Guarding the frontiers: the biology of type III interferons

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Type III interferons (IFNs) or IFN-λs regulate a similar set of genes as type I IFNs, but whereas type I IFNs act globally, IFN-λs primarily target mucosal epithelial cells and protect them against the frequent viral attacks that are typical for barrier tissues. IFN-λs thereby help to maintain healthy mucosal surfaces through immune protection, without the significant immune-related pathogenic risk associated with type I IFN responses. Type III IFNs also target the human liver, with dual effects: they induce an antiviral state in hepatocytes, but specific IFN-λ4 action impairs the clearance of hepatitis C virus and could influence inflammatory responses. This constitutes a paradox that has yet to be resolved.

Upon infection by viruses, mammals (including humans) react by producing interferons (IFNs), which induce an antiviral state in infected and yet-uninfected cells to block viral replication and spread of the infection. Mammals possess three classes of IFNs: type I (IFN-α/β), type II (IFN-γ) and type III (IFN-λs). The direct antiviral effects of type II IFN are limited, but it has pleiotropic effects on a diverse set of immune cells promoting both adaptive and innate responses. Type I and III IFNs induce a strong antiviral state in responsive cells by initiating a transcriptional program that regulates the expression of several hundred genes. Whereas almost all nucleated cells respond to type I IFN, responses to type III IFNs are restricted to tissues with a high risk of viral exposure and infection, such as those at mucosal surfaces. This allows type III IFNs to selectively induce a strong antiviral state in high-risk tissues at a limited inflammatory cost for the host organism. Here we review the role of IFN-λ as the border guard of the body and discuss the putative roles of IFN-λ4. This paradoxical protein is highly antiviral in vitro but impairs clearance of hepatitis C virus (HCV) in vivo and might influence inflammatory processes in the liver.

Discovery and nomenclature

The type III IFN family was discovered by two teams1,2. The groups chose different naming conventions but subsequently agreed upon the IFN-λ nomenclature3. We use the current nomenclature here and include the now abandoned nomenclature in parentheses. We list protein names, but the names of their encoding genes are generally equivalent, with the exception that Greek letters are replaced by Latin letters (for example, the IFN-λ1 protein is encoded by the IFNλ1 gene). In humans, the type III IFN family consists of four members: IFN-λ1 (IL-29), IFN-λ2 (IL28A), IFN-λ3 (IL-28B) and IFN-λ4 (ref. 4). Mice have two functional genes encoding IFN-λ (Ifnl2 and Ifnl3) and two Ifn1 pseudogenes Ifn1l-P1 and Ifn1l-P2 (Fig. 1). The IFN-λ receptor complex is composed of the specific IFN-λ receptor chain 1 (IFN-λR1 (IL28RA)) and the shared IL-10 receptor chain 2 (IL-10R2 (IL-10Rβ)).

IFN-λ receptor engagement and signaling

Engagement of the IFN-λ receptor complex by any of the four ligands leads to activation of the receptor-associated tyrosine kinases JAK1 and TYK2, which then phosphorylate specific tyrosines in the intracellular domain of the receptor (Fig. 2). This event creates docking sites for STAT1 and STAT2 signaling molecules, which leads to their recruitment and subsequent phosphorylation1,2,5. The phosphorylated STATs recruit IFN regulatory factor 9 (IRF9), which together form IFN-stimulated gene factor 3 (ISGF3), which enters the nucleus and drives the transcription of IFN-stimulated genes (ISGs). Despite using different receptors, both type I and III IFNs activate ISGF3 (ref. 6) and therefore induce highly similar transcriptional responses6–9. IFNAR2 (part of the type I IFN receptor complex (Fig. 2)) interacts directly with the IFN-induced protein Usp18, which inhibits the response to IFN-α10 but not to IFN-β or IFN-λ11,12, thereby creating, at least in human cells, a negative feedback loop for IFN-α. In accordance with this, IFN-β and IFN-λ elicit prolonged responses in cell cultures, whereas the response to IFN-α is shorter in duration13,14. However, a different study showed an inhibitory effect of Usp18 on IFN-λ induction of some (but not all) ISGs tested in the mouse system13.

The crystal structure of IFN-λ reveals a four-helix bundle structure typical of class II cytokines, with the closest structural homolog of IFN-λ being interleukin 22 (IL-22)16, but it is not clear whether this reflects a common evolutionary origin or convergence necessitated by the fact that both cytokines utilize IL-10R2. The binding site on the IFN-λR1 receptor chain is well conserved among all four IFN-λs16,17, whereas the binding site on IL-10R2 is poorly defined.

Type III IFN responsive tissues

In mice, the type III IFN response is restricted largely to mucosal epithelial tissues, with the lung epithelium responding to both

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Type I and III IFNs18–20 and intestinal epithelial cells responding exclusively to type III IFNs19. Mouse liver shows no expression of IFNAR1 and no responsiveness to IFN-λ in vivo,22,23 but liver cells derived from mice require the IFNAR1 receptor for efficient control of viral replication24. In humans, mucosal epithelial tissues as well as the liver respond to type III IFNs25,26. Type III IFN responses in immune cells are still being investigated and are discussed below. Differential splicing of the IFNL1 gene gives rise to three mRNAs. Variant 1 leads to the expression of the functional receptor; variant 2 lacks a part of the intracellular domain and is nonfunctional; variant 3 encodes only the extracellular part of the receptor27. The product of variant 3 is released from cells and can act as a decoy receptor and potentially downregulate type III IFN responses28, but the physiological role of splice variants 2 and 3 are not yet well established. Interestingly, epigenetic silencing seems to block the induction of IFNL1R1 in nonresponsive tissues29, but more work is needed to understand the mechanisms regulating IFNL1R1 induction and the physiological role of its splice variants.

Expression of type III IFNs

As would be expected from an antiviral cytokine, and much like the type I IFNs, type III IFNs can be induced by a wide range of viruses in different cell types30–32. Type III IFN can be expressed in a variety of primary human cell types of the hematopoietic lineage33–37, but these cell types also produce type I IFN in abundance. Among nonhematopoietic cells, epithelial cells are potent producers of type III IFNs38–40. In mouse models, type III IFNs seem to be the primary type of IFN found in the bronchoalveolar lavage in response to influenza A virus infection41.

The induction of IFNs is mediated by pattern-recognition receptors that recognize the invading virus and initiate a transcriptional response through the transcription factors NF-κB, IRF3 and IRF7 (ref. 42). Early studies on the genes encoding type III IFNs have shown that they have binding sites for the transcription factors NF-κB, IRF3, IRF7 and AP-1 in their promoter regions43–44 and can therefore be coexpressed with type I IFNs. Additionally, it was suggested that induction of IFNL1 resembles that of IFNB1, as it seems to be well induced by both IRF3 and IRF7, whereas IFNL2 and IFNL3, similarly to IFNA, are more dependent on IRF7 and seem to have delayed expression kinetics.

It later became clear that the expression of type I and type III IFNs is not regulated by identical mechanisms and could differ among the cell types and stimuli tested. IFN11, unlike IFNB1, possesses a cluster of distal NF-κB sites that are necessary for maximal IFNL1 transcription and could stimulate IFNL1 gene induction in an IRF-independent manner45. In colon and respiratory epithelial cells, ZEB1 was identified as a selective repressor of IFNL1 transcription, suggesting a key difference between regulation of type I and III IFN expression46,47. Furthermore, induction of type III IFNs can also be initiated via the signaling adaptor MAVS when it is associated with peroxisomes48. The involvement of peroxisomes in the induction of type III IFNs is interesting, as RIG-I–like receptor signaling via MAVS on peroxisomes does not drive the induction of type I IFNs but induces expression of ISGs49. In human hepatocytes infected with HCV or treated with poly(I:C), the induction of IFNL2 and IFNL3 was dependent on IRF3 and IRF7, whereas the induction of IFNL1 was also dependent on NF-κB50. Finally, another group has identified Med23, a subunit of the mediator complex51, as a direct interaction partner for IRF7 (ref. 52). Med23 and IRF7 synergistically increase IFNL1 transcription but have no effect on IFNB1.

Type I and type III IFNs and the mucosal immune response

The role of type III IFN has been assessed in a number of viral infections, either through the addition of recombinant IFN-λ or in IFN-λ–deficient mice. The redundant and unique roles of type I and type III IFNs have also been investigated. Not surprisingly, viral tropism is the major determinant of the relative contribution of each IFN type. Gut epithelial cells respond exclusively to type III IFN21, and the type III IFN system mediates control of epitheliotropic viruses, such as rotaviruses, in a nonredundant fashion21. Reoviruses initiate their infection in the gut epithelia but can penetrate the epithelial layer and infect cells in the lamina propria or cause a systemic infection in mice. Type III IFN restricts the initial replication in the gut epithelium and diminishes the shedding of virus through feces.
but the type I IFN system is indispensable for the prevention of systemic infection. Thus, both IFN systems are required in a nonredundant fashion for control of reovirus infection. Similarly, in norovirus infection, type I IFN restricts systemic spread of the virus, but virus control in the gastrointestinal tract is achieved only in the presence of type III IFN signaling. Surprisingly, type III IFN can eliminate norovirus even in the absence of an adaptive immune system. The protective effect of type III IFN is counteracted by gut commensals, which explains why wild-type mice with a functional type III IFN system are still susceptible to norovirus. Sterilizing the gut by antibiotic treatment renders wild-type mice with a functional type III IFN system are still susceptible to norovirus. This indicates a higher degree of redundancy of the type I IFN system is indispensable for virus control in the gastrointestinal tract is achieved only in the presence of type III IFN signaling. Surprisingly, type III IFN can eliminate norovirus even in the absence of an adaptive immune system. The protective effect of type III IFN is counteracted by gut commensals, which explains why wild-type mice with a functional type III IFN system are still susceptible to norovirus. Sterilizing the gut by antibiotic treatment renders wild-type mice with a functional type III IFN system are still susceptible to norovirus.

The compartmentalization of type I and III IFNs is less black and white in the respiratory tract, where there is a degree of redundancy between the two IFN systems. Studies on mice deficient in IFN-α1, IFNAR1 or both showed that mice lacking both (receptor double-deficient mice) were highly susceptible to respiratory infections, whereas single-deficient mice were largely as resistant as wild-type mice. This indicates a higher degree of redundancy of the two IFN systems in the respiratory tract than in the gastrointestinal tract. Infection of airway epithelial cells in vitro and subsequent measures of the IFN-induced transcriptional signature by microarray showed that the influenza-induced IFN signature was abolished only in receptor double-deficient epithelia, thus confirming the redundancy observed in vivo. Thus, airway epithelia express receptors for both IFN types, whereas some or all gut epithelial cells seem to express only IFN-α receptors.

It remains to be seen whether there are niches in the respiratory tract where cells express only IFN-α1 and where type III IFNs have unique roles, as in the gut. One may speculate that the gut, with its high risk of uncontrolled inflammation owing to the vast burden of microbe-associated molecular patterns derived from commensals, is a compartment where it is desirable to keep local responses ‘below the radar’ of systemic immunity. In contrast, in the lungs, which are not sterile but certainly have a bacterial burden several orders of magnitude below that of the intestines, the compartmentalization of the two IFN systems is less strict. During influenza virus infection, mouse airway epithelia produce higher amounts of type III IFNs than type I IFNs. Therefore, as long as lung epithelial cells are the main IFN producers, the response may be dominated by type III IFNs. However, once immune cells start producing IFN, the response may shift toward being driven by type I IFN. Thus, the division of labor between type III and type I IFNs in the lung might not be as strict as in the gut but seems to follow a similar pattern.

Taken together, observations in the mouse model paint a clear picture of a type III IFN defense system that is active at the borders, defending the mucosal lining against the frequent challenge from viruses. It does so at a substantially lower risk of immune-associated pathology than the type I IFN system would impose, as discussed below. We believe that the lessons learned from the mouse model hold largely true for humans, where type III IFN has an extended role providing protection also against hepatotropic viruses.

**Effects of type III IFN on immune cells**

Although it is largely accepted that IFN-α1 expression is more restricted than that of IFNAR1 and IFNAR2, there is still some controversy about which immune cells produce or respond to type III IFN and what functional consequences type III IFN signaling has in immune cells. A consensus is emerging showing that human and mouse plasmacytoid dendritic cells (pDCs), as well as some conventional DC subsets (BDCA3+ in humans and CD8+ in mice), produce type III IFNs. Similarly, only some subsets of myeloid immune cells, such as human monocyte-derived macrophages, produce type III IFNs and some dendritic cell subsets in humans and in mice, respond directly to type III IFN. For natural killer (NK) cells, reports have come to opposite conclusions about the ability of type III IFNs to influence IFN-γ secretion by NK cells. However, adoptive transfer experiments in mice using IFN-α1–deficient (Ifnar1−/−) NK cells...
concluded that direct type III effects on NK cells are required for maximal production of IFN-γ and antitumor activity. Given the controversial findings, there are essentially three possibilities. One is that the type III IFN effects on NK cells are detected only in combination with other stimuli. A second is that only specific NK cell maturation stages are responsive to type III IFN. Or third, the effects of type III IFN observed are indeed indirect.

Finally, type III IFN can suppresses IL-1 and IL-17 responses as well as neutrophil recruitment in arthritis and other inflammation models, suggesting a direct effect on neutrophils, which express IFN-AR1. These effects are reminiscent of the antagonism between type I IFN and IL-1 described in tuberculosis. Effects on T cells and B cells were also reported, but it is still unclear whether these effects are direct or indirect through type III IFN–induced changes in antigen-presenting cells, as described above. Several reports suggest that type III IFN ‘favors’ IL-12 induction, which indicates a role in promoting type 2 over type 2 immune responses, and might be helpful for the induction of strong antiviral responses from type 1 helper T (Th1) cells and CD8 T cells.

Owing to the restricted IFN-AR1 expression by immune cells, the immunomodulatory effects of type III IFNs are limited, which is in contrast to the ubiquitous activity of type I IFN during responses to infection. The wider range of activity of type I IFN is crucial for the control of systemic viral infection, but the risk of increased disease severity is always looming. Multiple reports describe the deleterious effects of inappropriate type I IFN responses during infection, including massive induction of proinflammatory cytokines and production of apoptosis-inducing molecules on immune cells during acute infection, and also in chronic disease, where the source of disease-promoting type I IFN often appears to be pDCs. Furthermore, the blockade of adaptive immune responses by excessive production of type I IFN during chronic infection has been described in detail for T cells, and to a lesser degree for B cells.

These findings all point in a similar direction: type III IFN is the antiviral weapon of choice when a local mucosal response is sufficient to control the virus and when immune-mediated inflammation is a real risk. Only in severe or systemic infections would it be appropriate to ‘pull out the big guns’, with a systemic response and strong immune activation, with the associated risk of immune-mediated damage or paralysis of adaptive immunity that may compromise or kill the infected organism (Fig. 3).

**Type III IFN and coinfection**

Type I IFN can exaggerate disease and impair clearance in the case of infection with bacteria such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*, most probably due to type I IFN suppression of macrophage activation by IFN-γ. In human, macrophages, IFN-γ and TLR activation synergize to activate a series of important antibacterial molecules, and this is antagonized by type I IFN. However, type III IFN acts in a markedly different manner, by increasing macrophage responsiveness to IFN-γ. The net result is that type III IFN ‘favors’ the production of Th1 cytokines such as IL-12, as described above. This is interesting, as most comparisons between type I IFN– and type III IFN–mediated gene induction show no or only minor quantitative differences. However, further studies are required in this area.

The difference between the effects of type I and type III IFNs might be important especially in the context of viral-bacterial coinfection, which is seen frequently during influenza virus infections, for example, and can lead to severe disease. Therefore, type III IFN is keeping the borders clear of viral infection without causing widespread immune activation, but it could also be better than type I IFN in steering clear of interfering with the antibacterial immune responses required during polymicrobial exposure. Furthermore, it needs to be explored whether type III IFN interferes with Th17 responses, which is particularly important during coinfections involving viruses and extracellular bacteria.

**Why does IFN-λ4 impair clearance of HCV?**

HCV causes chronic infection in approximately 75% of people, whereas the remaining individuals manage to clear the infection within the first year. The host genetic background is important for both spontaneous and treatment-induced clearance of the virus. In 2009, several independent consortia, using rather different HCV-infected cohorts, mapped the major genetic determinant of HCV clearance in response to treatment with IFN-α plus ribavirin to the type III IFN loci. The same association was found in spontaneous clearance of the virus. However, different single nucleotide polymorphisms (SNPs) were identified as the best predictor of treatment outcome: rs8099917 (shortened hereafter to 917) best predicted the outcome in Asians and people of European descent, whereas rs12979860 (shortened hereafter to 860) was the best predictor in a US cohort of mixed ethnicity. Both SNPs were originally described as IL28B-related (i.e., IFNL3-related), and they...
were surprisingly not in complete linkage disequilibrium (meaning that they are not inherited together), as would be expected if they represented the same underlying causative genetic defect. What was more surprising was that only one of the SNPs identified was located within the encoding regions of known type III IFNs (changing Lys70 in IFN-λ3 to arginine, which had no effect on the function of IFN-λ3)92. These findings led to the speculation that these SNPs represented changes in the IFNL3 promoter region and caused differences in IFN-λ3 expression. However, subsequent studies came to divergent conclusions about the SNPs’ influence on IFN-λ3 expression, and this issue is still not fully resolved.

A breakthrough came with the discovery of the IFNL4 gene situated within the region determining HCV clearance. It was found that the SNP rs368234815 was superior to SNP 860 in predicting treatment outcome in individuals of African ancestry. The TT allele of rs368234815 disrupts the open reading frame of IFN-λ4 and is protective in terms of HCV, whereas the ancestral ‘ΔG’ allele encodes a functional IFN-λ4 and impairs HCV clearance. This finding suggests that IFN-λ4 is the causative agent of HCV clearance failure, but it does not explain why the SNP 917 is a powerful predictor of the treatment outcome in several other populations. The discovery of a second SNP acting in combination with the ΔG SNP resolved this discrepancy. The SNP rs117648444 (shortened hereafter to 444) represents a nonsynonymous change in the coding region of IFN-λ4, where a proline residue is replaced by serine, resulting in two versions of IFN-λ4: the fully active IFN-λ4–P70 and a much less active IFN-λ4–S70 (ref. 93). By combining the ΔG and 444 SNPs, one can stratify patients into three groups: (i) those having no IFN-λ4, (ii) those having IFN-λ4–P70 and (iii) those having IFN-λ4–S70. Compared to the single SNPs described previously, this stratification led to a significant improvement in the predictive power of genotyping and reflects the existence of a set of distinct haplotypes in humans (a haplotype is a set of tightly linked alleles that are likely to be inherited together). Before the discovery of the IFNL4 gene, elegant work identified the probable causative haplotype by massive parallel sequencing94. In people of European ancestry, this haplotype is specifically tagged by SNP 917 and encodes the IFN-λ4–P70 variant, whereas the 860 SNP marks several haplotypes encoding both the P70 and S70 variants of IFN-λ4 but not the frameshift mutation. In both cases, the patient group having IFN-λ4–S70 is somewhat neglected, and the apparent discrepancy can be resolved by genotyping and stratification as described above. Notably, this has been done only for one European HCV-infected cohort so far, and its repetition in different cohorts will be important. In particular, the impact of the IFN-λ4–S70 variant might differ among diseases and ethnic backgrounds. In summary, the higher rate of HCV clearance in patients encoding the IFN-λ4–S70 variant than in patients with IFN-λ4–P70 strongly suggests that the activity of the IFN-λ4 protein causes, by yet unknown means, poor HCV clearance.

The effect of IFNL4 genotype is not restricted to IFN-based therapies for HCV; it also extends to direct-acting antiviral-based treatments95–99. Furthermore, IFNL4 genotype also influences the reactivation of cytomegalovirus (CMV) in immune-suppressed patients100,101. The data are clear but paradoxical. IFN-λ4 signals through the same receptor that other members of the type III IFN family do, and its effect is highly similar. Furthermore, IFN-λ4 is antiviral in vitro102. Nevertheless, having a functional IFNL4 gene renders humans less capable of clearing chronic infections such as HCV and CMV. It is likely that IFN-λ4 directly or indirectly influences inflammatory responses and, thereby, viral clearance. Several studies have reported increased liver inflammation and fibrosis103–106 and increased degranulation activity of lymphocytes107 in HCV patients with the protective IFNL4 genotype (the TT allele, which destroys the open reading frame of IFNL4), suggesting that IFN-λ4 impairs HCV clearance but diminishes liver inflammation and fibrosis. Other studies did not find any association or had directly conflicting data but used smaller cohorts. Interestingly, a study has found that decreased liver inflammation and fibrosis was associated with having a functional IFNL4 gene in a nonalcoholic fatty liver disease cohort, indicating that the effect of IFN-λ4 on inflammation could be independent of viral infection104. However, this finding needs confirmation from other studies. We are currently incapable of offering a mechanistic explanation.
The IFNL4 gene became a liability during human evolution
A report identified IFN-λ4 sequences in most mammalian species, with the notable exception of rodents. Phylogenetic analyses (Fig. 4) reveal that the IFN-λ4 family constitutes a separate clade in the type III IFN tree. In contrast, IFN-λ1–3 do not form separate clades but group according to species. This suggests that the ancestor of mammals had an IFNL4-like gene and a second gene that was independently duplicated several times during mammalian evolution, giving rise to IFNλ1, IFNλ2 and IFNλ3. Thus, mouse IFN-λ2 cannot be considered a strict ortholog of human IFN-λ2, etc., similarly to what is seen for IFN-α.

It is worth noting that in humans, IFN-λ4 shares only ~30% identity to IFN-λ3, but despite the low sequence conservation, it has preserved its ability to signal. This shows that until the appearance of the ‘TT-pseudogenizing’ allele in humans, IFN-λ4 was under purifying selection to preserve its ability to signal via the IFN-λ receptor complex. This was confirmed by a bioinformatics analysis that found clear purifying selection acting on all nonhuman IFNL4 genes. The frameshift mutation in human IFNL4 was introduced approximately 55,000 years ago, just before the ‘Out of Africa’ scenario, and was positively selected almost immediately. This observation raises several interesting questions. Why did IFNL4 suddenly become a liability? What drove (and still drives) the pseudogenization of IFNL4? Importantly, IFNL1R1 exhibits a selection against nonsynonymous substitutions, showing a clear evolutionary pressure in favor of maintaining a functional type III IFN system while specifically eliminating the IFNL4 gene.

Clinical application of type III IFN
PEGylated IFN-λ1 first entered into clinical trials against HCV, and the initial trial design aimed at replacing PEGylated IFN-α with pegylated IFN-λ1. A successful phase II trial showed that IFN-λ1 was as effective or more so than PEGylated IFN-α and had significantly fewer hepatoxic adverse events. However, the successful development of several direct-acting antivirals is changing the therapeutic landscape for HCV, and IFN is likely to have a less dominant role in the future. However, several phase III trials are currently underway in which PEGylated IFN-λ1 is combined with direct-acting antivirals (telaprevir, asunaprevir or daclatasvir). Type III IFN could represent an attractive therapeutic option for the treatment of several direct-acting antivirals is changing the therapeutic landscape for HCV, and IFN is likely to have a less dominant role in the future. However, several phase III trials are currently underway in which PEGylated IFN-λ1 is combined with direct-acting antivirals (telaprevir, asunaprevir or daclatasvir). Type III IFN could represent an attractive therapeutic option for the treatment of several direct-acting antivirals.

Concluding remarks
Type III IFN clearly has an important role in protecting the epithelial surfaces from viral infection, but a number of interesting issues remain to be addressed. Given that lung epithelia respond to both type I and type III IFN, what is the distribution of work between these two systems in the respiratory epithelia? Humans are frequently challenged by several infections caused by viruses such as influenza virus or coronaviruses (telaprevir, asunaprevir or daclatasvir). Type III IFN could represent an attractive therapeutic option for the treatment of several direct-acting antivirals. The authors declare no competing financial interests.

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