

Interferon lambda 4 signals via the IFN λ receptor to regulate antiviral activity against HCV and coronaviruses

Ole J Hamming¹, Ewa Terczyńska-Dyla¹,
Gabrielle Vieyres², Ronald Dijkman³,
Sanne E Jørgensen¹, Hashaam Akhtar¹,
Piotr Siupka¹, Thomas Pietschmann²,
Volker Thiel^{3,4} and Rune Hartmann^{1,*}

¹Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark, ²Institute of Experimental Virology, TWINCORE-Centre for Experimental and Clinical, Infection Research, Hannover, Germany, ³Institute of Immunobiology, Kanton Hospital, St. Gallen, Switzerland and ⁴Vetsuisse Faculty, University of Zürich, Zürich, Switzerland

The *IFNL4* gene is a recently discovered type III interferon, which in a significant fraction of the human population harbours a frameshift mutation abolishing the IFN λ 4 ORF. The expression of IFN λ 4 is correlated with both poor spontaneous clearance of hepatitis C virus (HCV) and poor response to treatment with type I interferon. Here, we show that the *IFNL4* gene encodes an active type III interferon, named IFN λ 4, which signals through the IFN λ R1 and IL-10R2 receptor chains. Recombinant IFN λ 4 is antiviral against both HCV and coronaviruses at levels comparable to IFN λ 3. However, the secretion of IFN λ 4 is impaired compared to that of IFN λ 3, and this impairment is not due to a weak signal peptide, which was previously believed. We found that IFN λ 4 gets N-linked glycosylated and that this glycosylation is required for secretion. Nevertheless, this glycosylation is not required for activity. Together, these findings result in the paradox that IFN λ 4 is strongly antiviral but a disadvantage during HCV infection.

The EMBO Journal (2013) 32, 3055–3065. doi:10.1038/emboj.2013.232; Published online 29 October 2013

Subject Categories: immunology; microbiology & pathogens

Keywords: coronavirus; genetics; hepatitis C virus; interferon lambda; interferon therapy

Introduction

Type III interferon or interferon lambda (IFN λ) is a recently discovered group of interferons (Dumoutier *et al*, 2003; Kotenko *et al*, 2003; Sheppard *et al*, 2003). Although IFN λ s are clearly interferons (Ank *et al*, 2006; Doyle *et al*, 2006; Zhou *et al*, 2007), they signal via a complex consisting of the IFN λ R1 and IL-10R2 receptor chains and share both structural features and the IL-10R2 chain with the IL-10 family of cytokines (Gad *et al*, 2009). Type III interferons distinguish

themselves in being highly tissue specific. The IFN λ R1 receptor chain is expressed on cells of epithelial origin and a yet not clearly defined small subset of haematopoietic cells (Mennechet and Uze, 2006; Mordstein *et al*, 2010; Pott *et al*, 2011). The liver is of particular interest to this report. In humans, hepatocytes express IFN λ R1, and thus respond to IFN λ (Dickensheets *et al*, 2013; Wang *et al*, 2013). Humans possess four IFN λ genes (*IFNL1*, *-L2*, *-L3* and *-L4*) as well as a pseudogene (*IFNL3P1*) (Lasfar *et al*, 2006; Fox *et al*, 2009). Whereas the *IFNL1*, *-L2* and *-L3* genes were described in 2003 (Kotenko *et al*, 2003; Sheppard *et al*, 2003), the *IFNL4* gene was described recently and the *IFNL4* gene has been inactivated in large part of the human population by a frameshift mutation (Prokunina-Olsson *et al*, 2013). Phase 2 of clinical trials using pegylated IFN λ 1 against hepatitis C virus (HCV) infection has recently been completed (Ramos, 2010), and it has now entered the phase 3 trials. IFN λ s are interesting pharmaceuticals, as the rather specific expression pattern of the IFN λ R1 receptor should reduce the adverse effects compared to the type I IFN treatment.

The responses to the current standard treatment for HCV infection, which consists of pegylated interferon- α 2 combined with ribavirin (pegIFN- α 2 RBV), depend both on the viral genotype and on the genetics of the patient. Rather unexpectedly, single-nucleotide polymorphisms (SNPs) located within and around the *IFNL3* gene were discovered as powerful predictors of treatment outcome as well as the likelihood for spontaneous clearance of the virus (Ge *et al*, 2009; Thomas *et al*, 2009). Extensive studies of the genetic region around the *IFNL3* gene revealed the existence of a novel gene, the *IFNL4* gene, which harbours a dinucleotide variant (ss469415590, TT or Δ G), where the TT allele leads to a frameshift thus inactivating the gene, and the Δ G allele results in a functional *IFNL4* gene (Prokunina-Olsson *et al*, 2013). In humans, the TT allele is strongly positively associated with HCV clearance as well as with positive treatment outcome (Bibert *et al*, 2013; Prokunina-Olsson *et al*, 2013). Thus, disruption of the *IFNL4* gene is beneficial for humans in the context of HCV infection, though the reason for this remains unclear.

The transfection of cells with an expression plasmid encoding IFN λ 4 induced STAT1 and STAT2 phosphorylation, but the authors were unable to detect any significant secretion of the IFN λ 4 protein, which was ascribed to a very weak signal peptide (SP) in IFN λ 4 (Prokunina-Olsson *et al*, 2013). In addition, the authors produced recombinant IFN λ 4 inactive protein using insect cells. However, this protein was purified from cell lysates and not from the media as it is normally done with secreted proteins, and it appears likely that the protein was not properly folded. The lack of IFN λ 4 secretion together with the clear observation of intracellular IFN λ 4 protein led to the suggestion that IFN λ 4 could signal via an intracellular receptor (Booth and George, 2013;

*Corresponding author. Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds vej 10, Aarhus 8000, Denmark. Tel.: +45 28992578; Fax: +45 86123178; E-mail: rh@mb.au.dk

Received: 8 July 2013; accepted: 30 September 2013; published online: 29 October 2013

Lupberger *et al*, 2013; Ray, 2013). Furthermore, the sequence of IFN λ 4 is similar to other IFN λ s within the first and last helices, which bind IFN λ R1, while the IL-10R2 binding region is poorly conserved. Thus, the authors questioned whether IFN λ 4 actually signals through IL-10R2.

We have expressed, purified and refolded IFN λ 4 from *E. coli* and show that this recombinant protein is active and signals via IFN λ R1 and IL-10R2, as do the other members of the type III interferon family. Furthermore, we show that IFN λ 4 has antiviral activity in human hepatocytes against HCV and in primary human airway epithelia (HAE) cells against human coronavirus strain 229E (HCoV-229E) as well as the novel coronavirus MERS-CoV. We demonstrated that IFN λ 4 gets secreted from mammalian cells, but with a substantially lower efficiency than what is seen for IFN λ 3. Our data suggest that the poor secretion of IFN λ 4 is not just a consequence of the weak IFN λ 4 SP, but it might be connected with the glycosylation of IFN λ 4.

Results

IFN λ 4 expression and purification

To investigate the properties of IFN λ 4, we cloned a codon-optimised cDNA encoding the mature form of human IFN λ 4 with an N-terminal 6 \times His tag followed by a tobacco etch virus (TEV) protease cleavage site into a pET-15b vector. This recombinant form of IFN λ 4 was expressed in *E. coli* and purified from inclusion bodies under denaturing conditions by metal-ion affinity chromatography. The protein was then refolded *in vitro* and purified to homogeneity by cation exchange chromatography (Figure 1A) followed by size-exclusion

chromatography (Figure 1B) (Dellgren *et al*, 2009). IFN λ 4 was eluted from the size-exclusion chromatography column at \sim 75 ml, consistent with the expected monomeric size of IFN λ 4. The purified protein has a size of 17 kDa (Figure 1C) corresponding to IFN λ 4 without the SP (residues 23–179 of IFN λ 4) (NCBI accession code AFQ38559).

Recombinant IFN λ 4 signals through IFN λ R1 and IL-10R2

The effect of the recombinant IFN λ 4 was tested in HL-116 cells. These cells were stably transfected with IFN λ R1 and a luciferase reporter under the control of the IFI6 promoter (Uze and Monneron, 2007). Recombinant IFN λ 4 is highly active and activates the IFI6 promoter in a concentration-dependent manner comparable to IFN λ 3 (Figure 2A). Further, we verified the activity of IFN λ 4 in HepG2 cells, which express IFN λ R1 naturally. HepG2 cells were treated with IFN α 2, IFN λ 3 or IFN λ 4, and the induction of the well-known interferon-stimulated genes (ISGs) *MX1*, *IFIT1* and *OASL* was monitored by qPCR (Figure 2B). All three interferons clearly induced all three genes. In fact, we observed comparable induction by IFN λ 3 and IFN λ 4. Recombinant IFN λ 4 is thus a highly active interferon.

To determine the receptor complex utilised by IFN λ 4, we used HEK293 cells. These cells express low levels of IFN λ R1 and normally respond very poorly to type III interferon (Meager *et al*, 2005). They do, however, express IL-10R2. In our assay, we introduced a luciferase reporter under the control of the Mx1 promoter in order to measure the interferon activity, and at the same time, we introduced IFN λ R1 by transfection and/or knocked down IL-10R2, using specific siRNA (Figure 2C). The expression of IFN λ R1 by transfection renders them highly responsive to both IFN λ 4 and IFN λ 3. However, this signal is largely lost when IL-10R2 is knocked down using siRNA (Figure 2C). The IFN α -mediated signalling was not significantly affected by either overexpression of IFN λ R1 or knock-down of IL-10R2. To confirm these results, we repeated the experiment now blocking IL-10R2 with a specific antibody which has previously been shown to block IL-10R2 signalling in relation to IFN λ (Sheppard *et al*, 2003). The IL-10R2 antibody did not result in any activation of the reporter gene on its own, but both IFN λ 3 and IFN λ 4 signalling were sensitive to the IL-10R2 antibody, whereas IFN α signalling was unaffected. These results conclusively demonstrate that IFN λ 4, like the other members of the type III interferon family, signals via a heterodimeric receptor complex consisting of IFN λ R1 and IL-10R2.

Evaluating IFN λ 4 binding to IL-10R2 using structural modelling

Since IFN λ 3 and IFN λ 4 interact with the same receptor complex, we made a sequence alignment in Clustal W (Figure 3A) and generated a homology model of IFN λ 4 (homIFN λ 4) using the SWISS-MODEL Workspace with IFN λ 3 as a model (Figure 3B). The overall structure of homIFN λ 4 is similar to that of IFN λ 3 (Figure 3C), as is expected for a homology model. The following observations validate the accuracy of the model. Cys76 and Cys178, which are not present in IFN λ 3, are in close proximity in homIFN λ 4, and with minor local rearrangements, they could form a disulphide bridge (Figure 3B). Furthermore, two conserved disulphide links are expected to exist in IFN λ 4, connecting

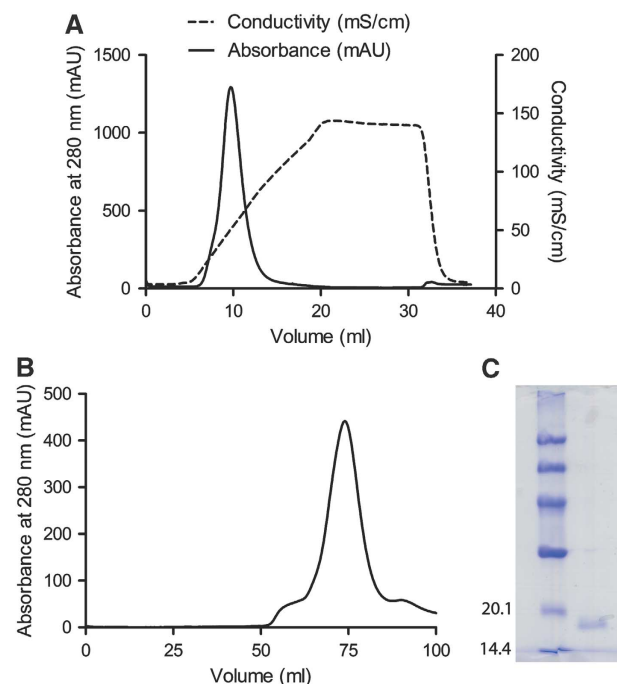


Figure 1 Purification of recombinant human interferon lambda 4. (A) Refolded IFN λ 4 was loaded on an anion exchange column (5 ml Hi trap SP FF). The protein was eluted from the column using a salt gradient and the fractions of the peak at \approx 10 ml were collected. (B) The fractions collected from the ion exchange column were applied to a gel filtration column (Hi load Superdex 75). The fractions around the peak at \approx 75 ml were pooled. (C) A Coomassie stained 12% SDS-PAGE gel of the purified protein.

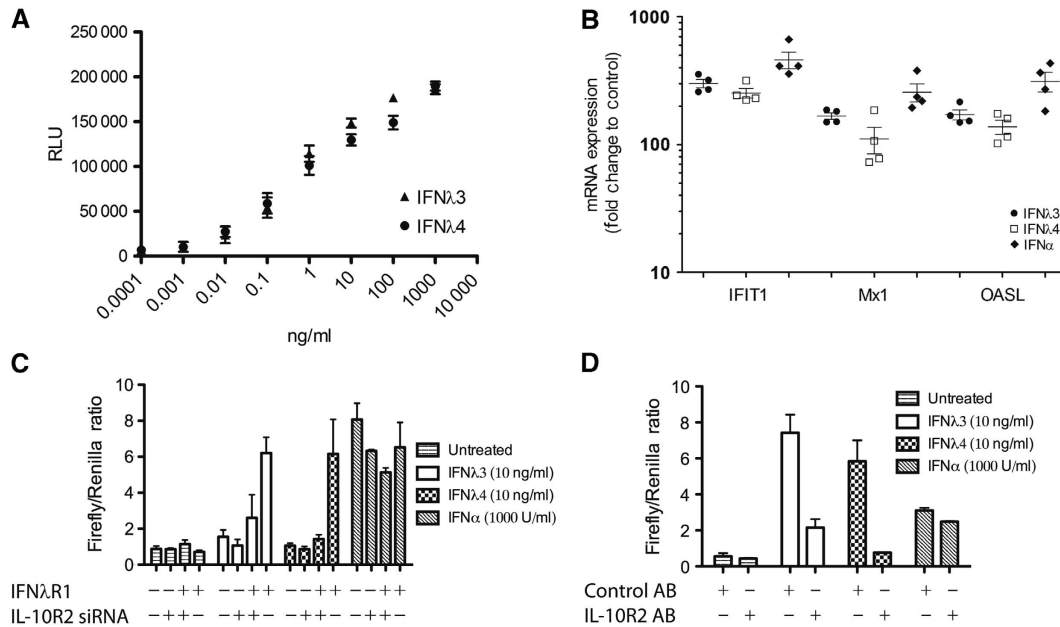


Figure 2 Activity of recombinant human interferon lambda 4. (A) The activity of IFN λ 4 was tested and compared to IFN λ 3 in HL-116 reporter cells. Dose response of IFN λ 3 and IFN λ 4 was performed in triplicate. Mean and s.d. are shown. (B) HepG2 cells were treated with IFN α (1000 U/ml), IFN λ 3 (10 ng/ml) or IFN λ 4 (10 ng/ml). After 4 h, the level of the interferon-induced genes, *IFIT1*, *MX1* and *OASL*, was quantified by qPCR, four independent experiments are shown, mean and s.e.m. are plotted. (C) HEK293 cells were transfected with the IFN λ R1 and/or treated with siRNA against IL-10R2 as indicated. The cells were also transfected with *Renilla* and Firefly luciferase reporter constructs. The Firefly construct is IFN inducible whereas the *Renilla* is constitutively expressed. The cells were subsequently either treated with IFN λ 3, IFN λ 4 or left untreated, treatment with IFN α was performed as a control. (D) HEK293 cells were transfected with IFN λ R1 as well as *Renilla* and Firefly luciferase reporter constructs (as in C). The cells were then treated with anti IL-10R2 antibody or control antibody followed by interferon treatment as indicated.

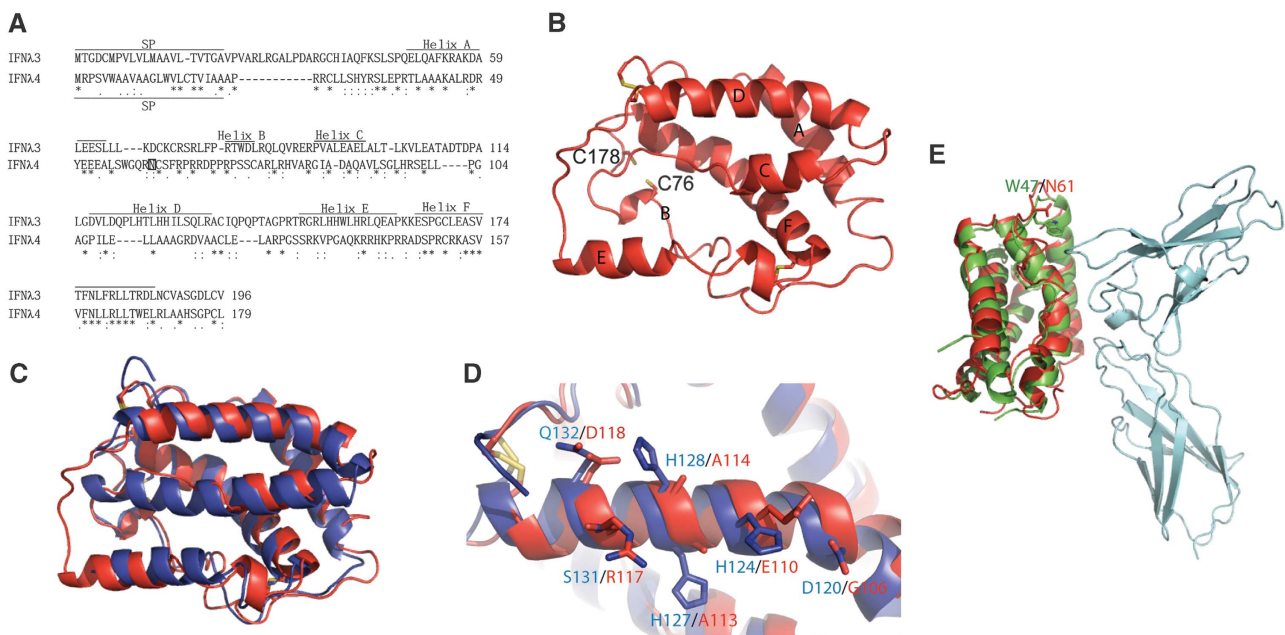


Figure 3 Homology model of human interferon lambda 4. (A) Alignment of IFN λ 3 and IFN λ 4 using Clustal W. The position of the signal peptide (SP) in IFN λ 3 and in the model of IFN λ 4 shown in (B) are shown. A possible N-linked glycosylation site at Asn61 is marked by a square in IFN λ 4. (B) Homology model of IFN λ 4 (homIFN λ 4) generated by the Swiss model server using IFN λ 3 (PDB entry code HHC3) as a template. The position of the individual structural elements is denoted as A-F. The cysteines are shown as yellow sticks. The positions of C76 and C178 that could form a disulphide bridge specific for IFN λ 4 are shown. (C) Superimposition of IFN λ 3 (blue) and homIFN λ 4 (red). (D) Comparison of helix D of IFN λ 3 (blue) and homIFN λ 4 (red). The residues that are expected to interact with IL-10R2 are labelled in blue for IFN λ 3 and red for IFN λ 4. Disulphides are shown in yellow. (E) Superimposition of homIFN λ 4 (red) onto IFN λ 1 (green) bound to IFN λ R1 (cyan). N61 of homIFN λ 4 (red) and W47 of IFN λ 1 (green) are shown in sticks and labelled.

Cys27 to Cys122 and Cys62 to Cys152. In both cases, homIFN λ 4 is compatible with the formation of these disulphide links. Moreover, superimposing homIFN λ 4 onto the structure of IFN λ 1 bound to IFN λ R1 clearly shows that the homIFN λ 4 structure is compatible with the IFN λ R1 binding.

As noted by Prokunina-Olson *et al*, the residues in helices A and F, which bind IFN λ R1, are well conserved between IFN λ 3 and IFN λ 4, whereas the D-helix, which is expected to bind IL-10R2, is quite different (Figures 3A and D). Yet our data clearly show that both IFN λ 3 and IFN λ 4 use IFN λ R1 and IL-10R2 for signalling. The model of IFN λ 4 suggests a conservation of the helical structure and the way this is presented to IL-10R2. The conserved residues in helix D are primarily hydrophobic residues, which dock helix D to the rest of the structure and thus, determine the steric conformation of this helix. This conservation is most likely crucial for the activation as both receptor chains need to be engaged simultaneously. It is, however, important to remember that IL-10R2 is a shared chain that is capable of binding several different cytokines (IL-10, IL-22 and IL-26 and the IFN λ s). The chain is thus, promiscuous, allowing itself to interact with different ligands (Logsdon *et al*, 2012).

To evaluate whether the structure of homIFN λ 4 is compatible with binding to IFN λ R1, we superimposed the structure of homIFN λ 4 onto the structure of IFN λ 1 in the IFN λ 1: IFN λ R1 complex (PDB entry code: 3OG6). Figure 3E shows that the overall structure of homIFN λ 4 is very similar to IFN λ 1 in the receptor-bound conformation and there are thus no obvious reasons why IFN λ 4 would not bind IFN λ R1. The glycosylation site N61 in IFN λ 4 is equivalent to W47 in IFN λ 1. W47 interacts weakly with IFN λ R1, but is located at

the periphery of the interaction site away from the membrane and is situated in a loop between the A- and B-helices (Miknis *et al*, 2010). We believe that this position offers sufficient flexibility to allow for simultaneous glycosylation of N61 and receptor binding.

IFN λ 4 possesses strong antiviral activity

As the ss469415590 SNP Δ G leading to the expression of IFN λ 4 is associated with poor spontaneous HCV clearance and a negative response to pegIFN- α /RBV treatment, we decided to test the effect of recombinant IFN λ 4 against HCV infection. Huh7-Lunet hCD81-Fluc cells were transfected with a HCV genome (JcR2a, encoding luciferase as a reporter), and the 4-h post-transfected cells were treated with IFN α , IFN λ 3 or IFN λ 4 for 72 h. All interferon treatments resulted in a concentration-dependent decline in HCV replication (Figures 4A and B). In the Huh7-lunet cells, IFN λ 4 is slightly weaker than IFN α but at the same level as IFN λ 3. The experiment was repeated in HepG2 cells, which were treated with the indicated interferons for 48 h. In HepG2 cells, the antiviral activity of all three interferons is at the same level. Thus, using two different liver cell lines, we do not see any measurable difference between IFN λ 3 and IFN λ 4.

The IFN λ R1 chain is primarily expressed on cells of epithelial origin, and it is thus here that IFN λ mostly exerts its effect. We therefore decided to investigate the effect of IFN λ 4 in an epithelial cell system. For this study, we used primary HAE cultures. This system is based on primary human bronchial epithelial cells grown in air-liquid interface to obtain fully differentiated pseudostratified HAE layers, and it reflects many characteristics of the conducting human

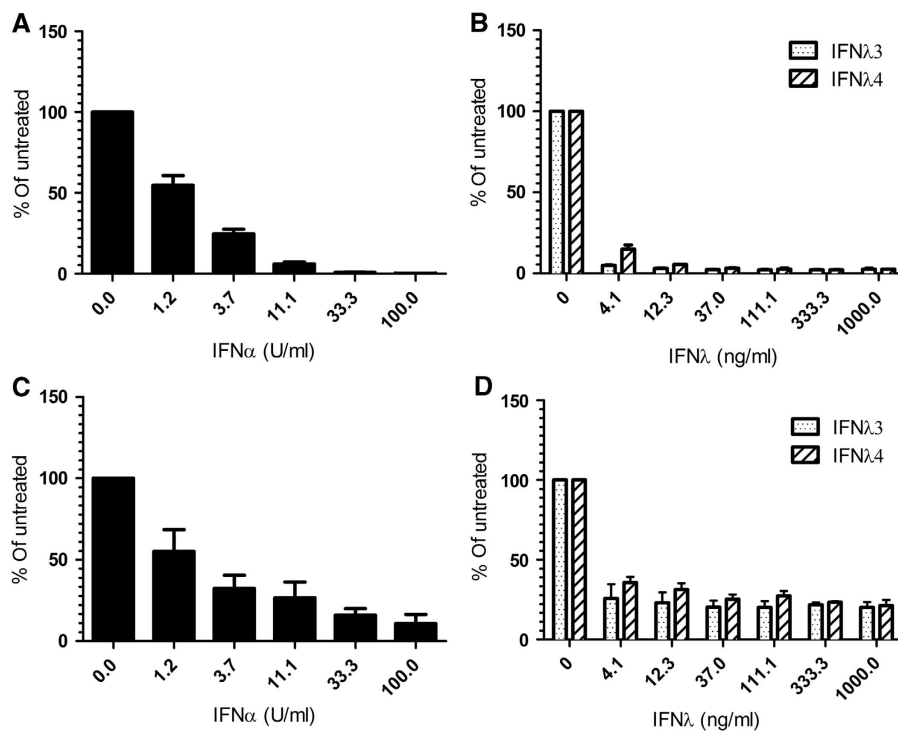


Figure 4 Antiviral effect of recombinant human Interferon lambda 4 against hepatitis C virus. (A, B) The antiviral effect of IFN α (A), IFN λ 3 or IFN λ 4 (B) against replication of the HCV JcR-2a chimaera in the Huh7-Lunet N hCD81-FLuc cell line is shown. (C, D) The antiviral effect of IFN α (C), IFN λ 3 or IFN λ 4 (D) against replication of the HCV JcR-2a chimaera in the HepG2 cell line is shown. The plot shows the average of three independent experiments with the *Renilla* luciferase activity normalised to the untreated control. Background luciferase activity is 0.2% for (A, B) and 2.4% for (C, D).

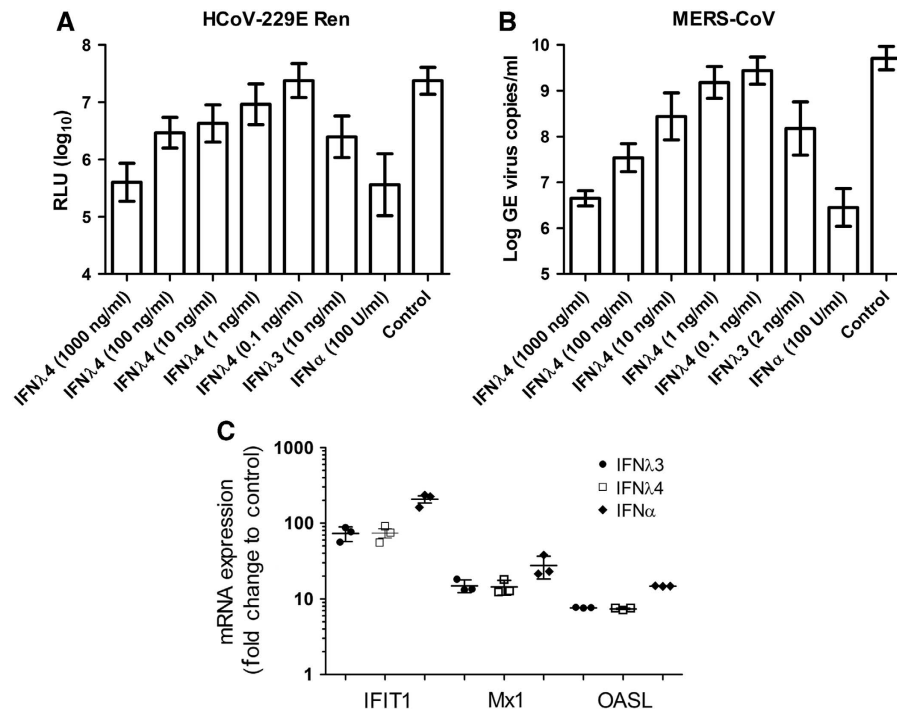


Figure 5 Antiviral effect of IFN λ 4 in human airway epithelial (HAE) cell culture. (A) The antiviral effect of IFN α , IFN λ 3 and IFN λ 4 against the human coronavirus HCoV-229E expressing luciferase was tested in human HAE cultures. For each data point, triplicate measurements were performed on three different donors, mean and s.d. are shown. (B) The antiviral effect of IFN α , IFN λ 3 and IFN λ 4 against the coronavirus MERS-CoV was tested in human HAE cultures. As in (A), experiments were performed in triplicate on each of the three donors, mean and s.d. are shown. (C) HAE cell cultures were treated with IFN α (100 U/ml), IFN λ 3 (10 ng/ml), IFN λ 4 (10 ng/ml) or IFN λ 4 (100 U/ml). After 72 h, the level of the interferon-induced genes *IFIT1*, *MX1* and *OASL* were quantified by qPCR, mean and s.e.m. are indicated.

airways, such as the presence of basal, secretory, columnar and ciliated cell populations and a physical barrier, that is, the mucus (Kindler *et al*, 2013). The HAE represents the entry port of human respiratory virus infection and is especially well suited for investigating the role of IFN λ s. HAE cultures derived from three separate donors were treated with IFN α 2, IFN λ 3 or IFN λ 4 prior to exposure to a human coronavirus 229E expressing luciferase upon replication (HCoV-229E-luc, 4000 plaque-forming units (PFUs)) (van den Worm *et al*, 2012). As can be seen in Figure 5A, treating the HAE culture with IFN α , IFN λ 3 or IFN λ 4 reduces replication of HCoV-229E-luc. IFN α is the strongest interferon, whereas IFN λ 3 and IFN λ 4 are equally strong. In addition, we observed a concentration-dependent effect of IFN λ 4.

We then performed an experiment testing the effect of IFN α 2, IFN λ 3 and IFN λ 4 against the novel and highly pathogenic coronavirus MERS-CoV (4000 PFUs). Again, we observed a concentration-dependent effect of IFN λ 4. To further investigate this effect, we looked at the induction of *Mx1*, *OASL* and *IFIT1* by qPCR in the HAE cells treated with IFN α , IFN λ 3 or IFN λ 4. All three interferons induced all three genes, and the induction by IFN λ 3 and IFN λ 4 is at the same level, whereas IFN α is slightly higher. Thus, there is a good agreement between the antiviral activity measured and the induction of ISGs.

Poor secretion of IFN λ 4 is not due to a weak SP

It was hypothesised by Prokunina-Olson *et al* that poor secretion of IFN λ 4 was due to a non-functional SP. Thus, we made chimaeric proteins of IFN λ 3 with the SP of IFN λ 4 and *vice versa*. HEK293 cells were then transfected with these

constructs, and the protein secretion was evaluated by western blots of both the media and the cells (Figure 6A). For both the MYC- and FLAG-tagged constructs, IFN λ 3 is present in the media regardless of whether it has its own or the IFN λ 4 SP. Contrary to this, IFN λ 4 is not detectable by western blot in the media regardless of the SP. Thus, the poor secretion of IFN λ 4 cannot solely be ascribed to the SP.

In the case of IFN λ 4, we observed two bands in the transfected cells at around 18–19 and 20–22 kDa, respectively (Figure 6A, left panels). The bottom band corresponds to the expected size of IFN λ 4 with the MYC or FLAG tags. As IFN λ 4 is predicted to contain a single N-linked glycosylation site at Asn61 (marked with a square in Figure 3A and labelled in Figure 3E), the upper band could be due to glycosylation. To test this, we treated the cell lysates from the IFN λ 4-transfected cells with PNGase F that cleaves N-linked glycosylation between the asparagine and the innermost *N*-acetylchondrosamine of high mannose, hybrid and complex oligosaccharides. As can be seen on the right in Figure 6A, treatment with PNGase F resulted in a single band at 18–19 kDa, showing that IFN λ 4 gets glycosylated.

To test whether active IFN λ was secreted from the transfected cells, we added the supernatant from the transfected cells in Figure 6A to HEK293 transfected with an interferon-inducible luciferase reporter system, with and without the expression of IFN λ R1. This resulted in a clear signal from both IFN λ 3 and IFN λ 4, which was dependent upon IFN λ R1 (Figure 6B). In order to estimate how much IFN λ 4 is secreted, we titrated the supernatants from IFN λ 3- and IFN λ 4-transfected cells (Figure 6C), and here we observed a substantially lower activity in the supernatant from IFN λ 4-transfected cells

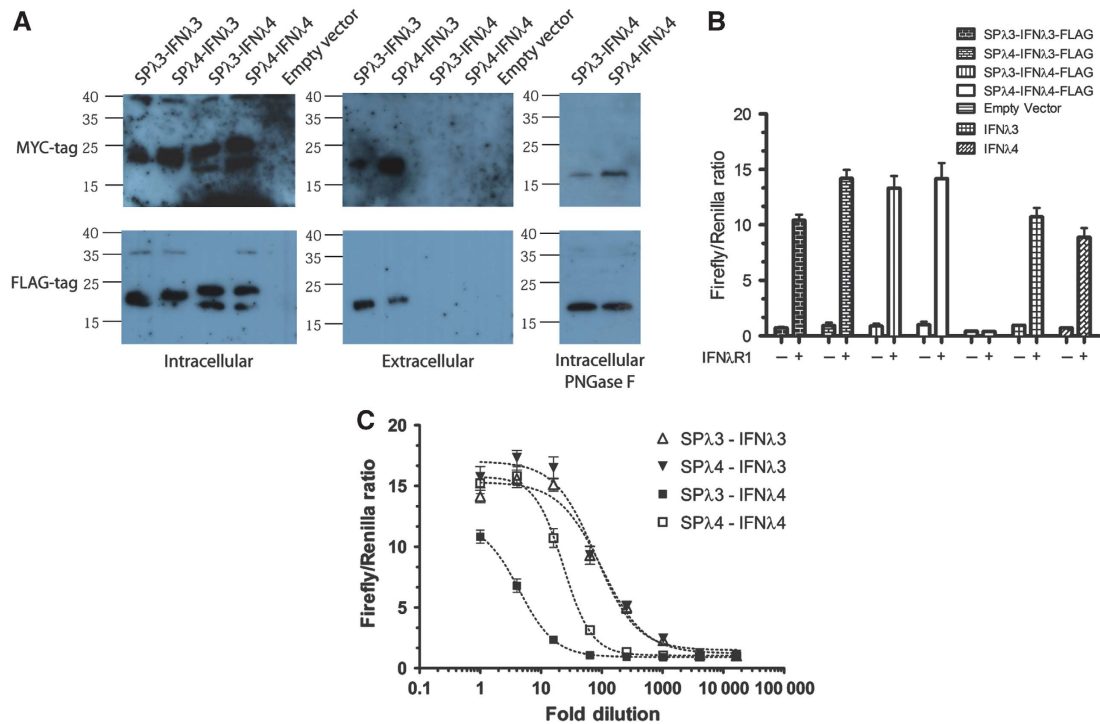


Figure 6 Secretion of human interferon lambda 4. (A) Constructs of IFN λ 3 and IFN λ 4 containing either the IFN λ 3 or IFN λ 4 signal peptide were transfected into HEK293 cells using pcDNA3.1 as a control. Western blots were performed on the intracellular and extracellular fractions as shown, using antibodies against the MYC or FLAG tag. For the cell fraction, the samples of IFN λ 4 were also subjected to treatment with PNGase F to confirm the presence of N-linked glycosylation. (B, C) The extracellular supernatants from HEK293 cells transfected with the FLAG-tagged constructs from (A) were added to HEK293 cells transfected with the pEF2 vector containing IFN λ R1 as well as the interferon inducible luciferase reporter system (see Figure 2C), in order to measure IFN λ activity present in the extracellular supernatants. In (B), the extracellular fraction was undiluted whereas in (C) a serial dilution was performed.

as compared to IFN λ 3-transfected cells (5- to 6-fold difference in EC50 values). Thus, IFN λ 4 is secreted at substantially lower levels. Swapping the SPs made no difference for IFN λ 3, which was equally well produced with its own or the SP of IFN λ 4. In the case of IFN λ 4, adding the SP of IFN λ 3 lead to lower levels of secreted interferon activity.

Glycosylation of IFN λ 4 is required for secretion but does not influence activity

As described above, IFN λ 4 contains a potential N-linked glycosylation site, and we observed a fraction of the intracellular protein which had a size suggesting post-translational modifications. Thus, we wanted to address the glycosylation state of the secreted IFN λ 4. As IFN λ 4 levels were too low to be detected using standard western blotting, we first refined the detection of IFN λ 4 in the media of transfected cells using acetone precipitation (Figure 7A). The western blot revealed IFN λ 4 of a size consistent with glycosylation, and this result was confirmed with PNGase F treatment. Next, we made a mutant of IFN λ 4 where the glycosylated asparagine residue N61 was mutated to aspartate (IFN λ 4 N61D). HEK293 cells were transfected with empty vector, IFN λ 4, IFN λ 3 or IFN λ 4 N61D, and the intracellular and extracellular fractions were analysed by western blotting (Figure 7B). IFN λ 4 N61D only gives one band on the western blot of the intracellular fractions corresponding to the unmodified IFN λ 4. Neither IFN λ 4 nor IFN λ 4 N61D is detectable in the extracellular fraction by standard western blotting. However, when we carry out acetone precipitation on the media before western blotting, we see a clear band for IFN λ 4,

but not for IFN λ 4 N61D, showing that this mutation further impairs the secretion of IFN λ 4. This is also reflected in the IFN λ activity (performed as in Figure 6C), where the activity in the supernatant of cells transfected with IFN λ 4 N61D is greatly decreased compared to that from cells transfected with IFN λ 4 (Figure 7C).

As the *E. coli*-produced IFN λ 4 is fully active and contain no glycosylation, this cannot be a prerequisite for activity. However, the question arose whether the glycosylated IFN λ 4 is active or whether low levels of unglycosylated protein that is undetectable by western blotting even after acetone precipitation mediate the activity. To exclude that non-glycosylated IFN λ 4 could be the source of the interferon activity, we incubated media from IFN λ 4-transfected cells with Concanavalin A (Con A) beads. Con A is a lectin that binds terminal α -D mannose and α -D glucose found on high mannose and hybrid N-linked glycans. Media from cells transfected with IFN λ 4 or empty vector were incubated with Con A beads. In the IFN λ 4-transfected cells, there was interferon activity in the input before addition of the Con A beads and this activity was removed after incubation with the beads (Figure 7E). This shows that the glycosylated IFN λ 4 is the source of the measured interferon activity. We attempted to elute IFN λ 4 from the beads using standard elution buffer but without success, as seen by the lack of activity (Figure 7D) and protein (Figure 7E) in the eluate. Nevertheless, we were able to confirm that IFN λ 4 was bound to the Con A beads by boiling these beads in SDS page buffer and performing a western blotting (Figure 7E).

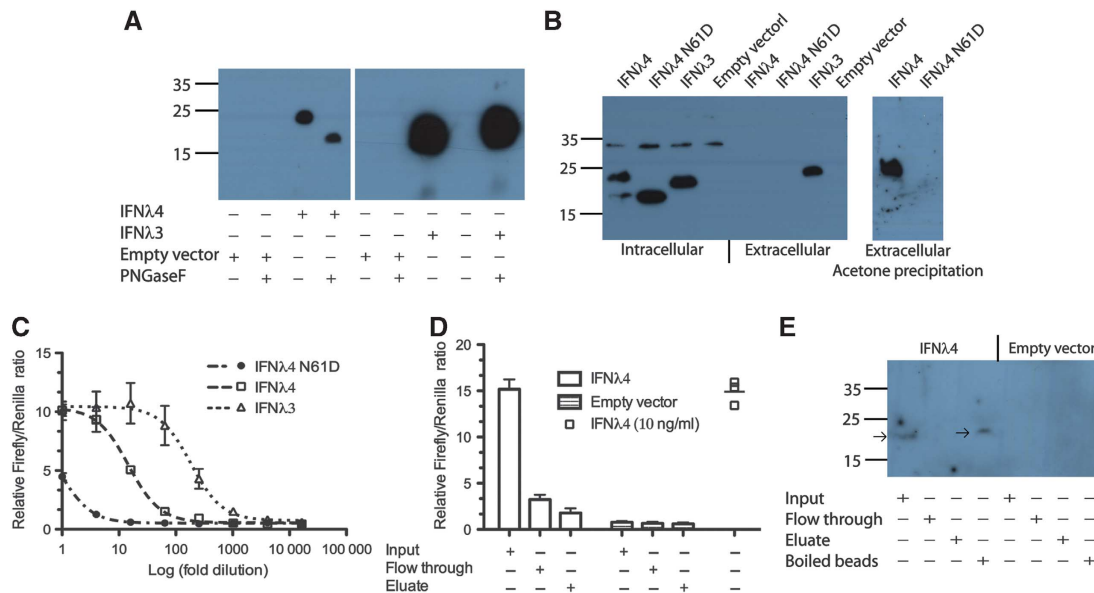


Figure 7 The secreted glycosylated IFN λ 4 is active. (A) Western blot of acetone precipitated media from cells transfected with empty vector or FLAG-tagged IFN λ 3 or IFN λ 4. Where indicated the sample was subjected to deglycosylation using PNGase F. (B) Western blot of intracellular and extracellular fractions of cells transfected with empty vector or FLAG-tagged IFN λ 3, IFN λ 4 or the IFN λ 4 N61D mutant. (C) HEK293 cells were transfected with *IFN λ R1* as well as the interferon inducible luciferase reporter system (see Figure 2C). The cells were subsequently treated with the media from (B) and the luciferase activities were measured. (D) Measurement of IFN λ activity was performed as in (C). Input: media before addition of Con A beads, Flow through: flow through from the Con A beads and Eluate: eluate from the Con A beads. (E) Western blot of the samples from (D) as well as the boiled Con A beads. The arrows show the position of IFN λ 4.

Discussion

IFN λ 4 signals through the IFN λ R1:IL-10R2 receptor complex

We produced recombinant IFN λ 4 protein in *E. coli* and did not observe any substantial difference in the behaviour of IFN λ 4 compared to the other isoforms of IFN λ during purification. First, we tested the activity of IFN λ 4 in a standard reporter gene assay, utilising a luciferase gene under the control of the IFI6 gene promoter (Uze and Monneron, 2007). The resulting dose response curves were comparable to IFN λ 3 and IFN λ 4. Furthermore, we tested induction of individual ISGs by both IFN λ 3 and IFN λ 4, and again we observed comparable levels of induction by both isoforms. Thus, we conclude that IFN λ 3 and IFN λ 4 are equally strong in inducing ISGs.

Based upon the low sequence similarity between IFN λ 4 and other isoforms of IFN λ in the region known to bind IL-10R2, Prokunina-Olsson *et al* (2013) understandably questioned whether IFN λ 4 uses this receptor chain for signalling. First, we confirmed the use of the IFN λ R1 receptor chain by IFN λ 4, as IFN λ 4 signalling was restored in HEK293 cells upon transfection with *IFN λ R1*. Next, we demonstrated the involvement of IL-10R2 both by siRNA knockdown and by blocking the IL-10R2 chain by a specific antibody that has been used to define the receptor usage of the other IFN λ s (Sheppard *et al*, 2003). Thus, IFN λ 4 leads to the activation of an interferon response and mediates antiviral effects through the canonical IFN λ receptor complex composed of IFN λ R1 and IL-10R2. However, these results do not exclude the possibility that IFN λ 4 can signal through other types of cytokine receptors, but it would indicate that if such a signalling existed it would not involve the regulation of classical ISGs.

IFN λ 4 is a disadvantage in the context of HCV infection despite possessing a strong anti-HCV activity

We measured the antiviral activity of IFN λ 4 against HCV, HCoV-229E and MERS-CoV and compared it to the antiviral activity of IFN λ 3 and IFN α 2. To our surprise, the antiviral activity of IFN λ 3 and IFN λ 4, respectively, was indistinguishable in all viral infection models tested. For HCV, we tested two different hepatic cell lines, Huh7 and HepG2, and in neither case did we observe any difference between IFN λ 3 and IFN λ 4. Likewise, using primary HAE cells for the infection with either HCoV-229E or MERS-CoV, we did not observe any difference between IFN λ 3 and IFN λ 4. This is remarkable as the sequence identity between the two isoforms is only 29% (Prokunina-Olsson *et al*, 2013), and our preliminary bioinformatics studies reveal that the protein sequence of IFN λ 4 is well conserved among mammals (data not shown). Thus, there must have been an evolutionary pressure to keep IFN λ 4 as a functional protein throughout the mammalian evolution until the sudden introduction of a frameshift mutation in humans. Since the inactivation of the *IFNL4* gene is strongly correlated with increased likelihood of spontaneous clearance of HCV as well as with a positive response to the treatment with type I IFN, it appears that the production of IFN λ 4 protein is actually a disadvantage during HCV infection. Furthermore, there appears to be a positive selection in humans for the frameshift mutation abolishing IFN λ 4 production (Prokunina-Olsson *et al*, 2013). Whether this selection is solely driven by HCV is currently not known. IFN λ 4 production could even be beneficial in the context of other viral infections. It was thus recently reported that the SNPs that have been shown to be favourable for the treatment outcome as well as the likelihood for spontaneous clearance of HCV are associated with poor recovery from hepatitis B virus infection (Kim *et al*, 2013).

How a functional interferon suddenly becomes a liability during HCV infection is a paradox that we are currently unable to explain. As discussed above, the induction of ISGs occurs through the canonical IFN λ receptor complex, but we cannot exclude that IFN λ 4 has activities outside the induction of ISGs, which could be mediated through an as yet unidentified receptor. However, our data suggests that IFN λ 4 is highly active against HCV despite the fact that it has been shown to be a predictor of poor response to HCV. The current data cannot exclude indirect genetic effects, and thus it is not firmly proven that the IFN λ 4 protein is the causal agent for the poor prognosis of HCV patients with a functional *IFNL4* gene. Furthermore, no evidence for the presence of the IFN λ 4 protein in HCV patients exists to date. However, if one assumes that the IFN λ 4 protein is the causal agent, this would suggest a complicated relationship between IFN λ 4 and HCV in humans, where IFN λ 4 somehow impairs a full immune response towards HCV. We have produced fully functional IFN λ 4 protein which should be used for further studies of IFN λ 4 on hepatic and immune cells. Furthermore, it will be important to address whether HCV is driving the selection of the TT allele (non-functional IFN λ 4) in humans, and if the introduction of the TT allele changes susceptibility towards other viral infections.

Poor secretion of IFN λ 4 is not determined by the SP

The inability of the IFN λ 4 protein to be properly secreted by cells was previously reported, and the authors speculated that this might be due to a weak SP (Prokunina-Olsson *et al*, 2013). We produced chimaeric cDNAs where we had swapped the SPs between IFN λ 3 and IFN λ 4. Here, we observed that the IFN λ 4 was retained within the cells regardless of which SP was used, and likewise the secretion of the mature IFN λ 3 protein was not significantly affected by the SP used. By both immunoprecipitation and acetone precipitation, we were able to show that IFN λ 4 get secreted, but with much lower efficiency than what seen for IFN λ 3, which is also reflected by the reduced activity of media from IFN λ 4-transfected cells compared to media from IFN λ 3-transfected cells.

Glycosylation of IFN λ 4 is required for proper secretion and does not interfere with activity

We tested for the presence of intracellular IFN λ 4 by western blots of cell lysates and observed two isoforms of IFN λ 4. Digestion with PNGase F, which removes N-linked glycans, revealed that this was due to incomplete glycosylation of IFN λ 4. By using acetone precipitation to concentrate the protein in the media, we were able to show that all secreted IFN λ 4 protein appeared to contain the N-linked glycosylation. This is in agreement with the current dogma that proteins need to complete their glycosylation before being exported to the extracellular media. It is not clear to us how the cell senses the difference between proteins, which are glycosylated like IFN λ 4 and proteins that are not glycosylated like IFN λ 3. We produced a glycosylation-deficient mutant of IFN λ 4 (IFN λ 4 N61D), and observed that the secretion of this mutant was greatly impaired, confirming that the N-linked glycosylation is needed for proper secretion. These results also suggested that IFN λ 3 and IFN λ 4 use different pathways for secretion, and that removing the N-linked glycosylation

site is not sufficient to make IFN λ 4 shift to the secretion pathway used by the non-glycosylated IFN λ 3.

The question whether the glycosylation impairs activity was also raised. As the *E. coli*-produced protein is fully active, it is obvious that the glycosylation is not required for activity, but could it interfere with receptor binding? Our structure modelling suggested that the sugars were attached outside the receptor-binding site, and the activity that we recovered from the supernatant of IFN λ 4-transfected cells, which appeared only to contain glycosylated IFN λ 4, suggested that the sugars did not interfere with activity. However, to confirm this result, we used Con A beads to deplete the media from glycosylated IFN λ 4. As this led to an almost complete loss of activity, we conclude that the IFN λ 4 secreted from HEK293 cells is both glycosylated and active.

The poor processing and secretion of the IFN λ 4 protein are currently what makes it stand out in comparison to the other IFN λ proteins, and our data suggest that the block in secretion takes place after the translocation to the Golgi, as the SP appears to be efficiently cleaved off. The lack of secretion of IFN λ 4 led several news and views papers to suggest the presence of an intracellular receptor. Our data clearly demonstrate that IFN λ 4 does use the normal receptor situated at the cellular membrane, although we cannot formally exclude the presence of an intracellular receptor. However, we consider it likely that the activation of the interferon pathway, which was observed after transfection of HepG2 cells with IFN λ 4 expressing plasmids (Prokunina-Olsson *et al*, 2013), is due to low levels of secreted IFN λ 4.

Materials and methods

Protein expression, purification and refolding

IFN λ 4 (NM_001276254, amino acids 23–179) preceded by a 6 \times His tag followed by a TEV protease cleavage site was codon optimised for *E. coli* and purchased from Invitrogen. This construct was cloned into the pET-15b vector using Fastdigest KpnI (Thermo Scientific, catalogue number FD0524) and Fastdigest XhoI (Thermo Scientific, catalogue number FD0694). BL21 (DE3) *E. coli* cells transformed with the plasmids were grown at 37°C in Luria Bertani medium containing 100 μ g/ml ampicillin and 100 μ l antifoam A concentrate (Sigma-Aldrich, catalogue number A5633) under continuous shaking until an OD₆₀₀ of 0.8–1. Protein expression was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside and incubated for another 4 h at 37°C. Refolding and purification were performed as previously described (Dellgren *et al*, 2009).

Plasmids

The pEF2-IFN λ 3 and the pEF2-IFN λ R1 vectors were kind gifts from Professor Sergei Kotenko (UMDNJ-New Jersey Medical School, Newark, USA). The human *IFNL4* gene (NM_001276254), including the SPSP, was purchased from Invitrogen. The following constructs were generated using Accupol (Amplicon, catalogue number 210302) following the manufacturer's instructions: IFN λ 4_FLAG (Template: IFN λ 4, forward primer: gcttggtaccatcgccgagtgctcgg, reverse primer: agttctagatcactgtcctcctgtaatccgacccaggcaggccc), IFN λ 3_FLAG (Template: pEF2-IFN λ 3, forward primer: gcttggtaccatcgccgagtgctcgg, reverse primer: agttctagatcactgtcctcctgtaatccgacccaggcaggccc), IFN λ 3SP_IFN λ 4_FLAG (Template: IFN λ 4, forward primer: gcttggtaccatcgccgagtgctcgg, reverse primer: agttctagatcactgtcctcctgtaatccgacccaggcaggccc), and IFN λ 4SP_IFN λ 3_FLAG (Template: pEF2-IFN λ 3, forward primer: gcttggtaccatcgccgagtgctcgg, reverse primer: agttctagatcactgtcctcctgtaatccgacccaggcaggccc), as well as IFN λ 4_MYC (Template: IFN λ 4, forward primer: gcttggtaccatcgccgagtgctcgg, reverse primer: agttctagatcactgtcctcctgtaatccgacccaggcaggccc), IFN λ 3_MYC (Template: pEF2-IFN λ 3,

forward primer: gcttggtaccatgaccggggactgc, reverse primer: agttctagatcacagatcctcactaactcagtttctgctcactccgacacacaggtccccactggc), IFN λ 3SP_IFN λ 4_MYC (Template: IFN λ 4, forward primer: gcttggtaccatgaccggggactgc, reverse primer: agttctagatcacagatcctcactaactcagtttctgctcactccgacacacaggtccccactggc), and IFN λ 4SP_IFN λ 3_MYC (Template: pEF2-IFN λ 3, forward primer: gcttggtaccatgaccggggactgc, reverse primer: agttctagatcacagatcctcactaactcagtttctgctcactccgacacacaggtccccactggc). The following PCR programme was used 1: 95°C for 5 min 2: 30 cycles of 95°C for 1 min, 59°C for 1 min and 72°C for 1 min and 45 s 3: 72°C for 7 min. All the constructs were cloned into the pEF2 vector using Fastdigest Kpn I (Thermo Scientific, catalogue number FD0524) and Fastdigest XbaI (Thermo Scientific, catalogue number FD0684) following the manufacturer's instructions.

The IFN λ 4 mutant IFN λ 4 N61D was generated by site-directed mutagenesis using IFN λ 4_FLAG in the pEF2 vector as a template. The reaction was performed using PfuUltra II with the primers (gctggggcagcgcgactgctctccgccccc and gggggcgaaggagcagtcgcgctgccccc agc) according to the manufacturer's instructions. The following PCR programme was used 1: 95°C for 5 min 2: 30 cycles of 95°C for 1 min, 59°C for 1 min and 72°C for 5 min and 45 s 3: 72°C for 7 min.

Cell culture

Unless otherwise stated, all cells were grown in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. Furthermore, the Huh-7 cells were supplemented with 2 mM L-glutamine, and kept under blasticidin selection (5 μ g/ml). The HL-116 cell line was supplemented with hypoxanthine, thymidine and aminopterin, and 400 μ g/ml G418. Cells were maintained at 37°C with 5% CO $_2$. HAE cells were generated as previously described (Kindler *et al*, 2013) and were maintained for 2 months.

Activity assay on HL-116 cells

The activity of IFN λ 4 and IFN λ 3 was tested in HL-116 cells, an HT1080 derived cell line containing a luciferase reporter gene controlled by the interferon inducible IFI6 promoter. Furthermore, the HL-116 cells were stably transfected with IFN λ R1 to render them responsive to IFN λ (Uze and Monneron, 2007). To measure the interferon activity, 1×10^4 HL-116 cells were plated in a 96-well plate and treated in triplicates for 3 h with 8 dilutions of IFN λ 3 or IFN λ 4 in a concentration range covering 0.0001–1000 ng/ml. The cells were lysed, and the luciferase activity was quantified using the DualGlo luciferase assay system (Promega).

Quantitative real-time PCR

HepG2 cells were seeded at a density of 2×10^5 cells per well in 12-well plates and incubated for 24 h. Then, fresh media was added with the indicated interferons. The cells were incubated for 4 h and then lysed, and RNA was purified using an extraction kit (Omega) according to the manufacturer's instructions. cDNA synthesis and analysis by real-time quantitative PCR were performed as previously described (Melchjorsen *et al*, 2009). This reference also lists primer sequences. The crossing points of the amplification curves were determined using the second derivative method on the Roche LightCycler software 3.5 (Roche). The data obtained from the Light Cycler were normalised using the mathematical model described by Pfaffl (2001). The experiments were performed in quadruplicates. For the untreated control, the mean of the quadruplicates was used to calculate fold induction for the other samples.

siRNA and transfection

For transfection experiments, 1×10^5 HEK293 cells per well were seeded in a 24-well plate in DMEM supplemented with 10% FBS and left to rest for 24 h. After 24 h, the cells were transfected with siRNA against IL-10R2 or control siRNA (ON-TARGETplus Pool, Thermo Scientific) using Lipofectamine 2000 (Invitrogen). Eighteen hours post transfection, media was changed to fresh media supplemented with 10% FBS; and 24 h post transfection, cells were transfected with the pEF2 plasmid encoding IFN λ R1, Firefly luciferase under the control of the *Mx1* promoter (Jorns *et al*, 2006), *Renilla* luciferase under the control of the β -actin promoter and siRNA against IL-10R2 or control siRNA. After 6 h, the media was

changed to fresh media supplemented with 10% FBS, and the cells were left to rest for the next 12 h. Eighteen hours post transfection cells were induced with 10 ng/ml of IFN λ 3, IFN λ 4 or 1000 U/ml of IFN α 2 (Chemicon) for 24 h. After 24 h, the cells were washed with PBS and lysed with Passive Lysis Buffer (Promega). Lysates were spun down at 10 000 r.c.f. for 2 min at 4°C, and the cleared lysates were used for the measurement of luciferase activities (Dual-Luciferase Reporter Assay System, Promega).

Neutralisation assay

HEK293 cells were seeded in 48-well plates in a concentration of 4×10^5 cells per ml in DMEM supplemented with 10% FBS. After 24 h, the cells were transfected using Lipofectamine 2000 (Invitrogen) with plasmids coding IFN λ R1, Firefly Luciferase under the control of the *Mx1* promoter and *Renilla* Luciferase under the control of the β -actin promoter. Twenty hours post transfection cells were incubated for 1 h in media containing IL-10R2 (R&D Systems) or control antibody in a concentration of 6 μ g/ml, after which the cells were induced with 10 ng/ml of IFN λ 3 or IFN λ 4 or 1000 U/ml of IFN α 2a. After 24 h of induction, the cells were lysed with Passive Lysis Buffer (Promega), and cleared lysates were used for the measurement of luciferase activities (Dual-Luciferase Reporter Assay System, Promega).

SDS page gel electrophoresis and western blotting

The proteins were run on 12% SDS-PAGE gels. Gel staining was done using Coomassie brilliant blue. Western blotting was performed using a PVDF STAR 0.45 μ m transfer membrane (applied-chem) using SuperSignal West Dura extended duration Substrate (Thermo Scientific). The membrane was exposed to MG-SR plus medical film (Konica Minolta), which was developed on an AGFA CURIX 60 film processor. The antibodies used were Mouse MYC antibody (Mycl-9E10 mouse hybridoma), Mouse monoclonal anti-FLAG[®] M2 antibody (Sigma-Aldrich, catalogue number F3165), IL-10R2 antibody from goat (R&D Systems, catalogue number AF874) and rabbit polyclonal GAPDH antibody (Santa Cruz Biotechnology, catalogue number FL-335). Anti-mouse IgG, horse-radish peroxidase-linked species-specific whole antibody from sheep (GE Healthcare, catalogue number NA931) and Polyclonal swine anti-rabbit immunoglobulins/HRP (Dako Cytomation, catalogue number P 0399).

Alignment of IFN λ 3 and IFN λ 4 and the model of IFN λ 4

The alignment of human IFN λ 3 (NCBI accession code: NP_742151.2) and human IFN λ 4 (NCBI accession code: AFQ38559.1) was performed in Clustal W2 using the default settings (Larkin *et al*, 2007). The full-length proteins including the SPs were used. The model of IFN λ 4 was generated in the SWISS-MODEL workspace (Bordoli *et al*, 2009) using the sequence of IFN λ 4 without the SP and the structure of human IFN λ 3 (PDB entry code: HHC3) as a model. Structural superimposition was performed in pymol (DeLano, 2008).

HCoV-229E infection of HAE

Human bronchial epithelial cells were isolated from patients (>18 years old), who underwent bronchoscopy and/or surgical lung resection in their diagnostic pathway for any pulmonary disease and that gave informed consent. This was done in accordance with the local regulation of the Kanton St. Gallen, Switzerland, as part of the St. Gallen Lung Biopsy Biobank (SGLBB) of the Kanton Hospital, St. Gallen, which received approval by the ethics committee of the Kanton St. Gallen (EKSG 11/044, EKSG 11/103). HAE cultures were prepared as previously described (Dijkman *et al*, 2009). HAE cultures were used 28 days post exposure of the apical surface to air for infection studies. IFN α A/D (I4401, Sigma Aldrich), IFN λ 3 or IFN λ 4 was added to the basolateral medium 4–16 h prior to infection, after which the basolateral medium was replaced, and 20 000 PFUs of HCoV-229E-*ren* were applied apically. At 24 h post infection *Renilla* luciferase activity was determined from cell lysates infected with HCoV-229E-*ren*.

The MERS-CoV infection was performed as previously described (Kindler *et al*, 2013).

HCV replication

The Huh7-Lunet N hCD81-FLuc cell line was generated from the Huh7-Lunet N hCD81 parental cell line (Bitzegeio *et al*, 2010) by lentiviral gene transfer as previously described (Gentsch *et al*,

2011). It constitutively expresses the *Firefly* luciferase gene (FLuc), which is used in our assay as a marker for cell viability.

In all, 4×10^6 Huh7-Lunet N hCD81-FLuc cells or 6×10^6 HepG2-CD81/mi122 cells (Narbus *et al*, 2011) were electroporated with 5 μ g of *in vitro*-transcribed JcR-2a RNA as previously described (Haid *et al*, 2010). The JcR-2a construct corresponds to the full-length infectious HCV Jc1 chimaeric clone (Pietschmann *et al*, 2006), expressing a *Renilla* luciferase reporter gene (Reiss *et al*, 2011). Electroporated cells were resuspended into 20 ml complete medium and seeded in 96-well dishes (100 μ l/well). Four hours post electroporation, the cell medium was replaced by serially diluted IFN α 2b (Introna[®], Essex Pharma), IFN λ 3 or IFN λ 4. For each dilution, triplicate wells were used. Cells were lysed 48 (HepG2 derived) or 72 h (Huh7-lunet) post electroporation in passive lysis buffer (Promega), and *Renilla* luciferase activity was measured to evaluate HCV replication (Vieyres and Pietschmann, 2013).

Activity of secreted IFN λ 3 and IFN λ 4

For transfection experiments, HEK293 cells were seeded in 24-well plates (1.5×10^5 cells/well) or 6-well plates (7×10^5 cells/well) in DMEM supplemented with 10% FBS and left to rest for 24 h. After 24 h, cells were transfected using Lipofectamine 2000 (Invitrogen) either with plasmids coding IFN λ s (6-well format) or co-transfected with plasmids coding IFN λ R1, *Firefly* Luciferase under the control of the *Mx1* promoter and *Renilla* Luciferase under the control of the β -*actin* promoter (24-well format). Six hours post transfection, cells transfected with IFN λ s were given fresh media (DMEM, 10% FBS and 100 U/ml Penicillin and 100 μ g/ml Streptomycin). Twenty hours post transfection, media from cells transfected with IFN λ s was harvested, spun down at 500 r.c.f. for 8 min and added to cells co-transfected with IFN λ R1 and Luciferases in different dilutions. After 24 h, the cells were washed with PBS and lysed. Lysates were centrifuged at 10 000 r.c.f. for 2 min at 4°C, and cleared lysates were used for the measurement of *Firefly* activity (Dual-Luciferase Reporter Assay System, Promega).

Transfection of HEK293 cells using polyethylenimine

In all, 8×10^6 HEK293 cells were seeded in a 15-cm dish and transfected with IFN λ 3-FLAG, IFN λ 4-FLAG or empty vector (pcDNA3.1). After 5–6 h, the media was changed to media without serum, and the cells were transfected using 40 μ g DNA per dish using polyethylenimine (PEI). In all, 40 μ g DNA was mixed with media without antibiotics and serum to a concentration of 1.5 ml.

In all, 120 μ l of PEI was mixed with media without antibiotics and serum to a concentration of 1.5 ml. The DNA and PEI were mixed and left for 15–20 min at RT before addition to the cells. The cells were incubated for 18 h, after which the media was isolated by centrifugation at 7000 r.p.m. for 10 min.

Immunoprecipitation

In all, 8×10^6 HEK293 cells were grown in 15 cm dishes using 20 ml of media and transfected as described. The supernatants were incubated with 100 μ l of ANTI-FLAG[®] M2 Affinity Gel (SIGMA-Aldrich, catalogue number A2220) for 3 h. The beads were spun down by centrifugation at 8000 r.p.m. for 1 min. The supernatant was removed, and the beads were washed two times in 0.5 ml PBS containing 2% Triton X-100. The beads were then incubated in the elution buffer (PBS containing 2% Triton X-100 and 500 μ g of FLAG peptide (SIGMA-Aldrich, catalogue number F3290) for 30 min. The

beads were precipitated by centrifugation at 8000 r.p.m. for 1 min, and the supernatant was isolated and analysed by western blotting.

Deglycosylation

Deglycosylation was performed using Glycerol Free PNGase F (New England Biolabs, catalogue number P07055). For deglycosylation, 9 μ l of the cell lysate was mixed with 1 μ l of 10x Glycoprotein denaturing buffer and denatured by heating at 100°C for 10 min. Then 5 μ l H₂O, 2 μ l of G7 reaction buffer, 2 μ l 10% NP-40 and 1 μ l PNGase F was added. This mixture was incubated at 37°C for 15 h and analysed by western blotting.

Acetone precipitation

The media was mixed in a 1:4 ratio of media to cold (–20°C) acetone. The samples were vortexed and incubated at –20°C for 60 min. The protein was precipitated by centrifugation at 6000 r.p.m. for 45 min. The supernatant was decanted, and the protein pellet was resuspended in PBS.

Concanavalin A

In all, 2 ml glucose-free DMEM (Sigma) from IFN λ 4-FLAG and mock (pcDNA3.1) transfected cells was incubated for 30 min with 100 μ l of Concanavalin A (Con A) beads. The beads and the media were added to a column, and the flow through was collected. The beads were washed with PBS before incubation with 1 ml of elution buffer (glucose-free DMEM supplemented with 500 mM glucose) for 30 min. The activity of the flow through and eluate was investigated in HEK293 cells co-transfected with plasmids coding IFN λ R1, *Firefly* Luciferase under the control of the *Mx1* promoter and *Renilla* Luciferase under the control of the β -*actin* promoter (24-well format) as previously described. The protein content was evaluated in the flow through, eluate and on the beads by western blotting. The beads were boiled in SDS loading buffer for 10 min before loading on the gel.

Acknowledgements

We wish to thank Dr Sergei Kotenko for the kind gift of the IFN λ R1 and IFN λ 3 expression plasmids, Dr Georg Kochs for the *Mx*-Luc reporter plasmid and Dr Gilles Uzé for the gift of HL-116 cells expressing the IFN λ R1. We are also in debt to Lisbeth Heilesen and Dr Hans Henrik Gad for critical reading of the manuscript. This work was funded by the Danish Cancer Society (grant: R20-A927; RH), and the Danish Council for Independent Research, Medical Research (grant 11-107588; RH); the Swiss National Science Foundation (project 31003A_132898; VT), the 3R Research Foundation Switzerland (project 128-11; VT and RD), and the Deutsche Forschungsgemeinschaft (Priority Programme (SPP) 1596; VT).

Author contributions: OJH, ET-D, GV, RD, SEJ, PS and HA designed, performed and analysed the experiments; TP, VT and RH supervised research; OJH and RH conceived the project and prepared the manuscript; all authors commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Ank N, West H, Bartholdy C, Eriksson K, Thomsen AR, Paludan SR (2006) Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections *in vivo*. *J Virol* **80**: 4501–4509
- Bibert S, Roger T, Calandra T, Bochud M, Cerny A, Semmo N, Duong FH, Gerlach T, Malinverni R, Moradpour D, Negro F, Mullhaupt B, Bochud PY (2013) IL28B expression depends on a novel TT/G polymorphism which improves HCV clearance prediction. *J Exp Med* **210**: 1109–1116
- Bitzegeio J, Bankwitz D, Hueging K, Haid S, Brohm C, Zeisel MB, Herrmann E, Iken M, Ott M, Baumert TF, Pietschmann T (2010)

- Adaptation of hepatitis C virus to mouse CD81 permits infection of mouse cells in the absence of human entry factors. *PLoS Pathog* **6**: e1000978
- Booth D, George J (2013) Loss of function of the new interferon IFN-lambda4 may confer protection from hepatitis C. *Nat Genet* **45**: 119–120
- Bordoli L, Kiefer F, Arnold K, Benkert P, Battey J, Schwede T (2009) Protein structure homology modeling using SWISS-MODEL workspace. *Nat Protoc* **4**: 1–13
- DeLano W (2008) The PyMOL Molecular Graphics System, Ver 1.2r3pre

- Dellgren C, Gad HH, Hamming OJ, Melchjorsen J, Hartmann R (2009) Human interferon-lambda3 is a potent member of the type III interferon family. *Genes Immun* **10**: 125–131
- Dickensheets H, Sheikh F, Park O, Gao B, Donnelly RP (2013) Interferon-lambda (IFN-lambda) induces signal transduction and gene expression in human hepatocytes, but not in lymphocytes or monocytes. *J Leukoc Biol* **93**: 377–385
- Dijkman R, Koekkoek SM, Molenkamp R, Schildgen O, van der Hoek L (2009) Human bocavirus can be cultured in differentiated human airway epithelial cells. *J Virol* **83**: 7739–7748
- Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, Yao L, Liu H, Barahmand-pour F, Sivakumar P, Chan C, Birks C, Foster D, Clegg CH, Wietzke-Braun P, Mihm S, Klucher KM (2006) Interleukin-29 uses a type I interferon-like program to promote antiviral responses in human hepatocytes. *Hepatology (Baltimore, MD)* **44**: 896–906
- Dumoutier L, Lejeune D, Hor S, Fickenscher H, Renauld JC (2003) Cloning of a new type II cytokine receptor activating signal transducer and activator of transcription (STAT)1, STAT2 and STAT3. *Biochem J* **370**: 391–396
- Fox BA, Sheppard PO, O'Hara PJ (2009) The role of genomic data in the discovery, annotation and evolutionary interpretation of the interferon-lambda family. *PLoS One* **4**: e4933
- Gad HH, Dellgren C, Hamming OJ, Vends S, Paludan SR, Hartmann R (2009) Interferon-lambda is functionally an interferon but structurally related to the interleukin-10 family. *J Biol Chem* **284**: 20869–20875
- Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* **461**: 399–401
- Genzsch J, Hinkelmann B, Kaderali L, Irschik H, Jansen R, Sasse F, Frank R, Pietschmann T (2011) Hepatitis C virus complete life cycle screen for identification of small molecules with pro- or antiviral activity. *Antiviral Res* **89**: 136–148
- Haid S, Windisch MP, Bartenschlager R, Pietschmann T (2010) Mouse-specific residues of claudin-1 limit hepatitis C virus genotype 2a infection in a human hepatocyte cell line. *J Virol* **84**: 964–975
- Jorns C, Holzinger D, Thimme R, Spangenberg HC, Weidmann M, Rasenack J, Blum HE, Haller O, Kochs G (2006) Rapid and simple detection of IFN-neutralizing antibodies in chronic hepatitis C non-responsive to IFN-alpha. *J Med Virol* **78**: 74–82
- Kim SU, Song KJ, Chang HY, Shin EC, Park JY, Kim do Y, Han KH, Chon CY, Ahn SH (2013) Association between IL28B polymorphisms and spontaneous clearance of hepatitis B virus infection. *PLoS One* **8**: e69166
- Kindler E, Jonsdottir HR, Muth D, Hamming OJ, Hartmann R, Rodriguez R, Geffers R, Fouchier RA, Drosten C, Muller MA, Dijkman R, Thiel V (2013) Efficient replication of the novel human betacoronavirus EMC on primary human epithelium highlights its zoonotic potential. *mBio* **4**: e00611–e00612
- Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP (2003) IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* **4**: 69–77
- Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948
- Lasfar A, Lewis-Antes A, Smirnov SV, Anantha S, Abushahba W, Tian B, Reuhl K, Dickensheets H, Sheikh F, Donnelly RP, Raveche E, Kotenko SV (2006) Characterization of the mouse IFN-lambda ligand-receptor system: IFN-lambdas exhibit antitumor activity against B16 melanoma. *Cancer Res* **66**: 4468–4477
- Logsdon NJ, Deshpande A, Harris BD, Rajashankar KR, Walter MR (2012) Structural basis for receptor sharing and activation by interleukin-20 receptor-2 (IL-20R2) binding cytokines. *Proc Natl Acad Sci USA* **109**: 12704–12709
- Lupberger J, Felmlee DJ, Baumert TF (2013) Interferon-lambda polymorphisms and hepatitis C virus clearance revisited. *Hepatology (Baltimore, MD)* **58**: 439–441
- Meager A, Visvalingam K, Dilger P, Bryan D, Wadhwa M (2005) Biological activity of interleukins-28 and -29: comparison with type I interferons. *Cytokine* **31**: 109–118
- Melchjorsen J, Kristiansen H, Christiansen R, Rintahaka J, Matikainen S, Paludan SR, Hartmann R (2009) Differential regulation of the OASL and OAS1 genes in response to viral infections. *J Interferon Cytokine Res* **29**: 199–207
- Mennechet FJ, Uze G (2006) Interferon-lambda-treated dendritic cells specifically induce proliferation of FOXP3-expressing suppressor T cells. *Blood* **107**: 4417–4423
- Miknis ZJ, Magracheva E, Li W, Zdanov A, Kotenko SV, Wlodawer A (2010) Crystal structure of human interferon-lambda1 in complex with its high-affinity receptor interferon-lambdaR1. *J Mol Biol* **404**: 650–664
- Mordstein M, Neugebauer E, Ditt V, Jessen B, Rieger T, Falcone V, Sorgeloos F, Ehl S, Mayer D, Kochs G, Schwemmler M, Gunther S, Drosten C, Michiels T, Staeheli P (2010) Lambda interferon renders epithelial cells of the respiratory and gastrointestinal tracts resistant to viral infections. *J Virol* **84**: 5670–5677
- Narbus CM, Israelow B, Sourisseau M, Michta ML, Hopcraft SE, Zeiner GM, Evans MJ (2011) HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle. *J Virol* **85**: 12087–12092
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45
- Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R (2006) Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci USA* **103**: 7408–7413
- Pott J, Mahlakoiv T, Mordstein M, Duerr CU, Michiels T, Stockinger S, Staeheli P, Hornef MW (2011) IFN-lambda determines the intestinal epithelial antiviral host defense. *Proc Natl Acad Sci USA* **108**: 7944–7949
- Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, Hergott D, Porter-Gill P, Mumy A, Kohaar I, Chen S, Brand N, Tarway M, Liu L, Sheikh F, Astemborski J, Bonkovsky HL, Edlin BR, Howell CD, Morgan TR et al (2013) A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet* **45**: 164–171
- Ramos EL (2010) Preclinical and clinical development of pegylated interferon-lambda 1 in chronic hepatitis C. *J Interferon Cytokine Res* **30**: 591–595
- Ray K (2013) Hepatitis: New gene IFNL4 is associated with impaired clearance of HCV. *Nat Rev Gastroenterol Hepatol* **10**: 63
- Reiss S, Rebhan I, Backes P, Romero-Brey I, Erfle H, Matula P, Kaderali L, Poenisch M, Blankenburg H, Hiet MS, Longrich T, Diehl S, Ramirez F, Balla T, Rohr K, Kaul A, Buhler S, Pepperkok R, Lengauer T, Albrecht M et al (2011) Recruitment and activation of a lipid kinase by hepatitis C virus NS5A is essential for integrity of the membranous replication compartment. *Cell Host Microbe* **9**: 32–45
- Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J, Ostrand C, Dong D, Shin J, Presnell S, Fox B, Haldeman B, Cooper E, Taft D, Gilbert T, Grant FJ et al (2003) IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* **4**: 63–68
- Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB, Carrington M (2009) Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* **461**: 798–801
- Uze G, Monneron D (2007) IL-28 and IL-29: newcomers to the interferon family. *Biochimie* **89**: 729–734
- van den Worm SH, Eriksson KK, Zevenhoven JC, Weber F, Züst R, Kuri T, Dijkman R, Chang G, Siddell SG, Snijder EJ, Thiel V, Davidson AD (2012) Reverse genetics of SARS-related coronavirus using vaccinia virus-based recombination. *PLoS One* **7**: e32857
- Vieyres G, Pietschmann T (2013) Entry and replication of recombinant hepatitis C viruses in cell culture. *Methods* **59**: 233–248
- Wang Y, Li J, Wang X, Ye L, Zhou Y, Ho W (2013) Induction of interferon-lambda contributes to Toll-like receptor-3-activated hepatic stellate cell-mediated hepatitis C virus inhibition in hepatocytes. *J Viral Hepatitis* **20**: 385–394
- Zhou Z, Hamming OJ, Ank N, Paludan SR, Nielsen AL, Hartmann R (2007) Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. *J Virol* **81**: 7749–7758