synthesis by regulating tRNA abundance, limiting West Nile virus, dengue virus, and Zika virus replication, all (+) ssRNA viruses, but having little effect on (–) ssRNA viruses (118). Interestingly, SLFN 11 control of HIV-1 infection is independent of type I IFN signaling (119). IFNβ induced SLFN14 exhibits antiviral activity in mouse macrophages, limiting infection with influenza virus or the DNA virus, varicella-zoster virus (120).

Besides the duration of type I IFN signaling influencing whether there is inhibition or enhancement of viral replication (105, 112), cell environmental factors also contribute to a type I IFN response. In a mouse model of vesicular stomatitis virus infection, high salt levels augment type I IFN signaling through the non-canonical p38 pathway (121). In neurons, viral infection may cause pain hypersensitivity; type I IFNs elicit pain sensitization in neurons, by promoting MAPK interacting kinase phosphorylation of eukaryotic initiation translation factor (122).

CONCLUSIONS AND FUTURE EXPECTATIONS

Though over sixty years have elapsed since the original discovery of IFNs, in recent years, there has been mounting evidence for the critical roles of type I IFNs as immune regulators in multiple biological systems. The mechanisms of induction of type I IFNs and their subsequent biological responses are complex, due in part to the large number of family members, both cell type-dependent and independent biological responses, and varying influences in different disease settings. As identified above, for acute and chronic virus infections, type I IFN signaling can have distinct and sometimes contrasting biological effects. In malignancies, type I IFNs induce antiproliferative and antineoplastic effects but may also upregulate PD-L1 expression, thereby limiting an anti-tumor immune response. In some autoimmune diseases, such as SLE, the persistent exposure of immune cells to endogenous IFNα appears associated with pathogenesis. On the other hand, IFNβ provides therapeutic benefits in MS. Regardless of whether type I IFN associated responses contribute to favorable or poor outcomes, it is clear that both canonical and non-canonical IFN signaling pathways are critical for type I IFN responses. In many cases, both canonical and non-canonical are activated in parallel, but it is possible that in certain cell-type and disease states a given pathway may play a predominant role. With the identification of the roles of non-canonical MAPK and mTOR pathways, the involvement of PKC and SLFN proteins, our understanding of how type I IFN signaling alters the transcriptome to produce proteins that affect changes in biological responses has increased dramatically. The discovery of new non-canonical pathways and effectors has substantially advanced the field, but other non-canonical pathways may have yet to be identified. Understanding how there is connectivity between the classical, canonical JAK/STAT signaling, and non-canonical pathways will provide the basis for further targeting of type I IFN signaling in different diseases.

AUTHOR CONTRIBUTIONS

All authors have contributed in the writing and editing of this review. All authors contributed to the article and approved the submitted version.

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REVIEW
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All About the RNA: Interferon-Stimulated Genes That Interfere With Viral RNA Processes

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Interferon (IFN) signaling induces the expression of a wide array of genes, collectively referred to as IFN-stimulated genes (ISGs) that generally function to inhibit viral replication. RNA viruses are frequently targeted by ISGs through recognition of viral replicative intermediates and molecular features associated with viral genomes, or the lack of molecular features associated with host mRNAs. The ISGs reviewed here primarily inhibit viral replication in an RNA-centric manner, working to sense, degrade, or repress expression of viral RNA. This review focuses on dissecting how these ISGs exhibit multiple antiviral mechanisms, often through use of varied co-factors, highlighting the complexity of the type I IFN response. Specifically, these ISGs can mediate antiviral effects through viral RNA degradation, viral translation inhibition, or both. While the OAS/RNase L pathway globally degrades RNA and arrests translation, ISG20 and ZAP employ targeted RNA degradation and translation inhibition to block viral replication. Meanwhile, SHFL targets translation by inhibiting -1 ribosomal frameshifting, which is required by many RNA viruses. Finally, a number of E3 ligases inhibit viral transcription, an attractive antiviral target during the lifecycle of negative-sense RNA viruses which must transcribe their genome prior to translation. Through this review, we aim to provide an updated perspective on how these ISGs work together to form a complex network of antiviral arsenals targeting viral RNA processes.

Keywords: interferon-stimulated genes, co-factors, viral RNA degradation, translation inhibition, RNA sensing

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INTRODUCTION

Organisms must constantly defend themselves against viral pathogens. In order to stem viral spread, cells must both signal the presence of viral infection and hinder their replication. One key first line of cellular defense in vertebrates is the type I interferon (IFN) response. Hosts possess sensors which recognize pathogen-associated molecular patterns (PAMPs) of invading viruses such as the viral replicative intermediate double-stranded RNA (dsRNA) and activate transcription factors such as IFN-regulatory factors 3 or 7 (IRF3/7) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). As a result, these transcription factors translocate to the nucleus to activate expression of type I IFN and other proinflammatory cytokines.

The type I IFN receptor is expressed ubiquitously on almost all cell types, allowing for IFN signaling in both infected and neighboring cells that are uninfected. Janus kinase-signal transducer and activator of transcription (JAK-STAT) is the predominant, canonical pathway that regulates ISG transcription. IFN binding to its cell surface receptor, comprised of IFN-α receptor 1 (IFNAR1) and IFN-α receptor 2 (IFNAR2), leads to phosphorylation of pre-associated JAK1. Phosphorylated JAK1 and tyrosine kinase 2 (TYK2) then phosphorylate the IFN receptor, which recruits STAT1/2 to be phosphorylated themselves. Phosphorylated STAT1/2 recruit IRF9 to form the transcription factor complex IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates to the nucleus where STAT1 is further phosphorylated for full activation. Within the nucleus, ISGF3 binds to IFN-stimulated response elements present in the promoters of IFN-stimulated genes (ISGs), which then effect an antiviral cellular environment [for a comprehensive review on IFN signaling, see (1)].

Interestingly, a growing body of evidence in recent years suggests a plethora of non-canonical mechanisms [for a comprehensive review on canonical and non-canonical regulation of ISG transcription, see (2)]. Non-canonical ISGF3 complexes containing unphosphorylated STAT2, unphosphorylated STAT1 and STAT2, or STAT2 and IRF9 only have been found to mediate expression of specific ISGs (3–6). Other transcription complexes such as STAT5-CrkL (7, 8) or transcription factors such as IRF1 (9) can induce ISG expression. Additionally, cytokines such as TNF-α can moderately induce a subset of ISGs through the NFκB protein complex and further synergize with type I or II IFN to jointly upregulate antiviral ISG expression (10–13). Surprisingly, cellular pathways that have no apparent connection to the innate immune response have been linked to ISG induction. For example, inhibitors of nucleotide synthesis have been shown to effectively upregulate ISG expression in a JAK-STAT-independent manner (14–17). Differences in the extent and timing of ISG upregulation likely depend on the complex interplay between these various mechanisms.

Broadly speaking, an ISG is any gene whose expression is induced by IFN signaling. Advances in RNA-sequencing (RNA-seq) technology have enabled the identification of ISGs across varied cell lines by measuring changes in the transcriptome in response to IFN stimulation. The online database INTERFEROME continues to catalog the results of such gene profiling studies (18). However, ISG expression is more nuanced in reality. A subset of ISGs are direct targets of IRF3/7 and can be induced with or without downstream IFN signaling (**Figure 1**) (1). Other ISGs are both basally expressed and IFN-inducible, while still others are cell-type specific (19, 20). Moreover, there are three types of IFN, wherein type I and III are the classic antiviral IFNs. Though type I and III IFNs bind to different receptors, they signal through the same JAK-STAT pathway, thus inducing a shared array of ISGs. Still, type I and III IFN signaling pathways are differentiated by expression kinetics and cell-type specific receptor expression [for a recent review, see (21)]. Tight regulation of ISG expression is necessary because dysregulation of the type I IFN response results in interferonopathies or systemic inflammation deleterious to the organism (22).

In addition to regulating their own expression, ISGs are well known for their inhibition of viral replication. They employ diverse mechanisms to block virtually every step of viral replication, though ISGs have been shown to target different viral life cycle stages for different viruses [for recent reviews on a broad range of antiviral ISGs, see (20, 23)]. For example, the IFITM family blocks viral entry of diverse viruses (24) while the Mx GTPases recognize diverse nucleocapsids and block their

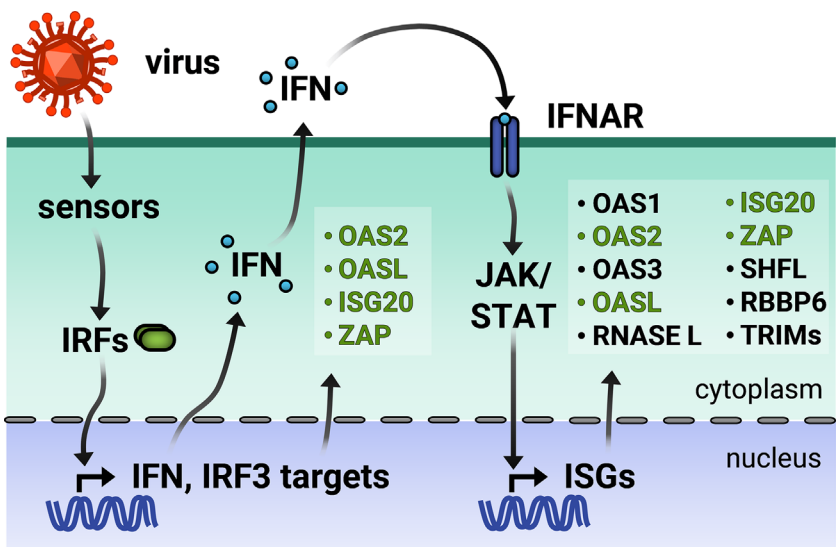

FIGURE 1 | Viral infection triggers the type I interferon (IFN) response. Virus entry triggers cellular pathogen sensors, which induce production of IFN and IFN regulatory factor 3 (IRF)-dependent genes. Type I IFN is secreted and binds to IFN-α receptor on the same and neighboring cells, activating production of a wider array of IFN-stimulated genes (ISGs). IRF3-dependent ISGs are colored in green. TRIMs is not inclusive of the entire TRIM family, but only those inducible by IFN.

nuclear import (25). TRIM5α disrupts retrovirus uncoating and targets several viral proteins for proteasomal degradation (26). However, these only represent a tip of the iceberg. Recent advances in systematic approaches have allowed us to unbiasedly uncover ISGs with previously uncharacterized antiviral activity. Compiled ISG libraries have facilitated focused loss-of-function or gain-of-function screens of hundreds of ISGs, illuminating the contribution of individual ISGs in varied viral contexts (27–30). Furthermore, as advances in omics approaches allow examination of cellular changes on a systemic level, attention is shifting to how ISGs interact and even synergize with one another (27, 31, 32). Moreover, detailed mechanistic studies are still needed in order to unravel their mode of action. As ISGs may employ different antiviral mechanisms against different viruses, studies in varied viral systems will illuminate how ISGs might recruit different cellular pathways or factors.

Rather than provide a comprehensive, surface-level view of myriad ISGs with their arrayed antiviral functions, we have chosen to focus on a subset of ISGs that interfere with viral RNA processes. Recent studies have provided an emerging view on the diversity and complexity of RNA-based mechanisms by which different ISGs inhibit viruses with an RNA genome. With the exception of retroviruses which replicate through a DNA intermediate, single-stranded RNA (ssRNA) viruses can generally be categorized as positive-sense and negative-sense. Positive-sense (+) ssRNA viruses possess genomes that generally resemble mRNA, in that it can be translated directly by host translation machinery. However, negative-sense (-) ssRNA viruses code their proteins in the reverse orientation. Therefore, they must package their own RNA-dependent RNA polymerases and transcribe their genomes before viral protein synthesis can occur. While there are many more ISGs that inhibit viral RNA processes (**Tables 1** and **2**), this review will highlight recent exciting work on a subset of ISGs that act in an RNA-centric manner to sense, degrade, or inhibit transcription or translation of both (+) and (-) ssRNA viral genomes (**Figure 2**). We have chosen the ISGs here because at the time of writing of this review, they have not been comprehensively reviewed in the antiviral innate immunity field, and exciting developments have either illuminated nuances of well-characterized mechanisms or uncovered entirely new mechanisms by which these ISGs inhibit viral replication. We synthesized diverse antiviral mechanisms of individual ISGs and provided hypotheses for how cellular co-factors mediate the distinct antiviral activities of these ISGs, which not many previously published reviews have done. We will begin our discussion with the 2'-5' oligoadenylate synthetase (OAS)/RNase L pathway, which both senses and degrades RNA, making it a potent early inhibitor of viral replication. We will then segue into ISGs that both degrade RNA and inhibit translation, such as ISG20 and zinc finger antiviral protein (ZAP), before focusing on a novel means of translation inhibition by Shiftless (SHFL). We will end with a discussion of E3 ligases that inhibit viral transcription. Though there are many additional ISGs that block these RNA-centric steps of viral replication, this review focuses on ISGs that possess multiple or seemingly contradictory antiviral mechanisms. Protein-protein interactions or cellular co-factors could explain diverse antiviral mechanisms of individual ISGs.

TABLE 1 | ISGs that must bind RNA to inhibit viral RNA processes.

Gene	Mode of inhibition	RNA motif	References
ADAR1	A-to-I sequence conversion	dsRNA	(33)
IFIT1, -2, -3, -5	Translation inhibition	Type 0 cap structure lacking 2'-*O*-methylation at the 5' end of ssRNA	(34, 35)
ISG20	RNA degradation, translation inhibition	Largely unknown; recognizes m^6A on HBV RNA	(36–38)
MDA5	RNA sensor	Long cytosolic dsRNA (> 1,000-2,000 bp)	(39–41)
OAS1, -2, -3	RNA sensor, synthesize 2-5A to activate RNase L	dsRNA	(40, 42)
OASL	RNA sensor, promotes RIG-I signaling and inhibits cGAS signaling	dsRNA	(43)
PARP12	Translation inhibition	Unknown	(44–46)
PKR	RNA sensor, translation inhibition	dsRNA	(40)
RIG-I	RNA sensor	short cytosolic dsRNA or ssRNA (10-300 bp) with 5'-triphosphate ends, enriched in poly-U/UC or AU sequences	(39–41)
RNase L	RNA degradation	ssRNA, cleaves at ^ in U^N, where N is any nucleotide	(47)
TRIM25	Translation inhibition	Unknown	(48–50),
ZAP	RNA degradation, translation inhibition, ISG synergy	ssRNA, CG dinucleotide	(23)
ZCCHC3	RNA sensor	dsRNA	(51)
ZNFX1	RNA sensor	dsRNA	(52)

Mode of inhibition, RNA motif or substrate preference, and references for the most recent reviews are listed for ISGs that bind RNA to inhibit viral RNA processes.

TABLE 2 | ISGs that inhibit viral RNA processes with no known dependence on RNA binding.

Gene	Inhibited process	Known mechanism(s)	References
RBBP6	Transcription	Competitively binds to viral RNA polymerase	(53)
SHFL	Translation	Inhibits -1 ribosomal frameshifting	(54)
TRIM22	Transcription	Prevents transcription factor binding to HIV-1 promoter	(55)
TRIM25	Transcription	Blocks IAV RNA elongation	(56)
TRIM32	Transcription	Targets IAV polymerase for degradation	(57)
TRIM69	Transcription	Sequesters VSV_{IND} P	(58, 59)
Viperin	Viral RNA synthesis	Synthesizes chain terminator from cytidine triphosphate	(60)

Viral RNA process inhibited (viral RNA synthesis, transcription, or translation) known mechanism(s), and references for the most recent reviews are listed for ISGs that inhibit viral RNA processes without a strict requirement for RNA binding.

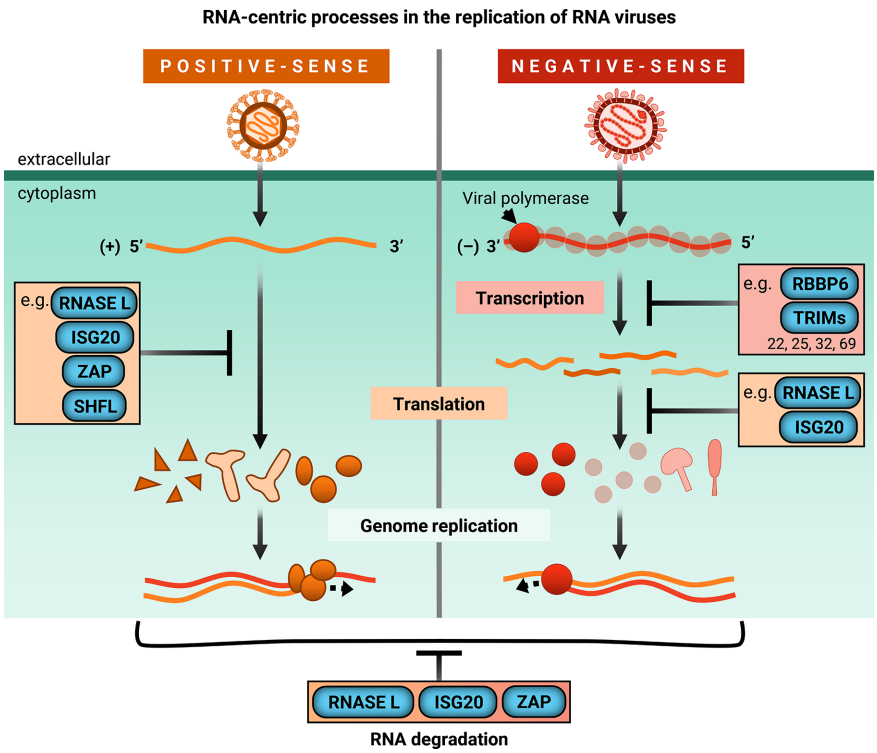

FIGURE 2 | ISGs inhibit RNA-centric processes in replication of RNA viruses. Upon infection, positive-sense single-stranded RNA genomes are directly translated by host machinery. Following expression of viral replicase proteins, genome replication occurs. However, negative-sense single-stranded RNA viruses bring their own RNA-dependent RNA polymerase and viral co-factors to transcribe their genomes before the viral mRNAs can be translated by host machinery for genome replication. ISGs covered in this review are shown to block global or viral translation (RNaseL, ISG20, ZAP, SHFL) and viral transcription (RBBP6, TRIMs). They can also inhibit viral replication by degrading cellular and/or viral RNA (RNase L, ISG20, ZAP). TRIMs that inhibit transcription are limited to those mentioned in this review, specifically TRIM22, TRIM25, TRIM32, and TRIM69.

OAS/RNASE L: SENSING VIRAL PAMP TRIGGERS GLOBAL RNA DEGRADATION AND TRANSLATIONAL ARREST

Degrading viral genomes presents one potent method of antiviral activity; digesting viral genetic material ensures that no further steps in replication can occur. However, the challenge lies within being able to control RNA degradation to ensure cellular survival or limit destruction within the host. The OAS/RNase L pathway is activated upon sensing the PAMP of dsRNA, serving two functions: sensing viral intruders and inhibiting viral replication by degrading RNA almost indiscriminately, inducing global translational arrest.

The OAS/RNase L pathway was one of the first ISG antiviral mechanisms to be identified and elucidated in the 1970s [reviewed in (61–64)]. Binding to dsRNA activates OAS, which then synthesizes 2'-5' oligoadenylates (commonly abbreviated as 2-5A) that in turn activate RNase L to cleave cytoplasmic RNA, thereby inhibiting viral replication (**Figure 3A**). Humans possess three catalytically active OAS genes (OAS1-3) and one inactive gene (OASL). Each OAS is composed of 1 (OAS1 and OASL), 2 (OAS2), or 3 (OAS3) basal OAS units (**Figure 3A**). While only the C-terminal OAS unit in each protein is catalytically active and responsible for synthesizing 2-5A, both active and inactive OAS units can still bind dsRNA. RNase L, present in cellular cytoplasm as an inactive monomer, forms a catalytically active dimer upon binding to 2-5A and cleaves RNA in a seemingly indiscriminate manner. Though OAS1-3 all carry signatures of positive selection, which is indicative of rapid evolution resulting from host-pathogen interactions, OASL does not. Furthermore, OAS1 displays much stronger signatures than OAS2 or OAS3 (65, 66). RNase L also carries signatures of positive selection and some of the positively selected residues are located within the RNA-binding domain (66).

In the past 10 years, great strides have been made in clarifying dsRNA substrate specificity and 2-5A synthesis activity of individual OAS isoforms due to the advent of CRISPR-Cas9 gene-editing techniques and generation of OAS knockout cell lines and mouse models. Moreover, sweeping improvements in genome-wide RNA-seq and in-depth proteomics have illuminated new intricacies of OAS/RNase L-mediated inhibition of viral replication, which we will review here.

OAS1-3: 2-5A Messenger Synthesis

Recent years have not only seen a flurry of biochemical and structural studies on individual OAS paralogs, which have advanced understanding of their RNA substrate specificity and

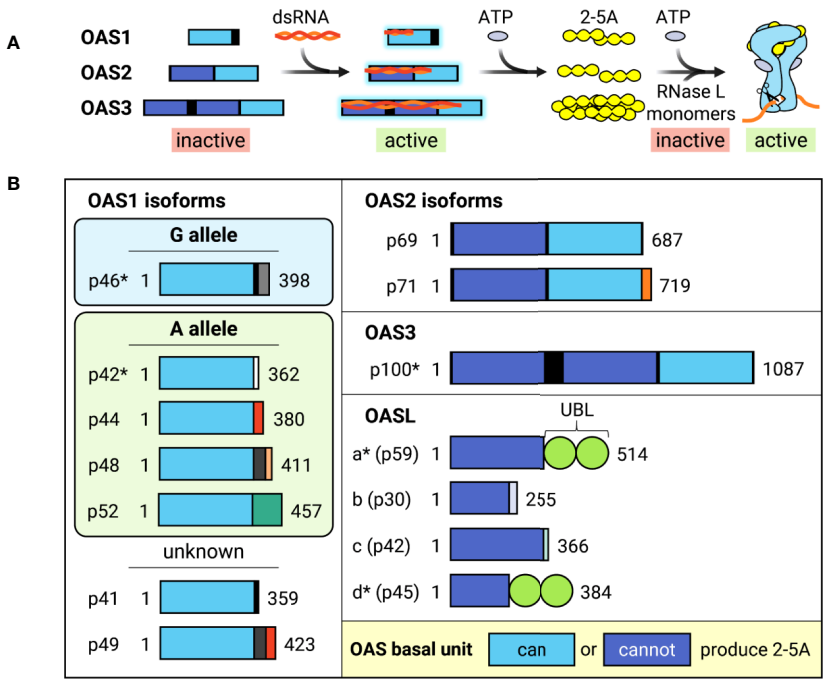

FIGURE 3 | Activation and dsRNA binding of OAS isoforms. **(A)** Diagram of how OAS is activated by viral dsRNA and synthesizes 2-5A to activate RNase L. Longer OAS isoforms are activated by longer dsRNA. Though all are able to synthesize 2-5A, OAS3 2-5A synthesis is necessary and sufficient for RNase L activation. RNase L exists as inactive free monomers, but dimerizes and activates upon binding to 2-5A and ATP, thereby cleaving RNA. Interestingly, IFN and some ISG transcripts are resistant to RNase L cleavage, resulting in preservation of antiviral signaling. **(B)** Schematic of known human OAS isoforms encoded by OAS genes OAS1, OAS2, OAS3, and OASL. Isoforms marked with * have demonstrated direct antiviral activity. Length of boxes approximates gene length, with different colored C-terminal boxes representing different coding sequences. Light blue OAS basal units are able to synthesize 2-5A, while dark blue OAS basal units are catalytically inactive. Most OAS1 isoforms are expressed by either G or A alleles, which differ by the inclusion of a G or A nucleotide at a splice acceptor site. However, OAS1 p41 and p49 have not been attributed to either G or A and are therefore labelled as "unknown." UBL, Ubiquitin-like domain.

activation, but also of their individual splice variants. Surprisingly, OAS3 has been identified as the main contributor to the OAS/RNase L pathway, as OAS3-mediated 2-5A synthesis has been demonstrated as necessary and sufficient for RNase L pathway (67).

Though there are four OAS genes in humans, alternative splicing of additional 3' exons generates 10 different catalytically active and 4 inactive isoforms (**Figure 3B**) (62, 68, 69). OAS isoforms can vary greatly in size and degree of catalytic activity within the same gene (69). Though OAS1 isoforms differ from each other in their C-terminal regions, all are able to synthesize 2-5A *in vitro* after incubation with poly(I:C), a dsRNA mimetic, with isoforms p42 and p46 expressed more highly in HEK293 cells (69). A yeast two-hybrid screen for p42 and p44, predicted to be expressed by all humans (70), revealed different binding partners, suggesting that OAS1 unique C-terminal regions may alter protein-protein interactions mediating isoform-specific functions (69). In support of this hypothesis, p46 possesses a CaaX prenylation motif that causes it to localize to mitochondria, whereas p42 lacks the CaaX motif and is cytoplasmic (71, 72). This difference in cellular localization is thought to contribute to their divergent impact on cellular respiration upon overexpression (72), and could contribute to their differential antiviral activity.

Genome-wide association studies of 2-5A synthesis activity revealed the presence of two OAS1 alleles which differ by the presence of a G or A at a splice acceptor site (70). Individuals with the G allele have high 2-5A activity and express the OAS1 p46 isoform, whereas individuals with 1-2 copies of the A allele have significantly lower OAS activity and express the OAS1 p42, p44, p48, and p52 isoforms (**Figure 3B**) (70, 73). This G/A single nucleotide polymorphism is associated with a variety of infectious diseases, such as the (+) ssRNA viruses West Nile virus (WNV) and hepatitis C virus (HCV), in addition to a hepatitis B virus (HBV)-associated autoimmune disease; wherein the G allele confers resistance and the A allele is associated with higher risk (74–78). A comprehensive study of OAS isoform-specific antiviral activity against dengue virus (DENV) found that out of all catalytically active isoforms, only OAS1 p42 and p46 and OAS3 p100 were able to block DENV replication through an RNase L-dependent mechanism (79). Furthermore, degree of antiviral activity was positively correlated with amount of RNase L activity, as measured by 28S and 18S rRNA cleavage, wherein OAS3 inhibited DENV replication more robustly than either OAS1 isoform.

This finding of RNase L-dependent antiviral activity of OAS3 was surprising at the time. OAS3 was thought to primarily

synthesize the minimal dimeric 2-5A molecule, composed solely of two adenylate groups, which poorly activates RNase L (80, 81). More recently, it was shown that not only does OAS3 require 3-4 logs lower concentration of dsRNA than OAS1 for 2-5A synthesis, but it also readily synthesizes 2-5As of 3 or more linked ATPs both *in vivo* and *in vitro* (82). Increased OAS3 sensitivity to dsRNA can be explained by its additional, catalytically inactive OAS units, which retain ability to bind dsRNA (83). Though catalytically inactive itself, deletion of the most N-terminal OAS unit in OAS3 (OAS3.DI) nearly abolished OAS3 catalytic activity and dsRNA binding (83). Given that OAS3 is thought to adopt an elongated conformation (82), dsRNA binding by OAS3.DI may determine OAS3 preference for longer dsRNA substrates, and may explain its greater dependency on dsRNA length for activation as compared to OAS1 (83, 84). This could help OAS3 discriminate self from non-self dsRNA, as long dsRNA is absent from uninfected cells (85). These studies culminated with the novel finding that OAS3 is the primary driver of RNase L antiviral activity (67). The replication of both DNA (vaccinia virus (VV)) and RNA viruses ((+) ssRNA: WNV, Sindbis virus (SINV); (-) ssRNA: influenza A virus (IAV)) was tested in OAS1-3 single KO cells. While wild-type WNV and SINV trigger the OAS/RNase L pathway, wild-type VV and IAV do not due to their active antagonism of the pathway. Therefore, mutant VV and IAV strains that lacked viral antagonists were used. OAS3 KO cells have negligible rRNA degradation and 2-5A production during both poly(I:C) treatment and viral infection, in stark contrast to OAS1 and OAS2 KO cells which had high rRNA degradation and 2-5A production (67). This generally correlated with inhibition of virion production, as OAS3 and RNase L single KO cells had significantly higher viral titers than parental and single OAS1 and OAS2 KO cells for all viruses tested. These data suggest that OAS3 is both necessary and sufficient to drive RNase L-dependent antiviral activity against diverse viruses.

Though OAS3 possesses a dominant role in RNase L-dependent antiviral activity, more antiviral and cellular roles of OAS1 and OAS2 are beginning to be elucidated. In fact, OAS1 and OAS2 are responsible for some antiviral activity against WNV and SINV, albeit to a lesser extent than OAS3/RNase L (67). OAS2 was found to inhibit the (+) ssRNA virus Zika virus (ZIKV) replication through positive regulation of IFN signaling (86). OAS2 may also play a role in lactation, as it was identified in a screen for genes with roles in mammary development (87). Moreover, a novel role for OAS1 and 2-5A synthesis was recently identified in the context of poly-ADP-ribosylation (PAR) and DNA damage-induced cell death (88). As part of the DNA damage response, poly-ADP-ribose polymerase 1 (PARP1) synthesizes PAR polymers to recruit and modify DNA repair proteins (89). Upon resolution of the response, PAR products are hydrolyzed and can trigger apoptosis. ADP-ribose is a known substrate of OAS (90) and addition of 2-5A linkage onto ADP-ribose effectively functions as a chain terminator of PARylation (88). As a result, OAS1 p42 protects cells from DNA damage-induced death by reducing PARP1-mediated PARylation. The absence of OAS1 dramatically increased PAR accumulation within cells upon H_2O_2 treatment, which was not rescued by a catalytically inactive OAS1 mutant (88). These data agree with the observation that cancer cells resistant to DNA-damaging therapies frequently highly express OAS1 (91, 92), as it is hypothesized OAS1 confers resistance to DNA damage-induced cell death.

RNase L: RNA Degradation and Global Translational Arrest

Upon activation by 2-5A, ubiquitous RNA degradation by RNase L simultaneously inhibits many facets of viral replication. Not only does RNase L activity directly degrade single-stranded cellular and viral RNAs, but it also promotes apoptosis, stimulates immune signaling, and induces rapid translational arrest (61).

Activation of RNase L quickly arrests global translation. This rapid translational arrest is traditionally attributed to degradation of transcripts involved in host translation machinery, as evidenced by degradation of 28S and 18S rRNA upon RNase L activation. However, closer examination revealed that at least 50% of 28S rRNA remains and overall tRNA levels are unchanged at the onset of global translation shut-off, challenging this prevailing hypothesis (93). Thus, degradation of translational machinery transcripts cannot explain early translational arrest (93). Another group presents the compelling hypothesis that translational arrest results from almost indiscriminate degradation of cellular RNA by RNase L, crippling gene expression by depriving ribosomes of substrates (94). Comparison of mRNA abundance in parental and RNase L KO A549s revealed reduction in almost all abundant mRNAs in parental, but not in RNase L KO cells after poly(I:C) stimulation. However, some mRNAs are resistant to RNase L cleavage, such as *IFN-β*. Furthermore, mRNAs that substantially increase in both parental and RNase L KO cells in response to poly(I:C) are highly enriched for ISGs such as *IFIT2*, *OAS2*, *MDA5*, and *RIG-I*, suggesting that antiviral mRNAs are resistant to RNase L turnover (94). Determinants of RNase L resistance have yet to be identified. These data are compatible with the independent observation that both type I and III IFN are still produced from seemingly translationally arrested cells (95). Together, these data support a model in which the rapid translational arrest by the OAS-RNase L pathway is doubly beneficial to the organism by both inhibiting viral replication and by permitting antiviral signaling to inhibit viral spread within the host.

RNase L structural studies have uncovered nuances of RNase L substrate selectivity. The first near full-length human RNase L crystal structure allowed for detailed analysis of RNase L dimerization, substrate recognition, and ribonuclease activity (96). In agreement with previous studies that found RNase L cleavage after UU and UA dinucleotides (97), recent structural analysis suggests that RNase L recognizes and cleaves the pattern UN^N (N: any nucleotide, ^: cleavage site) (96). Two different RNA-seq approaches have been utilized to identify RNase L substrates. One approach sequenced RNAs enriched for poly-A-tails after incubating lysates with either 2-5A or pre-activated RNase L. Their results suggest that RNase L selectively degrades

transcripts similar to those regulated by miRNAs, achieving a redundant outcome of suppression of mammalian cell adhesion and proliferation (98, 99). In contrast, another approach capitalized on the characteristic 2',3'-cyclic phosphate termini of RNase L cleavage products. They used a 2',3'-cyclic phosphate RNA-seq analysis in order to identify small RNA cleavage products (93). Here, it was found that most highly-upregulated reads map to tRNAs and Y-RNAs. Analysis of these RNAs revealed site-specific cleavage in both tRNAs and Y-RNAs, which may be shaped by post-transcriptional modifications. Interestingly, though cleavage sites of the tRNAs and other Y-RNAs followed UN^N specificity, the Y-RNA RNY4 was cleaved at CA^G. This unusual cleavage was not recapitulated in the absence of cellular proteins, suggesting that RNase L may acquire site-specificity by recognizing protein/Y-RNA complexes. In this way, RNase L could require co-factors to determine cleavage specificity. It is interesting to speculate that putative co-factors bound to ISG transcripts could also shield cleavage sites to enable their escape from RNase L recognition.

OASL

Though OASL lacks the ability to synthesize 2-5A and thus to participate in RNase L-dependent antiviral activity, it is still potently induced upon IFN stimulation and is also a direct target of IRF3 (100). OASL is composed of an N-terminal, catalytically inactive basal OAS unit followed by a tandem ubiquitin-like domain (UBL) (**Figure 3B**) (43) and possesses antiviral activity against several RNA viruses (27, 68, 101, 102). However, OASL promotes replication of the DNA virus, Kaposi sarcoma herpesvirus (KSHV) (103). These conflicting data can be reconciled by the recently uncovered divergent roles of OASL in both enhancing signaling of the dsRNA sensor RIG-I, thus inhibiting replication of RNA viruses (104), and inhibiting signaling of the cytoplasmic dsDNA sensor cGAS, hence promoting replication of DNA viruses (105, 106). Activation of RIG-I requires its simultaneous binding to dsRNA and poly-ubiquitin chains (107). OASL was shown to interact and colocalize with RIG-I, enhancing RIG-I signaling *via* its C-terminal UBL domain which acts as a poly-ubiquitin mimic to activate RIG-I (104). Furthermore, OASL antiviral activity is suggested to be completely RIG-I dependent, as its viral inhibition is abolished in the absence of RIG-I (104).

Meanwhile, two independent groups simultaneously identified the role of OASL in suppressing cGAS activity (105, 106). One group took a proteomics-based approach to uncover cGAS interactors in the context of herpesvirus infection, identifying OASL as a cGAS interactor and inhibitor (105). Another group utilized a targeted approach to assess the role of OASL during infection with varied DNA viruses, and verified the importance of OASL in promoting DNA viral replication *in vivo* by using a murine model for OASL KO (106). They observed that OASL deficiency results in increased IFN induction and reduced viral titers (106). Both groups found that the OAS-like domain is responsible for interacting with cGAS and that OASL-cGAS interaction is independent of cGAS DNA binding (105, 106). Enzyme inhibition kinetics experiments with OASL, cGAS, and the cGAS substrates ATP and GTP showed that OASL non-competitively inhibits cGAS production of its signaling molecule, cGAMP (106).

The OAS/RNase L pathway effectively inhibits viral replication by linking viral sensing to global inhibition of cellular processes. Widespread RNA degradation by activated RNase L globally not only arrests translation, preventing viruses from synthesizing new proteins, but also still allows for IFNs and several ISGs to be translated, promoting the establishment of an antiviral environment. Furthermore, the catalytically inactive OASL functions as a double-edged sword in its modulation of innate immune signaling, simultaneously inhibiting replication of RNA viruses while enhancing replication of DNA viruses.

MULTIFACETED RNA-DEPENDENT ANTIVIRAL MECHANISMS: FROM TARGETED VIRAL RNA DEGRADATION TO TRANSLATION INHIBITION

The following three ISGs are like the OAS/RNase L pathway in inhibiting viral translation, but dissimilar in their mechanism of inhibition. While the OAS/RNase L pathway employs global translation inhibition, ISG20, ZAP, and SHFL target specific RNA substrates. Furthermore, both ISG20 and ZAP have been shown to directly or indirectly degrade viral RNA, but their RNA degradation differs from OAS/RNase L in two major ways. They target specific viral RNA substrates for degradation, and this activity is independent of their inhibition of viral translation. The multiple, diverse, independent antiviral mechanisms of ISG20, ZAP, and SHFL can be explained through their recruitment of different co-factors, which this section will explore in depth.

ISG20

Antiviral activity of ISG20 has been attributed to two distinct mechanisms so far: degradation of viral RNA and translation inhibition, which can be direct or indirect. ISG20 was first identified as upregulated in response to IFN over 20 years ago (108). ISG20 is expressed in both the cell nucleus and cytoplasm, and is part of the DEDDh subgroup of the larger 3' to 5' DEDD exonuclease superfamily, which possesses a large exonuclease domain (EXO III domain) of about 150 amino acids that can confer DNase and/or RNase activity. The EXO III domain is characterized by three distinct exonuclease motifs defined by four invariant amino acids which lend this superfamily its name: three aspartate (D) and one glutamate (E) residue (109). The DEDDh subgroup also includes a conserved histidine residue. DEDDh exonucleases share a conserved fold and active site but have divergent substrate-binding sites, allowing them to recognize and thus degrade different substrates. Biochemical studies have shown that ISG20 degrades both ssRNA and DNA, with higher nuclease activity against RNA substrates (110).

Because ISG20 is a 3' to 5' RNA exonuclease, it has long been thought that ISG20 antiviral activity results primarily from degradation of viral RNA. In line with this hypothesis, ISG20 has been shown to exhibit antiviral activity primarily against RNA viruses (111). Overexpression and knockdown experiments

show that ISG20 widely suppresses viral replication of diverse (+) ssRNA viruses from *Togaviridae* (SINV), *Flaviviridae* (yellow fever virus (YFV), WNV, HCV), *Picornaviridae* (encephalomyocarditis virus, hepatitis A virus), and (-) ssRNA viruses from *Rhabdoviridae* (vesicular stomatitis virus (VSV)), *Orthomyxoviridae* (IAV), and *Bunyaviridae* (111, 112). ISG20 also inhibits human immunodeficiency virus-1 (HIV-1) and HBV, a DNA virus with an RNA replicative intermediate (36, 37, 113–115). However, ISG20 does not display pan-antiviral activity, as it fails to inhibit severe acute respiratory syndrome coronavirus (SARS-CoV), a member of the (+) ssRNA virus family *Coronaviridae* (38), and adenovirus, a DNA virus (111). The hypothesis that ISG20 degrades viral RNA is supported by reduced expression of various viral mRNAs and replicons in the presence of catalytically active, but not catalytically inactive ISG20 (36, 111, 112, 116).

One long-standing question has been how ISG20 selectively degrades RNA, given that ISG20 does not have any apparent regulatory domains and that its overexpression does not decimate cellular RNA. It is thought that ISG20 may interact with cellular co-factors, supported by its cell-type specific inhibition of YFV (38). The cellular N^6-methyladenosine (m^6A) pathway has been linked to ISG20 antiviral activity in the context of HBV infection. Briefly, m^6A is the most common reversible post-transcriptional modification that occurs on cellular RNAs. The m^6A pathway involves three primary types of proteins—writers, erasers, and readers—which respectively add, remove, and bind m^6A (117). Readers affect stability, translation, and localization of m^6A mRNA (117). One main group of m^6A readers is the YT521-B homology (YTH) domain-containing proteins. The cytoplasmic YTH members (YTHDF1-3) were recently found to play redundant roles in mediating degradation of m^6A-mRNAs (118). HBV transcripts are methylated within the epsilon stem-loop structure (ϵ), which is present at the 3' end of all HBV RNAs and repeated twice in the pregenomic RNA (pgRNA) (37). The HBV polymerase binds the 5' ϵ in pgRNA to initiate packaging and reverse transcription. ISG20 was shown to inhibit HBV replication by binding to ϵ (115). Furthermore, ϵ contains a conserved m^6A consensus sequence that negatively regulates HBV RNA stability in a YTHDF2-dependent manner (119). It was then demonstrated that YTHDF2 and ISG20 interact in an HBV-independent manner and that depletion of YTHDF2 abolishes IFN-dependent HBV RNA degradation (37). They propose that m^6A modification of ϵ is recognized by YTHDF2, which then recruits ISG20 to target HBV pgRNA for RNA degradation (**Figure 4A**). Based on these data, YTHDF2 was identified as an essential ISG20 co-factor, marking the first time any group has identified a regulator of ISG20 substrate specificity.

Still, the role of RNA degradation in ISG20 antiviral activity remains open for debate. Multiple studies have shown that ISG20 overexpression inhibits viral replication in an exonuclease-dependent manner, and several have observed a corresponding decrease in viral RNA (38, 111–113, 120). Moreover, most studies on ISG20 have utilized the catalytically inactive mutant D94G (110) to support the hypothesis that ISG20 exonuclease

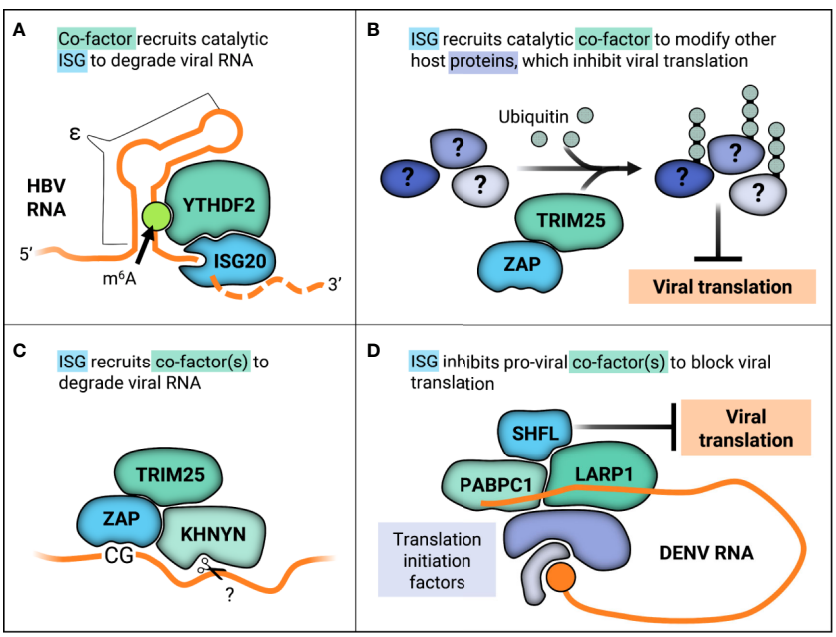

FIGURE 4 | ISGs work with co-factors in varied ways to inhibit viral replication through diverse mechanisms. **(A)** YTHDF2 recognizes m^6A methylation on the ϵ in HBV RNA transcripts, recruiting catalytic ISG20 to degrade HBV RNA. **(B)** ZAP recruits TRIM25 to ubiquitinate other host proteins, which may alter their native cellular or viral-associated activities in order to inhibit viral translation. **(C)** ZAP recognizes CG motifs in viral RNA and forms a complex with TRIM25 and the endonuclease KHNYN, which then putatively cleaves ZAP-bound RNA. **(D)** SHFL inhibits DENV replication by interacting with the viral RNA 3'UTR binding proteins PABPC1 and LARP1 and potentially inhibiting recruitment of further translation machinery.

activity is required for antiviral activity (38, 111–113, 116, 120, 121). This invariant residue is crucial to ISG20 structure as it helps coordinate an Mn^{2+} ion in the active site (122). It is unknown what other deleterious effects D94G may have on the overall structure of ISG20. One group mutated all Asp residues in the DEDDh catalytic motif to Gly (D11G, D94G, and D154G) and found that while all three mutations abolished exonuclease activity, only D11G and D94G also lost antiviral activity against HBV. Surprisingly, the exonuclease-deficient ISG20-D154G was still able to inhibit HBV replication (36). This suggests that ISG20 possesses both exonuclease-dependent and -independent antiviral activities.

Recently, two groups have independently found that ISG20 mediates antiviral activity through translation inhibition and not RNA degradation, but propose two divergent mechanisms (121, 123). One group proposes that ISG20-mediated translation inhibition is indirect, mediated through ISG20-dependent upregulation of other ISGs (123). They found that overexpression of ISG20 upregulates expression of many IRF3-dependent genes in mouse embryonic fibroblasts, such as IFIT1, an ISG that inhibits translation of viral RNA with non-2'O-methylated 5' caps (124). Furthermore, they showed that mutant alphavirus normally recognized and attenuated by IFIT1 is equally virulent as the wild-type virus in the absence of ISG20 *in vivo*, suggesting that ISG20 inhibits translation by promoting IFIT1 expression (123). On the contrary, the second group proposes that ISG20 is critical for discrimination of non-self nucleic acids from self to inhibit viral translation (121). By examining luciferase reporter activity, they found that ISG20 inhibited translation for all exogenous DNA of both viral and host origins. However, when a CMV-GFP cassette was integrated into the host genome, ISG20 failed to inhibit GFP produced from this context as it did when the identical cassette was transfected into the same cells. They also generated a series of mutants outside of the invariant DEDD residues to explore how non-exonuclease regions impact ISG20 antiviral activity. Notably, mutations predicted to affect phosphorylation and cellular trafficking but not exonuclease activity still lose antiviral activity against VSV. This loss of antiviral activity correlates with inability to inhibit translation of non-self nucleic acids. Contrary to previous findings, the second group did not find that ISG20 induces IFIT1 expression in HEK293T or U937 cells. They propose instead that ISG20 recruits foreign RNA to P bodies, sites of RNA storage, where translation is repressed in the absence of RNA degradation (121).

All in all, ISG20 effectively inhibits viral replication by degrading or inhibiting translation of specific RNAs. ISG20 co-opts the cellular m^6A pathway component, YTHDF2, to recognize and target modified HBV RNA (37). ISG co-factors such as IFIT1 may also be required in ISG20 translation inhibition, as ISG20 does not globally arrest translation like the OAS/RNase L pathway, but rather specifically targets non-self transcripts.

ZAP

ZAP, encoded by the gene *ZC3HAV1*, is a potent antiviral factor which broadly inhibits replication of HIV-1 (*Retroviridae*), HBV (*Hepadnaviridae*), the (-) ssRNA viruses Ebola virus (EBOV, *Filoviridae*) and IAV (*Orthomyxoviridae*), and (+) ssRNA viruses such as alphaviruses (*Togaviridae*), coxsackievirus B3 (CVB3, *Picornaviridae*), and Japanese encephalitis virus (JEV, *Flaviviridae*) (125–132). ZAP antiviral activity can be selective within viral families and genera, as not all flaviviruses and picornaviruses tested are sensitive to ZAP (126, 131). It also post-transcriptionally regulates expression of cellular mRNA (133) and restricts retrotransposition of human retrotransposons (134, 135). ZAP possesses four N-terminal zinc fingers (136) that directly bind to viral RNA, are required for antiviral activity, and dictate its mostly cytoplasmic and stress granule localization (134, 137–139). Phosphorylation of this minimal antiviral N-terminal region by glycogen synthase kinase 3β enhances ZAP antiviral activity, though its mechanistic contribution remains unclear (140). Alternative splicing results in multiple splice variants which differ from one another in expression, localization, and antiviral activity. ZAP is also known as PARP13, due to poly-ADP-polymerase 13 (PARP13), due to the inclusion of a catalytically inactive C-terminal PARP-like domain in its long splice variant (ZAPL). In addition to a short splice variant that lacks this C-terminal domain (ZAPS), alternative splicing of a 121 aa extension of exon 4 results in two additional splice variants ZAPM and ZAPXL, whose antiviral activities are similar to ZAPS and ZAPL, respectively (141).

ZAP targets viruses primarily by two distinct antiviral mechanisms, namely viral translation inhibition (for the (+) ssRNA viruses SINV and JEV) and viral RNA degradation (for HIV-1, HBV, the (-) ssRNA virus EBOV, and the (+) ssRNA viruses CVB3 and JEV). These disparate mechanisms can be explained in part by recruitment of co-factors and differing viral contexts. ZAP inhibition of SINV translation has been linked to its disruption of the interaction between translation initiation factors eIF4A and eIF4G (142). This disruption does not affect global translation seeing as polysome profiles were unchanged when ZAP was overexpressed (142). ZAP is also able to repress translation of a luciferase reporter containing the minimal ZAP responsive fragment in the SINV genome without promoting degradation of the reporter (142). More recently, the E3 ligase tripartite motif-containing protein 25 (TRIM25) was uncovered as a novel ZAP co-factor in the context of alphavirus infection (47, 143). TRIM25 is absolutely required for inhibition of viral translation by ZAP, as ZAP is unable to inhibit translation of a replication-deficient reporter virus in TRIM25-deficient cells (47). Not only is TRIM25 putatively required for ZAP recognition of its RNA substrates, as TRIM25 knockdown decreases ZAP association with luciferase reporter RNA, but also TRIM25 ubiquitin ligase activity is essential for ZAP antiviral activity (47, 143). Curiously, though TRIM25 ubiquitinates ZAP, TRIM25 still contributes to ZAP antiviral activity in the presence of a ubiquitination-deficient ZAP mutant (47), suggesting that TRIM25-mediated ubiquitination of host factors other than ZAP is critical for the inhibitory effects (**Figure 4B**). Moreover, it is likely that K63-linked ubiquitination is required for ZAP antiviral activity, as overexpression of a ubiquitin K63R mutant unable to form K63 linkages reduces ZAP inhibition of SINV replication (143). The identity of these TRIM25 substrates that function in ZAP antiviral

activity remains to be discovered, as does how they contribute to viral translation suppression.

Apart from inhibiting viral translation, ZAP also induces viral RNA degradation by recruiting an array of RNA helicases, the endonuclease KHNYN, and exosome components (144–147). ZAP selectively affects cellular transcripts, as it destabilizes the TRAILR4 mRNA and inhibits retrotransposition of endogenous retroelements such as Long INterspersed Element-1 (LINE-1) and Alu (133, 134). ZAP substrate specificity determinants largely remained a mystery until it was demonstrated to inhibit HIV-1 with synonymous, elevated CG dinucleotide mutations (HIV^{CG}) but not wild-type HIV-1 (148). Interestingly, only elevation of CG dinucleotides in the 5' third of the HIV-1 envelope gene caused ZAP susceptibility (149). Solving the crystal structure of ZAP in complex with CG-rich RNA revealed that ZAP has a CG-dinucleotide specific binding pocket and binds to ss nucleic acids (150, 151). ZAP preference for CG-rich substrates could explain in part why many RNA viruses infecting mammals and other vertebrates, such as IAV and SARS-CoV-2, exhibit CG suppression (152–155). ZAP can even sense CG dinucleotides within individual RNA transcripts of DNA viruses, as in the case of human cytomegalovirus (156). Here, CG suppression within the major immediate early transcript 1 confers ZAP resistance (156). However, CG suppression does not always confer resistance to ZAP as in the case of SARS-CoV-2 (154). Though initially identified in the context of translation inhibition, ZAP was also shown to form a complex with TRIM25 and the endonuclease KHNYN to inhibit HIV^{CG} (**Figure 4C**) and knockdown of KHNYN abolished HIV^{CG} sensitivity to ZAP (147).

All three ZAP antiviral activities of CG dinucleotide sensing, RNA degradation, and translation inhibition were linked in the context of JEV infection, wherein ZAP bound CG-rich regions of JEV RNA and inhibited translation at early time points without RNA degradation, and degraded RNA in an exosome-dependent manner at later time points of a JEV replicon (131). Therefore, ZAP can block viral translation in the context of alphavirus infection, target viral RNA for degradation in the context of retrovirus infection, and do both in the context of JEV infection.

To complicate matters further, ZAP splice variants also display differences in antiviral activity. ZAPL is more antiviral than ZAPS (141, 157). This boost to antiviral activity is attributed to its PARP-like domain. Not only does the PARP-like domain carry signatures of positive selection (157), but it also has a prenylation motif that targets ZAPL to endolysosomes (158, 159). Addition of this prenylation motif to ZAPS increases its antiviral activity, though not to the same extent as ZAPL (159). Curiously enough, ZAPL's catalytically dead PARP triad motif is required for its antiviral activity; its replacement with the canonical active PARP motif abolishes ZAPL antiviral activity, though it remains unclear how this inactive motif is required (160). Furthermore, ZAPL is constitutively expressed in cells, while ZAPS expression is induced by innate immune signaling (141, 159). Studies conflict as to how ZAPS contributes to innate immune signaling. Though one group showed that ZAPS stimulates RIG-I dependent IFN response upon stimulation with a RIG-I RNA agonist (161), others found that ZAP mediates a RIG-I-independent antiviral response to retroviruses and HBV (128, 162, 163). More recently, ZAPS was shown to negatively regulate the type I IFN response by binding to and stimulating the degradation of *IFN* mRNAs; ZAP-deficient Huh7 cells had a higher and more prolonged IFN response upon treatment with a RIG-I agonist (159). On the other hand, ZAPS was found to synergize with other ISGs, wherein 31 ISGs have a statistically significant increase in antiviral activity in the presence of ZAP (32). In addition to its role as a co-factor in ZAP translation inhibition and CG sensing, TRIM25 may also modulate expression of ZAP isoforms by regulating alternative splicing, wherein TRIM25 is required for efficient expression of ZAPS (156).

All in all, ZAP inhibition of viral replication layers in complexity through its diverse mechanisms of translation inhibition and RNA degradation, recruitment of varied co-factors, and further differences between splice variants. The differing C-termini, expression kinetics, and cellular localization between splice variants could facilitate recruitment of divergent co-factors to enable different antiviral roles. As the PARP-like domain of ZAPL lacks any catalytic activity, it likely acts as an interaction domain to recruit specific co-factors that might be ADP-ribosylated to effect the RNA-centric antiviral mechanisms of ZAP.

SHFL

SHFL, variously referred to as C19orf66, RyDEN, IRAV, or FLJ11286, is a 291 amino acid protein that is predicted to consist of eight α-helices and seven β-strands and possess both a nuclear export and localization signal, a zinc-ribbon domain, and a coiled-coil motif (164). SHFL binds nucleic acids and shows greater preference for ss nucleic acids and for RNA over DNA *via* fluorescence polarization assays (165). No catalytic activity is currently attributed to SHFL.

In uninfected cells, SHFL resides primarily in the cytoplasm in punctate structures, associating with both stress granule and P body proteins in HEK293 and Huh7.5 cells (165, 166), but was also more recently identified as an antiviral effector counteracting replication of RNA viruses (27, 28, 164, 167). For example, SHFL broadly inhibits replication of members of the (+) ssRNA virus family *Flaviviridae*, including all four DENV serotypes, WNV, ZIKV, and HCV (164–166, 168). SHFL also inhibits the virion production of chikungunya virus and SINV, members of the (+) ssRNA virus family *Togaviridae*. However, SHFL selectively inhibits members of another (+) ssRNA family, *Picornaviridae*, inhibiting replication of encephalomyocarditis virus but not poliovirus or enterovirus 71 (164, 165). SHFL selective inhibition also extends to DNA viruses, as its overexpression inhibits virion production of *Adenoviridae* member human adenovirus type 3, and *Herpesviridae* members KSHV and herpes simplex virus-1 (HSV-1), but not HSV-2 (164, 169).

A recently proposed mechanism for SHFL antiviral activity is suppression of viral translation. In line with this hypothesis, SHFL associates specifically with DENV RNA (164) and co-immunoprecipitates with other RNA-binding proteins that bind to mRNA 3' UTRs such as PABPC1, LARP1, MOV10, and UPF1 (164, 165). Given that PABPC1 is critical for translation, overexpression of SHFL suppresses translation of a DENV replication-deficient

luciferase reporter (164). Co-immunoprecipitation and immunofluorescence techniques were used to show that SHFL interacts with MOV10 and UPF1 even in the presence of RNase A, though the interaction was diminished (165). It is likely that SHFL mediates viral translation inhibition by interacting with the viral RNA 3'UTR binding proteins such as PABPC1 and LARP1 to block recruitment of further translation machinery (**Figure 4D**).

A separate, better characterized mechanism that SHFL utilizes to block viral translation is its broad inhibition of -1 programmed ribosomal frameshifting (-1PRF) which is crucial for many viruses to control protein expression levels (54). SHFL inhibits HIV-1 replication by altering the Gag to Gag-Pol protein ratio *via* inhibition of -1PRF, wherein knockdown of SHFL results in increased Gag-Pol expression without obviously altering either Gag or capsid expression (54). Noticeably, these data are in agreement with previous findings of unchanged p24 levels upon SHFL overexpression, which were originally interpreted as evidence that HIV-1 is not inhibited by SHFL (164). SHFL was also demonstrated to inhibit both viral and cellular mRNAs -1PRF signals in the context of a dual luciferase reporter construct (54). It is important to note that overall cellular translation and protein expression and ISG expression are unchanged in the presence of SHFL overexpression or knockdown in Huh7.5 cells (166), supporting the notion that SHFL antiviral activity is not due to modulating ISG expression or alteration of global translation (166).

However, SHFL may also act on some viruses independent of its direct effects on viral translation. For example, SHFL associates with the flavivirus replication complex in both DENV and ZIKV infections and interacts specifically with nonstructural protein 3 (NS3) in an RNA-independent manner (165, 168). By doing so, SHFL induces lysosomal-mediated degradation of NS3 in ZIKV-infected cells. SHFL is thought to inhibit HCV replication by interfering with the HCV-induced remodeling of the ER, which generates a membranous web that scaffolds assembly of viral replication complexes (166).

Taken together, SHFL inhibits viral replication by regulating translation through -1PRF-dependent and independent mechanisms and by specific antagonism of viral proteins and structures. SHFL displays both RNA-independent and -dependent interactions with other proteins, relying on RNA for its interaction with cellular RNA helicases MOV10 and UPF1 while interacting with flavivirus NS3 in the absence of RNA. These varied requirements for protein-protein interactions may reflect SHFL's diverse antiviral mechanisms.

RING IN VIRAL TRANSCRIPTION: UBIQUITIN LIGASE-DEPENDENT AND -INDEPENDENT INHIBITION

The really interesting new gene (RING) proteins are the most abundant family of E3 ligases, characterized by their N-terminal catalytic RING domain. E3 ligases occupy the final step in cellular ubiquitination. Ubiquitination of a protein can alter its cellular fate depending on the type of linkage, ranging from proteasomal degradation to scaffold formation for assembly of cellular signaling complexes (170). In order for ubiquitin to be ligated to an acceptor lysine, it must be sequentially activated by the E1 enzyme, carried by the E2 conjugating enzyme, and finally ligated to an acceptor lysine by one of over 600 human E3 ligases. Though many of the antiviral effectors mentioned in this section have other known cellular and antiviral roles, this section will focus on how IFN-inducible RING ligases inhibit viral transcription by both ligase-dependent and -independent mechanisms.

RBBP6: Ligase-Independent Viral Mimicry

Retinoblastoma binding protein 6 (RBBP6) is a RING E3 ligase that inhibits transcription of the (-) ssRNA virus EBOV (*Filoviridae)* (53). RBBP6 was identified in an affinity-purification mass spectrometry screen to map host-EBOV protein-protein interactions as the most robust host interactor with the EBOV transcription regulator viral protein 30 (VP30) (53). RBBP6 competes with the EBOV nucleoprotein for binding to VP30 in an RNA-independent manner; the minimal RBBP6 interaction motif is sufficient to inhibit viral transcription, demonstrating a ligase-independent antiviral mechanism for RBBP6 (53). However, full-length RBBP6 causes dose-dependent decrease of VP30 protein in a manner dependent on the RBBP6-VP30 interaction, potentially suggesting a ligase-dependent antiviral mechanism (53). Curiously, RBBP6 also causes a dose-dependent decrease of VP30 mRNA independent of RBBP6-VP30 interaction. This suggests that RBBP6 either degrades VP30 mRNA through an uncharacterized exonuclease domain or recruits co-factor(s) that possess exonuclease activity (53). Knockdown of RBBP6 enhances EBOV RNA synthesis and replication. Taken together, these results suggest that RBBP6 inhibits EBOV replication through a three-pronged approach, and that both ligase-dependent and -independent antiviral mechanisms and exonuclease-dependent mechanism may be waiting to be further characterized.

TRIMming Viral Transcription

The tripartite motif containing proteins (TRIM) are the largest group of RING E3 ligases and constitute an important family of proteins in the type I IFN response (171, 172). There are over 70 human TRIM proteins, many of which are induced by type I IFN (171, 173). Interestingly, the rapid expansion of the TRIM family coincides with the development of adaptive immunity, suggesting that TRIMs may have evolved to play a role in immune regulation (171). These proteins typically possess three conserved domains at the N-terminus—a catalytic RING domain, one to two B-box domains that are thought to function in higher order oligomerization, and a coiled-coil domain that allows TRIMs to dimerize and potentially oligomerize (174). Most TRIMs directly inhibit viral replication by targeting viral components for degradation, or indirectly inhibit by modulating innate immune signaling (175, 176).

Multiple TRIM members have been found to inhibit viral transcription *via* both ligase-dependent and -independent mechanisms. TRIM22 does both, though its ligase activity is required to inhibit HBV transcription, it inhibits HIV-1

transcription independent of its ligase activity (177–179). TRIM22 inhibits HBV core promoter activity, which is critical for HBV pgRNA synthesis and hence viral transcription and reverse transcription (177, 180). A single point mutation in its RING domain abolishes its inhibition of viral replication, strongly implicating ligase activity in anti-HBV effects of TRIM22 (177). Meanwhile, TRIM22 inhibits HIV-1 basal transcription independent of its ligase activity by indirectly preventing the transcription factor specific protein 1 (Sp1) from binding to the HIV-1 promoter, thus facilitating HIV-1 latency (181, 182). As TRIM22 neither directly interacts with Sp1 nor binds to the HIV-1 promoter, it is possible that TRIM22 recruits another co-factor to alter chromatin state or stimulate Sp1 post-translational modification (181). Two independent TRIMs inhibit IAV transcription, a (-) ssRNA virus. TRIM32 depends on its ligase activity, ubiquitinating the core component of the IAV RNA polymerase complex and targeting it for degradation, subsequently reducing polymerase activity (57). On the other hand, TRIM25 restricts IAV RNA synthesis in a ligase-independent manner by binding to viral ribonucleoproteins and blocking RNA chain elongation (56).

TRIM69 is a more recently identified ISG and antiviral effector that shares high homology with TRIM25. Recently, two independent groups found that TRIM69 inhibits replication of the Indiana strain of VSV (VSV_{IND}), a (-) ssRNA virus, in ligase-independent manner (58, 59). Both groups identified TRIM69 through targeted screens using complementary approaches, either overexpressing an array of known ISGs or knocking down VSV-induced host genes (58). In addition, both groups found that TRIM69 inhibition of VSV is highly specific, as overexpression of TRIM69 fails to inhibit the New Jersey strain of VSV (VSV_{NJ}) or other negative-strand RNA viruses such as SeV, rabies virus, or IAV (58, 59). VSV_{IND} sensitivity to TRIM69 was mapped to a short peptide sequence within the viral phosphoprotein P by serial passaging VSV_{IND} in the presence of TRIM69 overexpression and sequencing escape mutants (58, 59). VSV_{NJ} differs from VSV_{IND} at five out of six amino acids within this TRIM69 P sensitivity determinant, potentially explaining differential resistance between VSV strains. TRIM69 physically associates with VSV_{IND} P but does not require its ligase activity or target it for degradation. Instead, TRIM69-inhibition of VSV_{IND} requires its multimerization in order to sequester VSV_{IND} P into filamentous structures, thus disrupting viral replication machinery.

To summarize, RING E3 ligases combat viral transcription in myriad ways. Many do not rely on their ligase activity to inhibit transcription by directly binding to components of viral transcription machinery to inhibit protein-protein interactions, such as RBBP6, TRIM25, and TRIM69. Others ubiquitinate viral components to target them for degradation, such as TRIM22 and TRIM32.

DISCUSSION

Inhibition of viral replication by ISGs grows more nuanced as every new study promises to uncover new facets of antiviral or pro-viral activity. Even well-characterized ISGs such as OAS/RNase L, ISG20, and ZAP have had new aspects of their antiviral mechanisms come to light in recent years. RNA viruses present a plethora of unique viral RNA processes that host cells can identify and inhibit. They rely on their own viral RNA-dependent RNA polymerases to transcribe and replicate genomic RNA, generating dsRNA intermediates that host cells recognize as foreign. Each RNA-centric antiviral mechanism mentioned in this review affords specific advantages and disadvantages. Blocking viral translation is especially effective against positive-stranded RNA viruses, which must translate their incoming genomes before any further steps in viral replication can occur. Likewise, inhibiting viral transcription is especially effective against negative-stranded RNA viruses, which prioritize transcribing their genomes upon entry. While degrading genomes outright appears to be the most straightforward and universal way to inhibit replication of RNA viruses, RNA degradation presents its own set of challenges of distinguishing foreign from self RNA. In the case of OAS/RNase L, dsRNA sensing by OAS leads to virtually indiscriminate degradation of viral and host RNAs, curtailing viral replication but also killing the infected cell. Some especially pathogenic viruses such as Middle East respiratory syndrome coronavirus (MERS-CoV) circumvent RNA degradation by enzymatically degrading 2-5A, thus preventing RNase L activation (183). Meanwhile, other ISGs, such as ZAP, recognize specific motifs in the viral genome distinct from host genomes so only viral RNA is selectively targeted. However, ZAP specificity for CG dinucleotides may have driven selection against high CG content in RNA viral genomes, thus potentially rendering ZAP ineffective (152, 153).

The perpetual arms race between antiviral effectors and viruses has likely driven the development of multilayered mechanisms of viral inhibition. Some individual ISGs have acquired multiple antiviral mechanisms, enabling them to circumvent viral evasion. For example, ZAP is still able to inhibit replication of SARS-CoV-2, a positive-sense ssRNA virus with highly suppressed CG content (154). This suggests that ZAP inhibits SARS-CoV-2 not by CG sensing alone and that there are likely additional sequence or structural motifs that are recognized and targeted by ZAP. Another way ISGs may prevent viral evasion is through the use of co-factors, which could function as a natural "antiviral cocktail" (**Figure 4**). By using co-factors, host cells employ a multipronged attack on viral replication that could help minimize evasion by RNA viruses. ISGs that work in concert to recognize specific viral RNA motifs could make it difficult for viruses to simultaneously mutate all recognizable motifs in their genomes. Though it is known that TRIM25 functions as a ZAP co-factor (47, 143), that both TRIM25 and ZAP bind RNA (48, 139, 184, 185), and that RNA binding is crucial for their antiviral activity (136, 185), it remains unexplored whether TRIM25 and ZAP work together to recognize viral RNA substrates or motifs or both. TRIM25 and ZAP putative cooperation in viral recognition could help explain why many alphaviruses have not acquired ZAP resistance and remain acutely sensitive to its inhibition (126, 141).

In-depth characterization of ISG antiviral mechanisms and methods of viral evasion has been facilitated by rapid expansion of CRISPR-Cas technologies. For example, identification of OAS3 as necessary and sufficient for RNase L activation was enabled by generation of single OAS KO cell lines (67). CRISPR-Cas technologies have also enabled the discovery and interrogation of functions of novel ISGs and their splice variants or polymorphisms (186). Genome-wide CRISPR-Cas9 KO and targeted ISG overexpression screens have been used to great effect, identifying novel host factors and highlighting important antiviral ISGs to characterize [reviewed in (20)]. One exciting new avenue for RNA-centric ISG identification lies at the intersection of chemical biology. Generation of nucleotide analogs that can be incorporated into RNA, cross-linked to proximally bound proteins, and immunoprecipitated for subsequent proteomic analysis enables the identification of novel RNA-binding proteins (187). Variations on this approach have been used several times in the context of positive-sense RNA virus infections by *Togaviridae* and *Flaviviridae* family members to elucidate new host-virus interactions (49, 188–190). It is not always feasible to target host factors required for viral replication, since these host factors may also be essential for cell survival. Furthermore, overexpression of any given protein may yield false phenotypes that are not biologically relevant. In contrast, *in situ* labeling and identification of endogenous RNA-binding proteins offers a more specific and minimally disruptive approach with fewer effects on cell viability. Cross-referencing ISGs with novel viral RNA-binding proteins could yield promising candidates to characterize and open up new horizons for antiviral exploration.

AUTHOR CONTRIBUTIONS

EY and ML determined the scope and focus of the review. EY drafted the manuscript and generated all figures. ML provided critical feedback on the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Shared and Unique Features of Human Interferon-Beta and Interferon-Alpha Subtypes

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Type I interferons (IFN-I) were first discovered as an antiviral factor by Isaacs and Lindenmann in 1957, but they are now known to also modulate innate and adaptive immunity and suppress proliferation of cancer cells. While much has been revealed about IFN-I, it remains a mystery as to why there are 16 different IFN-I gene products, including IFNβ, IFNω, and 12 subtypes of IFNα. Here, we discuss shared and unique aspects of these IFN-I in the context of their evolution, expression patterns, and signaling through their shared heterodimeric receptor. We propose that rather than investigating responses to individual IFN-I, these contexts can serve as an alternative approach toward investigating roles for IFNα subtypes. Finally, we review uses of IFNα and IFNβ as therapeutic agents to suppress chronic viral infections or to treat multiple sclerosis.

Keywords: type I interferon, interferon-beta, interferon-alpha, interferon-omega, human, primate

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INTRODUCTION

Type I interferons (IFN-I) are monomeric cytokines that are best known for their antiviral activity but that also suppress proliferation of cancer cells and modulate innate and adaptive immune responses. IFN-I were first discovered as an antiviral factor by Isaacs and Lindenmann in 1957 and were subsequently revealed to include IFNβ and multiple subtypes of IFNα (1, 2). We now know that human type I IFNs comprise a family of 17 functional genes and 9 pseudogenes clustered on chromosome 9 (3) that encode 16 proteins: IFNβ, ϵ, -κ, -ω, and 12 subtypes of IFNα (**Figure 1**). Since protein sequences for mature IFNα1 and IFNα13 are identical, we will collectively refer to them as IFNα1.

IFNβ may be considered the "primary" IFN-I because it is expressed by all nucleated cells and may be expressed in isolation of all other IFN-I (except IFNα1, discussed below). Two IFN-I genes are selectively expressed in specific organs or by specific cell types: IFNϵ is hormonally regulated and primarily expressed in the female genital tract (4) and has recently been reviewed elsewhere. IFNκ is primarily expressed by keratinocytes (5) where it has recently been shown to have a role in protection against cutaneous herpes simplex virus (6), papilloma virus (7), and cutaneous lupus erythematosus (8). Like IFNϵ, IFNκ is constitutively expressed (9). By contrast, IFNκ expression is activated and suppressed by TGFβ and ERK1/2 kinases, respectively (7, 10).

While IFNω is the least studied IFN-I in human biology, feline IFNω is well characterized and licensed as a veterinary antiviral therapeutic. In felines, IFNω is leukocyte specific (11, 12). While little is known about IFNω expression patterns, the presence of neutralizing autoantibodies is

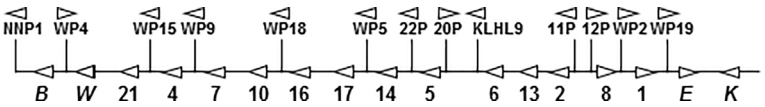

FIGURE 1 | Gene map of the human IFN-I gene cluster. Above the line are pseudogenes for IFNν (NNP), IFNα subtypes, IFNω, and for the functional KLHL9 gene. On the line are the 17 functional type I IFN genes. Genes for IFNα subtypes are labeled only by number.

indirect proof that it is expressed and suggest a role in human disease. For example, in 2006, Meager et al. reported that 100% of *AIRE*-deficient patients with the autoimmune polyendocrinopathy syndrome have high titers of neutralizing autoantibodies against both IFNω and IFNα (13). More recently, Bastard et al. reported that ~1% of patients with severe Covid19 has selective neutralizing auto-antibodies against IFNω (14), suggesting that the importance of this type I IFN is in viral infections is underappreciated.

Mature IFNβ and eleven of the 12 IFNα subtypes are 166 a.a. in length (IFNα2 is 165 a.a. due to deletion of D44) with a MW of ~20 kD. IFNε and IFNω are 187 a.a. and 174 a.a., respectively, both due to an elongated carboxy-terminal, while IFNκ is 179 a.a. due to an insertion following residue 116. As shown in **Supplemental Figure 1** (15), IFNβ and IFNω share 31%–38% and 55%–60% identity with all IFNα subtypes, respectively, whereas identity among the IFNα subtypes ranges from 76%–96%. IFNβ, IFNω, and two IFNα subtypes are glycosylated; IFNβ at N80 (16), IFNω at N78 (17), IFNα2 at T108 (18), and IFNα14 at N72 (19).

Despite sharing only ~30% identity across all IFN-I, the three-dimensional structures are remarkably similar (20, 21). The salient structural features of all IFN-I, which are reviewed in detail by Walter et al. in this series include: 1) cylindrical proteins that consist of five 11-24 residue α-helices (labeled A–E), each parallel to the long axis of the cylinder; 2) Loops that connect the helices, of which the AB loop is relatively long and includes three short 3_{10} helices (22, 23); 3) conserved bonding including disulfide bridges (one in IFNβ, two each in IFNω and all IFN subtypes) and a network of hydrogen bonds to form and stabilize the tertiary structure; 4) IFNAR2 binding residues in Helix A, the AB loop and Helix E, and IFNAR1 binding sites spaced among helices B–D and the CD loop (21).

All IFN-I signal through a heterodimeric receptor that is comprised of two subunits, IFNAR1 and IFNAR2. In the classical model of IFN signaling, IFN first binds IFNAR2 forming a high-affinity binary complex which then recruits IFNAR1 to form a functional ternary structure that triggers phosphorylation of Jak1 and Tyk2-initiating "canonical" signaling (24). In canonical IFN-I signaling (**Figure 2**), activation of Jak1 and Tyk2 is followed by phosphorylation of STAT1 and STAT2, which trimerize with IRF9 to form the transcription factor interferon-stimulated growth factor-3 (ISGF3) (25). Once assembled, ISGF3 translocates to the nucleus and binds to interferon stimulated response elements (ISRE) to promote transcription of interferon stimulated genes (ISGs). Through this canonical pathway, many genes are highly susceptible to shifts in expression with small amounts of IFN-I, thus earning the label of "robust" ISGs (26). Robust ISGs include most antiviral effectors from which the name "interferon" was derived.

Non-canonical IFN-I signaling includes cell-specific pathways such as those mediated by STAT1 homodimerization, other STAT family members, and MAP- or PI_3-kinases (**Figure 2**). To better characterize these pathways, Urin and colleagues used HeLa cell signaling-component deletion mutants to show that except for the formation of STAT1 homodimers or STAT2/IRF9 heterodimers, non-canonical signaling depends on phosphorylation of both STAT1 and STAT2 (27). For the most part, non-canonical signaling induces "tunable" ISGs (26), which exhibit gradual rather than steep dose-response curves, and higher IFN concentrations for peak expression (26). Non-canonical pathways such as suppression of cell proliferation best correlates with the stability of the IFN/IFNAR1/IFNAR2 ternary complex [defined as (IFN-I K_D IFNAR1* IFN-I K_D IFNAR2)] (24). Non-canonical signaling also mediates expression of chemokines and cytokines that modulate innate or adaptive immunity, transcription factors that modulate cell phenotype, and some antiviral responses. As examples, APOBEC3, a cytidine deaminase that blocks HIV replication in macrophages, and IRF1, a transcription factor that mediates IFN-dependent and -independent viral immunity (28–31), share characteristics of tunable ISGs. While IFNAR2-independent signaling has been reported in mice (32), there are no data to controvert the current model that both IFNAR1 and IFNAR2 are necessary for signaling in humans.

Why there are so many IFN-I genes, and specifically so many IFNα subtypes, remains a mystery. As would be predicted by their common use of a shared receptor, evidence to date points to quantitative rather than qualitative differences among the IFN-I. In other words, differences in gene expression, antiviral, or antiproliferative activity at subsaturation are equalized by dose adjustments or in the extreme, by receptor saturation. Thus, while their evolutionary history and expression patterns suggest that at least some IFN-I serve specific functions, very few have been defined. Here, we focus on differences among IFNβ and the IFNα subtypes to propose a model by which patterns of expression mirror their evolutionary history, and thus provide an alternative approach toward deciphering their roles in human biology.

EVOLUTION OF TYPE I INTERFERONS

Types I and III IFNs evolved from a common ancestor gene that shares the 5-exon/4-intron organizational structure of the IL-10

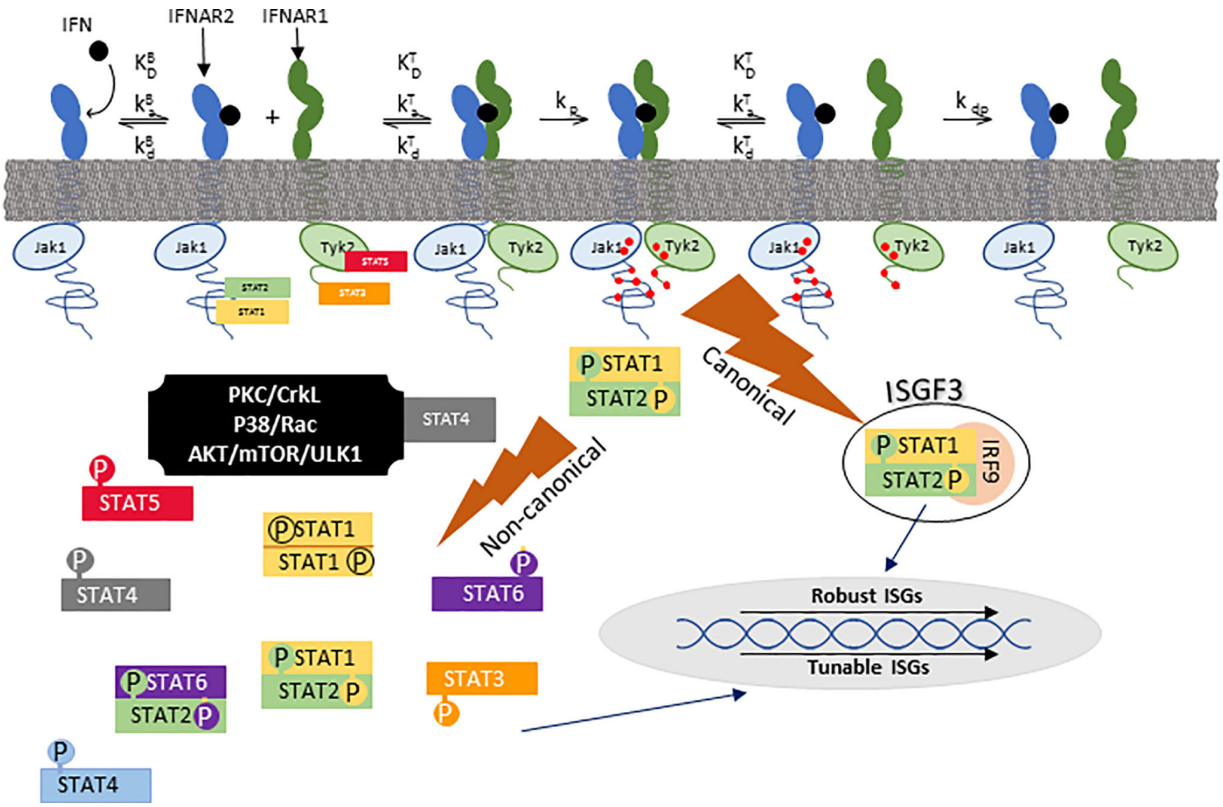

FIGURE 2 | Canonical and noncanonical IFN signaling. IFN first binds to IFNAR2 after which the IFN/IFNAR2 binary complex recruits IFNAR1 to form a functional ternary signaling complex (IFN/IFNAR1/IFNAR2). Following that, Jak1 and Tyk2 kinases, which are pre-associated with IFNAR2 and IFNAR1 respectively, phosphorylate each other and tyrosine residues on each receptor (red dots) upon which STAT (signal transducers and activators of transcription) family members dock. Canonical signaling consists of a trimer of pSTAT1, pSTAT2, and IRF9 which is referred to as ISGF3 (interferon-stimulated gene factor 3). ISGF3 translocates to the nucleus to bind ISRE (interferon-stimulated response elements) to stimulate transcription of robust ISGs. There are many non-canonical signaling pathways, one of which is formation of phosphorylated STAT1 homodimers that bind to GAS (gamma activation site) promoter elements. k_a and k_d are association and disassociation rates, respectively. K_D is the equilibrium disassociation constant (k_d/k_a). k_p and k_{dp} are rates of phosphorylation and dephosphorylation, respectively. K^B and K^T refer to binary (IFN/IFNAR2) and ternary (IFN/IFNAR2/IFNAR1) complexes, respectively. This figure was adapted from **Figure 1** of (24).

family of cytokines. The intronless IFN-I genes of all higher order primates evolved and diversified from those of cartilaginous and bony fish. As shown in **Figure 3A**, IFNκ was the first to evolve from the "most recent common ancestor" (MRCA), followed by IFNβ. Both were present ~200 million years ago (MYA) before eutherians and marsupials diverged. IFNε arose from IFNβ, which later duplicated to give rise to IFNω and the IFNα genes (15). Primate IFN-I are highly divergent from other mammals. For example, in bats and ungulates, IFNω emerged as a multigene subtype (33) while primates have one functional IFNω gene and multiple IFNα subtypes.

The first *IFNA* gene appeared 95–105 MYA, which through duplication and conversion gave rise to an expanded set of IFNα subtypes in a subset of placental mammals (15). *IFNA* gene duplication and conversion that occurred before speciation gave rise to a conserved cluster of IFNα subtypes that are dissimilar, but that are shared across species. Conversely, duplication after speciation gave rise to variant clusters that are highly similar within each species but are not shared across species. As shown in **Figure 3B**, the first IFNα subtypes that are present in humans and simiiforms—*IFNA13*, *-A2*, *-A8*, and *-A21*—were present before the divergence of new world and old world monkeys (NWM and OWM) 65-47 MYA. NWM have one gene each for *IFNA13* (syntenic with *IFNA13* in monkeys and apes), *IFNA2* and *IFNA21*, and two genes each that are similar to *IFNA8* and *IFNA5* in higher order primates. Subsequently, *IFNA13* duplicated to give rise to *IFNA1* (present in OWM and apes), and *IFNA5*, *IFNA6*, and *IFNA14* arose to complete the set of *IFNA* subtypes that are conserved during primate evolution (**Figure 3B**, blue background). The subset of human *IFNA* subtypes that are variant among primates (pink background) arose after orangutans and the other great apes diverged. It has been proposed that *IFNA4*, *IFNA10*, *IFNA17* are products of partial conversions from *IFNA14* or *IFNA21 (IFNA4*, *-A10*, and *-A17)* (15) and that *IFNA10* may have converted *IFNA7* or vice versa (34).

Based upon a detailed analysis of human polymorphisms in sub-Saharan African, Asian, and European populations, Manry et al. (35) found the fewest polymorphisms in *IFNA6*, *-A8*, *-A13*, and *-A14*. Exclusion of *IFNA1* from this group appeared to be

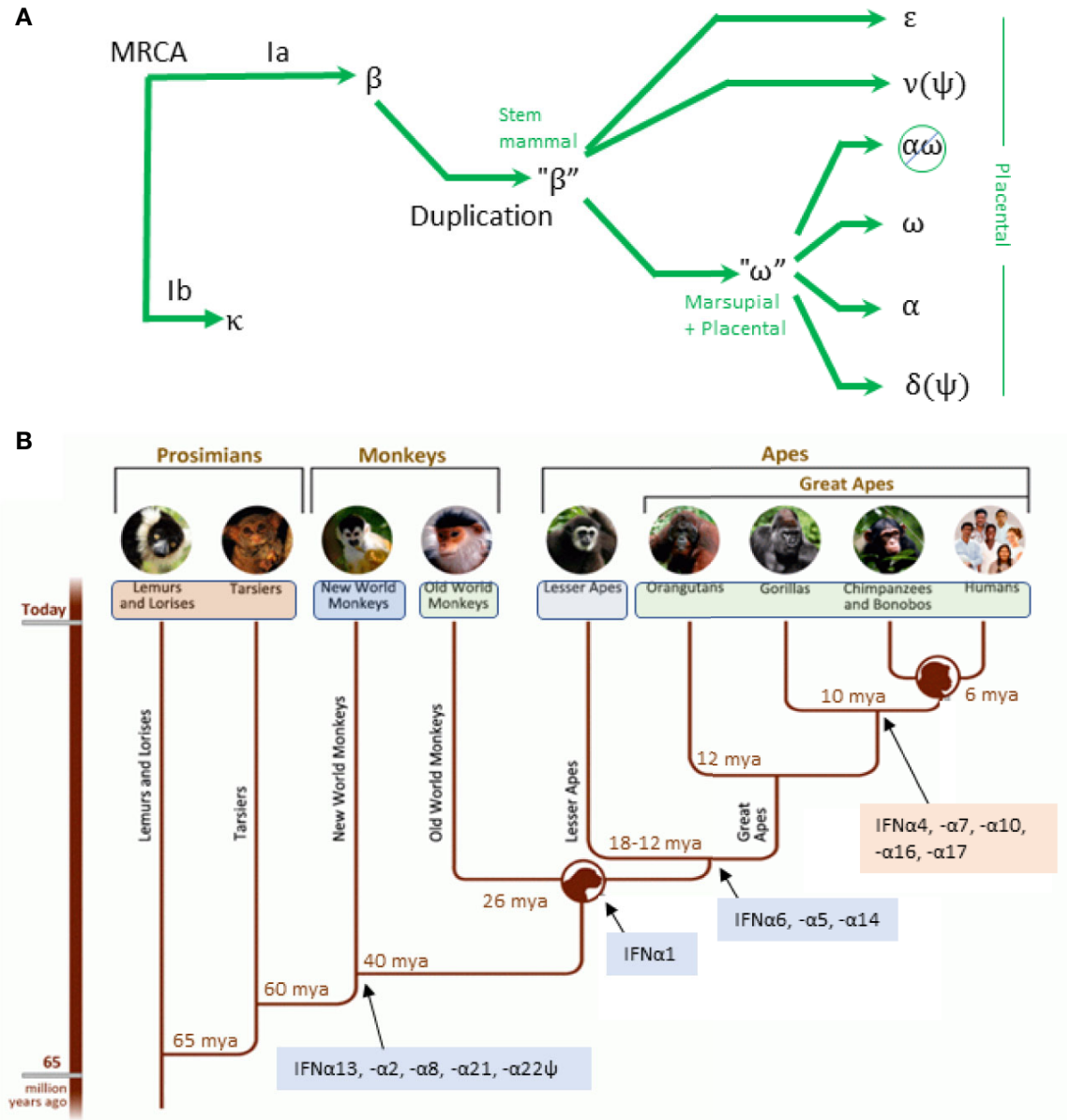

FIGURE 3 | Evolution of IFN-I. **(A)** Simplified evolution of type IFN-I in mammals adapted from Krause and Petska. The most recent common ancestor (MRCA) gave rise to IFNκ and a progenitor for IFNβ. A duplicate of the IFNβ progenitor gave rise to IFNε, IFNν (a pseudogene in mammals), and a progenitor for IFNω. The IFNω progenitor gave rise to the remaining subtypes. In simiiforms, IFNαω is deleted and IFNδ is a pseudogene. **(B)** Evolution of IFN subtypes from simians to homininae showing conserved (blue) and variant (orange) subtypes. Figure adapted from: http://humanorigins.si.edu/evidence/genetics.

based on the A137V substitution (residue 114 of the mature peptide), that is predicted to have no damaging effects, and in our experience, is not functionally different from A137 IFNα1 (36). Manry et al. concluded that these evolutionarily conserved subtypes have have undergone selection against nonsynonymous variants. Taken together, the conserved cluster may have evolved to counter pathogens common that threatened the MRCA to OWM and great apes, and there is a selective advantage for having two genes, *IFNA1* and *IFNA13*, that express IFNα1.

REGULATION OF TYPE I INTERFERON EXPRESSION BY IRF3 AND IRF7

Comparing promoter regions and transcription factor usage provides insight toward specialized roles for the different IFN-I. The interferon regulatory factor (IRF) family members are the dominant transcription factors that regulate IFN-I expression. While IRF1, -2, -5, and -8 have been shown to regulate IFN-I expression, this review will focus on the two most important members, IRF3 and IRF7.

IFNβ is expressed after stimulation of pattern-recognition receptors (PRRs) such as RIG-I-like receptors (RLRs) and toll-like receptors (TLRs) by pathogen-specific molecular motifs referred to as pathogen associated molecular patterns (PAMPs) [reviewed in (37)]. Once activated, PRRs trigger signaling cascades that activate assembly of the "enhanceosome," which consists of the transcription factors ATF-2/c-Jun, NFκB (p50/65 heterodimer) and two interferon response factor (IRF) dimers [**Figure 4** (39)] that bind to four promoter regulatory domains (PRDs). Based primarily on mouse models, it was initially thought that PRDs III and I required either IRF7 homodimers or IRF3/IRF7 heterodimers for a functional enhanceosome (40).

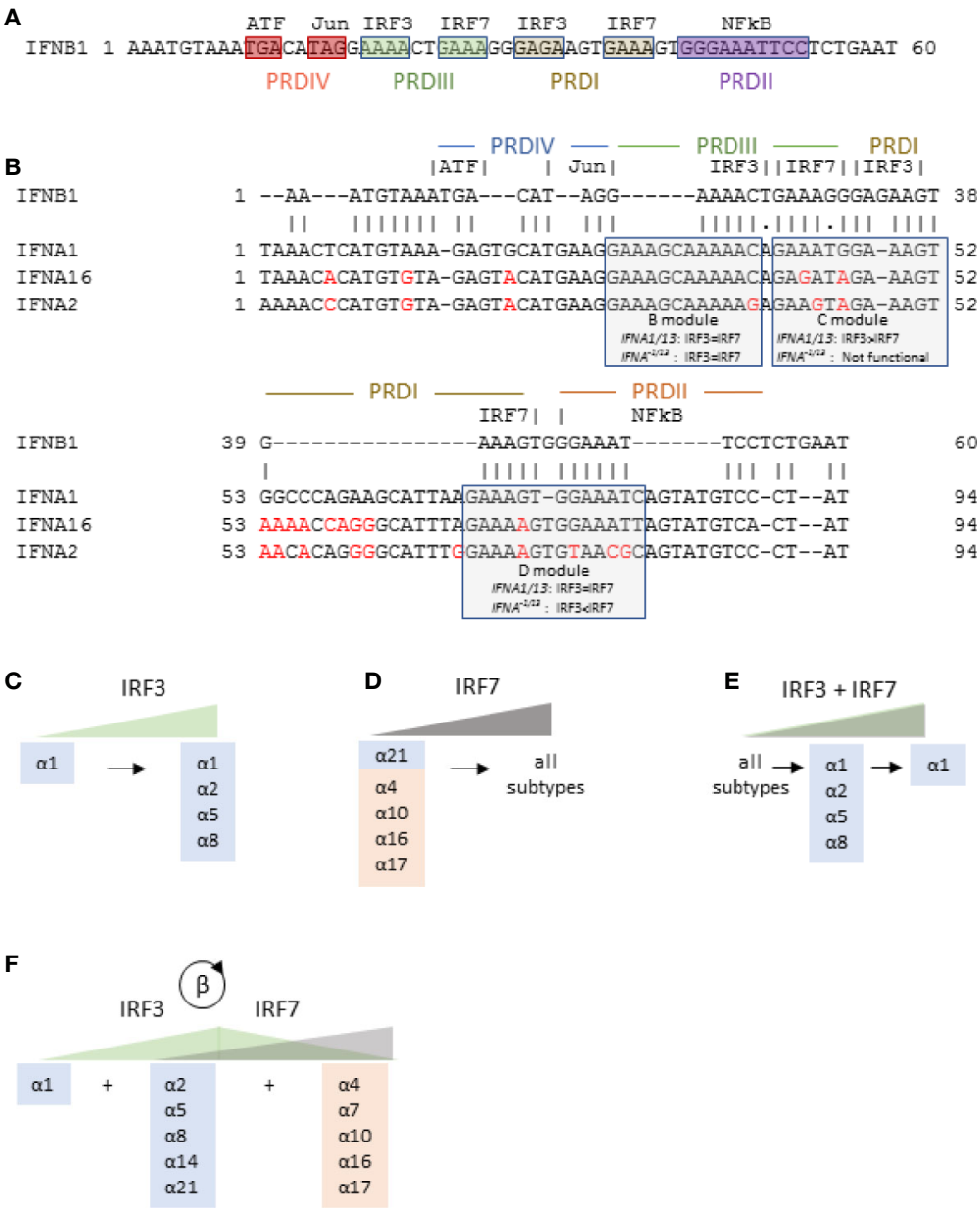

FIGURE 4 | *IFNB1* and *IFNA* gene transcription is controlled by IRF3 and IRF7. **(A)** Promoter region of *IFNB1* gene showing the four promoter regulatory domains (PRD), all of which must be engaged for gene transcription. **(B)** Promoter regions of *IFNA1*, *IFNA16*, and *IFNA2* aligned with the promoter region of *IFNB1* showing the three IRF regulatory modules and their relative sensitivity to IRF3 and IRF7. Differences from *IFNA1* promoter are shown in red. The promoter region of *IFNA16* is representative of *IFNA21* and the variant subtypes (*IFNA17*, *IFNA16*, *IFNA10*, *IFNA7*, *and IFNA4)*. The *IFNA2* promoter region is representative of all IFNA$^{-1/13}$ conserved subtypes except *IFNA21*. **(C–E)** Model of differential regulation of human *IFNA* genes. Blue and orange shading show evolutionarily conserved and variant IFNα subtypes, respectively, *IFNA* genes expressed in response to increasing levels of activated IRF3 alone **(C)**, IRF7 alone **(D)**, or IRF3 and IRF7 together **(E)** as described by Genin et al. (38). **(F)** Proposed model of IFNα subtype expression in the context of initial activation of IRF3 followed by IRF7 expression (and subsequent activation) in response to a forward feedback loop initiated by IFNβ.

In most cells, however, basal IRF7 expression is low while IRF3 is ubiquitously expressed. Thus, in most cells, viral PAMPs trigger activation of IRF3, which homodimerizes to complete the functional enhanceosome and initiate transcription of IFNβ. Subsequently, autocrine/paracrine IFNβ increases expression of IRF7 (a robust ISG) in infected and bystander cells—a well-documented critical step in a forward feedback loop for IFNβ to enhance its own expression (41).

The critical importance of IRF3 toward initiating IFN expression is emphasized by the number of pathogens with gene products that antagonize its activation (41) and by reports that cells from IRF3-deficient patients express little or no IFNβ (42, 43). The critical importance of the IRF7-mediated forward feedback loop is supported by an *in vitro* study in which the percentage of IFNβ-expressing cells after viral infection was dependent on cell density, and secretion of IFNβ (44), and reports that IRF7 deficient patients poorly express IFNβ (45, 46). By contrast, cells that constitutively express IRF7, as is the case for macrophages and plasmacytoid dendritic cells (pDC) (47) highly express IFN-I in response to synthetic ligands (imiquimod or CpG oligonucleotides) or pathogens such as influenza (48, 49). Taken together, the IFNβ-IRF7 forward feedback loop is a sentinel at the early stages of viral infection in local environments that enhances the antiviral state of common target cells for viral infection such as respiratory or gastrointestinal epithelium.

After the crystal structure of the *IFNB1* enhanceosome was published, Genin et al. described promoter regions of the *IFNA* genes (38) and modulated cellular expression of IRF3 and IRF7 to determine their effects on IFNα subtype expression. **Figure 4A** shows the promoter region of *IFNB1*, and **Figure 4B** shows the *IFNB1* promoter region aligned to representative *IFNA* subtypes up to −30 bp from the transcription start site. Overall, the *IFNA* promoter regions align well to that of *IFNB1* with 95% identity excluding several insertions and three short deletions. As shown in **Figure 4B**, the insertions into the *IFNA* promoters shift the IRF binding sites, referred to as modules B, C, and D, 5' from the transcriptional start site such that the B module ends half-way through *IFNB1* PRDIII, the *IFNA* C module straddles *IFNB1* PRDIII and PRDI, and the *IFNA* D module straddles *IFNB1* PRDI and PRDII (to which NFκB binds in the *IFNB1* promoter). Among the three modules, only module B, which is equally responsive to IRF3 and IRF7, is essentially identical among all the subtypes. By contrast, module C, which preferentially binds to IRF3, is functional only in the *IFNA1 (and IFNA13)* promoter. Module D also differs between *IFNA1/13* and the other subtypes. For *IFNA1*, module D binds equally to IRF3 and IRF7, while for all the other *IFNA* subtypes, module D preferentially binds to IRF7. Binding of IRF3 to *IFNA1* promoter modules C and D explains why *IFNA1* and *IFNB1* can be co-expressed in the absence of any other *IFNA* subtypes (38, 49–51).

The promoter regions of the *IFNA* subtypes other than *IFNA1* (which we will refer to as $IFNA^{-1/13}$ or $IFN\alpha^{-1/13}$ for the gene and protein, respectively) cluster into two groups. The first cluster consists of *IFNA4*, *-A7*, *-A10*, *-A16*, *-A17*, and *-A21*, (represented by *IFNA16* in **Figure 4B**). Note that this set includes all the evolutionarily variant *IFNA* subtypes (15) along with *IFNA21*, from which the variant subtypes may have arisen. The substitutions in the C modules of these subtypes renders them nonfunctional, and the 73G/A substitution in their D modules renders them more sensitive to IRF7. The B, C, and D modules are identical among the *IFNA* subtypes in this cluster.

The second cluster of $IFNA^{-1/13}$ subtypes is represented by *IFNA2* and includes *IFNA5*, *-A6*, *-A8*, and *-A14*. These are all evolutionarily conserved subtypes. The C module for this cluster is also non-functional, and their D modules include the 73G/A substitution that renders them more sensitive to IRF7. Unlike the cluster represented by *IFNA16*, however, there are substitutions in the B and D modules that may affect their relative sensitivity to IRF3 and IRF7 (52).

Based on analysis of the *IFNA* promoter regions and expression studies with EBV-transformed B cells, Genin et al. proposed a model for differential regulation of the *IFNA* genes by either activation of IRF3 or IRF7, or by co-activation of both IRF3 and IRF7 Genin, 2009 #71} (15, 52). In this model, low activation of IRF3 is sufficient to induce expression of IFNα1, while increased IRF3 activation may induce expression of IFNα2, -α5, and -α8 (**Figure 4C**). Similarly, increasing levels of IRF7 activation will first induce expression of IFNα21 and the evolutionarily variant subtypes followed by the remaining subtypes (**Figure 4D**). Co-activation of IRF3 and IRF7 at low levels induces expression of all subtypes, but coactivation increases, IRF3 inhibits IRF7 and thus limits the number of subtypes expressed (**Figure 4E**).

PATTERNS OF HUMAN TYPE I INTERFERON EXPRESSION IN RESPONSE TO SYNTHETIC LIGANDS AND VIRAL INFECTION

To characterize expression patterns of IFNα subtypes in response to synthetic ligands or viral infection, transcripts are usually measured with RT-qPCR. **Table 1** summarizes human IFNβ and IFNα subtype expression patterns reported in the literature. As predicted by Genin et al., IFNα1 is co-expressed with IFNβ after activation of IRF3 with poly I:C. Additionally, when potently stimulated, pDC (which constitutively express IRF7) express all IFNα subtypes, while weaker stimulation of IRF7 with CpG B class oligodeoxynucleotides (ODN) induced expression of a set of IFNα subtypes that share the IRF7-sensitive promoter region exemplified by *IFNA16* (**Figure 4B**). By contrast, stimulation of cells that do not constitutively express IRF7 with viral RNA or the synthetic analog poly I:C primarily induces expression of a core set of conserved subtypes. **Table 1** also suggests the possibility that specific pathogens such as influenza virus, HIV, or hepatitis C may preferentially induce IFNα5.

Of particular interest is the report by Zaritsky et al., who infected the U937 histiocytic cell line with Sendai virus at low and high multiplicity of infections (MOI). While the U937 cells

TABLE 1 | Reported expression patterns of human IFN-I.

Cell type	Stimulus		Conserved cluster						Variant cluster					CC	Reference
		β	α1	α8	α2	α6	α5	α14	α17	α16	α10	α7	α4	α21	
PBMC	poly I:C	X	X		X			X			X				(49)
	CpG B-D class	X	X	X	X			X		X	X	X		X	(53)
	Imiquimod	X	X		X			X							(53)
	Sendai Virus[a]	nd	X	X	X			X			X			X	(19)
	Hepatitis C virus		X				X								(54)
Mo	poly I:C	X	X					X							(49)
MDM	poly I:C	X						X			X				(49)
	CpG D class	X						X			X				
	M. tuberculosis	X	X												(50)
MDDC	poly I:C	X	X												(49)
	RSV	X	X	X	X			X						X	(55)
pDC	poly I:C, LPS	X	X				X	X							(49)
	Imiquimod	X	X	X	X	X	X	X	X	X	X	X	X	X	
	CpG A, C, D	X	X	X	X	X	X	X	X	X	X	X	X	X	
	CpG B class	nd							X			X	X	X	(48)
	IAV H1N1	nd	X	X	X	X	X	X	X	X	X	X	X	X	(48)
	HIV	nd	X	X	X	X	X	X	X	X	X	X	X	X	(56)
	HIV	nd	X	X	X		X	X							(57)
Calu3[b]	IAV H5N1 IAV pH1N1 SARS-CoV MERS-CoV	X					X								(58)
BEAS2B	RSV	X	X												(51)
Lung explants	IAV H3N2	nd	X	X	X			X	X		X				(59)
U937[c]	Sendai Virus (low MOI)	X	X	X	X	x	x	x	x	x	x	x	x	x	(60)
	Sendai Virus (high MOI)	X	X	X	X		X	X		X				X	

[a]*Expression patterns determined by mass spectrometry.*

[b]*Expression patterns determined by RNAseq, which may be insensitive to detecting highly identical transcripts.*

[c]*Lower case "x" refers to the IFNα subtypes that were not expressed after IFNAR2 blockade (see text).*

PBMC, peripheral blood mononuclear cells; Mo, monocytes; MDM, monocyte derived macrophages; MDDC, monocyte derived dendritic cells; pDC, plasmacytoid dendritic cells; poly I:C, polyinosinic-polycytidylic acid; CpG, CpG oligodeoxynucleotides; HIV, human immunodeficiency virus; SARS-Cov, Severe adult respiratory syndrome coronavirus; MERS-CoV, Mideast respiratory syndrome coronavirus; MOI, multiplicity of infection. Blue and orange shading show evolutionarily conserved and variant IFNα subtypes, respectively.

expressed all IFNα subtypes after infection at low MOI, expression was limited almost exclusively to the conserved set after infection at a high MOI. Furthermore, while IFNAR2 blockade (which repressed the IFNβ-IRF7 forward feedback loop) did not affect the expression pattern in the high MOI infection, it significantly repressed all subtypes except IFNα1, -α2, and -α8 after low MOI infection (60). Taken together, these studies support the model of Genin et al. in which activated IRF3 alone induces expression of conserved IFNα subtypes (**Figure 4C**), and IRF7 alone first induces IFNα21 and variant subtypes and subsequently induces expression of all subtypes (**Figure 4D**). In the context of the IFNβ-IRF7 forward feedback loop, however, **Table 1** suggests that conserved subtypes are first expressed, followed by variant subtypes (**Figure 4F**).

It is important to note that the evolutionarily conserved or variant IFNα subtype clusters are not expressed *en bloc*. One possible explanation is that unlike the variant subtypes, the B and D promoter modules vary by one or two bp, which may affect their relative sensitivity to IRF3 or IRF7 (52). Another factor is that IRF3 and IRF7 are not the only mediators of subtype expression. For example, a set of *IFNA* transcripts is regulated by a competing endogenous RNA (ceRNA) network. Kimura and colleagues first described stabilization of *IFNA1* transcripts by a natural antisense transcript (NAT) that spans the coding region and extends well beyond the 3' poly-A UTR (61). They subsequently determined that the *IFNA1* NAT includes binding sites for microRNA-1270 (i.e., a microRNA response element) which otherwise represses *IFNA1* transcript levels. Additionally, NAT for *IFNA8*, *-A10*, *-A14*, and *-A17* (Kimura et al., personal communication) also sequester miRNA-1270 to enhance their transcript levels (62).

IFNβ, THE HIGH-AFFINITY SENTINEL

In addition to its evolutionary emergence as the first non-tissue specific IFN-I and its high sensitivity to IRF3/IRF7, IFNβ also has exceptionally high affinities for IFNAR1 and IFNAR2 (K_D = 0.1 uM and 0.1 nM, respectively). As estimated by the product of IFNAR1 and IFNAR2 affinities (K_D IFNAR1 * K_D IFNAR2), the stability of the IFNβ/IFNAR1/IFNAR2 ternary complex is 10-fold higher than for IFNω and at least 50-fold higher than the highest affinity IFNα subtypes, IFNα14 and IFNα6 (**Figure 5**).

A

IFN	K_D	
	IFNAR1 (uM)	IFNAR2 (nM)
κ	0.34	21
ε	2.2	70
α1	0.76	220
α8	0.53	1.6
α2	3.5	1.7
α6	0.83	0.6
α5	0.94	3.8
α14	0.68	0.7
α17	5	0.4
α16	1.1	1.1
α10	4.3	0.4
α7	1.3	0.7
α4	3	2.1
α21	1.25	5
ω	1	2
β	0.1	0.1

B

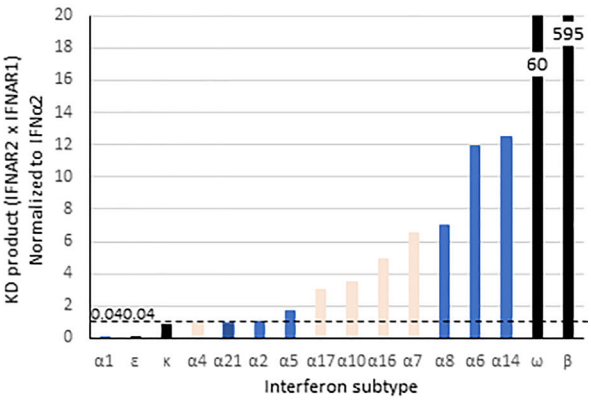

FIGURE 5 | Binding affinities of IFN-I. **(A)** Equilibrium disassociation constants for the IFN-I. IFNα subtypes are from (63); IFNβ from (64), and IFNε, -κ, and -ω from (65). **(B)** Product of K_D for IFNAR1 and IFNAR2, normalized to IFNα2. Highlighting and bar colors indicate conserved (blue) and variant subtypes (orange).

As reviewed elsewhere in this series, a consequence of its high affinity is more effective internalization of ternary receptor complexes (66) into early endosomes where signaling may be amplified and prolonged, or more rapidly terminated due to shuttling of IFNAR1 to proteasomes for degradation (67).

A second consequence of the high affinity that IFNβ has for the receptor is that unlike the other IFN-Is, signaling is unaffected by ubiquitin-specific protease-18 (USP18). USP18 is a deubiquitinating enzyme that deconjugates the ubiquitin-like interferon-stimulated gene-15 (ISG15) from its target proteins (68). Conversely, ISG15 prevents ubiquitination and proteolytic degradation of USP18, thus stabilizing its expression (69). Unrelated to its enzymatic function, USP18 is shuttled by STAT2 to IFNAR2, which sterically blocks binding of Jak2 to interfere with recruitment of IFNAR1 to assemble a stable ternary complex (68, 70, 71). Since USP18 is an ISG (72), this inhibitory function is considered a negative feedback regulator of IFN signaling. Due to its very high affinity for IFNAR1, however, IFNβ can override USP18 and recruit IFNAR1 to form a ternary complex to initiate signaling (70). Thus, the negative feedback regulation by USP18 is selective and is presumed to affect all IFN-I other than IFNβ. To our knowledge, selective inhibition has been demonstrated by comparing IFNβ induced signaling with that of IFNα2, but not higher affinity IFNAR1 ligands such as IFNα6 or -α8, or those with higher IFNAR1 × IFNAR2 K_D products such as IFNω or IFNα14 (**Figure 5**). The critical importance of USP18-mediated inhibition of IFN signaling is exemplified by pseudo-TORCH syndrome, a severely incapacitating or fatal "interferonopathy" in patients deficient in ISG15, USP18, or with a mutation to the STAT2 binding site for USP18 (69, 73, 74).

Two additional qualities of IFNβ bear discussion. First, in addition to IFNκ (9), IFNβ also binds to highly sulfated proteoglycans (PG), proposed to be mediated through a heparin binding site in an arginine-rich region of IFNβ that spatially separates the binding sites for IFNAR1 and IFNAR2 (75). PG binding of IFNβ may sequester it to buffer IFN-I signaling, which can be reversed by desulfation or shedding the IFNβ-bound PG (75) which may result in a depot effect. Second, amino acid residues 25-27 uniquely contain the sequence motif NGR which binds CD13. Asparagine residues undergo spontaneous deamidation, which may be increased during oxidative conditions. Deaminated NGR gives rise to DGR, which binds to αVβ3 and possibly other integrins that similar to CD13, are expressed in blood vessels during angiogenesis (76) and by some cancer stem cells (77) and mediates tumor invasion (78). It is proposed that CD13 or αVβ3 in tumors or tumor vasculature may sequester IFNβ and thus limit its antiproliferative effects (79). In addition to these biologic effects, binding of IFNβ to abundant PG and integrins (in addition to its propensity to stick to plastic) may limit its detection in biological fluids or tissue culture supernatants.

IFNα1, THE LOW-AFFINITY SUBTYPE

As discussed above, IFNα1 stands apart from the IFNα$^{-1/13}$ subtypes for its responsiveness to IRF3, for having two genes (*IFNA1* and *IFNA13*) on chromosome 9, and for the low frequency of polymorphisms in either of those genes. Most remarkable, however, is the low affinity of IFNα1 for IFNAR2, at least 100-fold lower than most other IFNα subtypes while it binds with relatively high affinity to IFNAR1 (**Figure 5**). **Figure 6** shows the protein sequences of the IFNα subtypes aligned to IFNα1, with secondary structures and receptor contact points. Residues 20-35 cover most of the AB loop, including two 3_{10} helices. In this span, two substitutions in IFNα1 contribute to the low affinity of IFNα1 for IFNAR2: F27S, which decreases its

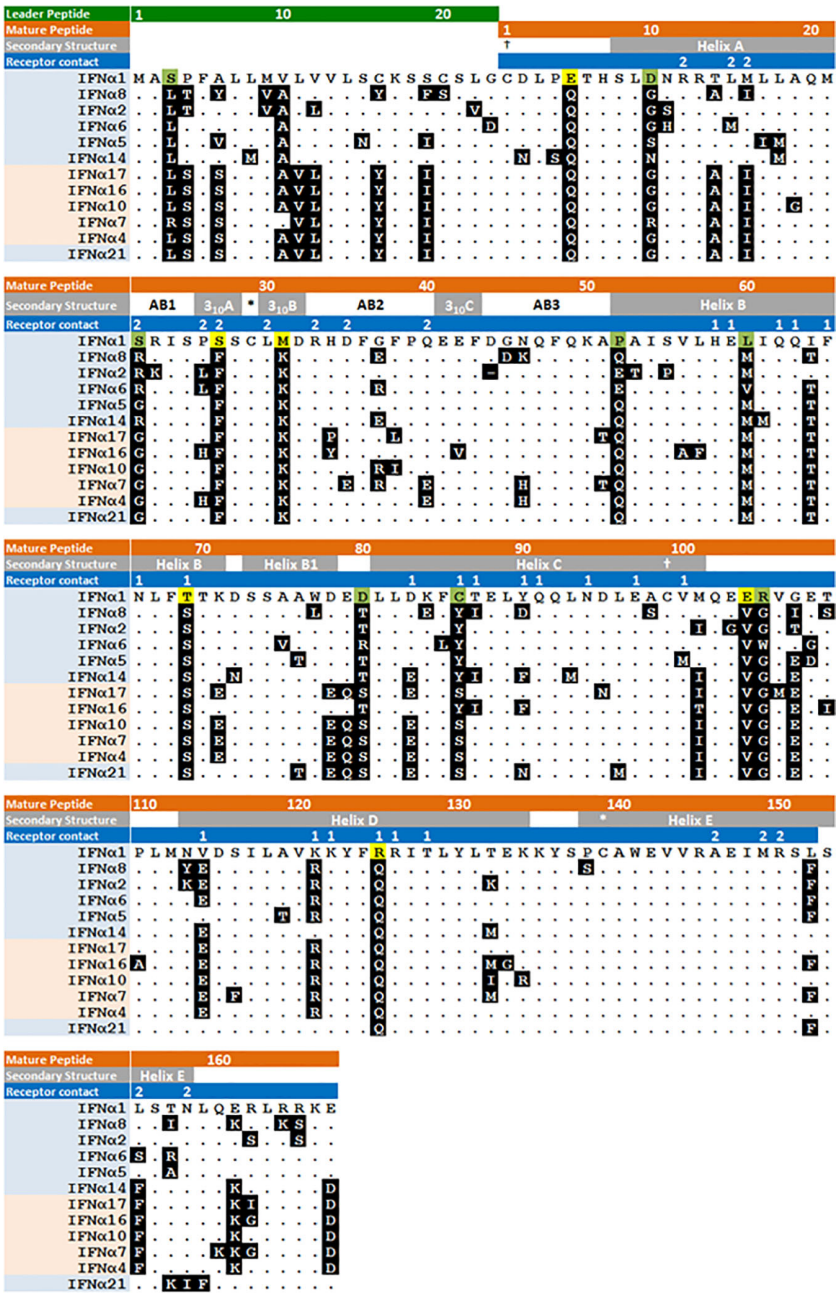

FIGURE 6 | Amino acid sequence of human IFNα subtypes. IFNα subtypes are shown in order of arrangement on chromosome 9 with evolutionarily conserved and variant subtypes highlighted in blue and pink respectively. Secondary structure and IFNAR1/2 contact residues, labeled 1 and 2 respectively, are shown in the gray and blue highlighted text. Amino acids are shown with IFNα1 as the comparator, showing those that are unique to IFNα1 and otherwise identical among all the *IFNα*$^{-1/13}$ subtypes, or otherwise varies among the other *IFNα*$^{1/13}$ subtypes. * and † indicate cysteine disulfide bonds. Figure modified from (80).

affinity for IFNAR2 by 4-fold as the polar side chain of serine is predicted to disrupt the hydrophobic interaction otherwise stabilized by phenylalanine (80), and R22S, which together with S27 decreases affinity by ~14-fold (65). Although not a contact point, the substitution K31M in IFNα1 may also contribute to its decreased affinity for IFNAR2 by disrupting the second 3_{10} helix.

While the low affinity of IFNα1 for IFNAR2 suggests the possibility of a qualitative difference in signaling or functional outcome, the evidence to date only supports a quantitative difference. Reports of IFNAR2-independent signaling in mice (32) have not been replicated in human cells, for which it has been reported that both IFNAR1 and IFNAR2 are essential for signaling and gene expression (27). Additionally, while IFNα1

also has unique substitutions at contact points for IFNAR1 that may affect its conformation at the SD2-SD3 hinge that affect binding affinity (81), conformational changes do not necessarily indicate an effect in IFN signaling (25).

The substitutions that decrease the affinity of IFNα1 for IFNAR2 also decrease its affinity for B18R, a soluble receptor antagonist encoded by vaccinia virus. According to this model, secreted B18R (or other poxvirus orthologues) block high affinity IFN-I from binding their receptors, while leaving these low affinity IFNs relatively unaffected (65). Similarly, the organ-specific IFN-I, IFNκ, and IFNϵ also bind to IFNAR2 and B18R with low affinity. While IFNκ and IFNϵ may protect against poxviruses that infect local environments (skin and female reproductive tract), IFNα1 may defend against invasive strains such as variola. It is intriguing to speculate that the low frequency of polymorphisms in human *IFNA1* and *IFNA13* (35) is a consequence of a selective advantage toward surviving smallpox.

Among the IFN$\alpha^{-1/13}$ subtypes, there are fewer substantial differences in their peptide sequences. **Figure 6** shows the shared residues that account for the high levels of identity among the evolutionarily conserved subtypes (**Supplementary Figure 1**) and differences in the unstructured C-terminal tail that contribute to higher antiviral and antiproliferative potencies of IFNα8 (82). Since the receptor contact points are conserved, variation in their binding affinities is apparently due to substitutions in adjacent residues.

THERAPEUTIC USES OF TYPE I INTERFERON

The antiviral and antiproliferative activities of interferons led to the development of their use as therapeutics. In 1986, IFNα2b (Intron A®, Merck Sharp & Dohme) was the first IFN-I approved for use in the United States (83). The current U.S. market for interferons, including IFNγ for chronic granulomatous disease and malignant osteopetrosis, has grown to $5B per year. **Table 2** shows the nine IFN-I licensed in the United States along with indications for use. As discussed elsewhere in this series of reviews (84), there are several ongoing clinical studies to test efficacy of IFN-I and IFN-III to treat Covid19.

IFNα2a or IFNα2b, which differ only at residue 23 (lysine or arginine, respectively), are prescribed for their antiviral or antiproliferative activity. These products are injectable preparations of either native or pegylated IFN proteins. Pegylation is modification of proteins with linear or branched polyethylene glycol to retards degradation and increase its serum half-life (85). While IFNα2 was used to treat chronic hepatitis C, it has been replaced with the highly specific inhibitors of HCV NS3/4A, NS5A, and NS5B proteins, which may be curative and are associated with fewer adverse events (86).

IFNβ was first approved for treatment of relapsing remitting multiple sclerosis in 1993 after showing an 18-34% reduction in relapse rate. The efficacy for IFNβ was considered to be due suppression of viral infections that are associated with relapses and to direct immunomodulatory effects that include reduction of pathogenic Th1 and Th17 CD4+ T cells, and to increases in IL-10 producing T_{reg} cells (87). All these may be mediated by increased expression of PD-L1 (CD274), an ISG that in mice is more responsive to IFNβ due to its high receptor affinity (88).

Therapeutic IFN-I has severe adverse events that are an obstacle to their use as therapeutics. The package inserts for pegylated IFNα includes black box warnings for the potential development of neuropsychiatric, autoimmune, ischemic, or infectious disorders. The package inserts also warn that treatment symptoms such as fever, fatigue, headache, myalgia, and nausea, which are usually associated with viral infections, are common side effects. More serious side effects can include cardiovascular and neurologic disorders, bone marrow, hepatic, and renal toxicity, and hypersensitivity reactions. Additionally, IFNβ for MS is associated with seizures, depression, suicide, and

TABLE 2 | Licensed IFN-I in the United States.

Proprietary Name	Proper Name	Dosage Form	Dosage	Route	Indication	Expression System
Avonex	IFNβ-1a	30 μg/0.5 ml	30 μg per week	IM	Multiple sclerosis including relapsing-remitting and secondary active disease	CHO cells
Rebif	IFNβ-1a	8.8 μg/0.2 ml 22/44 μg/0.5 ml	22 or 44 μg 3 times per week	SC		CHO cells
Plegridy	IFNβ-1a	63/94/125 μg/ 0.5 ml	125 μg every 14 days	SC		CHO Cells
Betaseron	IFNβ-1b	0.3 mg	0.25 mg every other day	SC		*E. coli*
Extavia	IFNβ-1b	0.3 mg	0.25 mg every other day	SC		*E. coli*
Pegasys	Peg IFNα2a	180 μg	Adult: 180 ug per week Pediatric: 180 ug/1.73 m^2	SC	Chronic Hepatitis C, Chronic Hepatitis B	*E. coli*
Pegintron	Peg IFNα2b	50/80/120/150 μg/0.5 ml	Adult: 1.5 ug/Kg/ week Pediatric: 60 ug/m^2/ week	SC	Chronic Hepatitis C in patients with compensated liver disease	*E. coli*
Intron A	IFNα2b	10/18/25 MIU	Diagnosis Dependent	IV, IM, SC, IL	Hairy Cell Leukemia, Malignant Melanoma, Follicular Lymphoma, Condylomata Acuminata, AIDS-related Kaposi's Sarcoma, Chronic Hepatitis C, Chronic Hepatitis B	*E. coli*
Sylatron	Peg IFNα2b	200/300/600 μg	6 ug/Kg/week for 8 weeks then 3 ug/Kg/week for up to 5 years	SC	Melanoma with metastasis to lymph nodes–to begin within 84 days of surgical resection	*E. coli*

Peg, polyethylene glycol; MIU, million international units; BSA; IM, intramuscular; IV, intravenous; IL, intralesional; SC, subcutaneous; CHO, Chinese hamster ovary cells.

other psychiatric disorders. It is therefore not too surprising that as more selective therapeutic agents have been developed and licensed, use of IFN-I has become adjunctive rather than a primary treatment for chronic viral infections, cancer, or MS.

CONCLUSIONS

As reviewed here, most if not all reported biological differences among IFN-I are quantitative rather than qualitative. While the antiviral subtype that most potently neutralizes infection *in vitro* may vary according to pathogen (57, 59, 89, 90), these differences may be overcome by increasing doses (57, 91). Similarly, differences in antiproliferative activity are largely dose dependent (92). While this may also be true for modulation of cytokine expression (36), immunosuppressive activity (i.e., induction of PD-L1) may be dependent on the exceptionally high affinity of IFNβ for IFNAR1/2.

As for the IFNα subtypes, other than escape from poxvirus soluble receptor antagonists (such as B18R by IFNα1), any suggestion of specialized roles is inferred from their evolutionary history or expression patterns. It is therefore possible that the primary role of IFNα is to prolong or amplify the effects of IFNβ and that multiple IFNα subtypes simply provide multiple layers of redundancy, albeit with a range of receptor affinities. However, it is also possible that unique functions for IFNα subtypes have not been revealed because the common experimental approach of comparing treatment with individual IFN-I does not reflect the biological context in which defined patterns of IFNα are co-expressed together and with with IFNβ. These patterns are likely most relevant at sub-saturating doses, which may more accurately reflect the environment of structural cells where organ specific immune responses are initiated (93).

AUTHOR CONTRIBUTIONS

MW, SC, EL, and RR performed the literature searches and contributed to draft versions of the manuscript. RR wrote and revised the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.605673/full#supplementary-material

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REVIEW
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Context Is Key: Delineating the Unique Functions of IFNα and IFNβ in Disease

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Type I interferons (IFNs) are critical effector cytokines of the immune system and were originally known for their important role in protecting against viral infections; however, they have more recently been shown to play protective or detrimental roles in many disease states. Type I IFNs consist of IFNα, IFNβ, IFNε, IFNκ, IFNω, and a few others, and they all signal through a shared receptor to exert a wide range of biological activities, including antiviral, antiproliferative, proapoptotic, and immunomodulatory effects. Though the individual type I IFN subtypes possess overlapping functions, there is growing appreciation that they also have unique properties. In this review, we summarize some of the mechanisms underlying differential expression of and signaling by type I IFNs, and we discuss examples of differential functions of IFNα and IFNβ in models of infectious disease, cancer, and autoimmunity.

Keywords: type I interferons, infection, autoimmunity, cancer, IFNα subtypes, IFNβ

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INTRODUCTION

Interferons (IFNs) are cytokines that were originally discovered and named for their ability to interfere with viral replication (1). IFNs are grouped into three classes according to the receptor that mediates their effects: type I IFNs (the focus of this review), type II IFN (IFNγ), and type III IFNs (IFNλs) (2, 3). Broadly speaking, each IFN class signals through receptor-associated Janus kinases (JAKs), which activate various Signal Transducer and Activator of Transcription (STAT)-signaling pathways. Type I IFNs signal through the heterodimeric IFN-α/β receptor 1 (IFNAR1) and IFNAR2, which are associated with the JAKs tyrosine kinase 2 (TYK2) and JAK1, respectively (4). Canonically, activation of TYK2 and JAK1 leads to the formation of the IFN-stimulated gene (ISG) factor 3 (ISGF3) complex, composed of STAT1, STAT2, and interferon regulatory factor 9 (IRF9). The ISGF3 complex then translocates to the nucleus to regulate the expression of hundreds of IFN-stimulated genes. Type I IFN signaling can activate other STAT complexes, often in a cell-type dependent manner. Additionally, alternative signaling cascades, including the mitogen-activated protein kinase p38 pathway and the phosphatidylinositol 3-kinase pathway, are also required for optimal generation of type I IFN responses (4).

Type I IFNs have broad, pleiotropic effects that include antiviral activity, antiproliferative effects, and immunomodulatory properties. There is growing evidence that the overall outcome of type I IFN responses can be beneficial or detrimental for the host depending on the timing, magnitude, and source of IFN production, as well as the specific biological context (5). Moreover, despite

signaling through a shared receptor, type I IFN subtypes possess important functional differences, both *in vitro* and *in vivo*. The purpose of this review is to summarize the current understanding of differential type I IFN properties, focusing on the role of human and mouse IFNα and IFNβ in infectious disease, cancer, and autoimmunity. In particular, we seek to highlight the few examples that demonstrate or suggest differential activities for type I IFN subtypes *in vivo*.

TYPE I IFNS: A MULTIGENE FAMILY

Type I IFNs exist as a multigene family across many species (**Figure 1**) (6). IFNαs, IFNβ, IFNϵ, IFNκ, and IFNω are found in many species, whereas IFNδ and IFNτ are only found in pigs and cattle (7). In humans (HuIFN), the type I IFN genes are located on chromosome 9 and encode 13 IFNα subtypes and single forms of IFNβ, IFNϵ, IFNκ, and IFNω (7). Type I IFNs in mice (MuIFN) are located on chromosome 4, and likewise, consist of multiple genes with some differences compared to human. MuIFNs include 14 IFNα subtypes, IFNβ, IFNϵ, IFNκ, an IFN-like cytokine IFNζ (also known as limitin), but lack a functional IFNω, which is present as a pseudogene (8).

Phylogenetic analyses reveal that the type I IFN subtypes form clades consistent with mammalian speciation (7, 9, 10). For the most part, placental mammals possess single copies of the genes encoding IFNκ, IFNβ, and IFNϵ, and these unduplicated subtypes represent the first major clade within mammalian IFNs (11). IFNκ is the first subtype to diverge within mammalian type I IFNs and forms an outgroup, possibly the result of a unique evolutionary route for IFNκ relative to IFNβ and IFNϵ (11). IFNκ is additionally distinctive as the only

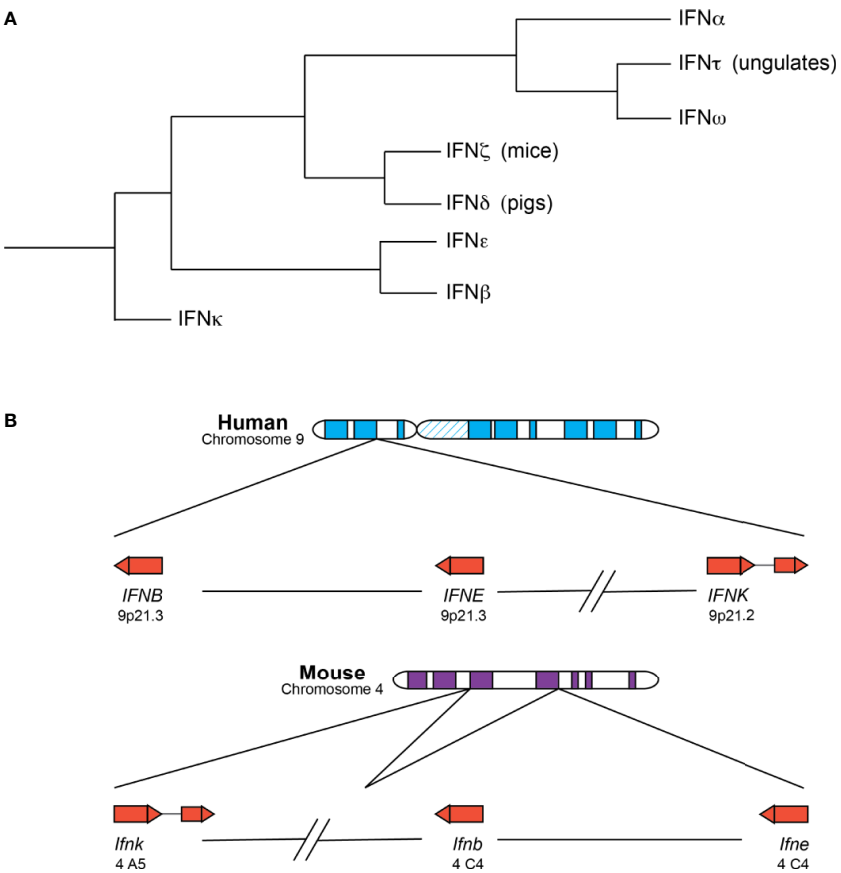

FIGURE 1 | Type I IFNs are a closely related family of related cytokines. **(A)** Depicted is a summary of existing phylogenetic analyses of the type I IFNs. The branches are not drawn to scale. IFNκ, IFNβ, and IFNϵ are mostly present in placental mammals as single copies and the first subtypes to diverge from the other type I IFNs. IFNβ and IFNϵ are especially similar and can be found within the same clade in some analyses. IFNδ and IFNζ are the next subtypes to diverge and are only found in pigs and mice, respectively. IFNτ and IFNω are closely related, despite their differences in function and distribution—IFNτ is only expressed in placental tissues of ungulate species and involved in pregnancy, whereas IFNω is found in many species and possesses the more canonical antiviral and immunomodulatory functions. IFNω and IFNα loci are expanded to include many subtypes in a number of species. **(B)** The chromosomal locations of human (top) and murine (bottom) IFNκ, IFNβ, and IFNϵ genes are depicted. The arrow direction indicates on which strand the gene is encoded: a left-to-right arrow depicts the forward or positive strand and a right-to-left arrow indicates the reverse or negative strand. IFNκ is the only subtype to contain an intron and is situated further away from the other type I IFNs, though its positioning relative to the other IFNs is different in mice and humans. IFNβ and IFNϵ roughly form the boundaries of the type I IFN locus, with the other type I IFNs falling between the two genes.

mammalian type I IFN that contains an intron, and for many species, the gene encoding IFNκ is situated further away from the IFN locus (7, 9, 11). Depending on the analysis, IFNβ or IFNϵ is the next subtype to diverge from mammalian type I IFNs, and in some analyses IFNβ and IFNϵ fall within the same clade, suggesting that these subtypes might be more closely related to each other than the other type I IFN subtypes (7, 9, 11, 12). The genes encoding IFNβ and IFNϵ are situated at the "beginning" and "end" of the type I IFN locus across many species, which is relatively conserved across mammalian species. IFNδ and IFNζ (limitin) are the next type I IFNs to diverge within mammalian IFNs and are only found in pigs and mice, respectively (7). However, recent identification of a putative HuIFNδ gene calls this into question (11).

The last subtypes to diverge are the IFNαs, IFNωs, and IFNτs. These subtypes are thought to be exclusively found in placental mammals and are usually situated between the IFNϵ and IFNβ genes within the type I IFN locus. IFNω and IFNτ are closely related, even though they possess different functions (7, 11). IFNτ is only found in placental tissues of ungulate species, is involved in pregnancy, and may have arisen from an IFNω subtype (10, 13). In contrast, IFNω is an antiviral and immunomodulatory molecule, like IFNα, and functional copies have been identified in humans and other animal groups including felines, pigs, cattle, serotine bats, and others but are not present in canines or mice (14). Notably, humans have only one IFNω, but there is evidence that IFNω is still expanding and diversifying in many species, including bats and pigs (15–17). Lastly, the genes encoding IFNα are found in all placental mammals and form species-specific clades, with some exceptions for closely related organisms (e.g. chimpanzees, humans, and gorillas); a combination of gene duplication and gene conversion events likely gave rise to the expanded IFNα genes present in many mammals (6). Of note, a recent study found that for some IFNα subtypes, such as HuIFNα6, α8, α13, and α14, amino acid-altering variation was more constrained in the human population, suggesting that they might perform non-redundant functions in host responses (18).

As sequenced genomes of other species become available, the phylogenetic clustering of some type I IFNs may change. However, the key point is that the multigene nature of type I IFNs is conserved across many species. Both IFNα and IFNω subtypes expanded independently and multiple times, suggesting that it is advantageous for the host to possess a large repertoire of at least several type I IFN subtypes. Unfortunately, the fact that type I IFNs expanded multiple times complicates directly applying results of IFN studies from animal models to clinical settings, and caution is warranted in drawing conclusions about specific human IFNα subtypes from studies of murine IFNα subtypes.

MOLECULAR MECHANISMS UNDERLYING DISTINCT FUNCTIONS OF TYPE I IFNS

Though type I IFNs possess many overlapping functions, it is now appreciated that the individual subtypes have different potencies of their shared functions and some unique functions *in vitro*. An important early example demonstrating this was the finding that HuIFNβ was 100-fold more potent than HuIFNα2 in inhibiting osteoclastogenesis through its ability to preferentially induce the chemokine CXCL11 (19). Since this observation, it is now appreciated that the pleiotropic activities ascribed to different type I IFN subtypes are the product of distinct patterns and kinetics of expression, as well as signaling differences that arise from differential binding affinities and susceptibility to negative feedback loops (20, 21). The ability of the type I IFN receptor to have fine-tuned responses to many ligands is likely advantageous considering the array of pathogens that have co-evolved alongside humans, mice, and other animals.

Differential Dependence on IRF3 and IRF7 for Transcription

Before examining the signaling and functional properties of IFN subtypes, it should be noted that type I IFNs are differentially induced downstream of pattern recognition receptor (PRR) signaling, except for IFNϵ, which is hormonally regulated (see below). PRR signaling converges on the phosphorylation and activation of the transcription factors IRF3 and IRF7, though other IRFs can be involved in IFN-dependent antiviral responses (22, 23). For most cell types IRF3 is constitutively expressed, whereas IRF7 is induced downstream of type I IFN signaling to then amplify and diversify the type I IFN response (22). The exception to this rule is plasmacytoid dendritic cells (pDCs), which constitutively express IRF7 and are thus poised to rapidly secrete large amounts of type I IFN (24). The promoters of specific type I IFN genes differ in their requirement of IRF3 or IRF7 binding for maximal transcription. Thus, the temporal regulation of IRFs dictates the expression of IFN subtypes.

Early in a response, IRF3 activation first induces transcription of MuIFNβ and MuIFNα4 *via* unique IRF3 binding sites within their promoters (25–31). For the most part, the other MuIFNα subtypes require both IRF3 and IRF7 for maximal transcription, and so they depend on type I IFN-mediated upregulation of IRF7 (32–34). Similar to mice, IRF3 also initiates human type I IFN responses by upregulating transcription of HuIFNβ and HuIFNα1, while the other HuIFNA genes require both IRF3 and IRF7 (35, 36). Altogether, these findings demonstrate that for most cell types, activation of constitutive IRF3 by PRR signaling initiates a first wave of HuIFNβ and HuIFNα1 (or MuIFNβ and MuIFNα4 for mice). Subsequently, a second, amplified wave of diverse IFNα subtypes follows that is IRF7-dependent. As the ratio of IRF3 to IRF7 or other IRFs changes over time, the repertoire of IFN subtypes expressed changes as well.

There are several intriguing deviations from this paradigm. First, the IFNβ promoter has additional response elements that make it responsive to NF-κB signaling through activating transcription factor 2 (ATF-2) and c-Jun, which allows other signaling pathways to augment IFNβ production (29, 37, 38). This unique promoter feature also permits IRF3-independent basal expression of low amounts of IFNβ in the absence of infection, which can have significant impact on mounting

successful innate immune responses against a variety of infections (39–47). IFNκ may have somewhat restricted expression, as it was named for its high expression in keratinocytes; however, other cell types, including immune cells and lung epithelial cells, can upregulate IFNκ expression (48–50). Further characterization is needed to determine which cells are capable of expressing IFNκ in different contexts. Lastly, IFNε is the most notable exception to the IRF-mediated IFN induction paradigm, as it is not regulated at all by PRR signaling and IRF3/7. Instead, it is constitutively expressed in the epithelium of reproductive organs and hormonally regulated, and this is reflected in its unique promoter (51–53).

Differential Binding Affinity Determines Signaling and Function

All type I IFNs bind to and signal through the heterodimeric receptor IFNAR1 and IFNAR2 to activate canonical JAK/STAT signaling pathways (4). A unique feature of type I IFN signaling is that the signaling outcome can vary depending on the cell type, specific ligand, and concentration of the type I IFN subtype. The molecular mechanisms that underlie the plasticity of type I IFN signaling have been extensively reviewed elsewhere, so only key features will be outlined in this review (20, 54, 55).

In general, IFNAR2 is the primary ligand binding receptor subunit and binds type I IFNs with high affinity (typically nanomolar affinity); IFNAR1 is subsequently recruited to the receptor-ligand complex and binds with relatively lower affinity (approximately micromolar affinity) (54). HuIFNβ has the highest natural binding affinity to the type I IFN receptors with picomolar affinity for IFNAR2 and nanomolar affinity for IFNAR1, whereas HuIFNα2 possesses nanomolar affinity for IFNAR2 and micromolar affinity for IFNAR1 (56–58). This higher affinity interaction may enable IFNβ to uniquely signal through IFNAR1 in an IFNAR2-independent manner, but further work is needed to corroborate this finding and to determine if other receptors are involved in this phenomenon (59, 60). Engineered IFNα2 and IFNω mutants that mimic the range of affinities for the receptor complex have demonstrated that type I IFN signaling outcomes can be directly linked to IFN affinity to the receptor complex. Hence, type I IFN mutants that acquire IFNβ-like affinity acquire IFNβ-like potency (61, 62).

In line with these findings for IFNα, IFNβ, and IFNω, recent work showed that HuIFNε and HuIFNκ bound IFNAR2 with particularly weak affinity and demonstrated approximately 1000-fold decreased potency in ISGF3-mediated gene expression compared to HuIFNα2, whereas their affinity for IFNAR1 was comparable to other type I IFN subtypes (63). HuIFNε and HuIFNκ also bound the poxvirus antagonist B18R with weaker affinity relative to the other IFN subtypes, perhaps suggesting a fitness advantage for the host to have some weaker binding IFN subtypes in order to avoid virus inhibition (63). In influenza A virus (IAV) infection, HuIFNκ, but not IFNα or IFNβ, relied on chromodomain helicase DNA binding protein 6 (CHD6) to efficiently suppress viral replication (50). Moreover, induction of CHD6 was not dependent on STAT1, but rather, IFNκ signaled through the mitogen-activated protein kinase (MAPK) p38 and the transcription factor c-Fos to mediate its antiviral effects. Altogether, these findings suggest that in addition to having unique expression patterns, IFNε and IFNκ may possess additional biochemical and signaling features that grant unique properties *in vivo*.

Differential Sensitivity to Feedback Loops

The affinity of individual subtypes, as outlined above, is a key component in determining the signaling outcome from IFNAR1/2 engagement, but negative feedback loops are an additional level of regulation and fine-tuning. IFNAR1/2 surface abundance is typically quite low, and modulating the surface receptor expression is one means of regulating type I IFN signaling after type I IFN induction (64). Manipulation of a cell line's IFNAR expression demonstrated that the antiproliferative and proapoptotic activities induced by HuIFNβ are less sensitive to decreased receptor levels than those induced by HuIFNα2 (65, 66). The physiological relevance of receptor expression influencing type I IFN signaling is demonstrated in the number of IFN-dependent mechanisms that downregulate IFNAR1 and IFNAR2 levels. We will outline a few examples.

First, protein kinase D2 (PKD2) is a negative regulator activated downstream of IFN signaling. It phosphorylates IFNAR1, enabling interaction with a ubiquitin E3 ligase, and subsequent ubiquitination leads to endocytosis of the IFN signaling complex (67, 68). Endosomes with short-lived receptor-ligand complexes formed by lower affinity IFNαs are more likely to be recycled to the cell surface; endosomes with longer-lived complexes formed by higher affinity IFNβ ultimately fuse with the lysosome, but signaling can continue to take place as trafficking progresses through the endosomal compartment (69–72). Second, Suppressor of Cytokine Signaling 1 (SOCS1) can directly dampen the type I IFN response by interacting with TYK2 to disrupt TYK2-STAT signaling, but it also decreases surface levels of IFNAR1, which requires TYK2 for stability at the cell surface (73). Lastly, ubiquitin-specific peptidase 18 (USP18) can bind the cytoplasmic domain of IFNAR2 and interfere with IFNAR1 recruitment and ternary receptor complex formation without decreasing surface IFNAR2 levels (74, 75). The USP18-IFNAR2 interaction makes it so that only higher affinity ligands such as IFNβ are able to recruit IFNAR1 into the receptor complex, making the cell less responsive to weaker affinity type I IFNs (76, 77).

Key Principles for Differential Activities

Altogether, differential expression, binding affinity to the receptor, and downstream feedback loops enable IFNAR1/2 to have graded responses to multiple ligands. Redundancy and pleiotropy are key features of type I IFN responses. Essentially, any type I IFN subtype can induce robust (or redundant) properties, such as antiviral activity, even at low surface receptor density. In contrast, tunable (or pleiotropic) functions, like antiproliferative activity, are more heavily influenced by affinity of the ligand, receptor density, and intracellular negative regulators, and so higher affinity ligands, like IFNβ, tend to be more potent (21). However, as noted above, some type I IFN

subtypes may be able to signal through alternative pathways, in spite of or, more likely, because of possessing lower binding affinity. Understanding the molecular mechanisms underlying differential signaling by IFNs is an active area of research and how the differential activities of IFNα and IFNβ impact disease will be explored in the remaining sections.

INFECTIOUS DISEASES

Type I IFNs have been extensively studied in the context of infectious diseases, and this body of work includes most of the studies that have directly compared the functions of IFNα and IFNβ *in vivo*. In the following subsections we highlight key findings from animal models and human studies that have contributed to understanding the mechanisms of differential properties of IFNα and IFNβ in viral, bacterial, and parasitic infections.

Viral Infections

The important role that viral infections have served in helping us understand type I IFN biology cannot be understated. Viral infections were key instruments in the discovery of the antiviral properties of type I IFNs (1). It is now widely appreciated that type I IFNs play a much larger role in coordinating protective immunity beyond directly eliciting an antiviral state, including their role in DC maturation, augmenting antibody production by B cells, and improving cytolytic T cell effector functions (5). Intriguingly, type I IFNs can also play a detrimental role in certain contexts, such as persistent viral infections. Given their key roles in disease outcome, viral systems also include some of the clearest examples of differential functions of IFNα and IFNβ *in vivo* (**Table 1**). The following viral models collectively highlight that differential functions of IFNαs and IFNβ can profoundly influence disease pathogenesis and that the mechanisms underlying differential functions vary depending on the biological context.

Lymphocytic Choriomeningitis Virus

Lymphocytic choriomeningitis virus (LCMV) is a nonlytic, negative-strand RNA virus and a prototypic member of the *Arenaviridae* family, which are causative agents of hemorrhagic fevers in humans (100). The host genetics, viral strain, dose, and inoculation route all have profound impacts on host responses and disease outcome, and this remains true for the role of type I IFN responses during LCMV pathogenesis (101). LCMV infection serves as an excellent example of the pathogenic potential of type I IFNs.

LCMV-Clone-13 (Cl-13), which differs from its parent strain LCMV-Armstrong (Arm) by just three amino acids, causes a persistent infection, whereas LCMV-Arm is acutely and effectively cleared by immunocompetent mice (102). A clear pathogenic role for type I IFNs during persistent LCMV-Cl-13 infection has been established (78, 79, 103–105). Loss of IFNAR1 caused increased viral loads early during infection but ultimately restored splenic organization, decreased expression of the negative immune regulators IL-10 and programmed death-ligand 1 (PD-L1), increased protective adaptive immune responses, and accelerated clearance of persistent virus (78, 79, 105). While both LCMV-Arm and LCMV-Cl-13 infection led to high IFNα levels in the serum, only LCMV-Cl-13 induced significant serum IFNβ (79). In a seminal study, Ng and colleagues showed that the pathogenic activity of type I IFNs in persistent LCMV infection could be ascribed to just one subtype—IFNβ. Using monoclonal antibody (mAb) blockade and genetic deletion, they showed that IFNβ was dispensable for controlling early LCMV-Cl-13 viral loads, suggesting that IFNα or other subtypes mediate these antiviral responses (80). Instead, blockade of IFNβ but not IFNα improved splenic architecture, decreased infection of CD8α$^-$ DC, and enhanced antiviral T cell responses that led to clearance of persistent virus, mimicking many of the effects seen with IFNAR1 blockade. Altogether, persistent LCMV-Cl-13 infection serves as an important example that the type I IFN subtypes can have distinct properties *in vivo* that have profound impacts on viral pathogenesis.

As discussed above, LCMV-Cl-13 infection causes persistent infection in certain mouse strains (C57BL/6, BALB/C, C3H, or SWR/J); however, LCMV-Cl-13 infection of other strains (NZB, SJL/J, PL/J, NZO, or FVB/N mice) causes type I IFN- and CD8 T cell-dependent severe vascular leakage and death by about 6–8 days post infection (dpi) (81, 82, 106, 107). NZB.*Ifnar1*$^{-/-}$ but not NZB.*Ifnb*$^{-/-}$ mice were protected from LCMV-Cl-13 induced lethal vascular leakage, suggesting that IFNβ is dispensable for the detrimental effects of type I IFN in this model and that other subtypes like IFNα may drive this phenotype (81). However, this is challenged by the fact that blockade of IFNβ alone, pan-IFNα (α1, α4, α5, α11, and α13) alone, or combined pan-IFNα/β did not replicate the protection provided by anti-IFNAR1 treatment in FVB/N mice (82). The inability of IFNβ or IFNα blockade to phenocopy IFNAR1 blockade could be due to dosing issues, as the serum levels of IFNα were severely elevated (roughly 18-fold over IFNβ levels), involvement of IFNα subtypes not blocked by the mAb, or involvement other type I IFN subtypes altogether could be responsible for the lethal phenotype. Nevertheless, type I IFNs are clearly important host determinants of lethal LCMV infection, and the individual IFN subtype(s) responsible remains an open question.

Chikungunya and West Nile Viruses

Chikungunya virus (CHIKV) is a mosquito-transmitted, reemerging alphavirus that causes outbreaks of acute fever, rash, polyarthritis, arthralgia, and myositis (108). West Nile virus (WNV) is a mosquito-transmitted flavivirus that can cause encephalitis in severe cases (109). It is helpful to consider these models together because both models utilize a peripheral route of infection by inoculating the footpad subcutaneously (s.c.), and type I IFNs are essential for controlling both CHIKV and WNV, as *Ifnar1*$^{-/-}$ mice rapidly succumb to a severe, disseminated infection with either virus (83, 84, 88, 89). The collective evidence from these models suggest that IFNα and IFNβ play nonredundant protective roles.

Loss of IRF7, the master transcriptional regulator of IFNα subtypes, in acute WNV infection increased lethality and viral

TABLE 1 | Summary of IFNα and IFNβ functions in mouse models of viral infections.

Intervention	Clinical Outcome	Virological and Immune Characterization	Refs.
Lymphocytic choriomeningitis virus Cl-13, i.v. (persistent infection)			
Ifnar1$^{-/-}$ or αIFNAR1 mAb	Improved splenic architecture	V: ↑ viremia, ↑ tissue titers (early); ↓ viremia, ↓ tissues titers (late) I: ↓ IL10 (serum), ↓ PD-L1 expression (splenic cells), ↑ Ag^{+} CD4 T (spleen)	(78–80)
αIFNα mAb	ND	V: ND (early); no Δ viremia, ↑ splenic titer (late)	(80)
Ifnb$^{-/-}$ or αIFNβ mAb	Improved splenic architecture	V: no Δ (early); ↓ viremia, ↓ tissue titers (late) I: no Δ IL-10 (serum), no Δ PD-L1 expression (splenic cells), ↑ Ag^{+} CD4 T (spleen)	(80)
Lymphocytic choriomeningitis virus Cl-13, i.v. (lethal infection)			
NZB.*Ifnar1*$^{-/-}$ or αIFNAR1 mAb (NZB)	↓ vascular leakage, ↓ lethality (0%)	V: ↑ viremia that persists I: ↓ CTL activity, ↓ lung infiltrate, ↓ BALF cytokines	(81)
NZB.*Ifnb*$^{-/-}$	No Δ lethality	ND	(81)
αIFNAR1 mAb (FVB/N or NZO)	↓ vascular leakage, ↓ lethality (0%)	V: ↑ viremia that persists I: ↑ platelet count	(82)
αIFNβ mAb (FVB/N)	No Δ lethality	ND	(82)
αIFNα mAb (FVB/N)	No Δ lethality	ND	(82)
αIFNα and αIFNβ mAbs co-treatment (FVB/N)	No Δ lethality	ND	(82)
West Nile virus, s.c. (footpad)			
Ifnar1$^{-/-}$	↑ lethality (100%)	V: ↑ viremia, ↑ tissue titers	(83, 84)
Irf7$^{-/-}$	↑ lethality (100%)	V: ↑ viremia, ↑ tissue titers I: ↓ serum IFNα, ↓ IFNα mRNA (cells)	(85, 86)
αIFNα mAb	↑ lethality	ND	(86)
αIFNβ mAb or *Ifnb*$^{-/-}$	↑ lethality (100%)	V: ↑ viremia, ↑ tissue titers (some but not all tissues) I: no Δ Ab responses, no Δ brain infiltrate	(86, 87)
Chikungunya virus, s.c. (footpad)			
Ifnar1$^{-/-}$	↑ lethality (100%)	V: ↑ viremia, ↑ tissue titers	(88, 89)
Irf7$^{-/-}$	↑ foot swelling	V: ↑ viremia, ↑ tissue titers I: ↓ serum IFN, ↓ IFNα mRNA (tissue)	(90–91)
αIFNα mAb	↑ foot swelling	V: ↑ viremia, ↑ tissue titers	(90)
αIFNβ mAb or *Ifnb*$^{-/-}$	↑ foot swelling	V: minimal Δ viremia and tissue titers I: ↑ neutrophil infiltrate (foot)	(90)
Influenza A virus, PR/8/34 (H1N1), i.n.			
B6.*Mx1*.*Ifnar1*$^{-/-}$ (functional Mx1 KI)	↑ lethality	ND	(92)
B6.*Mx1*.*Ifnb*$^{-/-}$ (functional Mx1 KI)	↑ lethality	V: ↑ lung titer	(92)
Vaccinia virus, i.n.			
Ifnb$^{-/-}$	↑ weight loss, ↑ lethality	V: ↑ tissue titers	(93)
Friend retrovirus, i.v.			
Ifnar1$^{-/-}$	ND	V: ↑ viremia, ↑ spleen titer I: ↓ CD4 T%, ↓ CD8 T% (spleen)	(94)
Ifnb$^{-/-}$	ND	V: no Δ viremia, ↑ splenic titer I: ↓ CD4 T% (spleen)	(94)
rIFN α1, α4, α6, or α9 (B10.A×A.BY)F_1	ND	V: ↓ viremia, ↓ spleen titer (α1, α4, α9); no Δ titers (α6) I: ↑ Ag^{+} CD8 T (α1 only), ↑ NK activation (α1, α4, α9)	(95)
rIFNα2, α5, or α11 (B6 or (B10.A×A.BY)F_1)	ND	V: ↓ spleen titer (α11 only) I: ↑ NK activation	(96)
Hepatitis B virus, hydrodynamic injection i.v.			
rIFNα1, α2, α4, α5, α6, α9, or α11 (BALB/C)	ND	V: ↓ viremia (α4, α5); no Δ viremia (α1, α2, α6, α11) I: ↑ CTL and NK activity (α4, α5)	(97)
pIFNα, pIFNβ (hydrodynamic i.v.)	ND	V: ↓ viremia (pIFNα > pIFNβ) I: ↑ liver ISG induction (pIFNα > pIFNβ), no Δ T cell responses (pIFNα or pIFNβ)	(98)

The mouse genetic background is C57BL/6 unless otherwise specified.

↑, increased; ↓, decreased; Δ, change; αIFN, anti-IFN; Ag, antigen-specific; BALF, bronchoalveolar lavage fluid; CTL, cytotoxic lymphocyte (CD8 T cell); I, immune; i.n., intranasal; i.v., intravenous; ISG, interferon-regulated gene; KI, knock-in; mAb, monoclonal antibody; ND, no data; p, plasmid; r, recombinant; V, virological.

loads in both peripheral and central nervous system (CNS) tissues compared to WT animals (34, 85). Similarly, *Irf7*$^{-/-}$ mice infected with CHIKV developed worse clinical disease (foot swelling) and sustained high viral loads at the site of infection and sites of dissemination (90–92). The poor clinical outcome of *Irf7*$^{-/-}$ mice during WNV and CHIKV infection may be the result of decreased IFNα activity in the serum (85, 86, 91, 92). This postulation is supported by the observation that *Irf7*$^{-/-}$ mice produce little to no systemic IFNα activity when infected with a number of viruses, including Dengue virus (DENV), herpes simplex virus 1 (HSV-1), and encephalomyocarditis virus (EMCV), and this loss of systemic IFNα activity correlated with increased susceptibility to those infections (34, 110, 111). Pan-IFNα mAb blockade closely mimicked the clinical and virologic phenotype of *Irf7*$^{-/-}$ mice in CHIKV infection and phenocopied the lethality observed in WNV infection (86, 90). Altogether, these findings suggest that an important protective function of IRF7 is the production and amplification of IFNα responses and that IFNαs are important for controlling viral replication and dissemination.

In contrast with IFNα, the role of IFNβ *in vivo* is more varied and dependent on the biological context. *Ifnb*$^{-/-}$ mice are more susceptible than WT mice to WNV infection, and this increased lethality was accompanied with elevated viral burden in some but not all tissues (87). Specifically, WT and *Ifnb*$^{-/-}$ mice similarly controlled WNV replication in the spleen and serum, consistent with IFNα subtypes dominating serum IFN activity. WNV did replicate to a larger extent in the brain, spinal cord, and the draining lymph in *Ifnb*$^{-/-}$ mice compared to WT mice (87). An antiviral role for IFNβ has also been described for vaccinia virus and IAV infections (93, 94). In contrast to WNV infection, loss of IFNβ exacerbated CHIKV-induced disease but with minimal impact on viral burden at the inoculation site or distant tissues, suggesting that IFNβ may be important in restricting viral replication within certain but not all tissues (90). Rather, the increased disease severity of CHIKV-infected *Ifnb*$^{-/-}$ mice correlated with increased neutrophil accumulation at the site of infection, and depletion of neutrophils in *Ifnb*$^{-/-}$ mice reversed the disease exacerbation to WT levels. Altogether, these data from CHIKV and WNV infections point to the particular importance of IFNα subtypes in restricting viral replication and spread and highlight that the primary role of IFNβ varies depending on the specific context.

Human Immunodeficiency Virus 1 and Friend Retrovirus

Human immunodeficiency virus 1 (HIV-1) is a highly pathogenic retrovirus that leads to acquired immunodeficiency syndrome (AIDS). The relationship between type I IFNs and HIV-1 pathogenesis is complex, and it is outside the scope of the this review to cover all the protective and pathogenic functions, which have been extensively reviewed elsewhere (112–114). The purpose of reviewing HIV and Friend retrovirus (FV) infection is not to delve into whether type I IFNs have a net protective or pathogenic role, but rather, we seek to underscore that the IFNα subtypes are not equivalent in their antiviral or immunomodulatory properties *in vivo*.

Harper and colleagues evaluated the mRNA expression of specific IFNα subtypes in human pDCs following HIV-1 exposure (115). Intriguingly, they found an inverse relationship between the subtypes induced and their antiviral potency. HuIFNα1/13 and HuIFNα2 were highly expressed, but they demonstrated weaker antiviral activity *in vitro*, whereas HuIFNα6, α8, and α14 represented a smaller fraction of the IFNα subtypes induced but demonstrated the highest antiviral activity against HIV-1. Likewise, a study from Lavender and colleagues showed that therapeutic administration of HuIFNα14 was more beneficial than administration of HuIFNα2 in controlling HIV-1 replication in a humanized mouse model (116). The efficacy of IFNα14 was associated with increased ability to stimulate intrinsic immune responses including expression of tetherin and Mx2 as well as a greater frequency of TRAIL^{+} natural killer (NK) cells. Conversely, IFNα2 was superior in increasing the frequency of CD8^{+} T cells. An additional study used humanized mice that lack pDCs (Hu-PBL mice) and do not express much endogenous type I IFN during acute HIV-1 infection to study the impact of IFNα subtypes. They performed a single hydrodynamic injection of plasmid encoding different type I IFN subtypes (HuIFNα2, α6, α8, α14, or β) into Hu-PBL mice prior to HIV-1 infection (117). The authors found that all subtypes tested limited HIV-1 replication and prevented HIV-induced CD4^{+} T cell depletion by 10 dpi, but only HuIFNα14- and HuIFNβ-expressing mice demonstrated this protective effect out to 40 dpi. Altogether these findings demonstrate nonredundant functions of IFNα subtypes, with HuIFNα14 emerging as an intriguing subtype for further studies during HIV-1 infection.

Distinct properties of murine IFNα subtypes have also been observed in FV infection, a commonly used murine retrovirus model. A protective role for type I IFNs in controlling FV infection *in vivo* was demonstrated with *Ifnar1*$^{-/-}$ and *Ifnb*$^{-/-}$ mice both having increased viral loads in the spleen. However, only *Ifnar1*$^{-/-}$ mice showed a significant increase in viremia (95). These findings suggest that both IFNα and IFNβ protect against FV infection, but IFNα may be more important for controlling systemic infection and dissemination. Different potencies among IFNα subtypes have also been revealed. *Ex vivo* stimulation of FV-specific CD8^{+} T cells demonstrated differential activities among the IFNα subtypes. IFNα4, α6, and α9 had the strongest effects on CD8^{+} T cells, including inhibiting proliferation, stimulating cytokine production, and enhancing cytotoxicity (118). Treatment of FV-infected mice with MuIFNα1, α4, or α9, but not α6, significantly decreased viral loads, and subtype effectiveness was associated with different mechanisms (96). Only IFNα1 treatment correlated with activated FV-specific CD8^{+} T cells in the spleen, whereas NK cell activation was observed after treatment with all examined IFNα subtypes. Another study demonstrated that prophylactic administration of MuIFNα11, but not α2 or α5, significantly reduced viral loads by activating NK cells and ultimately provided long-term protection (6 weeks) (97). Together with the HIV-1 studies, retroviruses have proven to be effective tools for probing the diverse functions IFNα subtypes.

Hepatitis B and Hepatitis C Viruses

Hepatitis B (HBV) and hepatitis C viruses (HCV) are drastically distinct pathogens from a virological perspective—HBV is a double-stranded DNA virus belonging to the *Hepadnaviridae* family, whereas HCV is a positive-strand RNA virus and a member of *Flaviviridae*. However, both viruses display tropism for hepatocytes, and chronic infection with either virus can lead to liver failure, cirrhosis, and hepatocellular carcinoma (119). Beginning in the 1980s, derivatives of recombinant HuIFNα2 were used to treat chronic HBV and HCV, but treatment was successful in a limited subset of patients and severe side effects were common [reviewed in reference (120)]. These issues have led to the phasing out of type I IFN-based therapeutics in favor of direct-acting antiviral drugs (120). Though HuIFNα2-based therapeutics are the only approved type I IFN therapies for HCV or HBV treatment, pilot studies of IFNβ therapy in IFNα-nonresponding HBV or HCV patients suggest some beneficial effects of IFNβ as well (121–123). These findings suggest that other IFN subtypes in addition to IFNα2 may offer protective effects against hepatitis viruses.

Indeed, one study with the HBV hydrodynamic injection model demonstrated that prophylactic treatment with MuIFNα4 or α5 was more effective than other IFNα subtypes in decreasing HBV replication *in vivo*, and both α4 or α5 also increased effector NK and $CD8^+$ T cell frequencies in the liver and spleen (98). Hydrodynamic injection of plasmids expressing MuIFNα4, α5, or combined α4 and α5 was more effective than treatment with the respective recombinant proteins, highlighting the importance of long-lasting endogenous IFNα expression in the liver during HBV infection. Another study directly showed differential effects of IFNα4 and IFNβ in the hydrodynamic injection HBV model (99). Co-injection of a plasmid encoding MuIFNα4 with HBV DNA decreased HBV serum markers, elevated liver ISG expression, and reduced HBV^+ cells in the liver, whereas co-injection of an IFNβ-expressing plasmid demonstrated weaker inhibition of HBV and surprisingly led to a transient increase in HBV^+ hepatocytes. This increase in HBV^+ hepatocytes was not observed if the IFNβ plasmid was injected 14 dpi instead of co-injected with HBV (99). Even as the currently approved type I IFN therapies are being phased out of clinical use, these findings add to the accumulating evidence of distinct potencies and functions of IFNα and IFNβ subtypes in mouse models of relevant human pathogens.

IFNω Subtype Differences

IFNω is understudied compared to IFNα/β subtypes likely because mice lack a functional IFNω, but there is much *in vitro* evidence that it signals and functions similarly to IFNα/β (61, 124). Humans have only one IFNω subtype, but several species possess an expansion of IFNω genes (15–17, 125, 126). A number of these IFNω subtypes have been cloned from several species and have been demonstrated to be functional type I IFNs (127–129). Just as there is growing appreciation that expanded IFNα subtypes provide an evolutionary advantage beyond redundancy, it stands to reason that the expansion of IFNω genes likewise imparts a fitness advantage for those species. Indeed, a recent study compared two different IFNω subtypes from *Rousettus aegyptiacus* bats and found that IFNω9 displayed more effective antiviral activity against several RNA viruses *in vitro* compared to IFNω4 (130). Additionally, differences in expression and activity of porcine IFNω subtypes have also been demonstrated, with IFNω7 demonstrating the best antiviral activity *in vitro* (131). Several of these animals with expanded IFNω subtypes represent important reservoirs and transmitters of relevant human pathogens, so IFNω functional studies may provide valuable information on understanding the interactions between pathogens and their natural hosts.

Remarks on Viral Infections

When type I IFNs act on the proper cell type at the opportune time, they can induce an antiviral state, promote apoptosis of virally infected cells, coordinate recruitment of immune cells, enhance activation of antigen-presenting cells, and augment protective B and T cell responses. Not all IFNs are equal in their ability to induce these protective effects, and exploring this idea *in vivo* is an active area of research. Studies from infection with LCMV, WNV, and CHIKV have made it evident that endogenous IFNα subtypes are particularly important for limiting viremia and viral spread, likely due to their abundant activity in the serum in a number of viral infections. In peripheral tissues, IFNαs and IFNβ can exert important antiviral or immunomodulatory activity. Whether a particular subtype emerges as more important than others is likely going to depend on its biochemical properties, the cellular tropism of the virus, the source and magnitude of its induction, how long its expression is sustained, and the specific cell types responding to IFN.

If type I IFN signaling is sustained too long, immunosuppression and viral persistence can occur through the upregulation of negative immune regulators, like IL-10 and PD-L1. LCMV infection is a good example of this scenario, and strikingly, IFNβ was critical in promoting many detrimental features of type I IFN signaling in this model. We did not have space to discuss the growing evidence that type I IFNs can promote tissue damage during acute viral infections by promoting excessive inflammation and cell death [discussed in references (132, 133)]. This has been observed for mouse strains highly susceptible to influenza or coronavirus infection (134–136). The mechanisms responsible for these detrimental effects of type I IFN are an active area of research, but initial observations suggest that excessive or delayed IFN induction may play a role. It is also unknown whether specific IFN subtypes are responsible for these effects. Future studies exploring this possibility could have an important impact on human disease.

Bacterial Infections

Type I IFNs can play a pathogenic or protective role during bacterial infection depending on the pathogen. The mechanisms underlying the beneficial or detrimental roles during bacterial infection remain poorly understood and warrant further study. Below we explore some of the properties of type I IFNs during models of bacterial infections (**Table 2**). However, compared to the examples from viral infections, few of these studies directly compare the functions of IFNα and IFNβ. We draw attention to

TABLE 2 | Summary of IFNα and IFNβ functions in mouse models of bacterial infections.

Intervention	Clinical Outcome	Bacterial Load and Immune Characterization	Refs.
Mycobacterium tuberculosis (Mtb)			
129.*Ifnar1*$^{-/-}$ *Mtb* (H37Rv)	↓ lethality	B: ↓ lung titer I: ↓ iNOS expression (lung), ↓ IL-1β, IL-1α, IL-6 (lung)	(137)
129.*Ifnar1*$^{-/-}$ *Mtb* (HN878)	↓ lethality	B: ↓ lung titer	(138)
Ifnar1$^{-/-}$ or B6.*Sst1*^{S}.*Ifnar1*$^{-/-}$ *Mtb* (Erdman)	↓ lethality	B: ↓ lung titer I: ↓ IL-1Ra (lung), ↑ functional IL-1β activity (lung)	(139, 140)
Ifnar1$^{-/-}$ *Mtb* (H37Rv)	ND	B: ↓ lung titer I: ↑ IL-1α, IL-1β expression (lung myeloid cells *in vivo*), ↑ PGE2 in BALF	(141, 142)
Poly-ICLC (i.n.), *Mtb* (H37Rv)	↑ lethality, ↑ lung necrosis IFNAR1	B: ↑ lung titer (acute, chronic) I: ↑ CD11b^{+}F4/80^{+}GR1^{int} infiltrate (lung)	(142, 143)
***Salmonella enterica* serovar Typhimurium**			
Ifnar1$^{-/-}$ adult (i.v.)	↓ lethality	B: ↓ spleen CFU I: ↑ Mφ freq. (spleen), ↓ Mφ cell death (spleen)	(144)
Ifnb$^{-/-}$ adult (oral)	↓ lethality	B: ↓ liver CFU I: ↓ IL-10 mRNA, ↑ CXCL2 mRNA, ↑ MPO activity (small bowel)	(145)
Streptococci spp.			
Ifnar1$^{-/-}$ *S. pyogenes*, s.c.	↑ lethality	B: ND I: ↑ neutrophil infiltrate (lung)	(146)
129.*Ifnar1*$^{-/-}$ (adult) Group B, type V, i.p.	↑ lethality	B: ↑ blood and kidney CFU	(147)
Ifnb$^{-/-}$ (adult) Group B, type V, i.p.	↑ lethality	B: ND I: ↓ TNFα and IFNγ induction by peritoneal Mφ (*ex vivo*)	(147)
129.*Ifnar1*$^{-/-}$ *S. pneumoniae*, i.v. or i.c.	↑ lethality	B: ↑ blood CFU (i.v. and i.c. routes)	(147)
Ifnar1$^{-/-}$ *S. pneumoniae*, i.n. or i.p.	ND	B: ↑ blood CFU (i.n. route), no Δ viremia (i.p. route) I: ↑ lung permeability, ↓ tight junction mRNA (lung)	(148)
rIFNβ (i.n.), *S. pneumoniae*, i.n.	↓ lethality	B: ↓ blood CFU	(148)
AdIFNα (i.n.), *S. pneumoniae*, i.n.	↓ lethality	B: ↓ lung, ↓ spleen CFU I: ↓ neutrophil and Mφ infiltrate (lung), ↓ BALF TNFα, IL-1β, and CXCL10	(149)
Listeria monocytogenes			
Ifnar1$^{-/-}$ (various routes)	↓ lethality	B: ↓ liver, ↓ spleen CFU I: ↓ TRAIL expression (spleen), ↓ apoptosis (spleen), ↑ serum IL-12p70, ↓ serum TNFα and IL-6	(150–151)
Irf3$^{-/-}$ (i.v.)	↓ lethality	B: ↓ liver, ↓ spleen CFU I: ↓ IFNβ induction in Mφ (*ex vivo*), ↓ apoptosis (spleen)	(152)

The mouse genetic background is C57BL/6 unless otherwise specified.

↑, increased; ↓, decreased; Δ, change; Ad, adenoviral vector expression; B, bacterial load; BALF, bronchoalveolar lavage fluid; CFU, colony forming unit; dep., dependent; freq., frequency I, immune; i.c., intracranial; i.v., intravenous; Mφ, macrophage; MPO, myeloperoxidase; ND, no data; Poly-ICLC, polyinosinic-polycytidylic acid stabilized with poly-$_L$-lysine; s.c., subcutaneous; spp., species.

a few instances in which specific subtypes have been examined and highlight areas where this may be an interesting avenue to explore.

Mycobacterium Tuberculosis

Mycobacterium tuberculosis (*Mtb*) causes the disease tuberculosis and represents a global health burden. This intracellular pathogen primarily infects the lungs, and it can enter latency if it is not eliminated, persisting in granulomas (154). The actions of type I IFNs during *Mtb* infections are complex, and there are numerous examples of contradictory findings. Overall, there is strong evidence that type I IFNs are detrimental to the host, but depending on the timing of IFN induction, the bacterial strain, and host genetics, IFNs may occasionally benefit the host during infection [reviewed in reference (155)].

Numerous studies have shown a type I IFN-inducible transcriptional profile in blood isolated from patients with active tuberculosis, but this signature is typically absent in patients with latent infection or patients who have undergone successful treatment (156–158). Concordantly, infection with hypervirulent *Mtb* laboratory strains showed increased recruitment of type I IFN-producing pDCs and classical DCs and elevated expression of IFNα or IFNβ in the lung, depending on the study (138, 139, 159–162). Multiple studies with human and mouse models have shown that type I IFNs are associated with impaired IFNγ-mediated antibacterial effects, decreased expression of IL-1α and IL-1β, decreased production of prostaglandin E2 (PGE2), and upregulation of IL-10 (138–142, 159, 162–165). Type I IFNs are also associated with increased cell death of macrophages and increased recruitment of myeloid cells

permissive to *Mtb* infection (137, 143). Limited work has addressed the pathogenic potential of individual type I IFNs, but one recent study found that *in vitro* blockade of IFNα (subtypes unspecified), but not IFNβ blockade, significantly decreased intracellular *Mtb* bacterial load in a macrophage cell line (166). It remains to be determined if a similar effect could be observed *in vivo*.

Despite all of the evidence pointing to detrimental effects of type I IFNs in *Mtb* infection, type I IFNs may play a beneficial role in particular circumstances. First, several case reports have suggested that coadministration of IFNα with antimycobacterial therapy decreased bacterial burden in individuals who failed to respond to antimycobacterial therapy alone (167–170). However, these studies were employed before the pathogenic effects of type I IFNs were appreciated, and the mechanisms driving the apparent protection remain elusive. Second, in agreement with the findings that the detrimental effects of type I IFNs are largely due to inhibition of IFNγ, type I IFNs appear to be protective in contexts of IFNγ deficiency. Mice lacking both type I and type II IFN receptors displayed increased mortality and pathology compared to mice lacking only the type II IFN receptor in *Mtb* infection (171, 172). Mechanistically, type I IFNs may dampen recruitment of *Mtb*-permissible macrophages and suppress macrophages from entering an alternative activation state. In accord with these mice studies, administration of IFNα2b combined with antimycobacterial chemotherapy had beneficial effects in *Mtb*-infected children with underlying IFNγ signaling deficiencies (173, 174). It is unclear whether IFNβ can induce these effects as well. Further head-to-head comparison studies of IFNα and IFNβ are needed to determine if this protective effect of type I IFNs is unique to IFNα.

Type I IFNs may also benefit the host in infection with less virulent *Mycobacterium* strains, such as the bacille Calmette-Guérin (BCG) vaccine derived from *M. bovis* (175, 176). Administration of IFNα at the time of BCG vaccination (s.c.) in mice followed by intramuscular IFNα boosts (subtype not disclosed) promoted production of IFNγ, tumor necrosis factor (TNF), and IL-12, thus slightly increasing the protection seen upon re-challenge with *Mtb* intranasal (i.n.) compared to immunization with BCG alone (175). Moreover, the bacterial ESX-1 secretion system promotes type I IFN induction, and its recombinant expression in the BCG vaccine better protected against *Mtb* infection than other versions of the vaccine (176–179). *In vitro* data also highlight the complexity of type I IFN functions, as pretreatment of permissible cells with IFN before *Mycobacterium* infection can promote bacterial growth or increase immune activation, depending on the cell type and bacterial strain (180, 181). Thus, type I IFNs may play a protective role in vaccination with weaker *Mycobacterium* strains.

Salmonella enterica Serovar Typhimurium

Salmonella is a common, pathogenic genus of bacteria that causes acute gastroenteritis. Type I IFNs largely play a pathogenic role in *Salmonella* infection by promoting necroptosis and suppressing protective innate cell recruitment and proinflammatory responses. Deletion of IFNAR1 increased survival of adult mice infected (i.v.) with *S. enterica* serovar Typhimurium (*S.* Typhimurium) and decreased splenic bacterial loads (144). Additionally, splenic macrophages in *Ifnar1*$^{-/-}$ mice were resistant to *S.* Typhimurium-induced necroptosis *ex vivo*, and a follow-up mechanistic study further determined that type I IFN signaling impaired antioxidative stress responses to *S.* Typhimurium infection of bone marrow-derived macrophages (144, 182). IFNβ may be the dominant type I IFN subtype driving this necroptosis phenotype, as blockade of IFNβ, but not IFNα, prevented necroptosis and enhanced survival of bone marrow-derived macrophages during *S.* Typhimurium infection *in vitro* (144). It is unclear how many IFNα subtypes the antibody used blocks (clone: RMMA-1), so it is premature to rule out a contribution of IFNα. A role for IFNβ was further demonstrated in a separate study which showed that *Ifnb*$^{-/-}$ mice were more resistant to oral infection of *S.* Typhimurium, which was characterized by decreased bacterial burden, dampened expression of IL-10, and increased levels of CXCL2 and myeloperoxidase activity in the liver (145). Altogether, these findings suggest that IFNβ may play a detrimental role in *S.* Typhimurium infection by negatively regulating protective immune responses, but further studies are needed to rule out the involvement of other type I IFN subtypes.

Listeria monocytogenes

Listeria monocytogenes is an intracellular, pathogenic bacteria that causes sepsis and meningitis in immunocompromised and pregnant individuals (183). Many groups have shown that type I IFN signaling is detrimental to the host in systemic *L. monocytogenes* infection, but not in all routes of infection (150–153, 184, 185). Despite the important role that type I IFNs play in *L. monocytogenes* pathogenesis, the contribution of individual subtypes remains unknown. *Irf3*$^{-/-}$ mice displayed increased resistance to *L. monocytogenes* infection (60% survival), which almost phenocopied the resistance seen in *Ifnar1*$^{-/-}$ mice (80% survival) (152). Additionally, C57BL/6ByJ mice, which have a polymorphism in *Irf3* causing inefficient splicing of its mRNA, demonstrated reduced IFNβ induction and increased resistance to *Mtb* infection (186). These observations may suggest an important role for IFNβ in susceptibility to *L. monocytogenes* infection. However, these studies did not assess IFNα induction, and characterization of *Ifnb*$^{-/-}$ mice is needed to confirm this hypothesis. Mechanistically, loss of type I IFN attenuated *Listeria*-induced cell death in myeloid cells and lymphocytes *in vivo* and *ex vivo* (150, 152, 187, 188). Antigen-stimulated T cells were more sensitive to lysteriolysin O (LLO)-induced apoptosis after exposure to IFNα compared to cells only treated with LLO (150). Thus, a role for IFNα subtypes should not be discounted. Altogether, it is impossible to draw firm conclusions about the roles of individual type I IFNs in *L. monocytogenes* infection with the currently available information. Studies that specifically block IFNα or IFNβ in *Listeria* infection might yield important insight into the functions of type I IFN subtypes.

Streptococci Species

Streptococci species often colonize mucosal surfaces and skin of healthy individuals without causing disease, but they can cause a variety of serious diseases in immunocompromised individuals or newborns (189). Type I IFNs appear to play a protective role during infection with a variety of *Streptococci* species (146–149).

S. pneumoniae, an alpha-hemolytic species commonly known as pneumococcus, is an opportunistic pathogen that colonizes the mucosal surfaces of the upper respiratory tract and is a leading bacterial cause of otitis media, pneumonia, sepsis, and meningitis (190). Type I IFNs play a beneficial role during pneumococcal infection, though the route of infection matters (147, 148). Loss of IFNAR1 increased lung permeability by decreasing tight junction protein expression, which is consistent with increased bacterial titer in the blood if *S. pneumoniae* was inoculated *via* an i.n. route but not *via* an intraperitoneal (i.p.) route (148). IFNβ played a role in mediating these protective effects because pre-treatment of mice with recombinant IFNβ i.n. significantly increased survival following *S. pneumoniae* challenge and decreased blood bacterial titer. However, IFNα subtypes likely provide beneficial effects as well since a separate study showed that prophylactic or therapeutic administration (i.n.) of an adenoviral vector expressing IFNα enhanced survival after pneumococcal infection and decreased lung and spleen bacterial burden (149). It is unclear which IFNα subtype was used in this study, so more work is needed to determine if some IFNα subtypes are more potent than others.

A protective role of type I IFNs was also demonstrated in infection with the beta-hemolytic species *S. pyogenes* (group A streptococcus, GAS) and *S. agalactiae* (group B streptococcus, GBS) (146, 147). In GBS i.v. challenge, IFNβ transcript was more robustly induced in the spleen compared to IFNα4, and *Ifnb*$^{-/-}$ mice demonstrated increased lethality compared to WT mice (147). Additionally, *in vitro* GBS infection poorly activated peritoneal macrophages from *Ifnar1*$^{-/-}$ or *Ifnb*$^{-/-}$ mice compared to WT controls, suggesting that IFNβ may function to augment macrophage antibacterial properties. However, carefully controlled experiments need to be performed in order to determine if IFNβ is directly modulating macrophage activation or if IFNβ acts indirectly by influencing bacterial loads. The role of specific subtypes was not evaluated in GAS infection; however, macrophages and DCs were found to induce IFNβ downstream of unique pathways. Macrophages required IRF3, STING, TBK1, MyD88, and stimulation with streptococcal DNA, whereas DCs depended on MyD88, IRF5, and streptococcal RNA (146). It might be interesting to evaluate *Irf3*$^{-/-}$, *Irf5*$^{-/-}$, and *Ifnb*$^{-/-}$ mice in *S. pyogenes* infection to determine if the cellular source of IFN affects pathogenesis. Additionally, better characterization of the IFNα subtypes induced and their role in GAS and GBS is needed.

Remarks on Bacterial Infections

Similar to viral infections, type I IFNs can be either detrimental or beneficial to the host during bacterial infections, depending on the specific pathogen. The mechanisms underlying these divergent outcomes share many features with viral infections. The ability of type I IFNs to regulate cell death, suppress protective IFNγ responses, and/or upregulate IL-10 can account for the detrimental functions of type I IFNs during *Mtb*, *Salmonella*, and *L. monocytogenes* infection. These activities are reminiscent of the type I IFN-driven increases in IL-10 and PD-L1 observed in LCMV, as well as the increased cell death observed in acute influenza infection (132, 135). Even though a detrimental role for type I IFNs is well documented in *Mtb* infection, in special contexts type I IFNs may be able to serve a protective function. Of particular interest is the possibility of type I IFN serving as an adjuvant with certain, less virulent *Mycobacterium* vaccination strains. As is the case with some viral infections, the timing, magnitude, and cellular source of type I IFNs underlie these distinct outcomes. In the future it will be interesting to explore if these divergent phenomena are also due to differential induction or functions of type I IFN subtypes.

There are also examples of type I IFNs having a protective role in bacterial infections, such as with several *Streptococcus* species. This net beneficial effect may reflect many of the functions commonly observed in viral infections, such as coordinating protective immune cell recruitment and activation and promoting the right level of inflammation needed to clear the bacterial infection. The exact mechanisms underlying these protective effects are understood at a very general level and questions remain. Which cells do IFNs signal on to mediate these protective effects? What ISGs are responsible for mediating protection, and are they different from those acting in viral infections? Importantly, do specific type I IFN subtypes drive particular protective functions? We are only beginning to grasp how type I IFNs contribute to protective antibacterial immune responses, and there are many interesting avenues to explore relevant to human health.

Parasitic Infections

Parasites include single-cellular protozoa (e.g. *Plasmodium* and *Leishmania* species) and multicellular helminths, which include flatworms (e.g. *Schistosoma* species) and roundworms (e.g. *Ascaris* species) (191–194). Previously, parasite-host interaction studies have not investigated the functions of type I IFNs, but recent studies in malaria have identified both protective and pathogenic properties of IFNα/β [reviewed in references (195, 196)]. Below we explore the roles of IFNα and IFNβ during *Plasmodium* infection, the causative agent of malaria (**Table 3**).

Plasmodium Overview

Malaria initially presents as a wide variety of symptoms, including periodic fever, chills, headache, malaise, and muscle and joint aches, but as disease progresses severe anemia, blood acidosis, splenomegaly, acute respiratory distress syndrome, and spread to the brain are possible, which can be fatal (210). Infected mosquitoes transmit *Plasmodium* sporozoites to humans during a blood meal. The sporozoites initially infect hepatocytes, where they replicate as merozoites (liver stage), and eventually, merozoites enter the blood stream to infect red blood cells, where they begin asexual reproduction (blood stage) (191). Symptoms in humans usually begin developing several days after release of parasites into the blood. *P. falciparum* and

TABLE 3 | Summary of IFNα and IFNβ functions in mouse models of malaria infection.

Intervention	Clinical Outcome	Bacterial Load and Immune Characterization	Refs.
Liver-stage			
Ifnar1$^{-/-}$ *P. berghei* (ANKA) (early time points)	ND	P: ↑ parasitemia, ↑ liver titer I: ↓ ISG induction (liver)	(197)
Irf3$^{-/-}$ *P. berghei* (ANKA) (early time points)	ND	P: ↑ liver titer I: ↓ ISG induction (liver)	(197)
Irf7$^{-/-}$ *P. berghei* (ANKA) (early time points)	ND	P: ND I: ↓ ISG induction (liver)	(197)
Ifnar1$^{-/-}$ *P. yoelii* (Py17XNL)	ND	P: ↑ liver titer (bioluminescence) I: ↓ NKT cells (liver); no Δ NK, CD4, and CD8 T cells (liver)	(198)
Irf3$^{-/-}$ *P. yoelii* (Py17XNL)	ND	P: ↑ liver titer (bioluminescence)	(198)
Irf7$^{-/-}$ *P. yoelii* (Py17XNL)	ND	P: no Δ liver titer (bioluminescence)	(198)
Blood-stage			
Ifnar1$^{-/-}$ *P. chabaudi*	ND	P: ↓ parasitemia I: ↑ serum IFNγ	(199)
Irf7$^{-/-}$ *P. chabaudi*	ND	P: ↓ parasitemia I: ↑ serum IFNγ, ↑ IFNγ^{+} CD4 T freq. (spleen)	(199)
Ifnar1$^{-/-}$ *P. yoelii* (Py17XNL)	ND	P: ↓ parasitemia (late) I: ↑ serum Ab titer, ↑ Tfh cells and GC B cells (spleen)	(200)
rIFNα (18 hpi, i.v.), lethal *P. yoelii* (YM)	↓ lethality (0%)	P: ↓ parasitemia	(201)
rIFNα1/α8 (i.p.), *P. yoelii* (265 BY)	ND	P: ↓ parasitemia I: no Δ RBC count, ↓ reticulocytosis	(202)
rIFNα1/α8 (i.p.), *P. yoelii* (Py17XNL)	ND	P: ↓ parasitemia (early); trend ↑ parasitemia (late)	(202)
Cerebral-stage			
Ifnar1$^{-/-}$ *P. berghei* (ANKA)	↓ lethality (0%); ↓ cerebral hemorrhage	P: ↓ parasitemia (variable); ↓ brain titer I: ↑ serum IFNγ, ↑ IFNγ^{+} CD4 T (brain, liver, spleen); ↓ CD8 T infiltrate (brain), ↓ BBB leakage	(199, 203–207)
Irf3$^{-/-}$ *Irf7*$^{-/-}$ *P. berghei* (ANKA)	↓ lethality (0%)	P: ND	(203)
Irf7$^{-/-}$ *P. berghei* (ANKA)	↓ lethality	P: ↓ parasitemia, no Δ brain titer I: ↓ CD8 T infiltrate (brain)	(199)
rIFNβ (i.p.), *P. berghei* (ANKA)	↓ lethality	P: ND I: ↓ BBB leakage, ↓ CXCL9 (brain), ↑ CXCL10 (brain), ↓ T cell infiltrate (brain)	(208)
rIFNα1/α8 (i.p.), *P. berghei* (ANKA)	↓ lethality	P: ↓ parasitemia, ↓ brain titer I: ↓ Mφ, neutrophil, CD4 T, and CD8 T infiltrate (brain)	(209)

The mouse genetic background is C57BL/6 unless otherwise specified.

↑, increased; ↓, decreased; Δ, change; Ab, antibody; BBB, blood brain barrier; freq., frequency; GC, germinal center; hpi, hours post infection; I, immune; i.p., intraperitoneal; i.v., intravenous; LN, lymph node; Mφ, macrophage; ND, no data; P, parasite; RBC, red blood cell; Tfh, T follicular helper.

P. vivax are the most common species responsible for malaria disease in humans, and several *Plasmodium* species (*P. berghei, P. yoelii, P. chabaudi*, and *P. vinckei*) infect rodents and recapitulate various stages of human disease (210).

Liver-Stage Malaria

Two important studies recently revealed a protective role for type I IFNs in controlling liver-stage *Plasmodium* infection. First, Liehl and colleagues showed that all of the early upregulated genes in the liver from mice infected with *P. berghei* (ANKA) were classified as IFN-stimulated genes or linked to the type I IFN signaling pathway (197). Similarly, Miller *et al.* also uncovered an early type I IFN signature in the liver of mice infected with *P. yoelii* (Py17XNL) (198). Upon global IFNAR1 deficiency or conditional deletion of IFNAR1 on hepatocytes (Albumin-Cre), mice failed to control parasite replication in the liver (197, 198). These studies suggest that type I IFN signaling protects against malaria infection by controlling early parasite replication in the liver. Further characterization revealed that *Irf3*$^{-/-}$ mice, but not *Irf7*$^{-/-}$ mice, showed a similar early increase in liver parasite burden as *Ifnar1*$^{-/-}$ mice following *P. yoelii* (Py17XNL) infection (198). This is consistent with the observation that *Irf3*$^{-/-}$ mice demonstrated a more severe decrease in early liver ISG induction compared to *Irf7*$^{-/-}$ mice following *P. berghei* (ANKA) infection (197). Given that IRF3 is a key regulator of IFNβ induction, these findings could suggest that endogenous IFNβ is more important than IFNα subtypes for controlling parasite burden in liver stage malaria. Additional studies are needed to confirm this hypothesis.

Blood-Stage Malaria

There is conflicting evidence for whether type I IFNs have a net beneficial or detrimental effect during the blood stage of malaria. Evidence for a protective role is as follows. First, treatment of mice with recombinant hybrid HuIFNα1/α8, which has activity on murine cells, concurrent with *P. yoelii* (265 BY) infection

decreased early parasitemia, and the authors proposed that this was due to IFNα-dependent inhibition of reticulocyte (immature red blood cell) development, as opposed to direct anti-plasmodium effects (202, 211). Moreover, deletion of inflammasome components or some intracellular PRR sensing components decreased parasitemia and increased resistance to lethal *P. yoelii* infection through alleviation of SOCS1-mediated suppression of type I IFN responses (201, 212).

Other studies have demonstrated that type I IFNs might play a detrimental role during blood-stage malaria. First, a group showed that *Ifnar1*$^{-/-}$ and *Irf7*$^{-/-}$ mice better controlled parasitemia in non-lethal *P. chabaudi* infection compared to WT controls (199). Additionally, Sebina and colleagues showed that IFNAR1 deletion in *P. yoelii* (Py17XNL) infection increased pathogen-specific antibody titers and decreased parasitemia late in infection (17–21 dpi) (200). Mechanistically, type I IFN signaled on DCs to limit their activation of T follicular helper cells in an inducible T cell co-stimulator (ICOS) signaling-dependent manner, and this interaction ultimately influenced downstream germinal center B cell responses (200). However, it should be noted that IFNAR1 deletion in the Sebina *et al.* study also trended toward increased parasitemia early in infection (6–11 dpi), suggesting that these findings are not completely incongruous with the studies that found a protective role for type I IFNs. Altogether, type I IFNs might be detrimental in the blood stage malaria by impeding humoral immunity later in infection, but the *Plasmodium* strain and timing of IFN action may influence the overall effect of type I IFNs on disease outcome. It would be interesting to determine if this effect is dependent on certain type I IFN subtypes.

Cerebral Malaria

Similar to the blood stage, the role of type I IFNs during cerebral malaria remains controversial. Several independent groups have demonstrated that *Ifnar1*$^{-/-}$ mice are either completely or partially protected from lethal experimental cerebral malaria (*P. berghei* ANKA sporozoite infection), demonstrating a net pathogenic effect for type I IFNs in this context (199, 203–207). Loss of type I IFN signaling may increase IFNγ-producing CD4$^+$ T cells, reduce pathogenic CD8$^+$ T cell recruitment and/or activation in the brain, improve DC priming of CD4$^+$ T cell responses, or some combination thereof (199, 204–207). *Irf7*$^{-/-}$ mice only partially recapitulated the decreased brain pathology and protection from *P. berghei* (ANKA) lethality observed in *Ifnar1*$^{-/-}$ mice, but loss of IRF7 perfectly phenocopied the decreased parasitemia observed in *Ifnar1*$^{-/-}$ mice (199). These findings may suggest IFNαs are more important in promoting parasitemia, whereas IFNβ and IFNα might both contribute to brain pathology, but specific antibody blockade of type I IFN subtypes would confirm this hypothesis.

Paradoxically, a few groups have shown that systemically administering either recombinant IFNβ or hybrid IFNα1/α8 concurrently with infection alleviated cerebral malaria (*P. berghei* ANKA) (208, 209). Both IFN treatments reduced parasite burden in the brain and decreased infiltrating CD8$^+$ T cells in the brain compared to control mice, but only IFNα1/α8 treatment decreased blood parasitemia (208, 209). A more recent study identified receptor transporter protein 4 (RTP4) as a positive regulator of type I IFN responses, and *Rtp4*$^{-/-}$ mice were completely protected from *P. berghei* (ANKA) lethality and brain pathology (213). This protection in *Rtp4*$^{-/-}$ mice correlated with increased type I IFN responses in microglia isolated from the brain, suggesting a protective role for IFNs, but blockade of type I IFN signaling in *Rtp4*$^{-/-}$ mice is needed to confirm a causal link (213). Overall, an issue of magnitude and timing of IFN response might underlie these apparent discrepancies with the protective phenotypes of *Ifnar1*$^{-/-}$ mice (discussed below). Indeed, antibody blockade of IFNAR1 as late as 5 dpi was almost as protective as *Ifnar1*$^{-/-}$ mice, suggesting that the detrimental effects of type I IFNs occurred during priming of adaptive immune responses (199).

Remarks on Parasitic Infections

It is clear that the role of type I IFNs in malaria is complex and depends on the stage of *Plasmodium* life cycle. Type I IFNs seem to play a protective role during the liver stage, but there are contradictory findings from various models of blood-stage and cerebral malaria. Perhaps infection with some strains of *Plasmodium* yields suboptimal type I IFN production very early in infection, ultimately leading to delayed and higher levels later in infection when parasite burden is not effectively controlled. Proper intervention at either step would benefit the host, and this could explain why loss of IFN signaling or exogenous IFN treatment can both be protective. The contribution of individual IFN subtypes remains unclear, though divergent phenotypes in *Irf3*$^{-/-}$ and *Irf7*$^{-/-}$ mice suggest this could be an interesting question to explore. Importantly, genetic variants in *IFNAR1* have been associated with either greater or lower risk of severe malaria disease (205, 214–217). The impact of each genetic variant on IFNAR1 expression and function still need to be determined, but these findings suggest that type I IFNs are important regulators of malaria disease in humans.

Overall, parasitic pathogens are biologically very diverse, so data from other parasitic infection models are needed to begin drawing broad conclusions. A recent study demonstrated that the TLR4-IRF1-IFNβ axis played a protective role in mice infected with *Leishmania infantum* by dampening proinflammatory pathways and IFNγ production by CD4$^+$ T cells (218). RNA sequencing analysis of human samples revealed that upregulation of TLR4 and type I IFN pathways was associated with asymptomatic individuals compared to patients with visceral leishmaniasis (218). Another group found that *Ifnar1*$^{-/-}$ mice were more susceptible to *Toxoplasma gondii* infection (219). It would be interesting to know if IFNs are generally more important in single-cellular parasitic infections. That said, the multicellular helminth *Schistosoma mansoni* can induce a systemic type I IFN signature in mice and activate TLR3 in DCs *in vitro*, suggesting that a role for type I IFNs in parasitic worm infections is certainly possible (220, 221). Continued work to delineate the cellular sources and functions of type I IFNs in malaria and other

parasitic diseases may reveal novel opportunities for therapeutic intervention and help uncover novel functions of type I IFNs.

CANCER

The majority of reports from animal models and the clinic demonstrate that type I IFNs play an important protective role in enhancing anti-tumor immune responses and restricting tumor growth [reviewed in (222, 223)]. However, similar to persistent viral infections, the functions of type I IFNs in cancer can change throughout disease course, and there is evidence that, in certain contexts, IFN might act as a barrier to efficacious checkpoint-blockade therapy [reviewed in (224)]. Below we discuss the actions of endogenous IFNα/β and IFN-based therapies in animal models and clinical studies (**Figure 2**).

Animal Studies: Endogenous Type I IFN Activity

An early study showed that mice transplanted with human tumors and treated with neutralizing antibodies to type I IFNs demonstrated exacerbated tumor growth and metastasis compared to controls, suggesting a protective role for endogenous type I IFN activity (225). Since this finding, we now know that endogenous type I IFN can mediate tumor rejection through signaling on immune cells or tumor cells.

A seminal paper showed that type I IFN signaling on host hematopoietic cells was crucial for development of anti-tumor immune responses (226). Using conditional IFNAR1 deletion, bone marrow chimeras, and adoptive transfer experiments, a number of studies have shown that type I IFN signaling on several types of immune cells is important for immunity in

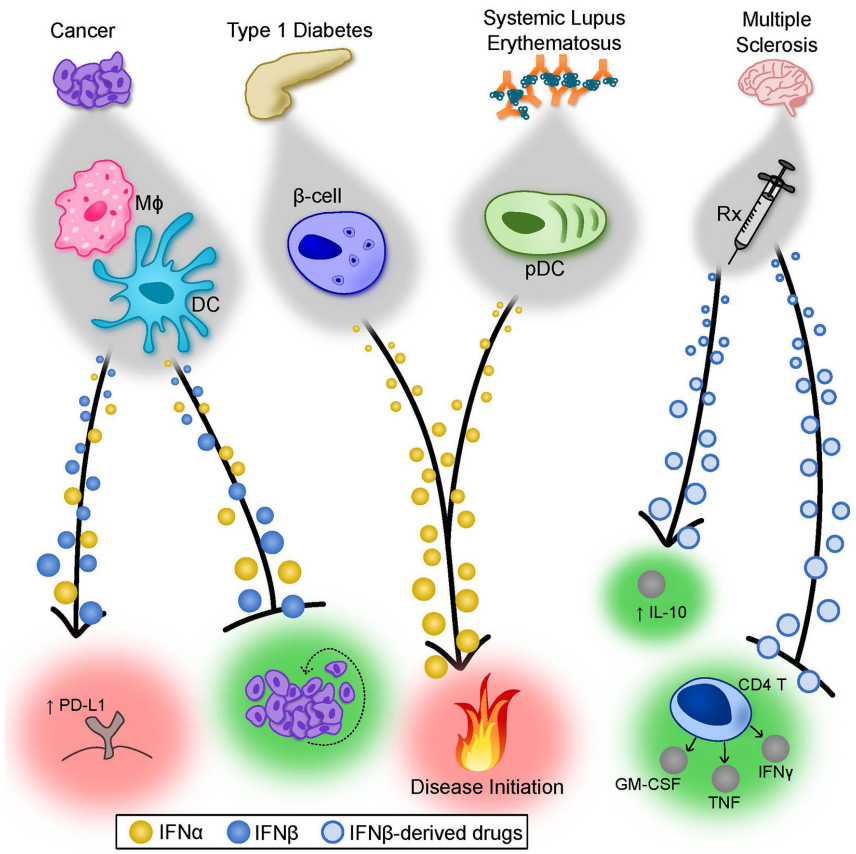

FIGURE 2 | Summary of the Properties of IFNα and IFNβ in cancer and autoimmunity. Type I IFNs display both unique and overlapping properties in various disease states. In cancer, depending on the tumor and degree of metastases, both IFNα and IFNβ can contribute to tumor rejection by directly limiting tumor cell proliferation (depicted) but also through modulation of antitumor immune responses (not depicted). In certain cases, type I IFNs can induce PD-L1 expression on tumor cells, suppressing immune-mediated killing of the tumor. The factors that cause type I IFNs to exert detrimental effects remain poorly understood. In T1D, there is evidence that IFNα subtypes play an important role in pathogenesis. Forced expression of IFNα by pancreatic β-cells accelerated the onset and severity of T1D in a mouse model, and patients receiving IFNα therapy for treatment of other diseases have a higher incidence of T1D. Similarly, immune complex-driven activation of pDCs induces robust IFNα production, which may participate in initiation of SLE. Finally, IFNβ-derived therapeutics have well-established efficacy for treating MS patients. Though still largely debated, the mechanism of protection mediated by IFNβ is complex and possibly includes limiting cytokine production from pathogenic $CD4^+$ T cells and augmenting IL-10 production in a number of cell types. β-cell, pancreatic β-cell; DC, dendritic cell; IL, interleukin; Mφ, macrophage; MS, multiple sclerosis; pDC, plasmacytoid dendritic cell; Rx, prescription drug; SLE, systemic lupus erythematosus; T1D, type I diabetes.

cancer. For instance, type I IFN signaling on DCs, but not granulocytes or macrophages, was required for rejection of highly immunogenic tumors (227). Additionally, *Itgax-Cre*$^{+}$ *Ifnar1*$^{fl/fl}$ (CD11c-Cre) mice showed diminished cross presentation by DCs to CD8^{+} T cells, which likely contributed to their failed tumor rejection (227, 228). In an NK cell sensitive tumor model, endogenous type I IFN was required for NK cell-mediated tumor rejection and homeostasis (229).

Other studies have shown that type I IFN signaling on tumor stromal cells may be important for controlling tumor burden. *In vivo*, both IFNα and IFNβ have antiangiogenic activity *via* signaling on vascular endothelial cells to downregulate growth factors such as vascular endothelial growth factor (230, 231). Stromal cells such as mesenchymal stem cells may play a role in controlling tumor growth by producing IFNα in order to enhance NK and CD8^{+} T cell responses (232). However, extended low level IFN signaling on tumor cells may render them resistant to apoptosis and immune-mediated killing (233, 234). These differences highlight the complexities of type I IFN actions and the need to delineate cell-type specific IFN signaling and consequent gene regulation.

Limited studies have directly compared the endogenous functions of individual IFNα/β subtypes in cancer models, but there have been a few studies conducted with IFNβ-deficient mice. *Ifnb*$^{-/-}$ mice showed expedited tumor growth, enhanced angiogenesis, and increased neutrophil infiltration to the tumor compared to WT mice (235–238). These findings demonstrate that endogenous IFNβ is important for the host anti-tumor response, but the specific signaling pathways downstream of IFNβ and cell types mediating these effects remain unclear. The direct contributions of endogenous IFNα remain uninvestigated, so much work is needed to fully characterize the contribution of endogenous IFN in tumor rejection.

Animal Studies: Type I IFN-Based Therapies

The possibility that IFNs might be therapeutically useful in cancer was first shown in the early 1970s, when crude preparations of were administered to mice with syngeneic tumors increased their survival compared to untreated mice (239, 240). IFN therapies have been quite effective against hematological cancers, including hairy cell leukemia and chronic myelogenous leukemia but vary in efficacy against solid tumors, such as melanoma [reviewed in (222, 223, 241, 242)]. Below we discuss various therapeutic strategies derived from either IFNα or IFNβ subtypes. Collectively, these studies show that IFNα and IFNβ are able to promote a similar range of immunomodulatory and antitumor effects, so studies that directly compare the activities of IFNαs and IFNβ are needed to discern if there are bona fide differential properties.

IFNα-Based Therapies

Derivatives of IFNα2b have long been used in the clinic, but toxicity issues are associated with systemic administration and persistent use. Consequently, many groups have sought ways to increase IFNα expression with more precision. An influential study developed RNA-lipoplexes encoding neoantigens or endogenous self-antigens, which yielded rapid and robust IFNα production by macrophages and DCs (IFNβ induction was not determined) (243). Importantly, these RNA-lipoplex vaccines were able to mediate rejection of several different types of aggressive tumors in mice (243). Another group developed a method to genetically modify human hematopoietic stem cells (HSCs) to express HuIFNα2b, but only in differentiated monocytes (244). The engineered HSCs were able to repopulate immunodeficient mice and effectively inhibit tumor progression in a murine breast cancer model (244). AcTakines (Activity-on-Target), which are optimized cytokines that only act on cells for which they are targeted, represent another interesting alternative to traditional IFN therapies. Indeed, CD20-targeted IFNα2b-derived AcTaferon reduced lymphoma and melanoma tumors engineered to express CD20 (245, 246). Increasing tumor cell production of IFNα is another approach, and a very recent study demonstrated that IFNα subtypes are not all equal in their antitumor properties. B16 melanoma cells were engineered to overexpress IFNα2, α4, α5, α6, or α9, but only IFNα2- and α9-expressing tumors were effectively controlled in an adaptive-immunity dependent manner (247). Other studies have used a variety of genetic engineering methods to augment IFNα production in the tumor microenvironment and improve antitumor immunity (248–251).

IFNβ-Based Therapies

Derivatives of IFNα2 have been the focus of most IFN-based therapies, but several studies have explored the effect of IFNβ during various models of cancer. IFNβ treatment of transformed human mammary epithelial cells *in vitro* led to a less aggressive state (252). Another group showed that treating mice with an anti-tumor antibody fused to IFNβ increased clearance of antibody-resistant tumor cells by increasing cross presentation by tumor-infiltrating DCs and activation of CD8^{+} T cells (253). Unfortunately, this treatment also upregulated the inhibitory molecule PD-L1 on tumor cells, but this negative effect was overcome with co-administration of anti-PD-L1 antibody (253). Another group transduced induced pluripotent stem cell (iPSC)-derived myeloid cells with an IFNβ-encoding lentivirus to treat disseminated gastric cancer (254). When injected into immunocompromised mice, the modified myeloid cells accumulated in the tumors and inhibited growth of the peritoneally disseminated cancer (254). Lastly, intratumoral injection of an mRNA encoding a fusion protein consisting of IFNβ and the ectodomain of transforming growth factor-β receptor II enhanced DC activation of CD8^{+} T cells *in vitro* and promoted rejection of the TC-1 tumor cell line *in vivo* (255).

Human Studies

The antitumor and immunomodulatory effects of IFNα therapy have been demonstrated in the treatment of a variety of cancers, and here we present a few representatives. IFNα-derived

therapies are the only approved adjuvant therapies in melanoma patients after surgical resection, and immunomodulatory actions, such as increased tumor-infiltrating cells and decreased circulating T-regulatory cells, are key mechanisms of action [reviewed in reference (242)]. After being replaced with tyrosine kinase inhibitors like imatinib, interest in IFNα-based therapy has recently reemerged for treatment of chronic myeloid leukemia (CML) [reviewed in reference (241)]. This is because there is evidence that IFNα therapy is able to target and sensitize the rare CML stem cell population to subsequent killing by chemotherapy, whereas imatinib is more effective against more differentiated CML progenitors (256, 257). Lastly, an analysis of matched primary breast cancer tumors and bone metastases revealed that primary tumor cells expressed *IRF7*, whereas metastases consistently demonstrated downregulation of *IRF7* expression (258). This may suggest that IRF7-mediated IFNα production in primary tumors is an important factor for limiting metastases, but further studies are needed to determine if this is an IFNα-specific effect or if there is also a role for IFNβ. Fewer clinical studies have been conducted with IFNβ-derived therapies, but there is evidence that IFNβ also plays a protective role in tumor rejection. Increased IFNβ mRNA expression significantly correlated with improved survival in patients with triple-negative breast cancer, though the mechanism is undetermined (252). *In vitro* studies have shown that IFNβ is more potent in inducing apoptosis in melanoma cells compared to IFNα (259). The relevance of this differential potency has yet to be extensively explored *in vivo*.

Detrimental Effects of Type I IFNs in Cancer

Despite all the evidence that type I IFNs can facilitate protective antitumor immune responses, IFNs can also impede cancer therapies. We provide just a few mechanistic examples. Persistent type II IFN signaling on tumors can result in PD-L1-dependent and PD-L1-independent resistance to immune checkpoint blockade, and the authors identified a role for type I IFNs in maintaining PD-L1-independent resistance (233). Radiation and chemotherapy stimulate immune-mediated destruction of tumor cells partly through induction of type I IFNs (260–264). However, recent work showed that conditional deletion of IFNAR1 on tumor cells enhanced responsiveness to radiation therapy through increased susceptibility to $CD8^+$ T cell-mediated killing (265). Lastly, oncolytic viruses can preferentially kill cancer cells, but tumor responsiveness to type I IFN activity confers resistance to this therapeutic method. One study showed that IFNα and IFNβ differ in their ability to confer resistance to oncolytic virus treatment *in vitro*. Exogenous IFNβ more effectively prevented oncolysis of human head and neck squamous cell carcinoma cells by vesicular stomatitis virus compared to IFNα, but differential effects were not observed for normal keratinocytes or endothelial cells (266).

Remarks on Cancer Studies

Collectively, this large body of cancer studies has shown that the roles of type I IFNs are complex and likely context specific. The extensive use of IFNα-derived therapies to treat a number of cancers in the clinic has greatly increased our understanding of the range of IFNα properties *in vivo*. Cancer models are uniquely advantageous for studying protective immunomodulatory effects of IFNs compared to infection models because pathogen load is not a confounding factor. Despite the large body of work suggesting the benefits of type I IFN signaling in cancer, the actions of specific IFN subtypes, for the most part, remain undefined. The beneficial effects of indirect activators of type I IFNs, such as the RNA-lipoplexes (discussed above) or STING agonists, may be due to their ability to induce multiple IFN subtypes with either overlapping or unique functions (222, 244). The heterogeneity of cancer makes it all the more important to appropriately stratify patients to ensure a beneficial effect of treatment.

AUTOIMMUNITY

Type I IFNs have emerged as critical mediators of autoimmunity. Patients with a variety of autoimmune diseases display serum type I IFN signatures, and IFN treatments for other diseases have correlated with the development of autoimmunity. These observations have led to the assumption that type I IFNs may contribute to autoimmunity pathogenesis. However, IFNβ-derived therapeutics have been used to treat multiple sclerosis, highlighting that caution is warranted in attempting to summarize the mechanisms of autoimmune disorders. Below we outline the current understanding of the roles of IFNα and IFNβ during systemic lupus erythematosus, type 1 diabetes, and multiple sclerosis (**Figure 2**). This is not an exhaustive analysis of autoimmune disorders, and active research is exploring the function of type I IFNs in other disorders, such as rheumatoid arthritis and Sjögren's syndrome (267, 268).

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects organs such as the skin, joints, kidneys, and CNS (269). A type I IFN gene signature in the blood of SLE patients is well established (270–272). Additionally, a number of genetic risk factors for SLE are associated with type I IFN production or signaling, including *IRF5, IRF7, IRAK1*, and *TYK2* [reviewed in reference (273)]. The majority of patients (70–80%) develop anti-nuclear autoantibodies (ANA), which form immune complexes with extracellular nucleic acids and induce production of type I IFN, especially IFNα, by pDCs (274). Type I IFNs promote disease by signaling on a variety of immune cells, including DCs, B cells, and T cells (275–277). It has been shown that IFNα or IFNβ treatment *in vitro* induced different transcriptional programs in DCs, with IFNα-primed DCs demonstrating increased phagocytic uptake of apoptotic cells and nucleic acids (278). Given the prevalence of IFNα in the serum of SLE patients and role of pathogenic responses to nucleic acids, the impact of IFNα versus IFNβ on DC activation in the context of SLE might be an interesting topic to interrogate.

A recent study from Klarquist *et al.* sought to dissect the effect of type I IFN signaling on $CD4^+$ T cells and B cells on the

development of T follicular helper cells, germinal center B cells, and plasmablasts. They found that IFN signaling decreased the threshold for B cell receptor signaling, increased MHC-II expression, and promoted germinal center B cell function, thus lowering the threshold for autoreactive B cell activation (276). They also found that type I IFN protected T follicular helper cells from NK cell-mediated death, thus further promoting B cell responses (276). Other studies suggest that IFNα may further drive SLE by increasing production of multiple TNF family members, such as BAFF and APRIL, which promote B cell survival and can drive SLE pathogenesis (279–281). Due to the apparent pathogenic role of IFNα during SLE, attempts have been made to neutralize type I IFNs in SLE patients (282–287). Both anti-IFNα and, more recently, anti-IFNAR1 therapies have been tested (282–287). Both treatment strategies showed disparate efficacy in patients, so further work is needed to clarify if this type of therapeutic intervention would be beneficial for patients. It might be that IFNα only plays a key role in the initiation and early stages of disease, so the disease stage may be important in stratifying patients [reviewed in reference (288)].

Type 1 Diabetes

Type 1 diabetes (T1D) is a chronic, autoimmune disease caused by the immune-mediated destruction of pancreatic β-cells that leads to insulin deficiency and hyperglycemia (289). A blood type I IFN signature in T1D patients precedes the development of autoantibodies and disease (290–293). One study detected a significant increase in expression of IFNα subtypes, but not IFNβ, in postmortem pancreas specimens from T1D patients compared to control subjects (290). Moreover, many genetic polymorphisms associated with T1D are involved in the type I IFN response such as *MDA5* and *TYK2 (*294–296). Altogether, these findings suggest a detrimental role for type I IFNs in T1D. A role for type I IFNs in the development of T1D is supported in animal models. An early study showed that forced constitutive IFNα expression by pancreatic β-cells in mice resulted in hypoinsulinemic diabetes and pancreatic inflammation (297). Additionally, non-obese diabetic (NOD) mice, a common model for T1D, showed elevated IFN-inducible transcripts in the pancreatic islets prior to disease onset, and treatment of young NOD mice with anti-IFNAR1 mAb delayed the onset and decreased the occurrence of T1D (298, 299). Collectively, these findings suggest that type I IFN signaling, especially in the pancreas, may play a key role in initiating T1D.

LCMV can be employed as a viral model of T1D, in which mice transgenically express LCMV glycoprotein (GP) under the control of the rat insulin promoter (*Rip*-LCMV) (300). Development of *Rip*-LCMV T1D is dependent on type I IFN (301, 302). Recent work showed that anti-IFNAR1 mAb treatment reduced blood glucose to normal levels and prevented destruction of pancreatic islets (302). Importantly, they also showed that pan-IFNα (α1, α4, α5, α11, and α13) mAb blockade, but not IFNβ blockade, was able to recapitulate the anti-IFNAR1 phenotype, demonstrating a distinct role for IFNα subtypes in promoting pathogenesis in the *Rip*-LCMV T1D model. A similar detrimental role for IFNα is suggested in human disease. IFNα therapy for HCV in individuals genetically predisposed to T1D induced or exacerbated the development of T1D (303). Moreover, a recent study showed that a subset of *AIRE*-deficient patients who developed autoantibodies specific for IFNα, especially IFN-α1/13, IFN-α5, and IFN-α14, were less likely to develop T1D, whereas patients who failed to generate these antibodies developed T1D (304). Altogether, animal and human studies suggest a detrimental role of type I IFNs in T1D, and IFNα subtypes appear to play a dominant role in disease development and pathogenesis.

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, autoimmune disease of the CNS in which immune cells target and destroy the myelin sheath surrounding neurons, leading to neurodegeneration (305). Similar to other autoimmune conditions, MS patients can show a serum type I IFN signature, but this signature is relatively low when compared to SLE patients (306, 307). However, in strong contrast to SLE and T1D, type I IFNs, do not appear to play a detrimental role. In fact, IFNβ was the first FDA-approved therapy for MS (308–311). However, due to its flu-like side effects and the availability of more effective treatments, it is no longer the preferred therapy for MS patients (312). Even though IFNβ treatment is currently less preferred in clinical use, animal models and clinical studies (discussed below) have revealed important insight into the properties of IFNβ *in vivo*.

In Vitro and Animal Studies

Experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, has provided mechanistic insight into the protective actions of IFNβ (313). Mice lacking IFNβ, IFNAR1, or IRF7 showed exacerbated clinical EAE compared to WT mice, perhaps due to greater T cell infiltration and increased proinflammatory cytokine production in the CNS (314–316). Unexpectedly, mice that lack IRF3 showed significantly lessened clinical disease compared to WT mice, and this seemed to be due to a cell-intrinsic defect in the development of T helper type 17 (T_H17) cells (317). Indeed, T_H17 versus T_H1 skewing can drastically influence the impact of IFNβ treatment in EAE. IFNβ treatment was effective in reducing EAE severity in T_H1-induced EAE but worsened disease in T_H17-induced EAE (318). Thus, depending on the skewing of the T helper responses and method of induction of EAE, IFNβ may be protective or pathogenic.

Many cell types respond to IFNβ therapy in EAE. Deletion of *Ifnar1* on myeloid cells including macrophages, monocytes, granulocytes, and microglia, but not neuroectodermal cells, resulted in increased severity of EAE symptoms, suggesting that IFNβ mediates its protective effects, in part, by acting on these cells (315). Mice treated with TLR3 or TLR7 agonists display reduced disease severity associated with increased type I IFN production by pDCs and other antigen presenting cells

(319, 320). Other reports have also suggested that IFNβ signaling on T cells curbs their pathogenicity (321, 322). Furthermore, type I IFN signaling on conventional DCs limited their migration to the CNS and prevented their activation of T_H17 cells during EAE (323, 324). The tissue resident antigen presenting cells in the CNS, microglia, may also play a role in the type I IFN response during EAE. Type I IFN signaling on microglia promoted clearance of myelin debris by increasing their phagocytic activity (325, 326). Finally, a study identified a role for type I IFN signaling on astrocytes to suppress CNS inflammation during EAE (327).

Clearly IFNβ is able to induce protective effects during EAE, and a recent report demonstrated that sustained low-dose IFNα1 delivery *via* an adeno-associated viral system prevented the onset of disease in EAE (328). This therapeutic effect was associated with regulatory T cell expansion, and myelin-specific effector T cells displayed reduced proliferative capacity, decreased proinflammatory cytokine production, and increased expression of IL-10 and PD-1 (programmed cell death protein 1) (328). Another study showed that a systemic high dose of MuIFNα11 was able to initially delay EAE in mice but ultimately caused significant toxicity and mortality; however, when IFNα activity was targeted to DCs (Clec9A-targeted AcTaferon), they found efficient protection from EAE (329). These findings suggest that IFNβ might not be unique in its ability to confer protection in EAE, but more work is needed to determine what factors cause IFNα treatments to yield detrimental effects or protective effects.

Human Studies

IFNβ was the first FDA-approved therapy for MS (308–311). However, due to its flu-like side effects and the availability of more effective treatments, it is no longer the preferred therapy for MS patients (312). Observations from patients suggest that IFNβ therapy likely acts through multiple mechanisms, such as influencing immune cell recruitment and activation. First, IFNβ treatment correlated with decreased new brain lesions and increased soluble VCAM-1 in patient serum, suggesting that modulating immune cell entry to the CNS is one potential mechanism of IFNβ therapy (330). In addition to impacting cell recruitment, IFNβ treatment may also regulate survival of immune cells since an increase in proapoptotic genes was observed in peripheral immune cells isolated from IFNβ-treated patients (331, 332).

Pathogenic T_H1 and/or T_H17 cells likely play an important role in MS, and IFNβ therapy may limit the proliferation of pathogenic T cells and modulate their cytokine production (332, 333). IFNβ therapy is likely more effective in individuals with a T_H1 driven disease, since high serum IL-17F levels correlated with a poor response to IFNβ therapy (318). A number of cell types are likely involved in protective IFNβ treatment. For example, IFNβ treatment of MS patients can induce IL-10 production by myeloid cells, but treatment can also suppress production of granulocyte-macrophage colony-stimulating factor (GM-CSF), IFNγ, and TNF by effector T cells (334–340). Additionally, in patients that responded to IFNβ therapy, treatment induced T regulatory cells that produced IL-10 and expressed PD-L1 (341, 342). Altogether, the protective mechanisms that underlie IFNβ therapeutic effects likely involve direct or indirect actions on effecter T cells. A better understanding of these mechanisms would likely reveal important information about the functional capacity of IFNβ *in vivo*.

Remarks on Autoimmune Studies

A large proportion of patients with SLE or T1D show a type I IFN signature in their blood, and many studies have shown that type I IFNs promote pathogenesis in these autoimmune disorders. There is strong evidence implicating the IFNα subtypes in initiation and progression of SLE and T1D, but at this time, a role for IFNβ cannot be entirely ruled out—direct functional comparisons of IFNα versus IFNβ would be needed to draw that conclusion. Altogether, the specific pathogenic functions of type I IFNs during autoimmune disorders are likely tissue specific. A recent study performed gene-expression profiling of structural cells from 12 different tissues and found that the responses of the cells to stimuli were tissue-specific, thus identifying the stroma as an important regulator of tissue-specific immune responses (343). While there is clear evidence that type I IFNs can modulate pathogenic autoimmune responses, it is important to know how systemic IFNα activity might promote cell-type specific effects in diseased versus nondiseased tissues in disorders like T1D that target a particular tissue, but also in diseases like SLE that have multi-organ effects.

In contrast, blood from MS patients do not display as robust a type I IFN signature as SLE or T1D patients, and many studies have demonstrated that IFNβ treatment has therapeutic properties in animal models of MS and in affected individuals. The protective functions of IFNβ are complex and likely include modulating immune cell recruitment and activation directly through action on immune cells and indirectly through action on brain resident cells. The functions of IFNαs in MS are less clear. There might be conditions, such as very low doses or when targeted to a specific cell type, in which IFNα subtypes are also protective. Careful comparison of IFNβ versus IFNα dose responses in EAE might uncover novel mechanisms for differential functions among type I IFNs *in vivo*.

CONCLUDING REMARKS

Whether type I IFNs have a net beneficial or detrimental effect on disease outcome depends on a variety of factors including the timing and magnitude of induction relative to disease onset, the duration of expression, the specific subtypes induced, the cell types responding, and likely other factors. Progress is needed in understanding the spatiotemporal induction of the various type I IFN subtypes *in vivo*, as well as the cell types responsible for type I IFN production. A lack of tools to differentiate between different subtypes has hindered progress in this area. Quantitative reverse transcription polymerase chain reaction has been a useful technique for quantifying specific IFN subtypes, and single-molecule array (Simoa) digital ELISA technology was demonstrated to detect IFN in blood with high sensitivity (344). However, there is a need for licensed antibodies against individual

subtypes that are able to neutralize in animal models and reliably stain tissue sections to more accurately determine the timing of expression at the tissue level.

Transcriptomic approaches have successfully differentiated type I and type III ISG signatures in organoid cultures (345). Because the effects of type I IFN are pleiotropic, there is a need to delineate the ISGs responsible for the protective and pathogenic functions of type I IFN subtypes in a given context and to understand how cell-type specificity might affect expression of those genes. A recent report profiled gene-expression networks of fibroblasts, endothelial, and epithelial cells isolated from multiple tissues and revealed tissue-specific signaling networks (343). A similar approach or spatial transcriptomics, which yields gene expression profiles in intact tissue sections, would be powerful tools to unravel the cell type-specific responses to different type I IFN subtypes *in vivo* (346).

Lastly, given that many type I IFN subtypes have expanded independently after mammalian speciation, there is a great need for tools to allow the study of human type I IFN subtypes in animal models. Immune-humanized mice and hybrid IFNAR (HyBNAR) mice, which transgenically encode variants of IFNAR1/2 that contain the human extracellular domains fused to the transmembrane and cytoplasmic segments of murine IFNAR, have both been used to study HuIFN in mice (347). These two systems are helpful in contexts where immune cells are the predominant sources of and responders to type I IFN or in studies administering exogenous HuIFN, but they do not permit loss-of-function studies, exclude the impact of endogenous IFN expression by stromal cells, and IFNAR1/2 transgenes are likely more highly expressed than endogenous IFNAR1/2. Overall, a concerted effort to address this lack of tools will go a long way toward increasing our ability to directly compare the expression and functions of distinct type I IFN subtypes, which will undoubtedly generate new strategies to augment or dampen the type I IFN pathway for biomedical purposes.

AUTHOR CONTRIBUTIONS

LEF and MCL conceptualized and drafted the manuscript, as well as created the figures and tables. LEF, MCL, and DJL all reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Importance of Type I and III Interferons at Respiratory and Intestinal Barrier Surfaces

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Interferons (IFNs) constitute the first line of defense against microbial infections particularly against viruses. They provide antiviral properties to cells by inducing the expression of hundreds of genes known as interferon-stimulated genes (ISGs). The two most important IFNs that can be produced by virtually all cells in the body during intrinsic innate immune response belong to two distinct families: the type I and type III IFNs. The type I IFN receptor is ubiquitously expressed whereas the type III IFN receptor's expression is limited to epithelial cells and a subset of immune cells. While originally considered to be redundant, type III IFNs have now been shown to play a unique role in protecting mucosal surfaces against pathogen challenges. The mucosal specific functions of type III IFN do not solely rely on the restricted epithelial expression of its receptor but also on the distinct means by which type III IFN mediates its anti-pathogen functions compared to the type I IFN. In this review we first provide a general overview on IFNs and present the similarities and differences in the signal transduction pathways leading to the expression of either type I or type III IFNs. By highlighting the current state-of-knowledge of the two archetypical mucosal surfaces (*e.g.* the respiratory and intestinal epitheliums), we present the differences in the signaling cascades used by type I and type III IFNs to uniquely induce the expression of ISGs. We then discuss in detail the role of each IFN in controlling pathogen infections in intestinal and respiratory epithelial cells. Finally, we provide our perspective on novel concepts in the field of IFN (stochasticity, response heterogeneity, cellular polarization/differentiation and tissue microenvironment) that we believe have implications in driving the differences between type I and III IFNs and could explain the preferences for type III IFNs at mucosal surfaces.

Keywords: interferons, type I interferon, type III interferon, interferon lambda, mucosal immunity, epithelial cells, respiratory epithelia, intestinal epithelium

INTERFERONS AND THEIR RECEPTORS

Type I Interferons

Interferons (IFNs) were first discovered to interfere with the replication of influenza virus sixty years ago by Isaacs and Lindenmann (1). Since their discovery, many studies in humans and animals have started to unravel the molecular details of how IFNs elicit an intrinsic antiviral program in cells to control viral replication and spread (2, 3). IFNs form a diverse family of cytokines composed of three types designated as type I, II, and III IFNs. In humans and mice, type I IFNs are the largest family consisting of multiple subtypes of IFN-α (13 in humans, 14 in mice), as well as IFN-β, IFN-ϵ, IFN-κ, IFN-ω (humans) and IFN-ζ (mice) (4, 5). Type I IFNs have a broad range of functions including anti-pathogen activities (antiviral, antibacterial and antifungal), anti-proliferative functions and the ability to modulate innate and adaptive immunity (6, 7). While type I IFNs are ubiquitously expressed, there is evidence of cell type specific expressions of some IFN-α subtypes (8, 9). Type I IFNs are sensed by cells through the binding of the heterodimeric receptor composed of the IFN-α receptor 1 (IFNAR1) and the IFN-α receptor 2 (IFNAR2), which are expressed on all nucleated cells (10). All 17 type I IFNs are capable of binding the receptor complex but they do so with different affinities (11).

Type II Interferons

The type II IFN family only has one member: IFN-γ. IFN-γ is produced predominantly by natural killer (NK) cells, natural killer T cells (NKT) and innate lymphoid cells (ILCs) and has been shown to be important for innate and adaptive immune responses (12). Additionally, it has been shown to play a key role in autoimmune and autoinflammatory diseases (13). IFN-γ binds to cells through the heterodimeric IFN- γ receptor 1 (IFNGR1) and the IFN-γ receptor 2 (IFNGR2) (14). Type II IFNs have been recently reviewed elsewhere (15) and this review will not focus on this cytokine.

Type III Interferons

In 2003, two groups simultaneously discovered three new cytokines in humans that were able to block viral infection: IL29, IL28A and IL28B also known as IFN-λ1, IFN-λ2 and IFN-λ3, respectively (16, 17). As these cytokines exhibited similar functions as type I IFNs but were structurally unique they were designated as a new class of IFNs, the type III IFNs. In 2013, a new type III IFN (IFN-λ4) was identified (18). While the function of IFN-λ1, λ2 and λ3 in protecting and resolving pathogen infection is broadly accepted, the precise function of IFN-λ4 remains disputed. This controversy arises from the fact that exogenously produced IFN-λ4 shows antiviral activity, however whether cells can produce IFN-λ4 on their own remains debated (19). It is known that genetic polymorphisms (SNPs) in IFN-λ4 have been associated with the protein expression of IFN-λ4 which then impacts hepatitis C viral load, spontaneous clearance of the virus, and response to treatment (20, 21). Importantly, recent studies have shown that several human populations have lost the expression of IFN-λ4 suggesting that it has been deleterious for humans during the evolution process (22). Mice only express IFN-λ2 and IFN-λ3 as both IFN-λ1 and IFN-λ4 are pseudogenes. This review will not focus on IFN-λ4 but a comprehensive description of its biological activities has been recently reviewed (19).

Similar to type I IFNs, type III IFNs are expressed by most cell types in the body, however they are sensed by a more limited number of cells leading to cell type specific responses (23–25). Type III IFNs bind to a heterodimeric receptor composed of the type III IFN receptor (IFNLR1, also known as IL-28Rα) and the interleukin 10 receptor 2 (IL-10R2). The IL10R2 receptor is not only used by type III IFNs but is also used by other IL-10 family members such as IL-10, IL-22 and IL-26 (16, 17). While IL-10R2 is widely expressed in most cell types, IFNLR1 has a limited expression and is found in epithelial cells (*e.g.* intestine, lung, vaginal, and hepatocytes) (23–26) and some immune cells (DCs, pDCs, NK cells and neutrophils) (27–31).

PRODUCTION OF IFNS

Production of Type I and III IFNs

Interferons are produced upon sensing of pathogen associated molecular patterns (PAMPs) by cellular pattern recognition receptors (PRRs). In a simplified view, as this is not the focus of this review, nucleic acids from the viral genome and intermediate products from virus replication are the main PAMPs for viruses. They are recognized by the Toll-like receptors (TLRs) and the RIG-like receptors (RLRs) (**Figure 1**). Activated PRRs recruit adapter proteins, such as Myeloid differentiation primary response 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), and mitochondrial antiviral-signaling protein (MAVS) (32). The adapter proteins activate a series of downstream proteins and transcriptional factors, like interferon regulatory 3/7 (IRF3/7) and NFκB. Activated IRF3/7 undergo dimerizations and translocate to the nucleus, where they bind to enhancer/promoters of IFN genes, subsequently inducing the production and secretion of both type I and III IFNs (**Figure 1**) (33). Further details on the molecular mechanism used by cells to sense PAMPs and produce IFNs can be found in recent reviews (34, 35).

Compartmentalization of PRRs for Production of Interferons

Pathogens can be sensed in different intracellular compartments depending on their mode of entry. In the case of viruses they can either infect the host by directly penetrating the plasma membrane or by being endocytosed and trafficking into the endosome compartment where they will be released into the cytosol (36). The site of entry will influence and dictate which PRR is most important for sensing viruses (TLR vs RLR) and as such will compartmentalize signal transduction leading to immune response and this could influence what kind of interferon is produced. This concept of compartmentalization of PRRs and downstream signaling has been pioneered through work on TLR4, which recognizes the bacterial component lipopolysaccharide (LPS). When TLR4 is located at the plasma membrane, stimulation of the receptor led to induction of pro-inflammatory cytokines through the MyD88 adaptor however, when TLR4 is

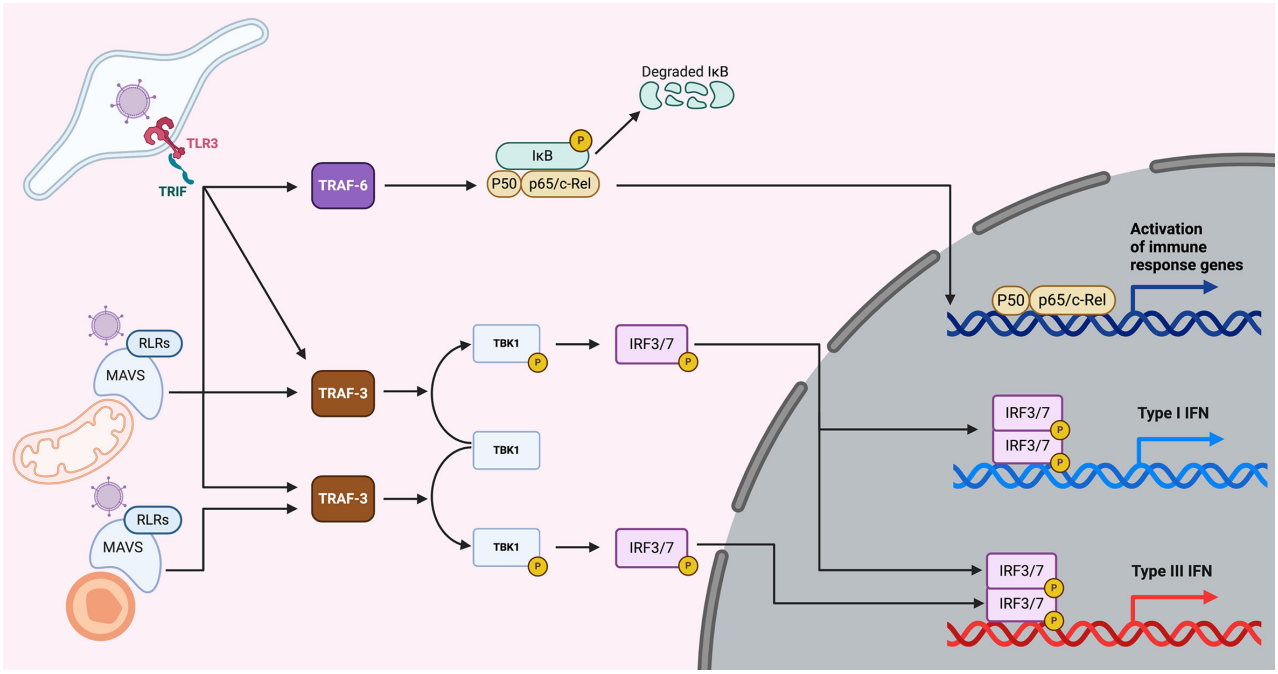


FIGURE 1 | Overview of IFN production upon viral infection. Upon virus entry into cells, viruses are sensed by the TLRs (endosomes) or the RLRs (cytoplasm). TLR3 senses dsRNA (a main component of viruses or viral replication) and is located in endosomes. Upon sensing of dsRNA molecules, TLR3 and its adapter TRIF lead to the induction of both the NFκB and the IRF3/7 pathways, which lead to the induction of both pro-inflammatory cytokines and type I and III IFNs. Viral PAMPs located in the cytosol are recognized by RLRs and upon activation recruit the adapter protein MAVS. When MAVS is recruited to mitochondria, NFκB and IRF3/7 are activated leading to the induction of pro-inflammatory cytokines and type I and III IFNs. However, when MAVS is recruited to peroxisomes the signaling cascade leads to the induction of type III IFNs only.

internalized into the endosomes it leads to the production of interferons by recruiting the adaptor TRIF (37). The RLR adaptor MAVS is located at both the peroxisomes and the mitochondria (**Figure 1**) (38). Studies have shown that the peroxisomal MAVS leads to the production of type III IFN only, while mitochondrial MAVS can produce both type I and III IFNs (**Figure 1**) (39), however recent studies have contradicted this view and suggested that both type I and III IFNs can be produced from peroxisomal MAVS (40). Similarly, TLR3 was shown to be localized on the basolateral side of polarized human intestinal epithelial cells (41). This polarized localization of TLR3 led to a higher induction of interferons when cells were infected basolaterally with TLR3 activating viruses or stimulated with TLR3 agonist as compared to the apical side (41). This compartmentalization of TLR3 is key for intestinal epithelial cells which are in constant contact with the commensal flora. Having PRRs polarized to the basolateral side allows intestinal cells to partially tolerate the presence of apical commensals while remaining highly responsive against enteric pathogens that have crossed the intestinal epithelial barrier. These pathogens are sensed by the basolateral PRRs and will lead to a potent type III IFN response. On the contrary, the apical microbes (commensals and pathogens) are poorly sensed because few PRRs are localized at the apical side of intestinal epithelial cells (41). Further studies are required to determine whether other PRRs and their adaptors can be compartmentalized leading to differences in the production of type I and III IFNs. Most importantly it is critical, while studying intrinsic immune response, to not only consider which PRR is involved in sensing a pathogen but to also consider from where, within a cell, it is signaling and to integrate this in a tissue-like environment to allow for proper intracellular distributions of PRRs.

Heterogeneity of Intrinsic Immune Response: Not All Infected Cells Produce IFNs

The textbook view of how PRRs sense pathogens and lead to the production of IFNs (**Figure 1**) would suggest that all infected cells in a population are equal: All cells will respond to pathogen infection and produce IFNs. However, recent studies have shown that each cell within a homogeneous cell population can respond differently. Work by O'Neal et *al.* showed that only a fraction of murine fibroblasts infected with West Nile virus produced IFN mRNA regardless of the viral load (42). Similarly, cell-to-cell variability was shown to regulate the ability of mouse fibroblasts infected with Sendai virus to produce IFN-β1 mRNA (43). Further studies have confirmed these observations and have shown that this heterogeneity is of cellular origin and not viral, and is due to intrinsic differences related to the activation and nuclear translocation of IFN regulatory transcription factors NFκB and IRF7 (44, 45). If IRF7 was not translocated into the nucleus then IFN-β1 was not made (44). These studies highlight that the ability of a virus to replicate and spread or be controlled

by host defenses can be directly linked to the proportion of cells in a population that produce and respond to IFN (45). Whether there is a similar heterogeneity in the production of type III IFN is unknown as no studies have directly addressed this question. It is legitimate to speculate that a similar heterogeneity would exist for type III IFN because of the high similarity in the signal transduction pathways which lead to IFN production. However, it will be interesting to address if the cells act in pairs and those that do not produce type I IFN also do not produce type III or if the production of the two IFNs will be regulated independently.

IFN-MEDIATED SIGNALING AND ISG PRODUCTION

The Importance of JAKs in Interferon Signaling

Following production and secretion of type I and III IFNs, these two cytokines will bind to their specific receptors in an autocrine (the secreting cells) and paracrine manner (the bystander cells) to activate complex signal transduction pathways which will induce transcriptional responses that will ultimately result in the development of an antiviral state in the stimulated cells (**Figure 2**). Both the type I and III IFNs induce the JAK/STAT signaling cascade leading to the induction of interferon stimulated genes (ISGs) (**Figure 2**) (26, 46–48). IFNs first bind one receptor chain with high affinity (IFNAR2 or IFNLR1), and then recruit the low-affinity chain (IFNAR1 or IL-10R2) to form a signaling-competent ternary complex (49–51). Upon binding, the extracellular part of the receptors induces the conformational change of the intracellular part of the receptor subunits, which causes receptor dimerization. Receptor dimerization activates receptor-associated Janus kinases (JAK), TYK2 and JAK1, which mediate the phosphorylation of tyrosine residues on the intracellular part of IFN receptors (52, 53). JAK1 is associated with IFNAR2 and IFNLR1 while TYK2 is associated with IFNAR1 and IL-10R2 (**Figure 2**) (54–58). JAK1 is critical for the activation and signaling of both type I and III IFNs. Importantly, JAK1 mutations have not been found in humans and are embryonic lethal in mice suggesting that they play a critical role in immune responses and development (59, 60).

Recent reports have uncovered that while TYK2 is required for type I IFN signaling, it is dispensable for type III IFN signaling (61, 62). Several mutations in the Tyk2 locus have been identified in

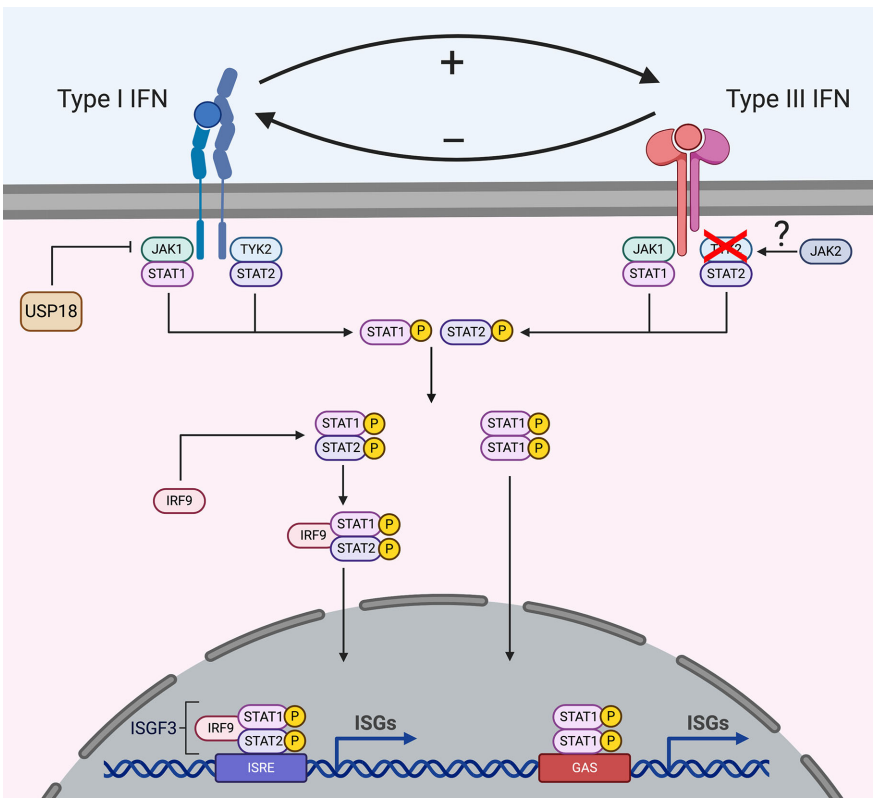


FIGURE 2 | Signal transduction downstream type I and type III IFN receptors. Upon binding to their receptors, IFNs induce the activation of the JAK/STAT signaling cascade. Both type I and III IFNs use JAK1 for their signaling, while type I IFNs also require TYK2 activation, type III IFNs signal independently of TYK2. Several studies suggest that type III IFNs use JAK2 for their signaling while type I interferons do not require JAK2. However, how JAK2 interacts with the receptor complex is currently unknown. Following JAK activation, STATs are recruited and activated which leads to their dimerization and binding to IRF9 forming the ISGF3 complex or homodimer complexes which translocates into the nucleus and drives ISG production. Some ISGs act as negative regulators and the ISG USP18 is known to regulate type I IFN signaling but not type III IFN signaling.

patients, however they do not show a high susceptibility to viral infections (61, 63). As TYK2 appears dispensable for type III IFN signaling, it is likely that in these patients the absence of increased susceptibility to pathogens is the result of type III IFNs providing first line protection, at least at mucosal surfaces. This model is supported by recent results where TYK2 knock-out murine intestinal epithelial cells treated with type III IFNs maintain their ability to produce ISGs and protect against virus replication (unpublished). Additionally, TYK2 knock-out mice pretreated with type III IFN prior to influenza infection were protected against viral infection in respiratory epithelial cells, while type I IFN pretreatment did not confer protection (unpublished). How signaling downstream the type III IFN receptor is transduced in the absence of TYK2 is unknown, however, it is tempting to speculate that another kinase takes over the function of TYK2. Interestingly, cells depleted of JAK2 or cells treated with specific JAK2 inhibitors are able to respond to type I IFNs and not type III IFNs suggesting that JAK2 could act in place of TYK2 for type III IFN signaling (**Figure 2**) (30, 39, 64).

STATs in Interferon Signaling

Following JAK activation and receptor phosphorylation, signal transducer and activator transcription (STAT) proteins are recruited to the complex. STATs are subsequently phosphorylated by JAKs and activated STATs form STAT1/2 heterodimers which bind to IRF9, forming the transcription factor interferon-stimulated gene factor 3 (ISGF3). ISGF3 transfers to the nucleus and binds interferon-stimulated response elements (ISREs), driving the transcription of interferon-stimulated genes (ISGs) (**Figure 2**). While STAT1/2 are the main proteins used in IFN signaling, other STATs are found to be activated and play cell type specific functions. Following type I IFN binding, STAT1–6 have all been shown to participate in the antiviral and anti-proliferative actions of these IFNs (65, 66). STAT1–3 are induced in all cell types, while STAT4–6 are cell type specific (67–69). However, which specific ISGs are produced upon STAT4–6 activation needs to be further investigated. Similar to type I IFNs, type III IFNs also induce STAT1–5 (**Figure 2**) (70, 71). However, to date, it remains unclear whether differences in the phosphorylation of the different STAT proteins are responsible for the differences in kinetics and magnitude of ISG expression observed between type I and III IFNs (See section "Interferon specific ISGs").

Negative Regulation of Interferon Signaling

Probably the most important step in mounting an antiviral response is the ability of cells to turn it off. Failure to arrest IFN signaling in tissues leads to inflammatory disorders in patients and interferonopathies (72). These disorders arise when cellular pathways fail to regulate IFN signaling and are often treated by blocking IFN signaling through the use of JAK inhibitors (73).

The suppressor of cytokine signaling (SOCS) (e.g. SOCS1 and SOCS3) are considered the most potent negative regulators used by cells to control type I IFN signaling as they can directly interact with TYK2 interfering with its activation (74). SOCS1 specifically acts by modulating the activity of IFNAR1 through downregulating TYK2 expression (75). Overexpression of SOCS1 in hepatic cells lines has been shown to also act on type III IFN leading to decreases in ISG production (76). Importantly, *in vivo* studies using SOCS1 knock-out mice showed increased ISG induction in the liver in response to type III IFNs while the lung and gut were only mildly affected (76). As lung and gut cells have been shown to be TYK2 independent, this suggests that either SOCS1 acts through another method to impact type III IFN signaling or that there are organ-specific differences in the regulation of IFNs.

JAK1 signaling can be regulated by the ISG ubiquitin-specific protease 18 (USP18). USP18 is induced upon both type I and type III IFN treatment, however it specifically regulates type I IFN signaling by binding to IFNAR2 (**Figure 2**) (77). Upon binding, USP18 acts as a negative regulator by preventing the interaction of JAK1 with IFNAR2 and thereby limiting type I IFN signaling. Interestingly, as type I IFNs bind to the receptor complex with different affinities, USP18 exerts its functions in a subtype dependent fashion with USP18 blocking IFN-α subtypes more than IFN-β1 (77–79). High USP18 levels are also suggested to be the reason that many hepatitis C infected patients show a refractory phenotype to IFN-α based antiviral therapy (80). Even though type III IFN signaling requires JAK1, it is not affected by USP18 as USP18 specifically targets and binds IFNAR2 and not IFNLR (81).

Regulation of Antiviral Functions

Beside activating the JAK/STAT signaling pathway, both type I and type III IFNs also induce the mitogen-activated protein kinases (MAPKs). Interestingly, in human intestinal epithelium cells, both type I and III IFNs activate MAPK signaling pathways however only type III IFNs require them for their antiviral functions. Intestinal cells treated with MAPK inhibitors blocked the ability of type III IFNs to control virus infection while type I IFNs' antiviral properties stayed intact (71). These observations suggest that to properly control viral infection, cells not only rely on ISGs made downstream JAK/STAT but that other parallel signaling pathways might be involved in determining the final outcome of infection by providing assistance to the main IFN-mediated antiviral signal. Whether this dependency on MAPK is intestinal cell specific and whether these differences participate in the regulation of ISG expression following type I and type III IFN stimulation of cells remains to be carefully addressed.

Interestingly, the signaling pathways downstream type I and III IFNs are interconnected and influence each other (82). Studies in human intestinal epithelial cells lacking either the IFNAR1 or IFNLR1 showed that the presence of a functional type III IFN receptor negatively regulates type I IFN signaling and antiviral activity, whereas the presence of type I IFN receptor positively reinforces type III IFN signaling and function (**Figure 2**) (82). These results suggest that studies which employ cells depleted of either IFN receptor, might show differences in responses to pathogens or signaling cascades that are not only due to the lack of the knocked-out receptor but also due to impaired signaling of the remaining receptor. Additionally, in tissues where one IFN receptor is naturally absent (*e.g.* murine intestinal cells which lack IFNAR, see section *Role of Type I and III IFNs in the Murine Intestine*), the properties of the remaining IFN receptor (*i.e.* IFNLR) could be weakened or enhanced.

Interferon-Specific ISGs

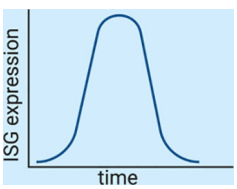
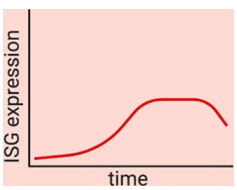
Over the past 17 years many studies have compared the differences between the ISGs induced upon type I and III IFNs stimulation in several mucosal tissues (*e.g.* intestine, lung and liver). These studies have revealed that while there is a core set of ISGs (*e.g.* IFIT1, MX1, USP18) induced in all tissues evaluated, there are others that may be tissue-specific (e.g. RSAD2 and GIP3 are highly induced in hepatocytes upon IFN treatment but are not induced in intestinal cells, where intestinal cells highly upregulate CXCL10 and BST2 which are absent in hepatocytes) (46, 47, 83, 84). However, defining which ISGs are specific to type I or type III IFN and which ones are tissue specific is very challenging. The reason for the difficulty in drawing a conclusive picture of type I vs. type III IFN signaling is that each study has used different amounts of IFNs to induce ISG production. Most importantly, evaluation of the IFN-mediated response was performed at different times post-IFN stimulation and this could severely impact which ISG is detected.

One of the predominant differences between the type I and the type III IFN-mediated immune response is that both cytokines induce ISG expression with very different kinetics. Human intestinal epithelial cells treated with either IFN-β1 or IFN-λ1-3 were shown to induce a similar set of ISGs but these ISGs were induced with different magnitudes and at different times post-IFN stimulation (46). Type I IFN showed a fast and strong induction of many ISGs compared to type III IFNs which showed a delayed induction of ISGs with a lower magnitude (46). This temporal induction of ISGs leads to a unique antiviral environment created by each IFN. These differences in the magnitude and temporal expression of ISGs appears to not be tissue-specific but intrinsic to both IFNs, as similar differences in ISG expression kinetics were also seen in respiratory epithelial cells and liver cells which also showed higher and faster induction of type I IFNs compared to type III IFNs (47, 83, 85–87). Importantly this delayed induction of ISGs by type III IFNs was not due to lower receptor levels, as overexpressing IFNLR1 did not lead to a faster induction of ISGs suggesting that type I and III IFNs uniquely regulate their signaling cascades (46).

IFN specific ISGs have been uncovered for type I and III IFNs in respiratory epithelial cells, liver cells, and intestinal epithelial cells (84–86). Studies in respiratory epithelial cells and liver cells revealed that IRF1 is induced both at the RNA and protein level only upon type I IFN treatment, however when cells were co-treated with type I and III IFNs the expression of IRF1 is prolonged suggesting that type III IFNs stabilize its expression (85, 86). The lack of induction of IRF1 and its proinflammatory downstream targets by type III IFNs has been suggested to explain why type III IFNs limit tissue damage following viral infection (85). Interestingly, mouse intestinal cells were found to produce type III IFN specific ISGs (*i.e.* Mmp7, Serpinb1a, and Csprs) (84). These IFN-λ2 specific genes were only found in the intestine and were not induced in the lung or bone marrow derived dendritic cells (BMDCs) following IFN treatment further supporting the model that tissues have unique sets of ISGs (84).

Our current understanding of type I and III IFN-mediated signaling suggest that while the main signal transduction pathways are very similar between both IFNs, there are unique differences between each cytokine (**Table 1**) that may provide IFN-specific control of pathogen infections. Although over the years, the signal transduction pathways downstream of the type I IFN receptor have been highly studied, many gaps are remaining in our understanding of the signaling pathways induced by type III IFNs. A systematic side-by-side comparison would be necessary to fully appreciate the differences in signaling pathways and the molecular mechanisms leading to antiviral function activated upon type I and type III IFN-mediated responses.

TABLE 1 | Similarities and differences of type I and III IFNs.

	Type I IFN	Type III IFN
IFN production	• Produced downstream TLR3, TLR4 (endosomes), RLRs, STING (32–35) • Produced by MAVS located on mitochondria (38, 39)	• Produced downstream TLR3, TLR4 (endosomes), RLRs, STING (32–35) • Produced by MAVS located on both mitochondria and peroxisomes (38, 39)
Receptor distribution	• Receptor expressed by all cells in the body (10)	• IFNLR receptor chain is only expressed in epithelial cells and in some immune cells (DCs, pDCs, NK cells and neutrophils) (23–31)
JAK/STAT signaling	• Requires JAK1 (JAK2 independent) (30, 39, 55, 57–59, 64, 72) • Signaling is TYK2 dependent (52–55)	• Requires JAK1 and JAK2 (30, 39, 72) • Signaling is TYK2 independent (61–63)
Other pathways	• Negatively regulated by IFNLR (82)	• Positively regulated by IFNAR (82) • Requires MAPKs for its antiviral activity (71)
Magnitude and kinetics of ISG induction	• High magnitude of ISG induction (46–48, 83, 85) • Fast induction and fast decrease in ISG expression (46–48, 83, 85)	• Low magnitude of ISG induction (46–48, 83, 85) • Slow but sustained induction of ISGs (46–48, 83, 85)
		
Negative regulators	• USP18 downregulates IFN-mediated signaling (77, 79, 81) • SOCS1 and 3 downregulate IFN-mediated signaling (74–76)	• SOCS1 downregulates signaling is some tissues (76)
IFN-specific ISGS	• IRF1 (85–87)	• Mmp7, Serpinb1a, and Csprs (84)

Heterogeneity in IFN Sensing: Not All Cells Respond to IFN

Upon interferon treatment it is accepted that signal transduction leads to the nuclear translocation of the ISGF3 complex and subsequent activation of ISGs in all treated cells (**Figure 2**). Nevertheless, recent studies have revealed that although a cell culture population is genetically homogeneous, cells within it could still respond differently to external stimuli, thus producing distinctive amounts of mRNA (88). It was originally thought that ISGs are produced in a binary manner, meaning that the presence of IFN switches them from an "OFF" to an "ON" state (or *vice versa*) (89). However, in recent years several groups have shown that seemingly homogeneous cell culture systems respond to type I IFN treatment in a heterogeneous manner. Mouse fibroblasts treated with IFN-β1 and analyzed in a single cell manner showed that ISG induction was asynchronous and that the magnitude of ISG induction varied between cells (44). Importantly, a subpopulation of IFN treated fibroblasts never responded regardless of IFN concentration indicating that part of the population became refractive to IFN stimulation (**Figure 3A**) (44). A similar subpopulation of non-responding cells was also found in both human liver cells and human airway epithelial cells stimulated with IFN-α (45, 90). In both of these human cell lines, the non-IFN responding cells were not defective in IFN sensing as sorting of the non-responding cells and re-stimulating them with IFN-α induced activation of ISGs with a similar proportion of cells responding and non-responding to IFNs (**Figure 3A**) (45, 90). Mathematical models have shown that a higher initial level of the transcription factor IRF9 determines the intensity and speed with which cells are able to respond to IFNs, and thus, differences in the levels of the ISGF3 complex members could play a key role in the responsiveness to IFNs (91). It is important to consider that differences in the basal levels of many proteins involved in signal transduction downstream the IFN receptors are likely to give rise to different outcomes upon IFN stimulation.

This heterogeneity in ISG induction is not exclusively found in type I IFNs, as recent studies have shown that 90% of a clonal population of mouse derived IECs responded to IFN-β1, whereas 55% of these cells responded to type III IFNs (92). This discrepancy between the number of cells that responded to type I and type III IFNs implies that different mechanisms regulate whether a cell responds or not to either IFN. This may provide a unique opportunity for cells that are normally responsive to both IFNs (e.g lung and gut epithelial cells) to favor one IFN over the other to promote an IFN-specific signaling/function. This lower cellular responsiveness against type III IFNs was also seen in human IECs, where even at very high concentrations type III IFN was never able to fully protect all cells from virus infection (85-90% inhibition) while type I IFN was (46). Histone deacetylases (HDACs) were described to play a role in regulating the sensitivity of epithelial cells to IFN-λ, as pretreatment of mouse intestinal cells with HDAC inhibitors significantly increased the number of IFN-λ responding cells. It is possible that the sensitivity of cells to IFNs is directly or indirectly regulated at the epigenetic level, and that a lack of synchronicity in these regulatory pathways causes delays or insensitivity to either or both type I and III IFNs.

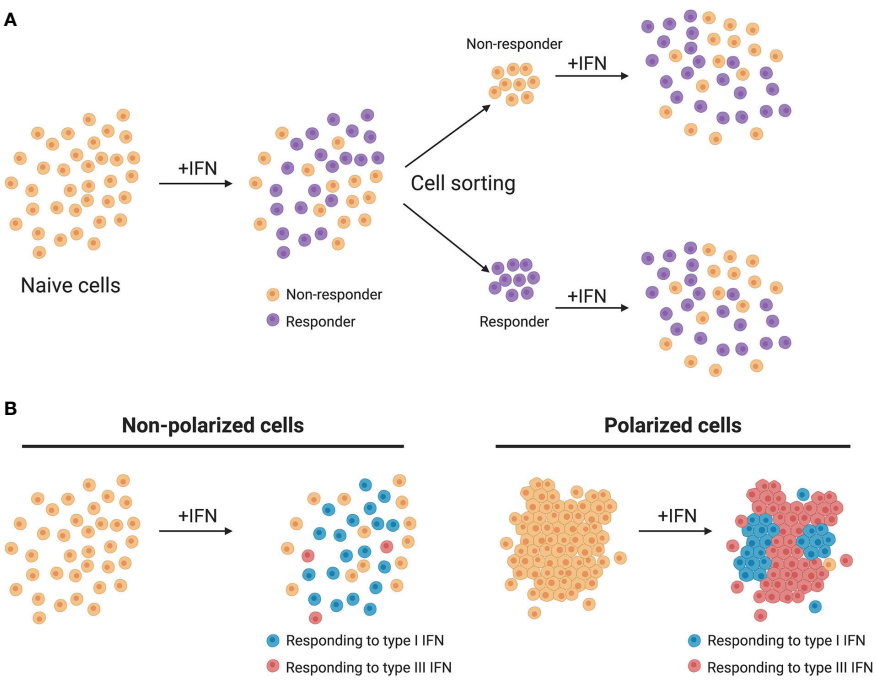


FIGURE 3 | Heterogeneity of IFN production and response. **(A)** Homogeneous cell cultures treated with IFN (+IFN) respond in a heterogeneous manner. Upon sorting and restimulation with IFN, non-responding cells display a similar distribution of responding cells as the naive population. **(B)** In murine models, non-polarized cells respond mainly to type I IFNs while polarized cells respond mainly to type III IFNs.

Furthermore, as type III IFNs act on epithelial surfaces it is important to consider their polarization state. Many experiments in laboratory settings use epithelial cells in sparse conditions whereas in the normal tissue environment they are tightly connected and polarized. The state of the cells is critical when evaluating responsiveness to IFNs as mouse intestinal cells have shown to be more sensitive to IFN-λ when reaching a polarized status (**Figure 3B**) (92) while human intestinal epithelial cells become less responsive to IFNs when polarized (71). Understanding the molecular mechanism of how within a population cell density, polarization status and epigenetic inheritance influence responsiveness to either IFN is a promising research axis that will help us to delineate the differences observed between different tissues and between different species.

IFN LAMBDA IN MUCOSAL IMMUNITY

The main difference that places type I and type III IFN apart lies in the fact that the type III IFN receptor expression is restricted to a subset of cells (23), providing these cells a unique way of protecting themselves against pathogen challenges. Research has focused initially on evaluating how type I and III IFNs control pathogen infections in the intestinal tract, the respiratory tract, the liver, the blood brain barrier and more recently the female reproductive tract. In this review we focus on the intestinal and respiratory epithelial cells because there is increasing evidence that type I and III IFNs are critical for both the intestinal and airway epithelium not only by mediating the antiviral response but also by impacting/regulating the epithelium themselves and by controlling and maintaining adaptive immune responses and the integrity of the epithelial barrier. More details on the role of IFNs in the female reproductive tract and the blood-brain-barrier can be found in a recent review (93).

Role of Type I and III IFNs in the Murine Intestine

The epithelial cells lining the intestinal tract play a unique role in regulating immune-homeostasis. These cells must be able to tolerate the huge commensal load present in the lumen of the gut and be responsive to invasive pathogens. In the intestinal tract, type III IFNs have been shown to play a key role in helping to maintain this balance and protecting the intestinal epithelial cells lining the gut from enteric pathogens while limiting excessive immune responses leading to tissue damage (24, 94–97). Upon enteric virus infection, murine IECs preferentially express type III IFNs over type I IFNs (95, 97). It has been shown that epithelial cells express higher levels of IFNLR1 and lower levels of IFNAR1 and IFNAR2 compared to the underlying lamina propria (95). This compartmentalization of the IFN receptors also favors IFN-λs as a first line defense against enteric pathogens (24). Using rotavirus as a model enteric virus, which predominantly infect epithelial cells, it was shown that mice lacking the type I IFN receptor were able to control rotavirus infection while mice lacking type III IFN receptor showed increases in virus replication, *de novo* virus production and damage to the intestinal epithelium (24, 97).

Type I IFNs are not dispensable for enteric infections. While they do not act to protect the epithelial surface, they play a key role in protecting against systemic spread of the viruses. Infection experiments using the enteric virus reovirus, which can also spread systemically following infection and replication in the GI tract, confirmed the critical role of type III IFN in protecting IECs against viral infection. Most importantly, it was shown that the type I IFN system was responsible for controlling the systemic dissemination of reovirus (95). Similar results were obtained using mouse norovirus (94).

The ultimate proof that type III IFN was the main player protecting IECs from enteric virus infection was provided by experiments performed in mice where the function of IFN-λ was only disrupted in IECs and by curing enteric infection using IFN-λs. Using mice with intestinal-specific conditional knock-out of the IFNLR1 it was shown that IFN-λ signaling in IECs is protective against enteric virus infections even in mice lacking an adaptive immune system (Rag-$1^{-/-}$) and that depletion of IFN-λ signaling from IECs resulted in an increase in norovirus, rotavirus and reovirus replication and fecal shedding (98). Complementarily, it was shown that administration of IFN-λs in mice could resolve persistent norovirus infection also in the absence of adaptive immunity (94).

Interestingly, recent evidence suggests that this spatial functional compartmentalization of IFNs at the intestinal epithelium, where the type III IFN is important for IECs protection while type I IFN is set to prevent systemic spread, is not genetically encoded but acquired in older animals. Adult mice only use the type III IFN receptor to control rotavirus infection in the gut (24, 95). On the contrary, neonatal mice appear to require both type I and III IFN receptors to efficiently protect IECs against rotavirus infection (99). Complementarily, while murine IECs do not respond to type I IFN *in vivo*, it was shown that they become responsive when isolated and stimulated with type I IFN cytokine *ex vivo* (24, 92). This observation is consistent with the fact that mouse intestinal organoids are also responsive to both IFNs (92).

The molecular origin for this reversion of IECs toward responsiveness to type I IFN is unknown. It is possible that following isolation from the intestinal epithelium, even when grown as organoids, IECs partially dedifferentiate and lose regulatory mechanisms that normally dampen the type I IFN mediated response. Another possible explanation is the presence of the commensal flora in the lumen of the gut, which is absent at birth but grows in number and complexity with time. The presence of these commensals might interfere with the type I IFN system in IECs. A relationship between IFNs and the microbiota was previously described, where commensals seem to negatively regulate the type III IFN-mediated clearance of persistent enteric virus infection (100). Additionally, IECs depleted of IFNAR1 result in a significant change of the microbiota composition likely as a result of changing the number of Paneth and Goblet cells in the epithelium (101). This is an interesting observation as it suggests that although type I IFN is not important to protect IECs against viral infection, the type I IFN pathways might still be active to regulate other functions and help promote homeostasis of the intestinal epithelium.

Role of Type I and III IFNs in the Human Intestine

Similar to murine cells, human intestinal epithelial cells respond to enteric virus infection by inducing a strong upregulation of type III IFNs transcripts while type I IFN transcripts are upregulated to a much lesser extent (41, 71, 102, 103). This leads to the preferential expression and secretion of type III IFNs, and thus to a protective effect of this cytokine on the surrounding epithelial cells expressing the type III IFN receptor (71). While the importance of type III IFN and dispensability of type I IFN in protecting IECs is well established in mice with the use of transgenic animals, it was only recently demonstrated that IFN-λs were keys to protect the human gut. It was shown that type III IFN controls SARS-CoV-2 infection of human intestinal epithelial cells. Human intestinal cells lacking the type I IFN receptor behaved similarly to wild type cells, whereas cells depleted of the type III IFN receptor showed increased virus infection, replication, and *de novo* virus production (96). All together this has led to the model in which the functions of type I and type III in the murine intestines are compartmentalized; type I IFNs protect the lamina propria and virus dissemination to the body and type III IFNs protect the epithelial surface itself (24, 95, 98).

Role of Type I and III IFNs in Respiratory Epithelial Cells

Similar to IECs, production of IFN-λs upon viral infection is a characteristic response of lung epithelial cells (95, 104). Overall, lung epithelial cells appear to favor the production of IFN-λs compared to type I IFN upon influenza A virus (IAV) infection (29, 104). Many studies using mice lacking either the type I or type III IFN receptors could show that either IFN was able to control infections by influenza viruses, respiratory syncytial virus or human metapneumovirus, suggesting that type I and III IFNs played a redundant function in the lung (25, 104). While the epithelial cells are the most responsive cells to type III IFNs due to the specificity of IFNLR1 expression, major differences in their ability to control virus infections are observed when comparing the upper and lower respiratory tract. In infection models where high doses of IAVs are used to infect the lower respiratory tract, both type I and type III IFNs are important to combat infection (25, 29, 104). On the contrary, if lower doses of IAVs are used and/or administered in a more physiological manner *via* nasal infection, the critical role of type III IFN for controlling IAV becomes much more apparent (29, 105). A recent study using mice lacking the type I or III IFN receptors in either neutrophils or epithelial cells specifically showed that each IFN had a unique effect in controlling influenza infection (29). This study revealed that upon influenza infection of respiratory epithelial cells, type III IFNs were produced first, and if the influenza virus load stayed low they were the most important IFNs used to clear the infection. Upon a greater viral load, type I IFNs were required to control the infection (29).

Role of Type I and III IFNs in Immune Cells at Mucosal Surfaces

An important growing concept in the field of type III IFNs, is that this cytokine is not only critical to control, clear and prevent pathogen infection at the level of the epithelium but it is also playing a role in providing long term immunity by stimulating adaptive immunity. Type III IFNs protect against long term infection and are required to reduce spreading of influenza virus to littermates (105) and are important for enhancing mucosal adaptive immunity by promoting antigen-dependent germinal center reactions in draining lymph nodes (106, 107). Additionally, a recent study has shown that mice lacking IFNLR1 showed impaired $CD8^+$ T cell and antibody responses following infection by a live-attenuated influenza virus (106). Influenza infection induced the release of IFN-λ, which triggered M cells to produce thymic stromal lymphopoietin (TSLP) in the upper airways. The release of TSLP then stimulated migratory dendritic cells and boosted antigen-dependent germinal center reactions in draining lymph nodes (106). The IFN-λ-TSLP axis also promoted production of the immunoglobulins IgG1 and IgA only when applied intranasally, suggesting that it required mucosal surfaces for its action (106).

IFN-λ acts on neutrophils to not only control virus infections but also fungal infections of the respiratory tract, as was recently highlighted in studies evaluating *Aspergillus fumigatus* infection in mice. Mice lacking the IFNLR1 were unable to activate a neutrophil response and showed higher fungal loads, a more aggravated disease in the lungs and severe fungal invasion (108). While these studies have clearly shown that murine neutrophils respond to IFN-λ and use it to help in pathogen clearance, the ability of human neutrophils to be activated in response to type III IFNs remains controversial (30, 31, 109). Whether tissue specific immune cells also play a role in regulating type I and III IFN responses in the intestinal epithelium have not been addressed.

Role of Type I and III IFNs in Maintaining Barrier Functions

Both type I and III IFNs have been shown to play a role in tightening barriers at mucosal interfaces. Following respiratory infection by *S. pneumoniae*, mice upregulated the IFN-β1 transcript, which was critical to control bacterial invasion (110). Mice lacking IFNAR1 or mice treated with a IFNAR neutralizing antibody showed an increase in bacteremia. Further studies showed that IFN-β1 induced the production of tight junction proteins and prevented transmigration of bacteria across the epithelial membrane (110). Similarly, type III IFNs were shown to protect human intestinal epithelial cells from *Salmonella enterica* serovar *Typhimurium* infection (111). Intestinal cells treated with both type I and III IFNs increased their barrier function and prevented the passage of dextran molecules through the epithelial membrane, however type III IFN was more efficient at blocking transmigration of *Salmonella* and the epithelial damage caused by *Salmonella* infection (111). Type I and III IFNs have also been shown to play a key role in tightening the blood-brain-barrier (BBB) which has been recently reviewed (93).

CONCLUSION AND FUTURE PERSPECTIVES

The type III IFN system is no longer considered just a redundant system to type I IFNs but it is now fully recognized as providing a

novel arsenal to the host to protect specific cells and tissues against pathogen challenges. The observation that type III IFNs can specifically provide efficient protection against pathogens in the gut and the lung (**Table 2**) (24, 95, 104), which could even be sterilizing in the absence of adaptive immunity, (94) has placed type III IFNs with a unique therapeutic potential. Within the current SARS-CoV-2 pandemic, it was quickly discussed that type III IFN could help to curtail viral replication while limiting the tissue damage that could be induced by type I IFN.

Seven type I IFNs (recombinant and pegylated IFN-α2a, IFN-α2b, IFN-β1a and IFN-β1b) have passed clinical trials and have been approved for use in treating virus infections (HBV, HCV), multiple sclerosis, leukemia, melanoma, and multiple myeloma (112). Currently three clinical trials are ongoing for type III IFNs, and while they are promising and have reported reduced side effects, they have still not been clinically approved for use in patients. IFN-α was key to early HCV treatment and its activity was improved by combining it with ribavirin and through pegylation (113). However, problems with non-responsive patients, drug toxicity and liver cells becoming refractive to IFN-α treatment has led it to become a second choice for HCV treatments (112). IFN-α has also been used to treat HBV where it has been shown to decrease viral loads in the blood and improve liver enzymes (114). While the pegylated form has also shown higher activity against HBV, patients experience similar side effects and loss of function as HCV patients. Recently, IFN-λ has been used in clinical trials against HCV, HBV and HDV. It has shown similar effects as IFN-α treatment in reducing viral loads, but patients describe less side effects. Longer term studies will be required to determine if patients also become refractory to IFN-λ treatment, however *in vitro* experiments suggest that IFN-λ does not lead to a loss of function even after cell cultures have had prolonged treatments which could be explained by the lack of negative regulators affecting type III IFN signaling (*e.g.* USP18) (79). Currently pegylated IFN-λ is in phase II clinical trials to assess its action against SARS-CoV-2. Many researchers are optimistic about its potential to act against SARS-CoV-2 as both IFNs have been shown to reduce replication however IFN-λ clears infection with less tissue damage that type I IFNs. However, we need to be careful as IFN-λ is not without risk as recent studies have highlighted that treatment with IFN-λ could prevent lung epithelial cell regeneration and favors bacterial superinfection (115, 116).

With age, mice do not rely on the type I IFN but rather the type III IFN to protect their intestinal epithelium against enteric pathogens (99). Additionally, when primary epithelial cells are isolated from the intestinal tract and cultured *in vitro* they regain responsiveness to type I IFNs (24). These observations suggest that the gut microenvironment (tissue specific immune cells, microbiota, hypoxia and peristalsis) is participating in regulating the IFN response in a precise manner. It was shown that during enteric virus infection of mice, innate lymphoid cells in the gut secrete IL-22 which can act on IECs to synergize the antiviral activity of type III IFN (97). It was shown that IL-22 enhances the expression of IFN-λ induced ISGs and this likely participates in amplifying the antiviral response (97). However, it is known that IL-22 is mostly a key cytokine for regulating cell proliferation and barrier function in the intestine (117). With the importance of type III IFN in regulating barrier function, it is now important to address whether the benefit of IL-22 is exerted *via* ISGs or due to improved tissue repair. Similarly, if IL-22 is also acting with IFN-λ in humans remains to be determined.

Hypoxia is a critical parameter of the gut which is often overlooked in infectious disease research. Hypoxia is not only required for the microbiota but it also influences the epithelial cells themselves (118). It was shown that hypoxia favors barrier function in human intestinal epithelial cells (119). As it is well established that hypoxia in the tumor microenvironment affects the response of immune cells (118), it is critical to start investigating whether hypoxia could impact immune response of intestinal epithelial cells upon infection.

An additional epithelium specific parameter that has been neglected up to now is the fact that epithelium surfaces are

TABLE 2 | Role of type I and III IFNs in respiratory and intestinal epithelial cells.

	Lung	Gut
Antiviral functions in epithelial cells	• Type III IFNs control viral infection in the upper respiratory tract (25, 109) • Both type I and III IFNs control viral infection in the lower respiratory tract (104) • Type III IFN acts first and type I IFN acts when infections persist (29)	• Type III IFNs act on epithelial cells to control infection (23–25, 71, 95, 96, 98, 99) • Type I IFNs act on lamina propria to prevent systemic spread (23, 24, 95, 99) • Type III IFNs can control virus infection in epithelial cells in the absence of adaptative immune response (94)
Organ-specific ISGs	• Unknown if lung epithelial cells produce ISGs that are not induced in intestinal epithelial cells	• Mmp7, Serpinb1a, and Csprs expressed in gut but not in lung following IFNλ2 treatment (84)
Importance of IFN signaling in immune cells	• Type III IFNs are needed to reinforce adaptative immune responses (30, 106) • Neutrophils depleted of IFNLR were unable to control fungal infections in the lung (108)	• Innate lymphoid cells produce IL-22 which synergize with type III IFNs to induce higher levels of ISGs and increase antiviral (rotavirus) protection of murine IECs (97, 117)
Barrier functions of epithelium	• Type I IFNs and IFNAR are required to maintain the lung epithelial barrier function following *S. pneumoniae* infection (110) • Chronic type III IFN stimulation of lung epithelial cells leads to loss of barrier function and bacteria infiltration (115, 116)	• Type I and III IFNs help to maintain the intestinal epithelial barrier function following *Salmonella enterica* serovar *Typhimurium* infection. Type III IFN is more potent in promoting barrier function (111)
Microbiota	• Currently unknown if bacteria play a role in shaping interferon responses in the lung	• Microbiota promote norovirus persistent infection via modulation of type III IFN signaling (100)

composed of multiple cell types. With the advance of both single cell sequencing technologies and organoid cultures, it is now possible to address whether different cell types lining epithelium surfaces mount a similar or distinct immune response upon pathogen challenges. Similarly, it will be important to investigate whether these different cell types will generate the same ISGs upon IFN stimulation to address if each individual cell type establishes a distinct antiviral strategy to preserve its cell type specific function. While there are single cell studies of viral infection in cell lines (120), understanding viral infection at the single cell level in the tissue or in organoids is in its infancy.

Finally, one of the most underappreciated parameters influencing IFN signaling is the stochastic response of cells following IFN stimulation and the heterogeneity in the generated response. Understanding this complex relationship between cell populations and IFN response requires not only biological approaches where the importance of different transcription factors will be addressed *via* genetic manipulation but also through the use of mathematical modeling to gain a system understanding of IFN signaling. This heterogeneity in response to IFN is even more complicated as the spatial location of an individual cell within a population seems to impact its response to IFN. It was shown that when murine IECs become confluent and polarized, they become more responsive to IFN-λ (92). This work should constitute a building block for future research directions as it is likely to have critical implications at mucosal surfaces as epithelium cells form a condensed polarized monolayer of cells. We can speculate, for example in the gut, depending on the intestinal section that you are looking at, depending on whether a cell is located in the crypt or villi region or if the tissue is damaged and there are microlesions, that there will be differences in how IECs respond to pathogens and secreted IFN.

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

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REVIEW
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The Role of Type I Interferons in the Pathogenesis and Treatment of COVID-19

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Type I interferons (IFN-I) were first discovered over 60 years ago in a classical experiment by Isaacs and Lindenman, who showed that IFN-Is possess antiviral activity. Later, it became one of the first approved protein drugs using heterologous protein expression systems, which allowed its large-scale production. It has been approved, and widely used in a pleiotropy of diseases, including multiple-sclerosis, hepatitis B and C, and some forms of cancer. Preliminary clinical data has supported its effectiveness against potential pandemic pathogens such as Ebola and SARS. Still, more efficient and specific drugs have taken its place in treating such diseases. The COVID-19 global pandemic has again lifted the status of IFN-Is to become one of the more promising drug candidates, with initial clinical trials showing promising results in reducing the severity and duration of the disease. Although SARS-CoV-2 inhibits the production of IFNβ and thus obstructs the innate immune response to this virus, it is sensitive to the antiviral activity of externally administrated IFN-Is. In this review I discuss the diverse modes of biological actions of IFN-Is and how these are related to biophysical parameters of IFN-I–receptor interaction and cell-type specificity in light of the large variety of binding affinities of the different IFN-I subtypes towards the common interferon receptor. Furthermore, I discuss how these may guide the optimized use IFN-Is in combatting COVID-19.

Keywords: type I interferon, COVID-19, signaling, differential activity, inflammation

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INTRODUCTION

Type I interferons (IFN-I) are a family of cytokines that bind the type I interferon receptor, constituted of two transmembrane subunits, IFNAR1 and IFNAR2 (**Figure 1**). The two receptors are constituted of an extracellular domain, which binds IFN-I, a transmembrane helix and an unstructured intracellular domain (ICD) that binds JAKs and STATs (1, 2). JAK1 is associated with IFNAR2 and TYK2 with IFNAR1. STAT1 and STAT2 (and maybe also other STATs) were found to be constitutively bound to the ICD of IFNAR2 (3–5). Binding results in close proximity of the intracellularly associated JAKs, JAK1 and TYK2, resulting in their activation through cross phosphorylation (**Figure 1**) (6, 7). This also results in receptor phosphorylation, which role is still under debate (3, 8–10). The phosphorylated STATs dissociate from the receptor and form homo and hetero dimers, which are transported to the nucleus, where they serve as transcription factors for a large number of genes. The most prominent effects are associated with STAT1/STAT2

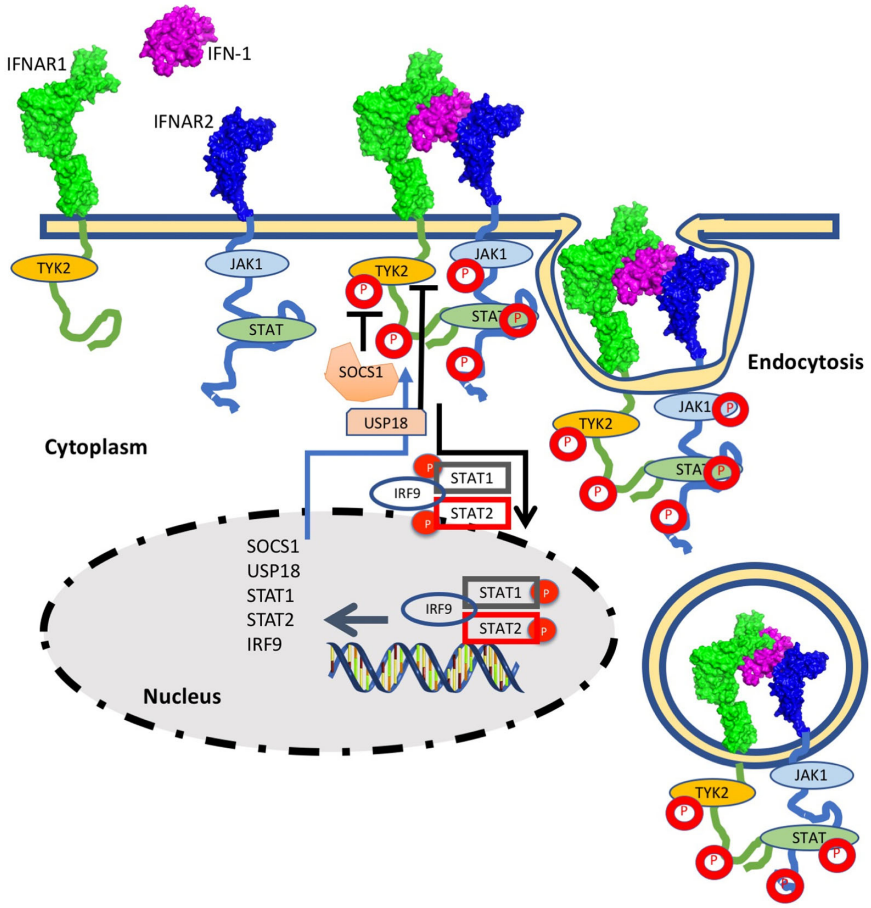

FIGURE 1 | The interferon response is initiated by IFN-I binding to the extracellular domains of IFNAR1 and IFNAR2. Following ternary-complex formation, the associated JAK kinases cross-phosphorylate each other as well as the associated STATs and tyrosine residues on the intracellular domains of the receptors. Upon phosphorylation the STATs are released and are transported to the nucleus. The STAT1/STAT2/IRF9 complex is strongest associated with IFN-I induced gene induction, albeit other STAT complexes are activated as well (see **Figure 2** for details). The STAT complexes serve as transcription factors for many IFN-I induced genes. Three main feedback mechanisms quell IFN-I activity: Receptor Ubiquitination, resulting in receptor endocytosis (which is initiated within minutes from IFN-I induction) and SOCS and USP18, which are IFN-I induced genes and thus their feedback relates to their production to high levels (which takes hours).

heterodimerization, which together with IRF9 form the interferon-stimulated gene factor 3 (ISGF3), which bind a distinct group of target genes harboring the interferon-stimulated response elements (ISRE). In addition to this, IFN-I drives STAT1/STAT1 and STAT3/STAT3 homodimerization, the formation of a STAT2/IRF9 binary complex and more (6, 10–12) (**Figure 2**). This leads to the transcription activation or suppression of over 1,000 genes, which drive a wide range of innate and adaptive immune functions. These, in turn respond against various pathogens, act as important regulators in tumor immunity and have a role in pathophysiology and autoimmune diseases (10, 13–18). STAT2 knockout cells still activate a STAT1/STAT1 response mediated by IRF1, while STAT1 knockout cells activate a STAT2/IRF9-induced response (10). Surprisingly, no change in the gene induction relative to wild-type cells was observed in STAT3 knockout HeLa cells, despite the strong IFN-I–induced phosphorylation of STAT3. However, as IFN-I responses are cell-type specific, a STAT3/STAT3-induced response may still be found in other cells than HeLa.

Due to this wide range of physiological responses, IFN-I has provided therapeutic benefits for multiple diseases, including multiple sclerosis, some cancers and viral diseases (hepatitis B and C) (19–21). Due to the efficient activation of antiviral activities by IFN-Is, most viruses have contemplated mechanisms to avoid its actions (22–24). For example, the Ebola virus, which outbreak in central Africa killed tens of thousands of people (25, 26), avoids IFN-I activity by producing the VP24 protein that binds the karyopherin alpha nuclear transporter. Thereby, it inhibits the nuclear transport of phosphorylated STAT1, rendering cells refractory to IFN-Is.

Another example of viral mechanisms that evolved to eliminate IFN-I functions in inducing innate immunity is given by the SARS corona virus, where both the production of IFNβ and the IFN-I induced signaling are attenuated. Recently, a

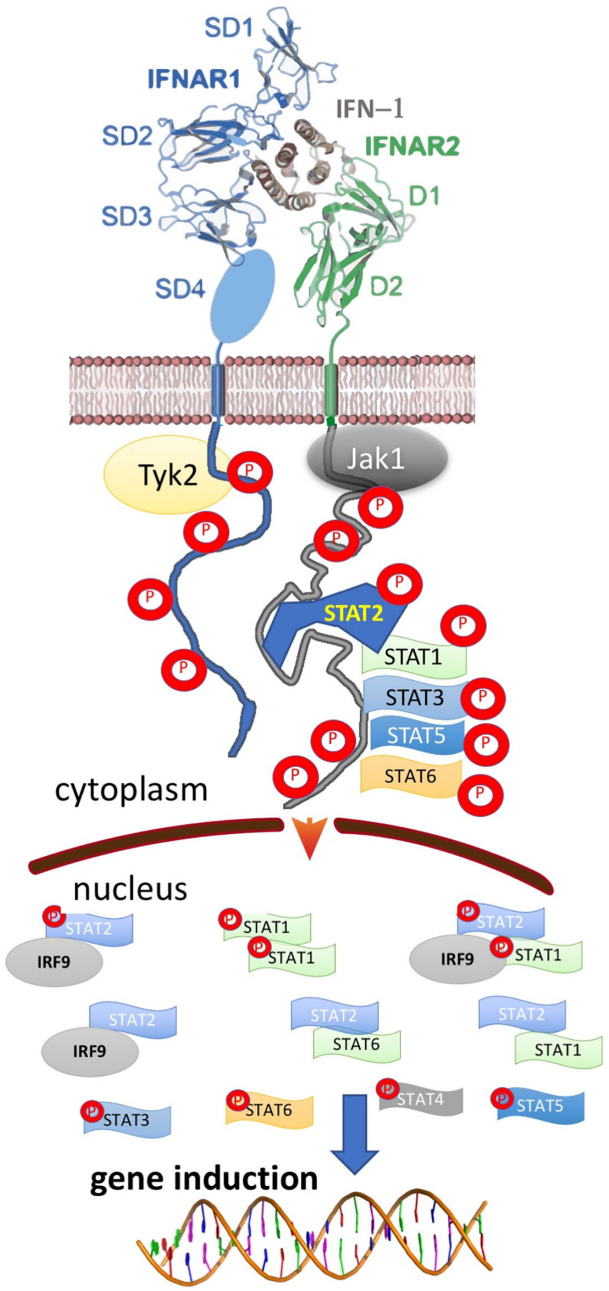

FIGURE 2 | Ternary, IFN-I/IFNAR1/IFNAR2 complex formation results in the activation of multiple STAT complexes that serve as transcription factors for different genes. The activated STATs and IFN-I regulated genes vary between different cells, IFN-I subtype, its concentration and duration of activation, result in a pleiotropy of responses.

more infective version of SARS has emerged, SARS-CoV-2 (which causes the COVID-19 disease). COVID-19 cases have been first reported by the end of 2019 in China, and rapidly became a world-wide epidemic with unprecedented consequences (27, 28). SARS-CoV-2 seems to have originated from horseshoe bats. Similar virus strains that circulate in bats in Hubei province in China may in the future cause further new zoonotic outbreaks (29). SARS-CoV-2 has 83% homology to the SARS-CoV virus that also spread from China in 2002 (30). SARS-CoV-2 proved to be much more infectious compared to the original SARS virus, resulting in a global epidemic. As IFN-I drives strong antiviral activities, the mechanisms SARS-CoV and SARS-CoV-2 combat IFN-I activities has been a matter of intense research, with at least 6 proteins being identified to counteract IFN-I functions in the SARS-CoV virus (31). In addition, IFN-Is were implicated in contributing to the severity of the cytokine storm, which is a major complication of SARS-CoV and SARS-CoV-2 and can lead to respiratory distress syndrome (ARDS) and death (31, 32).

In this review I will describe our current knowledge on the involvement of IFN-Is in the development of the COVID-19 disease, and how this relates to the different activities associated with type I interferons.

COMMON AND UNIQUE FEATURES OF TYPE I INTERFERON SIGNALING

Type I interferon receptors are found on all cell types, and are a major component of the innate immune system. Human type I interferons include 13 similar IFNαs with 80% homology between them and single IFNω, κ, ϵ and β, with lower homology (30–50%). All of them bind the receptor complex, composed of IFNAR1 and IFNAR2 at the same proximal location (1, 2, 33). Despite structural similarities among the ternary IFN-I-IFNAR1-IFNAR2 complexes, IFN-Is drive a range of different activities, dependent on the cell type and the interferon subtype (34). This apparent paradox has major implications for understanding the role of IFN-I in health and disease and its varied applications as a drug against a pleiotropy of diseases.

IFN-I signaling is initiated by binding of IFN-I to its receptor. It has been suggested that cytokine receptors are pre-associated, with ligand binding activating signaling through the induction of conformational changes (35). However, more recent single-molecule receptor tracking on life cells has clearly shown that for many of the cytokines, its role is to bring the receptors into close proximity, which drives signaling (36). This seems to be the case also for IFN-I induction, as shown both using single receptor tracking and mutational analysis (**Figure 1**) (37, 38). While structurally, the ternary ligand-receptor complex seems to be the same for all IFN-Is, the binding affinity differs by many orders of magnitude. The tightest binding IFN-I is IFNβ, which binds IFNAR1 with 100 nM affinity and IFNAR2 with sub-nanomolar affinity. The different IFNα subtypes bind IFNAR1 with 0.5 to 5 μM affinity and IFNAR2 with 1 to 100 nM affinity, with IFNα1 being the weakest binding IFNα (39, 40). Even weaker binding was measured for IFNϵ, with ~100-fold reduced affinity relative to IFNα proteins (15). Interestingly, IFNϵ is constitutively expressed by the reproductive tract epithelium and is regulated by hormones during the estrus cycle, reproduction,

menopause and by exogenous hormones. Thus, its mode of action is different from other IFN-Is (41).

These large differences in binding affinity between IFN-I subtypes were suggested to result in major differences in biological activity. To obtain a better insight into the molecular mechanisms of their actions, IFNα2 was engineered to cover the whole range of binding affinities of natural IFN-Is to both the high affinity (IFNAR2) and low affinity (IFNAR1) receptor chains (1). These studies have shown that indeed, the binding affinity to both receptors is a major determinant of IFN-I activity (42). Using both natural and engineered IFN-Is has shown that even weak binding IFN-Is activate the cellular antiviral program at very low (pM) concentrations (39). Moreover, the antiviral program was activated in all cell-lines tested. Despite the 50-fold higher affinity of IFNβ over IFNα2 towards binding IFNAR receptors, its potency to elicit an antiviral response is similar. For example, in WISH cells (originally thought to be of amniotic origin, but later found to be a HeLa (cervix cancer) contaminant) the EC_{50} for antiviral activity of IFNα2 is 0.3 pM, while the EC_{50} for IFNβ is 0.15 pM (43). WISH cells have been extensively used to characterize IFN-I activity, including for definition of IFN-I unit activity. An upper limit for antiviral potency was further verified by engineering an IFNα2 variant, YNS-α8-tail, with 50-fold tighter binding to IFNAR1 and 15-fold tighter binding to IFNAR2 in comparison to IFNα2 (thereby surpassing the receptor binding affinity of natural IFNβ). Still, the EC_{50} for antiviral activity is only 3-fold lower in comparison to IFNα2 (44, 45).

Conversely to antiviral activity, IFNβ is much more potent in activating the antiproliferative program relative to IFNα2, a result that was also verified using the IFNα2 variant, YNS-α8-tail (45). The EC_{50} for antiproliferative activity on WISH cells is 2 nM for IFNα2, 50 pM for IFNβ and 20 pM for YNS-α8-tail. A similar increase in antiproliferative potency was observed also for OVCAR3 and HeLa cells. Interestingly, while antiviral activity was observed in all cell lines tested, some cell lines were not susceptible to IFN-I induced antiproliferative activity (for example T47D and K562), independent on the concentration and subtype of IFN-I (45).

To better understand the molecular basis for this finding, IFN-I induced gene expression was monitored using various IFN-I subtypes or engineered mutants on the background of different cell-lines. These experiments showed that low concentrations of weaker binding interferons activate the expression of mostly antiviral genes. Higher concentrations of interferons activate also other genes, many of them related to immune-modulation (45). Examples for such genes are chemokines such as CXCL10 and 11, which are involved in chemotaxis of T cells and natural killer cells, induction of apoptosis, regulation of cell growth and more. We gave the term of "robust" for the common IFN-I induced program (including its antiviral activity) and "tunable" for the other programs induced by IFN-Is, which include between others antiproliferative and immunomodulatory activities (34). Further investigations into these two programs has shown that cells with low receptor numbers activate only the robust program, and that not all cell types execute the tunable program, conversely to the robust program that is common to all cells (46). Tighter binding IFN-Is at higher concentrations are essential for the activation of the tunable program. Genes upregulated by the robust program are mostly classical antiviral genes, such as MX1 and MX2, OAS1 and 2, PKR, IFIT1, 2 and 3, ISG15, and many more. **Figure 3A** shows a Venn diagram of RNAseq data for 4 different cell-lines induced with

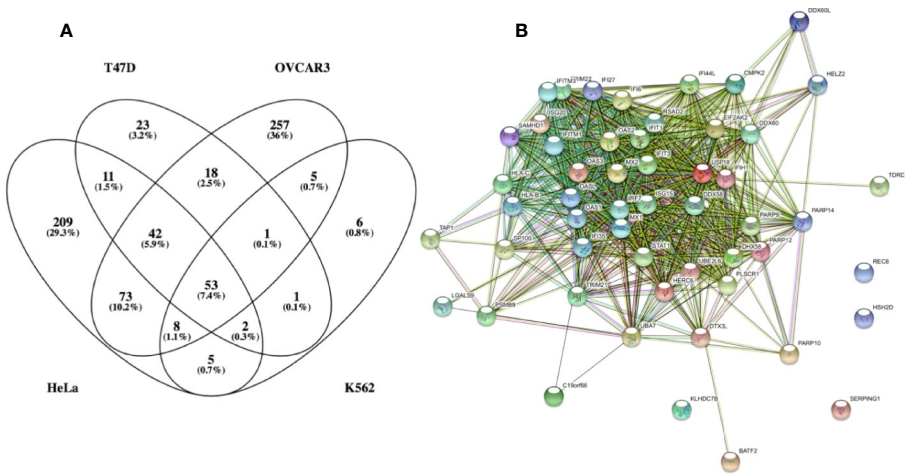

FIGURE 3 | Genes which expression was upregulated by over 3-fold in the following cell lines: HeLa, T47D, K562 and OVCAR3. **(A)** venn diagram of the upregulated genes. **(B)** STRING: functional protein association network analysis of upregulated genes in all 4 cell lines (53 genes). According to STRING and GO analysis, the commonly upregulated genes have a strong antiviral signature. The top GO terms (FDR $<10^{-25}$) are response to type I interferon, innate immune response, response to virus, defense response and immune system process. It is interesting to note that antiviral genes constitute most of the upregulated genes common to all 4 cell lines. Antiviral genes are also the majority of upregulated genes in K562 and T47D cells. Conversely, OVCAR3 and HeLa cells have many unique upregulated genes, many of them related to immunomodulatory functions, cell cycle, apoptosys and more.

IFN-I. The diagram shows that 53 genes are commonly upregulated by all 4 cell-lines. **Figure 3B** shows STRING protein interaction analysis of these common genes. Clearly, these form a tightly interacting mesh of gene products. Gene Ontology analysis shows these genes to have an extremely high signature for antiviral activity and IFN-I activation. Promoter analysis of common ISGs has shown them to be driven by the classical ISRE promoter sequence (45). Conversely, for tunable genes no clear promoter sequence was identified. The exact mechanism of how tunable genes are upregulated by IFN-I is thus not yet fully understood.

INTERFERON AND INFLAMMATION

From an immunological point of view, IFN-Is have three major functions: 1. To activate an antiviral state in infected and neighboring cells that limits spread of infection. 2. Modulate innate immune responses, including antigen presentation and natural killer cell functions while restraining pro-inflammatory pathways. 3. activating the adaptive immune system for the development of high-affinity antigen-specific T and B cell responses (47). As IFN-Is are highly active molecules, their expression and signaling potency is highly regulated. Opposing augmenting and suppressive signals are induced by host factors. Suppressive pathways include IFN-I activation of USP18, an ISG that suppresses signal transduction by reducing the ability of IFN-Is to form an active receptor complex (38, 48). A second inhibitory mechanism is the induction of SOCS1 and SOCS3, which KIR domain block the substrate binding groove on JAK, thereby inhibiting STAT phosphorylation (49). A third mechanism is by rapid endocytosis and subsequent lysosomal degradation of activated IFNAR complexes (50, 51) resulting in reduced receptor numbers (**Figure 1**). It has been demonstrated that a mutant in IFNAR1 (S535A and S526A in human and mouse respectively), which fails in IFNAR1 endocytosis through blocking its ubiquitination result in high incidence of inflammation (51, 52). At the transcriptional level, IFN-I response can also be regulated by miR-155, which is highly induced by pattern recognition receptors and inflammatory signaling, and suppresses the expression of over 100 genes. Between them genes related to the interferon pathway. It was shown that miR-155-deficient CD8(+) T cells had enhanced type I interferon signaling and were more susceptible to interferon's antiproliferative effect (53).

High basal IFN-I levels are implicated in various immunological diseases, such as systemic lupus erythematosus and more (18, 54, 55). However, IFN-I has also anti-inflammatory effects, as best demonstrated by their ability to suppress multiple-sclerosis (56). It is important to note that beneficial results in treating multiple-sclerosis were observed only for IFNβ but not for IFNα treatment (56). To see whether this relates to the higher receptor binding affinity of IFNβ, we established a transgenic mouse harboring the human interferon-receptors extracellular domains fussed to the mouse intracellular domains and compared the severity of EAE in a mice model upon treatment with IFNα2, IFNβ and the high-affinity engineered IFN-YNS-α8-tail. We found that the IFN-YNS-α8-tail had the strongest suppressive effect on the development of EAE (57). The effect was further enhanced by PASylation of IFN-YNS-α8-tail, which extends it plasma half-life by 10-fold. Interestingly, we found a tight relation between the increased levels of expression of PD-L1 in mice and the severity of the disease. These data show that tight binding IFN-Is induce preferential anti-inflammatory responses, at least in this MS mouse model. Another example for the immunosuppressive activity of IFN-I was shown for LCMV infection, which induces consistent IFN-I production including the immunosuppressive factors IL-10 and PD-L1 (58). In addition to the above, Interferons contribute to inflammasome activation through several different mechanisms, including caspase-11 expression and the IFN-I inducible GBP protein expression, which was reported to have an important role in caspase-11 activation and pyroptotic cell death (59).

IFN-Is have important roles in protecting the lung from spread of respiratory viruses. In addition to their direct role, IFN-Is have also been found to be critical in initiating lung inflammatory responses, by inducing recruitment and activation of immune responses, which have to be kept under control. IFN-Is have been shown to result in the production of chemokines such as CCL2 and CXCL10, which play important roles in the recruitment of monocytes/macrophages, T cells, NK cells, and DCs, therefore directly influencing inflammation in the lung (60). This varied effect of type I IFNs on T cells is partly dependent on the different STATs induced by type I IFNs. In the absence of IFN-Is, the detection of accumulating viral RNA and downstream processing of the signal is compromised, leading to viral spread and also to reduced inflammation in the lung. Interestingly, there is an age-related reduction of IFN-I production and ISG induction after viral infection, which may be related to the higher susceptibility of elderly population to lung infections (61).

A CONSTANT BATTLE BETWEEN THE INTERFERON SYSTEM AND VIRUSES

Viruses have developed many strategies to interfere with the synthesis of IFN-Is or the IFN-I induced responses. One of them, is the stimulation of turnover of the interferon receptors. Among other viruses implicated in accelerating the turnover of IFNAR1 are EBV, herpes simplex virus, hepatitis C and B viruses, vesicular stomatitis virus and the SARS coronavirus (62, 63). SARS-CoV has been shown to suppress IFN-I responses in the host through multiple mechanisms. A subdued IFN-I response diminishes antigen presentation and reduces the antiviral adaptive Th-1 immune response. IFN-Is communicate between cells against pathogens and have a critical role in the immune system, such as activating natural killer (NK) cells and macrophages. In addition, IFN-Is cause flu-like symptoms, which are observed in various diseases. These symptoms may have a role in alerting a person of his/her sickness, in order to limit disease-spread to other individuals. In SARS-CoV and

MERS-CoV, the induction of IFNβ is suppressed altogether. This dampening approach is highly associated with the disease severity and increased mortality (64). In the lethal cases of SARS-CoV or MERS-CoV infections, the increased influx of inflammatory cells is always observed. In a mouse model of SARS- CoV infection, imbalance in IFN-I and inflammatory cells were shown as the main cause of fatal pneumonia (65). In addition to these, SARS-CoV implements strategies to evade the immune response by antagonizing IFN-I induced signaling pathways. The ORF6 protein blocks the expression of STAT1-activated genes (66). SARS-CoV and MERS-CoV encode papain-like protease (PLP) that is able to impede the immune response function (67). In addition, SARS-CoV interacts with ISG15 and antagonizes the IFN-I-mediated antiviral response (68). The MERS-CoV ORF4b antagonizes the antiviral IFNβ production by inhibiting IRF3 and IRF7 (69). Also SARS-CoV inhibits activation of IRF3/7, slowing IFNβ production upon infection (70). While IRF3 is expressed in many different cell types, plasmacytoid dendritic cells are the only cells constitutively expressing IRF7 (47).

IFN-I treatment has been studied against MERS-CoV and SARS- CoV in numerous experiments, both *in vitro* and *in vivo*, and in combination or not with lopinavir/ritonavir, ribavirin, remdesivir, corticosteroids, or IFNγ. While IFNα and β were efficient *in vitro* and in certain animal models, their success in humans was less convincing [for review see, (71, 72)]. It should be noted that reduction in ARDS mortality (not related to SARS) was also found to be at best marginal upon treatment with IFN-I (73). Still, one has to consider that mice studies have shown the timing of IFN-I administration to be critical, with positive effects being observed if IFN-I was administered shortly after infection. Conversely, IFN-I failed to inhibit viral replication and resulted in unwanted side-effects when administered later in the disease circle (74, 75). These include elevated lung cytokine/chemokine levels, vascular leakage, and impaired virus-specific T cell responses. It is interesting to note that a knockout of the IFN-I receptor in mice resulted in its protection from lethal SARS-CoV infection. These findings have major implications on how to treat humans against SARS and MERS, and could have affected the outcome of the clinical studies.

Mode of Infection by SARS-CoV-2

The COVID-19 pandemic started in December 2019 in Wuhan, China. By the summer of 2020, thirty million cases were reported worldwide, with over 900,000 fatalities. As COVID-19 is closely related to the SARS-CoV virus, the interest in the effect of interferons on its disease progression, and its potential as a drug was immediate. Disease progression of COVID-19 goes through a number of stages. The initial stage, which last from 2 to 14 days (usually 5–6 days) from infection is asymptomatic. A certain proportion of patients never produce any symptoms (the percentage of those is under debate, but a range of 30–50% is most likely). Of those who develop symptoms, they are mostly mild (80% of those who develop symptoms). From the remaining 20%, about half will develop severe symptoms, which require hospitalization in intensive care units. The mortality rate, from those developing symptoms is 2% to 5%. The numbers given above are average, and change dramatically with age. At young age most of the infected people will be asymptomatic, while over the age of 70 about 80% will have symptoms. Moreover, as the age progresses, symptom severity increases (76). The major complication of severe infection is pneumonia, which can develop into acute respiratory distress syndrome (ARDS). In addition, COVID-19 has been linked to cardiovascular sequelae, such as myocardial injury, arrhythmias, cardiomyopathy and heart failure, acute kidney injury, neurological complications, and acute ischemic stroke (28). Developing severe symptoms and death is strongly related to background conditions. The strongest relation is to age, with the risk to people under 50 being very small, while the risk peaks for people over the age of 75. In addition, chronic kidney disease, chronic obstructive pulmonary disease, immunocompromised state, obesity, heart conditions and type 2 diabetes are linked to higher incidents of sever disease (76).

CoV-2 is presumed to infect people mostly though inhalation of viral particles, which can be airborne, in droplets or otherwise through infection through touching infected surfaces. The Spike protein on the CoV-2 surface binds to the human ACE2 protein, which serves as its receptor (**Figure 4**). The homotrimeric spike glycoprotein is made from S1 and S2 subunits. Its binding and subsequent cleavage by the host protease TMPRSS2 results in the fusion between cell and viral membranes and cell entry (77). Blocking the ACE2 receptors by specific antibodies voids viral entry (77–79). Interestingly, CoV-2 receptor-binding domain (RBD) exhibited significantly higher binding affinity to ACE2 than the SARS-CoV RBD, which was speculated to relate to the higher infectivity of COVID-19 in relation to SARS. After membrane fusion, the virus enters through the endosomal pathway and the viral RNA is released into the host cell. The viral RNA is then translated into viral polyproteins, which are cleaved into small products by viral proteases (papain-like protease [Plpro] and the main protease [Mpro]). Viral proteins and genome RNA are subsequently assembled into virions in the ER and Golgi and then transported and released out of the cell. The exact mechanism of viral self-assembly is still under intense investigation (80, 81).

Investigating ACE2 and the viral entry-associated protease TMPRSS2 expression levels in lung tissue and trachea has shown that TMPRSS2 is expressed in both tissues, while ACE2 is predominantly expressed in a transient secretory cell type (82). In addition, ACE2 and TMPRSS2 co-expressing cells were found within lung type II alveolar cells (which also release pulmonary surfactant), enterocytes, and nasal goblet secretory cells (83). Using single-cell RNA-sequencing, ACE2 and TMPRSS2 were found to be highly expressed also in the nasal goblet and ciliated cells (84). The inhaled virus likely binds to epithelial cells in the nasal cavity and starts replicating. The virus propagates and migrates down the respiratory tract along the conducting airways, and a more robust innate immune response is triggered. For about 80% of the infected patients, the disease will be mild and mostly restricted to the upper and conducting airways. Unfortunately, about 20% of the infected patients will progress to more severe disease and will develop pulmonary infiltrates and some of them will develop ARDS (85).

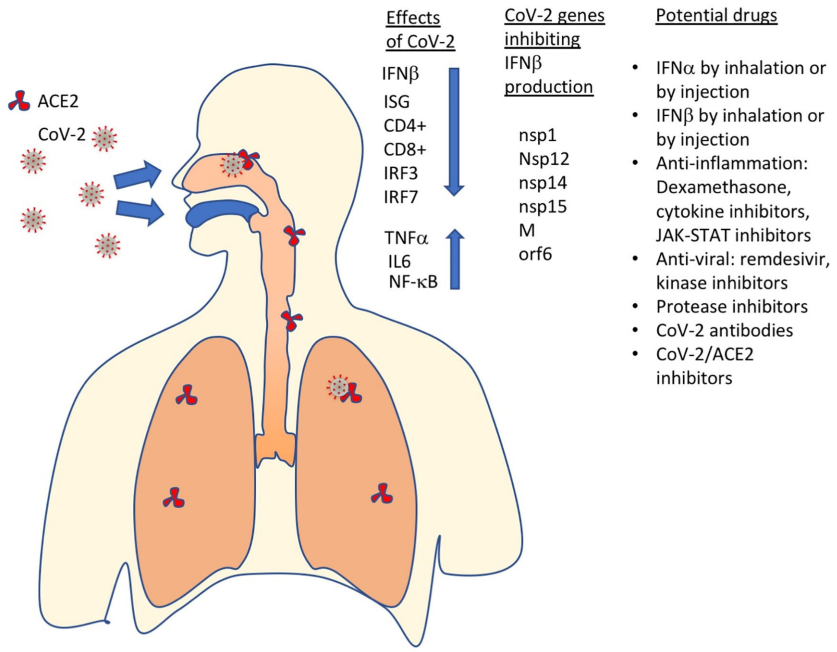

FIGURE 4 | SARS-CoV-2 has multiple effects on the immune system, including inhibition of IFNβ production, which results in ISGs not to be produced, CD4+ and CD8+ exhaustion and increased levels of pro-inflammatory proteins (TNFα, IL6, NF-kB). Currently, the most promising drugs against COVID-19 include IFN-Is, anti-inflammatory and antiviral drugs, protease inhibitors, antibodies, SARS-CoV2 – ACE2 (receptor) binding inhibitors and more.

Interferons and COVID-19

Like many other viruses, also SARS-CoV and SARS-CoV-2 have evolved mechanisms to reduce their exposure to IFN-I. In both viruses, mechanisms to block the production of IFNβ were identified. While the antiviral potency of IFN-Is on SARS-CoV is moderate, SARS-CoV-2 seems to be highly sensitive to IFN-I. This is evident by the significant reduction in viral replication observed following IFN-I treatment at both 24 and 48 h post-infection (86). In SARS-CoV-2–infected cells, IFN-I results in elevated STAT1 levels and ISG production (in contrast to SARS-CoV infected cells). This raises the question of why the innate immune system fails to combat SARS-CoV-2? The apparent answer to this is in the inhibition of IFNβ production by proteins of the SARS-CoV-2 virus. Within cells, RNA viruses are sensed by the innate immune system through three major classes of pattern recognition receptors (PRRs): Toll-like receptors (i.e. TLR-3, -7, -8), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) (87). To identify the molecular mechanisms that block IFNβ production through activation of IRF3/7, several research groups transfected cells individually with all the CoV-2 viral genes and with either RIG I, MDA5, or MAVS (88, 89). Among the 27 CoV-2 proteins transfected to cells, they identified nsp14 and orf6 as competent suppressors of IFNβ. Yuen et al. also identified nsp13 and 15, while Lei et al. identified nsp1, nsp12 and the M protein as potent inhibitors of the MAVS pathway, leading to inhibition of IFNβ production (**Figure 4**). Orf6 was between the strongest suppressors of IFNβ production in both studies. Orf6 was also the only SARS-CoV-2 gene suppressing the activity of an interferon-stimulated response element (ISRE) promoter in both studies. Lei et al. also identified nsp1 and nsp14 as potent inhibitors of the induction of an ISRE promotor. In another study, Li et al. showed that the viral ORF6, ORF8, and nucleocapsid proteins were strong inhibitors of IFNβ production, and through this of the IFN-I innate immune response (90). In this study, ORF6 and ORF8 also inhibited induction of transcription an ISRE promotor driving a luciferase as reporter, following IFNβ treatment.

In addition to the above-mentioned SARS-CoV-2 genes, ORF3b was implicated by Konno et al. as being a potent antagonist towards IFN-I production (91). An interesting civet in this study is the finding that a natural variant, with a longer ORF3b reading frame increased disease severity in two patients. In light of the much higher than expected coding capacity of the SARS-CoV-2 genome, where many more proteins than genes were identified (92), we may find even more proteins and peptides being involved in eliminating the innate immune response, including through inhibition of IFN-I activities.

Another mechanism by which SARS-CoV-2 inhibit antiviral functions of the cell is thought the activity of the papain-like protease (PLpro), which is essential for viral polyprotein processing. This gene was found to preferentially cleave the ubiquitin-like modifier interferon-stimulated gene 15 (ISG15),

which is an IFN-I induced gene with strong antiviral activity (93). This represents another layer of attenuation of IFN-I responses by SARS-CoV-2 and is similar to the mechanism previously identified for SARS-CoV (68).

Inhibition of IFNβ production by CoV-2 got further confirmation from measuring the levels of different cytokines in SARS-CoV-2–infected patients. An integrated immune analysis, including immune cell analysis, whole-blood transcriptomics and cytokine quantification on COVID-19 patients at 8 to 12 days after disease onset has shown an impaired IFN-I response that is a result of low IFN-I levels (94). This, in turn results in the low production of interferon-stimulated genes. Conversely, high levels of IL6 and TNFα were measured (**Figure 4**) (95, 96). This is in contrast to what is seen in patients infected with highly pathogenic influenza viruses. The high production of pro-inflammatory cytokines and low production of IFN-Is during SARS-CoV-2 infection suggests effective activation of NF-κB but not IRF3 and IRF7 (95). Impaired IFN-I production during severe COVID-19 may also lead to an imbalance in the pro-inflammatory versus pro-repair functions of airway macrophages. This was indeed seen in severely ill patients with COVID-19.

Other innate immune cells such as natural killer (NK) cells are also regulated by IFN-Is during coronavirus infection. Severe COVID-19 is associated with exhaustion of CD4+ and CD8+ T cells (97), which may be a result of deficient IFN-I production, as IFN-Is promote survival of T cells. An important issue to consider is that early production of IFN-Is promote efficient T cell responses, while a delayed response may inhibit T cell proliferation or their exit from lymphoid organs and thus cause their functional exhaustion. Indeed, T_{reg} cell counts in COVID-19 patients inversely correlate with disease severity (98, 99). Interestingly, transcriptomic analysis of blood, lung, and airways of CoV-2–infected patients showed that while IFNβ was indeed not highly expressed in either, a number of IFNαs were highly upregulated in the lung and airways but not in blood (100). Moreover, a clear IFN-I–induced gene expression profile was also detected for lung and airways, but not for blood (PBMCs). A similar finding of elevated IFNα but not IFNβ, during COVID-19 infection was also found by Wei et al. (101). In this study, the elevated IFN-I response was restricted to the stage in the disease were patients were in intensive care. In another study of 26 patients, of whom 5 did not produce IFN-I, those patients had higher viral load, required more aggressive medical intervention and their time of stay in the intensive care unit was longer that IFN-I producing patients (102).

PDCs are the most rapid and abundant IFN-I producers. PDCs express TLR7 and TLR9 which are important in sensing viruses. The response of PDCs to viruses, particularly IFN-I production, is significantly impaired with ageing while secretion of all other pro-inflammatory cytokines was comparable to that of younger individuals (103). This may relate to the master regulator for IFN-I production, IRF7, which expression, phosphorylation and nuclear translocation decreases with age. In addition, local neutrophil-mediated inflammation is increased with age, while cytotoxicity of NK cells induced by type I IFN-Is decreases in aged mice (104). In addition to age, other factors were also associated with reduced interferon responses. One of them is obesity, which is related to impaired IFNα and IFNβ responses, which may relate to inadequate response of obese people against viral infections (105).

Treating COVID-19 Patients With IFN-I

Clinical trials of using IFN-I for treating corona viruses has a long history. Already in 1983, intranasal human IFNα2 was given both before and after corona virus challenge, a strain that is causing common cold. The incidence of colds, the severity of symptoms and signs, and virus replication were all reduced in subjects receiving interferon as compared with those given placebo (106). For SARS-CoV, no randomized placebo-controlled trials have been performed to test the efficacy of IFN-Is, however, comparing the clinical outcome of patients treated with IFN-α (infacon-1) with patients at different locations (not a control group) that were not treated, has suggested clinical benefits (107). These studies have raised the hope that IFN-I may be a potent drug also against COVID-19. This hope was further exuberated by the observation that externally administrated IFN-I induced a strong antiviral response, much more than that observed for SARS-CoV (86). While some of the SARS-CoV-2 proteins may affect ISG production (most notably, ORF6 and 8, see above), the main defense of SARS-CoV-2 against IFN-I innate immunity seems to be the prevention of IFNβ production, which can be substituted by external administration.

A major problem in assessing the efficiency of IFN-I against COVID-19 is the lack of a good small animal model. While such models are now under development, they are still not perfect. In a recent study, mice were infected with a replication-deficient adenovirus containing human ACE2, and then infected with SARS-CoV-2. These mice developed pneumonia, severe pulmonary pathology, and high-titer virus replication in lungs. To test the role of IFN-I in disease development, IFNAR1 KO mice were infected with SARS-CoV-2, showing higher viral titer over time. Next, the mice were treated prior to infection with Poly I:C, a strong inducer of IFN-I. This resulted in significantly diminished clinical disease and induced more rapid virus clearance (108). These results suggest that at least in a mice model, IFN-I may benefit disease recovery.

Due to the lack of a good animal model, and the availability of clinically approved IFN-I therapies, multiple clinical studies have been conducted administrating different subtypes of IFN-Is using different routes of administration (for summary see **Table 1**). In a preventive study, nasal drops of IFNα1 were given to 2,944 healthy medical staff in Shiyan City hospital, Hubei Province for 28 days to prevent SARS-CoV-2 infections. None of them developed serious side effects or was infected with CoV-2. While the study lacked a control group from the same city, overall in Hubei province 3,387 medical staff were diagnosed with COVID-19 (109). The study thus gives an indication that IFN-I may help in preventing infection for high risk medical personal.

To test the benefit of subcutaneous injection of IFNβ on early stage patients, an open clinical trial was conducted with 127 patients, 86 were assigned to the combination of lopinavir, ritonavir, ribavirin, and three doses of 8 million international units of IFNβ, while the control group of 41 patients were given all the above except IFNβ. The median number of days from symptom onset to start of study treatment was 5 days. Patients

TABLE 1 | Summary of clinical trials conducted using IFN-is.

Study organizer	Aim of study	IFN subtype	Route of administration	Control group	Main findings	References
Shiyan City Hospital, Hubei, China	Preventive	IFNα1	Nasal drops	Health workers in different locations	Prevention of infection	(109)
Multi-center, Hong Kong	Hospital treatment of COVID-19 patients	IFNβ in combination with lopinavir, ritonavir, ribavirin	Subcutaneous injection	Patients not given IFNβ	Reduction in clinical symptoms	(110)
Wuhan, China	Hospital treatment of COVID-19 patients	IFNα2b in combination with arbidol	Nebulization to the lungs	Patients not given IFNα2b	Reduction in clinical symptoms	(111)
Imam Khomeini Hospital, Teheran, Iran	Hospital treatment of COVID-19 patients	IFNβ1a + standard care	Subcutaneous injection	Randomized clinical trial	No difference in clinical response, but lower mortality	(112)
Multi-center, Hubei, China	Hospital treatment of COVID-19 patients	IFNα2 + standard care	Inhalation	Retrospective study, historical control group	Early treatment reduced, while late treatment increased mortality	(113)
Synairgen, UK	Hospital treatment of COVID-19 patients	IFNβ	Inhalation	Controlled study	79% reduction in developing severe disease	ClinicalTrials.gov Identifier: NCT04385095

given also IFNβ had a significantly shorter median time from the start of treatment to negative nasopharyngeal swab (5–11 days) in comparison to the control group (8–15 days). Moreover, IFNβ reduced viral load and number of significantly ill patients relative to the control group, this without significant side-effects (110).

In a medical study on the effects of treatment with IFNα2b in a cohort of confirmed COVID-19 patients, some of the 77 participants were given nebulized IFNα2b with or without arbidol while others were given only arbidol. Treatment with IFNα2b with or without arbidol reduced the duration of detectable virus in the upper respiratory tract and reduced duration of elevated blood levels of IL6 and c-reactive protein, which are inflammatory markers (111). While the study did not include a standard care group, and all patients recovered, it still provides an indication of IFN-I efficiency.

The efficiency of IFNβ1a subcutaneously injected three times weekly for 2 weeks for treatment of severe COVID-19 was tested in a randomized clinical trial. All the patients (including the control group) received standard of care, including a range of other medicines (hydroxychloroquine, antibiotics, antiviral medicine and more). While the clinical response was not significantly different between the IFNβ1 and the control groups, the 28-day overall mortality was significantly lower (19% vs. 44%) in the IFNβ1 treated group (112).

In a retrospective study of patients receiving IFNα2 through inhalation, alone or in combination with other drugs at a relative early versus late stage of the infection, it was found that those receiving IFNα2 at an early stage had a significantly lower rate of mortality. In contrast, late interferon therapy increased mortality and delayed recovery (113). The study suggests a relation between the time of IFN-I treatment and its efficiency.

Synairgen, a UK-based company, performed a controlled clinical trial of inhaled IFNβ on 221 patients and reported that compared with placebo the odds of developing severe disease during the treatment period decreased by 79% for hospitalized patients receiving SNG001, and that patients who received SNG001 were more than twice as likely to recover from the virus during the treatment period versus those randomized to placebo. These are between the best results achieved so far in curing COVID-19.

More clinical trials are now under way to evaluate IFN-I efficiency, but clearly the initial trials have been encouraging. Moreover, due to the many years of experience in treating patients with IFN-Is, the availability of the drug and its relatively modest cost make it an excellent candidate for mass treatment, once approved. However, critical questions remain concerning the use of IFN-Is for COVID-19 and other diseases (**Figure 4**). These questions relate to the optimal IFN-I subtype, drug-concentration, duration of treatment, mode of treatment and at which frequency should it be given. Ample experience exists with subcutaneously administration, which is almost the only route IFN-Is were used in the clinic. Here, non-modified IFN-Is are usually administrated two to three times weekly, while PEGylated IFN-Is are administrated once per week or less. Injection of IFN-Is will result in a systemic response, where IFN-Is were shown to have antiviral functions as well as pro and anti-inflammatory functions. Contrary, if given by inhalation, it will directly target the epithelial, and thus replace the IFNβ, which production is inhibited by the virus. Administration as nasal drops of IFNα may be an excellent prophylactic method for people at high risk. Ideally, these questions could be answered using animal models. The problem is that the disease in those is not equivalent to that observed in humans. Due to the severity of the disease and the high proven safety of IFN-Is, more clinical trials on humans, testing the many open questions related to its best mode of administration may be the fastest way forwards.

The subtype to use is another important question. For multiple-sclerosis, IFNβ has been used for many years (114), as it seems to provide a better anti-inflammatory response than IFNαs. This may relate to its higher binding affinity to the interferon receptors, as has been demonstrated using a tight binding IFNα mutant (YNS-α8 tail), which binding affinity even surpasses that of IFNβ [see above (57)]. For combating viral

disease, most notable hepatitis C, IFNα2 has been most commonly used (115), which was later replaced by PEGylated (long plasma half-life) IFNα2 (116). Also, for cancers IFNαs were mostly used (117). A good clinical explanation of why specific IFN-I subtypes were used is often missing, and decisions of which interferon to use may often relate to availability rather than to efficacy. Moreover, due to the specie specificity of IFN-Is, one cannot deduce from mouse experiments, which IFN-I to use in humans, as the data are not transferable (57, 118). The main difference between IFNαs and IFNβ is that the later has a stronger potency to induce antiproliferative and immunomodulatory responses (tunable), while IFNα will provide a cleaner antiviral response (robust) without the additional responses associated with IFNβ. The open question is which is desired for COVID-19 treatment, where complications arise from the exuberated immune response.

Another, important parameter is the time of intervention by IFN-I, in early or late-stage COVID-19 disease. In a recent study in mice it has been shown that prolonged IFN-I and III signaling interferes with lung repair during influenza recovery, probably through p53 induction, which reduces epithelial proliferation and healing, while early treatment protects mice (119). In SARS-CoV-2 this is further complicated by the "cytokine-storm" symptoms of severe COVID-19, as indicated by elevated IL6 and TNF-alpha levels. Whether IFN-administration, particularly IFNβ suppresses or exacerbate the SARS-CoV-2 cytokine storm needs to be urgently determined, as to provide a guide for future application of IFN-I therapy in SARS-COV-2 treatment.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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