

IFN- λ s mediate antiviral protection through a distinct class II cytokine receptor complex

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We report here the identification of a ligand-receptor system that, upon engagement, leads to the establishment of an antiviral state. Three closely positioned genes on human chromosome 19 encode distinct but paralogous proteins, which we designate interferon- λ 1 (IFN- λ 1), IFN- λ 2 and IFN- λ 3 (tentatively designated as IL-29, IL-28A and IL-28B, respectively, by HUGO). The expression of IFN- λ mRNAs was inducible by viral infection in several cell lines. We identified a distinct receptor complex that is utilized by all three IFN- λ proteins for signaling and is composed of two subunits, a receptor designated CRF2-12 (also designated as IFN- λ R1) and a second subunit, CRF2-4 (also known as IL-10R2). Both receptor chains are constitutively expressed on a wide variety of human cell lines and tissues and signal through the Jak-STAT (Janus kinases–signal transducers and activators of transcription) pathway. This receptor-ligand system may contribute to antiviral or other defenses by a mechanism similar to, but independent of, type I IFNs.

The class II cytokine receptor family consists of 11 characterized receptors, which are used for signaling by members of the interleukin 10 (IL-10) and interferon (IFN) ligand families^{1–4}. The IFN family is divided into type I and type II IFNs. The type II group is represented by a single member, IFN- γ , which is encoded on human chromosome 12. The type I group contains 13 IFN- α members and a single member each of IFN- β , IFN- κ , IFN- ω and IFN- ϵ ^{5–7}. The genes encoding all of the type I IFNs are clustered on human chromosome 9. The family of IL-10–related cytokines consists of six members of cellular origin: IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26 as well as several viral cytokines^{1,3,4}. *IL22* and *IL26* are on human chromosome 12, next to the gene encoding IFN- γ (*IFNG*), and those for the remaining four members of the IL-10 family are clustered together on chromosome 1. The many immunomodulatory activities of type I and II IFNs are well characterized, whereas the functions of several IL-10–related cytokines have just started to be revealed. Nevertheless, it is clear that they play a role in the induction and regulation of the immune and inflammatory responses^{1,3,4,8}.

Viral infection induces a robust production and secretion of type I IFNs that leads to the expression of many genes and the generation of a multifaceted antiviral response. Type I IFNs are defined by their ability to induce antiviral protection, both within individual cells and mediated *via* the adaptive and innate immune systems^{9–12}. Type I IFNs exert

their biological activities through a specific cell-surface receptor complex composed of two chains, IFN- α receptor 1 (IFN- α R1) and IFN- α R2c^{13,14}. Receptor engagement leads to the activation of the IFN-stimulated regulatory factor 3 (ISGF3) transcription complex. ISGF3 is composed of latent transcriptional factors of the signal transducers and activators of transcription (STAT) family, STAT1 and STAT2, and of the interferon-regulatory factor (IRF) IRF9 (also known as ISGF3 γ or p48). ISGF3 regulates gene transcription by binding to an IFN-stimulated response element (ISRE)¹⁵. Studies in mice carrying disruptions in either subunit of the IFN- α R complex demonstrate an essential role for IFN- α signaling in the induction of antiviral resistance^{16–19}. However, the loss of antiviral protection to different viruses is variable in IFN- α R–deficient mice. For instance, infection with rotavirus proceeds similarly in mice with a disrupted or intact IFN- α R system²⁰, suggesting that a parallel, but as yet uncharacterized, mechanism of antiviral resistance may exist. One possibility is that antiviral functions are conferred by other cytokines acting through receptors of the class II cytokine receptor family.

In this report, we describe the identification, cloning and initial functional characterization of a family of cytokines, which we have designated IFN- λ s. We define the two essential receptor proteins, CRF2-12 (also designated as IFN- λ R1) and CRF2-4 (also designated as IL-10R2), that comprise the functional receptor complex for these ligands.

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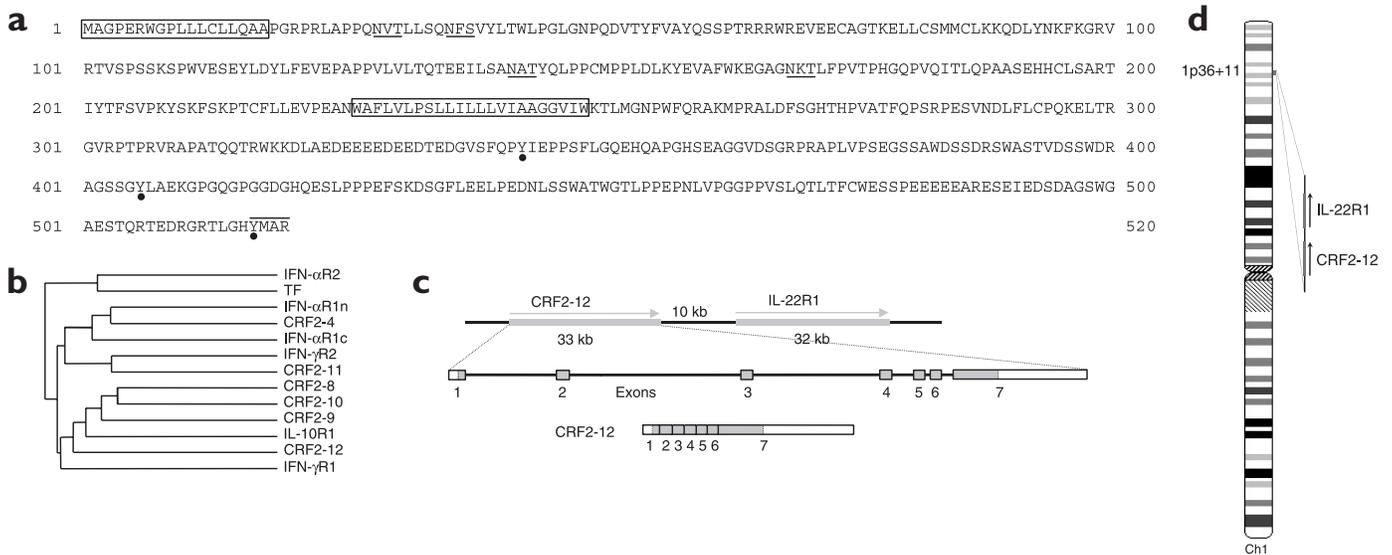


Figure 1. CRF2-12. (a) The deduced amino acid sequence of CRF2-12. Amino acid residues of the predicted signal peptide and transmembrane domain of CRF2-12 are boxed. Potential glycosylation sites are underlined and the C-terminal intracellular tyrosine-based motif, which is also similar to that within the IFN- α R2c intracellular domain and is likely to participate in STAT activation, is overlined. Three tyrosine residues within the intracellular domain, which are potential targets for phosphorylation, are marked with dots. (b) A phylogenetic tree was generated by alignment of amino acid sequences of the CRF2-12 extracellular domain and extracellular domains of other members of the class II cytokine receptor family. (c) Schematic exon-intron structure of the gene encoding CRF2-12 is shown. Coding regions of exons are shaded and the segments corresponding to 5' and 3' untranslated regions are left open. (d) Chromosomal localization of the gene encoding CRF2-12 (*IFNLRI*) and its close neighbor, the gene encoding IL-22R1 (*IL22R1*), as well as the ideogram of human chromosome 1 was generated from the NCBI database. The genes are transcribed in the direction indicated by the arrows.

We also provide an initial survey of their signaling through the Jak-STAT pathway. Finally, we demonstrate that one of the primary biological activities that is mediated by this family of cytokines is the induction of antiviral protection in a wide variety of cells, supporting their designation as novel and distinct interferons, the IFN- λ s.

Results

Cloning and characterization of CRF2-12

To identify possible additional members of the class II cytokine receptor family, a TBLASTN search of the GenBank and ENSEMBL databases was performed with consensus amino acid motifs drawn from the extracellular domains of receptors from this family. A genomic fragment from human chromosome 1 was identified as encoding an amino acid sequence with similarity to the consensus motif and with partial identity to the GenomeScan-predicted peptide Hs1_4548_30_10_1 (encoded by *LOC163702*). However, our prediction of the last exon encoding the intracellular domain of the receptor did not match the GenomeScan data. In addition, several expressed sequence tags (ESTs) were positioned in this genomic region representing the 3' untranslated region (3'-UTR) of an uncharacterized gene, which we predicted to be contiguous with *LOC163702* (see Methods for details). To test our predictions, several sets of primers were designed and used for reverse-transcribed-polymerase chain reaction (RT-PCR) with a human placental cDNA library as a template (see Methods). The resultant cDNA encoded a 500-aa receptor designated CRF2-12 (for member 12 of the class II cytokine receptor family, **Fig. 1a**). It possessed a characteristic signal peptide of about 18 aa and a 23-aa transmembrane domain (227–249 aa), which divides the receptor into a 208-aa extracellular domain and a relatively long 271-aa intracellular domain (**Fig. 1a**). Alignment of the extracellular domains of the class II cytokine receptors assigned CRF2-12 into a subgroup with several other receptors, CRF2-8 (also known as

IL-20R1), CRF2-9 (also known as IL-22R1), CRF2-10 (also known as IL-22 binding protein), IL-10R1 and IFN- γ R1 (**Fig. 1b**).

Mapping the sequence of CRF2-12 cDNA against the human genomic sequence revealed that the entire gene encoding CRF2-12 (*IFNLRI*) is composed of 7 exons (**Fig. 1c**) and its structure correlates well with the common conserved architecture of other class II cytokine receptor genes¹. The first exon contains the 5'-UTR and the signal peptide. Exons 2, 3, 4 and 5 and a part of exon 6 encode the extracellular domain. Exon 6 also encodes the transmembrane domain and the beginning of the intracellular domain. Exon 7 covers the rest of the intracellular domain and the 3'-UTR. *IFNLRI* is positioned in the vicinity of the gene encoding CRF2-9 (*IL22R1*), a member of the class II cytokine receptor family (1p36+11 chromosomal region) (**Fig. 1c,d**). Both *IFNLRI* and adjacent *IL22R1* are transcribed towards the telomere and positioned approximately 10 kb apart. All exon-intron and intron-exon splice sites within *IFNLRI* conform well to the consensus motifs (exon-GT-intron-AG-exon).

To determine which cell types expressed CRF2-12, we evaluated a panel of RNAs derived from various human tumor cell lines and tissues. The major CRF2-12 transcript appeared to be about 5-kb long and was variably expressed in all the cell lines examined (**Fig. 2a**). These included both hematopoietic (HL-60, K-562, MOLT-4 and Raji) and nonhematopoietic (HeLa S3, SW480, A549 and G-361) cell lines. Of these cell lines, Raji cells expressed the highest amounts of CRF2-12. CRF2-12 was also expressed by various normal tissues, including heart, kidney, skin, small intestine and lung (**Fig. 2b**). CRF2-12 mRNA was also present in skeletal muscle and liver (data not shown). Several CRF2-12 transcripts of different sizes other than the major transcript were observed in heart, skin and MOLT-4 cells, suggesting the possibility of alternative splicing and/or transcriptional termination variants. Therefore, our data demonstrate that CRF2-12 appears to be constitutively expressed across a broad range of cell lines and tissues.

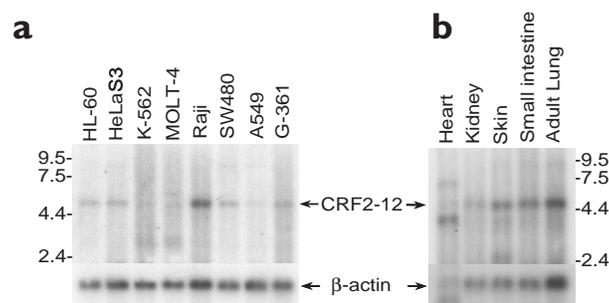


Figure 2. Expression pattern of CRF2-12 mRNA. Northern blotting was performed on two blots containing mRNA isolated from (a) human cancer cell lines (promyelocytic leukemia HL-60, epitheloid carcinoma HeLaS3, lymphoblastic leukemia MOLT-4, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549 and melanoma G361) and (b) normal human fetal tissues (heart, kidney, skin and small intestine) and adult lung. Arrows point to the CRF2-12 and the β -actin transcripts. Equal RNA loading was assessed by evaluating the expression of the gene encoding β -actin.

Expression of chimeric CRF2-12-IFN- γ R1 chain

Functional class II cytokine receptor complexes have two receptor chains, R1 and R2, with distinct and complementary functions^{1,2}. Although both R1 and R2 receptors are associated with Jak tyrosine kinases, only the R1-type subunits have long intracellular domains that can be phosphorylated on tyrosine residues after receptor engagement. Phosphorylation of R1 drives the recruitment of various signaling molecules and thus determines the specificity of cytokine signaling through activation of specific molecules, such as the STATs. R2 possesses a short intracellular domain and supports signaling by recruiting an additional tyrosine kinase to the receptor complex, but does not determine the specificity of signaling. The length of the CRF2-12 intracellular domain suggested that this receptor chain represented an R1-type subunit. Accordingly, CRF2-12 needs to dimerize with one of the R2-type subunits in order to generate a functional receptor complex. We speculated that either the CRF2-4 chain, which is ubiquitously expressed in tissues^{21,22}, or CRF2-11 (also known as IL-20R2), which also demonstrates a wide pattern of expression^{22,23}, are likely candidates to complement CRF2-12 in forming a functional receptor complex for a known or unidentified cytokine.

Chimeric R1-type receptors with their natural intracellular domains replaced by that of IFN- γ R1 signal through the IFN- γ pathway^{21,24,25}. We generated the chimeric CRF2-12-IFN- γ R1 (referred to hereafter as CRF2-12- γ R1) chain and expressed it with either of the intact R2 chains CRF2-4 and CRF2-11 in 16-9 hamster cells. To ensure that both receptors were expressed in each transfected cell, tandem vectors were constructed in which expression of CRF2-12- γ R1 and either CRF2-4 or CRF2-11 was controlled by separate promoters and polyadenylation signals. We then tested the ability of the resultant receptor complexes to transduce IFN- γ -like signaling upon treatment with different cytokines by testing for responsiveness to different ligands, utilizing electrophoretic mobility shift assay (EMSA) to detect STAT1 activation and flow cytometry to evaluate major histocompatibility complex (MHC) class I antigen expression. We did not detect signaling by IL-19, IL-20, IL-22 and IL-24 in cells expressing CRF2-12- γ R1 and either CRF2-4 or CRF2-11 chains (data not shown), suggesting that this receptor chain was involved in the signaling of another ligand.

The IFN- λ family

We identified a protein that demonstrated a limited similarity to members of both the type I IFN and IL-10 families (see Methods). Genes encoding two other closely related proteins were also identified by screening the sequence of the human genome for possible paralogs (Fig. 3). Genes encoding all three members of this cytokine family (Fig. 3a,b) are clustered on human chromosome 19 (19q13+13 region) and each is composed of five exons (Fig. 3c,d), resembling the structural organization of genes encoding IL-10-related cytokines¹ (whereas genes for recognized type I IFNs lack introns). The genes encoding

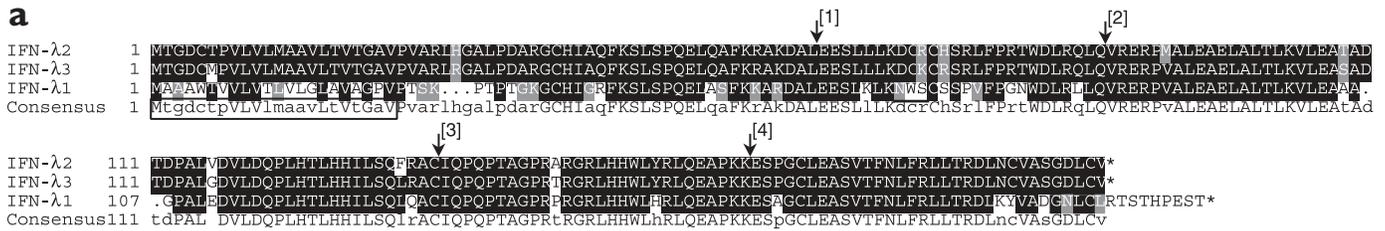
these cytokines, designated IFN- λ 1, IFN- λ 2 and IFN- λ 3 (tentatively designated as IL-29, IL-28A and IL-28B by HUGO), were cloned into the pEF-SPFL plasmid in-frame with the FLAG epitope (referred to hereafter as FL-IFN- λ sgene) and transfected into COS-1 cells, from whence the individual cDNAs were subsequently cloned.

The immunoblots of conditioned media from transfected COS-1 cells revealed that secreted FL-IFN- λ s migrated on the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel as a single band of about 22 kDa for FL-IFN- λ 2 and FL-IFN- λ 3 and as several bands in the region between 20 and 33 kDa for FL-IFN- λ 1, with a major band at about 33 kDa (Fig. 4). This observation suggests possible glycosylation of IFN- λ 1. There is a potential site for N-linked glycosylation (NTS) in IFN- λ 1 that is not present in IFN- λ 2 or IFN- λ 3 (Fig. 3a).

The functional IFN- λ R complex

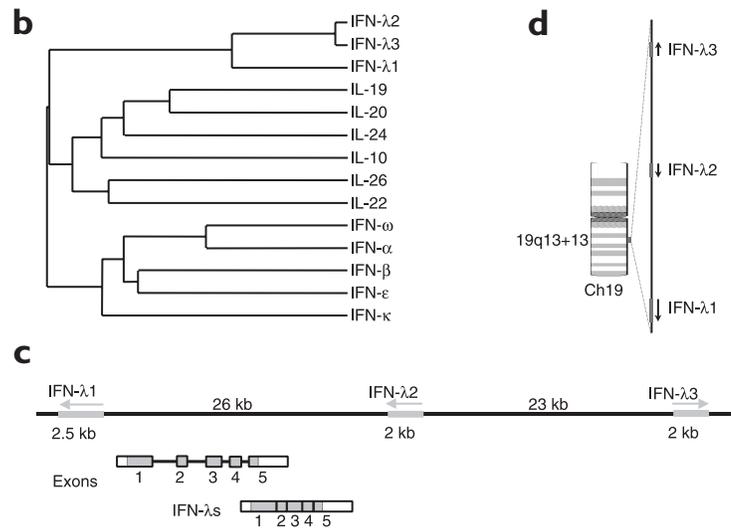
COS-1 cell-conditioned media containing FL-IFN- λ sgene were used to treat hamster cells expressing CRF2-12- γ R1 and either the CRF2-4 or CRF2-11 chains. All three proteins were able to induce STAT activation and up-regulate MHC class I antigen expression, but only in cells expressing CRF2-12- γ R1 and CRF2-4 chains (Fig. 5a,b and data not shown). Expression of CRF2-12- γ R1 or CRF2-4 alone was not sufficient to render cells responsive to IFN- λ treatment. IFN- λ cDNAs were subsequently cloned into the pEF2-SPFL plasmid by RT-PCR, in-frame with the FLAG epitope (FL-IFN- λ s). Conditioned media from COS-1 cells transiently transfected with plasmids containing either IFN- λ cDNAs (FL-IFN- λ s) or genes (FL-IFN- λ sgene) demonstrated indistinguishable biological activities and immunoblotting patterns (Figs. 4 and 5a,b and data not shown). Using flow cytometry, we were able to detect binding of FLAG epitope-tagged IFN- λ s to the cells expressing both CRF2-12- γ R1 and CRF2-4 chains (Fig. 5a, H and data not shown). Furthermore, *E. coli*-expressed IFN- λ 1 could compete with FL-IFN- λ s for receptor binding (Fig. 5a, H and data not shown). Parental cells or cells expressing either chain alone did not bind FL-IFN- λ s, as measured by flow cytometry (Fig. 5a, E-G).

The entire IFN- λ 1 cDNA was also cloned into the pcDEF3 vector. Conditioned medium of COS-1 cells transiently transfected with this expression vector (IFN- λ 1COS) competed for binding with FL-IFN- λ s and was positive in MHC class I induction experiments and in the EMSA experiments (Fig. 5a,b and data not shown), demonstrating that IFN- λ s were secreted with their own signal peptides and were biologically active. *E. coli*-produced recombinant IFN- λ 1 was also active in all the experiments described above (Fig. 5a,b and data not shown). Cytokines activate multiple STATs resulting in the formation of various homo- or heterodimeric STAT DNA-binding complexes with different mobility in EMSA^{15,21,25}. As shown on Fig. 5c, STAT DNA-binding complexes migrated as a single band and their mobility decreased when reacted with anti-STAT1, indicating that, as expected, IFN- λ induced formation of STAT1 dimers in hamster cells expressing CRF2-12- γ R1



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Figure 3. IFN-λs. (a) Sequence alignment of IFN-λ1, IFN-λ2 and IFN-λ3 proteins was generated. The consensus sequence is shown on the bottom. Amino acid residues are numbered starting from the methionine residue (signal peptide amino acids are included). Positions of corresponding introns are indicated by arrows. Predicted signal peptides are boxed. A potential glycosylation site in IFN-λ1 is underlined. (b) A phylogenetic tree for IFN-λ proteins with other class II cytokine receptor ligands. (c) Schematic gene structure and (d) chromosomal localization for IFN-λs genes as well as the direction of transcription (arrows) are shown. Coding regions of exons are shaded and the segments corresponding to 5' and 3' untranslated regions are left open.



and CRF2-4. We also demonstrated that a CRF2-4 neutralizing antibody was able to completely inhibit IFN-λ-induced STAT activation (Fig. 5b), emphasizing the requirement for CRF2-4 in assembling the functional IFN-λR complex.

Both CRF2-12 and CRF2-4 required for ligand binding

We next characterized the interaction between IFN-λ and its receptor by covalent cross-linking to demonstrate the requirement for both receptor chains in ligand binding (Fig. 6). Radiolabeled FL-IFN-λ1-P was cross-linked to itself in solution to determine whether it could oligomerize. Patterns of radiolabeled bands for cross-linked or untreated ligand were identical, suggesting that IFN-λ1 is a monomer in solution. Ligand-receptor cross-linking experiments were then performed with radiolabeled FL-IFN-λ1-P and the IFN-λR chains expressed on the surface of hamster cells (Fig. 6). Parental cells or cells expressing either receptor chain alone were unable to bind the ligand. The appearance of several labeled cross-linked complexes was observed only in cells expressing both CRF2-12-γR1 and CRF2-4 chains (Fig. 6, lane 6). The specificity of binding was shown by competition with an excess

of unlabeled IFN-λ1 (Fig. 6, lane 7). A major radiolabeled band in the region of 33 kDa corresponded to free FL-IFN-λ1-P, the ligand was bound to the cells but not cross-linked to receptors. Additional distinct cross-linked complexes migrating in the region just above 100 kDa, which were not present in the ligand cross-linked to itself in solution, were formed as a result of the interaction of IFN-λ1 with its receptor chains and, thus, contained various complexes of IFN-λ1 and either one or both receptor chains. The cross-linking experiments demonstrated that IFN-λ1 required the presence of both IFN-λR chains on the cell surface and was unable to interact with either chain expressed alone.

IFN-λ signal transduction and biological activity

Cytokines that utilize class II cytokine receptors for signaling primarily activate the Jak-STAT signal transduction pathway for the induction of cytokine-responsive genes^{1,2}. To define which STATs are activated by signaling through the CRF2-12 chain, we examined the activation of several STATs in HT29 cells transfected to express the chimeric receptor FL-IL-10R1-CRF2-12 composed of the FLAG epitope-tagged extracellular domain of the IL-10R1 chain linked to

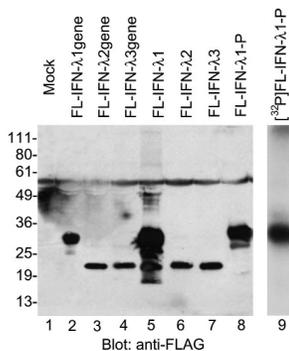


Figure 4. IFN-λs expression. Conditioned media from COS-1 cells transfected with the control plasmid pEF-SPFL (lane 1); plasmids encoding IFN-λ genes, pEF-FL-IFN-λ1 gene (lane 2), pEF-FL-IFN-λ2 gene (lane 3) and pEF-FL-IFN-λ3 gene (lane 4); plasmids encoding IFN-λ cDNAs, pEF-FL-IFN-λ1 (lane 5), pEF-FL-IFN-λ2 (lane 6) and pEF-FL-IFN-λ3 (lane 7); and FL-IFN-λ1-P purified from conditioned media by affinity chromatography (lane 8) were evaluated by immunoblotting with FLAG mAb. Lane 9 represents an autoradiograph of the SDS-PAGE gel containing radiolabeled FL-IFN-λ1-P. The molecular weight markers in kDa are shown on the left.

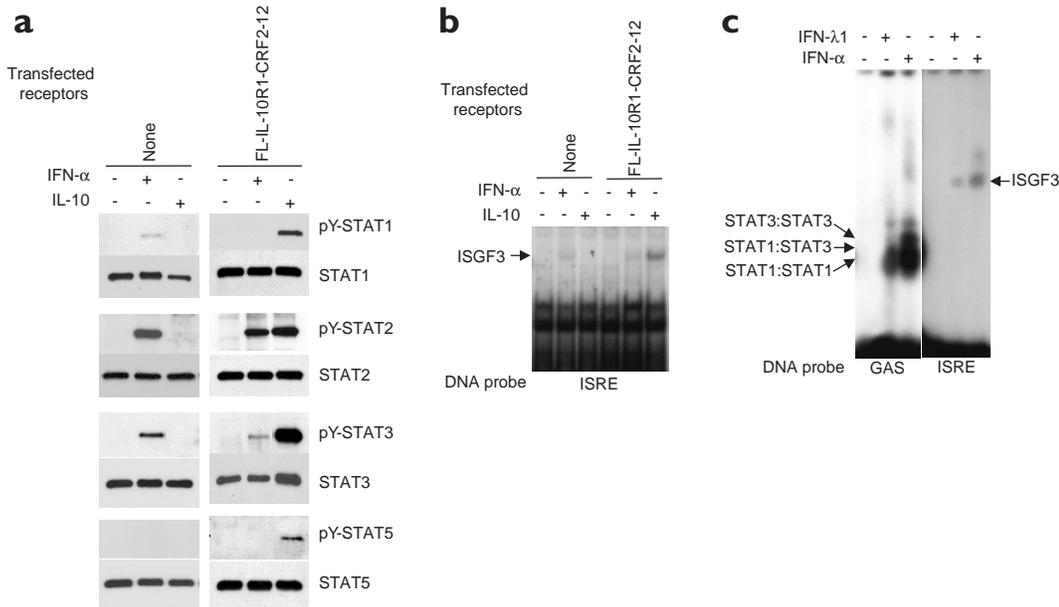


Figure 7. STAT activation performed on human cells. STAT activation in parental HT-29 cells or HT-29 cells expressing FL-IL-10R1-CRF2-12 in response to IFN- α and IL-10 was evaluated (a) by immunoblotting and (b) by EMSA with ISRE probe. (c) Intact A549 cells were also used in EMSA experiments with GAS and ISRE DNA probes as indicated. Cellular lysates were prepared and assayed for STAT (see Methods). Positions of STAT DNA-binding complexes in EMSAs are indicated by arrows.

The type I IFNs are defined by their ability to induce antiviral protection in a broad variety of cell types. We therefore evaluated the ability of IFN- λ 1 to induce cellular antiviral protection. As shown in **Fig 8b**, IFN- λ 1 induced antiviral protection in HT29, A549 and HaCaT cells infected with vesicular stomatitis virus (VSV) and in HT29 cells infected with encephalomyocarditis virus (EMCV) (data not shown). In antiviral assays, IFN- λ 1 demonstrated antiviral potency that was comparable to that of IFN- α ; it was of the order of 10^7 – 10^8 IFN- α -equivalent units per mg. Moreover, when HT29 cells were pretreated with 500 pg/ml of IFN- λ 1 prior to VSV infection, the titer of VSV in the conditioned media of infected cells (48 h after infection) dropped more than three orders of magnitude in comparison to the VSV titer produced by control untreated cells. In cells pretreated with 1250 pg/ml of IFN- λ 1, the

viral titer dropped more than six orders of magnitude, as determined by viral killing curve (data not shown).

Type I IFNs induce expression of many proteins, such as 2',5'-oligoadenylate synthetase (2',5'-OAS) and Mx proteins, which mediate antiviral protection. We demonstrated that IFN- λ 1 was capable of inducing transcription of type I IFN-responsive ISRE-controlled genes encoding 2',5'-OAS and MxA protein (**Fig 8c**). Thus, we demonstrated in intact cells that IFN- λ induced overlapping signaling and biological activities with those of IFN- α , which include up-regulation of MHC class I antigen expression and induction of antiviral protection, as well as induction of IFN-stimulated genes.

Because IFN- λ participated in the establishment of an antiviral state, we hypothesized that its expression should be modulated in response to viral infections. To determine whether *IFNL* expression is inducible by

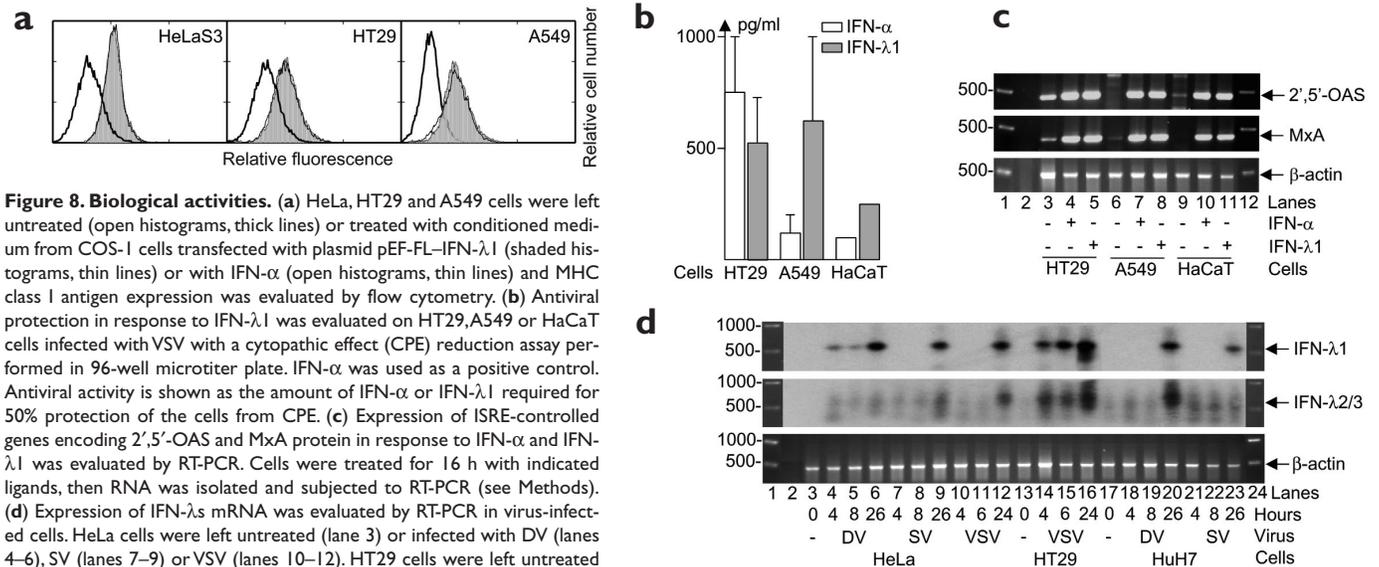


Figure 8. Biological activities. (a) HeLa, HT29 and A549 cells were left untreated (open histograms, thick lines) or treated with conditioned medium from COS-1 cells transfected with plasmid pEF-FL-IFN- λ 1 (shaded histograms, thin lines) or with IFN- α (open histograms, thin lines) and MHC class I antigen expression was evaluated by flow cytometry. (b) Antiviral protection in response to IFN- λ 1 was evaluated on HT29, A549 or HaCaT cells infected with VSV with a cytopathic effect (CPE) reduction assay performed in 96-well microtiter plate. IFN- α was used as a positive control. Antiviral activity is shown as the amount of IFN- α or IFN- λ 1 required for 50% protection of the cells from CPE. (c) Expression of ISRE-controlled genes encoding 2',5'-OAS and MxA protein in response to IFN- α and IFN- λ 1 was evaluated by RT-PCR. Cells were treated for 16 h with indicated ligands, then RNA was isolated and subjected to RT-PCR (see Methods). (d) Expression of IFN- λ s mRNA was evaluated by RT-PCR in virus-infected cells. HeLa cells were left untreated (lane 3) or infected with DV (lanes 4–6), SV (lanes 7–9) or VSV (lanes 10–12). HT29 cells were left untreated (lane 13) or infected with VSV (lanes 14–16). HuH7 cells were left untreated (lane 17) or infected with DV (lanes 18–20) or SV (lanes 21–23). Cells were collected at the indicated times after infection, RNA was isolated and RT-PCR was performed as described (see Methods).

viruses, HeLa cells, HuH7 hepatoma cells or HT-29 cells were incubated with Sindbis virus (SV), Dengue virus 2 (DV) or VSV for various time periods. Consistent with a role in antiviral protection, the expression of IFN- λ 1, IFN- λ 2 and IFN- λ 3 mRNAs was detected in a number of cell lines infected with various viruses (Fig. 8d). Moreover, monocyte-derived dendritic cells, which are important producers of IFN- α ²⁶, expressed IFN- λ 1 mRNA in response to treatment with the viral double-stranded RNA (dsRNA) mimic poly(I)•poly(C) (data not shown). Furthermore, we demonstrated that HT29 cells or A549 cells infected with EMCV secrete IFN- λ -specific activity capable of stimulating STAT1 activation through the CRF2-12- γ R1-CRF2-4 heterodimer (Fig. 5c and data not shown). Uninfected cells did not produce IFN- λ activity.

Discussion

We report here a widely expressed, IFN- α and IFN- β -independent, ligand-receptor system whose signals lead to the establishment of antiviral protection. Three closely positioned genes on human chromosome 19 encode distinct but highly paralogous proteins, which we have designated IFN- λ 1, IFN- λ 2 and IFN- λ 3 based on their ability to induce antiviral protection. We have also identified a receptor complex, which is utilized by all three IFN- λ proteins for signaling. This complex is composed of two subunits: a novel receptor designated CRF2-12 (or IFN- λ R1) and a second subunit, CRF2-4, which is also the shared receptor component within the IL-10 and IL-22 receptor complexes.

The members of the IFN- λ family occupy a space between the previously defined IL-10 family and the type I IFNs in terms of their evolutionary emergence. This suggests several possibilities. We have demonstrated here antiviral activities for IFN- λ , with signaling reminiscent of the type I IFNs (ISGF3 and STAT2 activation). The type I IFNs, however, have a number of other activities in the immune system beyond their direct antiviral action, such as direct antigrowth effects on some cell types and effects on natural killer cell activation, biasing of T helper cell function, B cell maturation and dendritic cell differentiation. Some of these effects derive from cell-specific signaling pathways. As the IFN- λ s are investigated, it is anticipated that a range of effects on immune and nonimmune system cells, perhaps comparable to the range of type I IFN activities, but distinct from the type I IFNs, will be revealed. In addition, it is possible that there will be found cross-coupling of IL-10 subfamily receptors with IFN subfamily receptors to constitute receptor complexes for orphan ligands from both families. Currently, receptors for IL-26, IFN- κ and IFN- ϵ are not defined. The fact that IL-26 is similar to IL-10, IL-22 and IFN- λ , which all share the CRF2-4 chain as a part of their specific receptor complexes, suggests that CRF2-4 is also the second chain for IL-26R, although the identity of the first chain is less clear. *IL26* lies adjacent to *IFNG* on human chromosome 12 and is induced after infection with herpesvirus Saimiri²⁷. It is interesting to speculate that IL-26 perhaps possesses antiviral activities.

We have also demonstrated that both chains of the IFN- λ R complex are required for ligand binding. IFN- λ -induced receptor heterodimerization results in engagement of the Jak-STAT signal transduction pathway, including the phosphorylation of STAT2 and activation of the ISGF3 transcription complex, functions previously held to be specific to the actions of type I IFNs, where STAT recruitment is largely mediated by the IFN- α R2c intracellular domain^{29,30}. It is interesting to note that there is a tyrosine-based motif (Tyr517-Met-Ala-Arg-Stop) at the C terminus of the IFN- λ R1 intracellular domain, similar to that present at the end of the IFN- α R2c intracellular domain (Tyr512-Ile-Met-Arg-Stop) and associated with STAT activation during IFN- α signaling³¹. This motif is also conserved in mouse IFN- λ R1 (Tyr-Leu-Val-Arg-Stop).

Thus, the IFN- λ and IFN- α signaling pathways overlap, at least in part. As a consequence, some of their biological activities are similar, including the induction of antiviral protection and up-regulation of MHC class I antigen expression described here. Although the amount of STAT activation appears to be weaker in response to IFN- λ than in response to IFN- α in intact human cells, IFN- λ was as potent as IFN- α in induction of biological activities.

As might be expected from proteins whose roles include antiviral activity, transcription of IFN- λ genes is induced by several viruses in a variety of cell lines and by poly(I)•poly(C) in dendritic cells (data not shown). Moreover, virus-infected cells produce IFN- λ activity. The kinetics of mRNA expression of IFN- λ genes in response to various viruses suggests that transcriptional activation of the genes may be regulated by a positive feedback mechanism, similar to that described for IFN- α / β genes, when synthesis of additional transcriptional factors is required for robust type I IFN production²⁸.

Future studies will determine the full extent to which the signal transduction pathways and biological activities of IFN- λ and IFN- α are redundant and where their distinct functions lie. Additional studies will also be necessary to determine whether IFN- λ can synergize with IFN- α in mediating resistance to viral infections or whether it plays an independent primary role in establishment of resistance to particular classes of virus.

Methods

CRF2-12: PCR, primers, cDNAs and expression vectors. CRF2-12 cDNA was cloned with the use of nested PCR as follows. A first round of PCR was performed with primers R12-1_F and R12-16_R with a human placental cDNA library (catalog number HL4025AH, Clontech, Palo Alto, CA) as template. The first round PCR product was used as a template for a second round of PCR amplification, with primers R12-2_F and R12-9_R to generate the extracellular domain of the CRF2-12 protein. This was subsequently cloned into plasmid pEF3-IL-10R1- γ R1²¹ with the use of *Kpn*I and *Nhe*I restriction endonucleases to excise the IL-10R1 coding sequence and replace it with that for CRF2-12, resulting in the plasmid pEF-CRF2-12- γ R1. Primers R12-12_F, R12-19_F, R12-8_R and R12-7_R and the same placental cDNA library were used for nested PCR (R12-12_F and R12-8_R primers for the first round and R12-19_F and R12-7_R primers for the second round) to clone the CRF2-12 intracellular domain into the pCR2.1 vector (Invitrogen, Carlsbad, CA), resulting in plasmid pCR2.1-CRF2-12_{IC}.

The FLAG epitope was introduced into plasmid pEF3-IL-10R1- γ R1 as follows. PCR was performed with primers 10R-11_F and 10R-Nhe_R with the use of plasmid pEF3-IL-10R1- γ R1 as a template. The resulting PCR product was cloned into plasmid pEF3-FL γ R2- α R2c²⁹ with the use of *Bam*HI and *Nhe*I restriction endonucleases, resulting in the plasmid pEF-FL-IL-10R1- α R2c. The CRF2-12 intracellular domain was then cloned from plasmid pCR2.1-CRF2-12_{IC} into plasmid pEF-FL-IL-10R1- α R2c with the use of *Nhe*I and *Nor*I restriction endonucleases, resulting in plasmid pEF-pEF-FL-IL-10R1-CRF2-12.

Plasmid pEF-CRF2-11 (or pEF2-IL-20R2) was obtained by cloning PCR-derived CRF2-11 cDNA fragment (first round: primers R11-2_F and R11-7_R and a human leukocyte cDNA library (catalog number HL4050AH, Clontech); second round: primers R11-3_F and R11-6_R and the PCR product of the first round as a template) into the pCDEF3 vector³² with the use of *Kpn*I and *Eco*RI restriction endonucleases.

Tandem vectors encoding two receptors, CRF2-12- γ R1 and either CRF2-4 or CRF2-11, in which the expression of each receptor is controlled by separate promoters and polyadenylation signals were generated as follows. The fragment containing the EF-1 α promoter, the CRF2-12 coding sequence and the bovine growth hormone (BGH) polyadenylation signal was released from the pEF-CRF2-12- γ R1 vector by digestion with *Bsa*I and *Bss*HIII restriction endonucleases and ligated into the *Bsa*I and *Mlu*I sites of either the pEF-CRF2-4³¹ or pEF-CRF2-11 plasmid. The resulting plasmids were designated pEF-CRF2-12- γ R1+CRF2-4 and pEF-CRF2-12- γ R1+CRF2-11, respectively.

IFN- λ : PCR, primers, cDNAs and expression vectors. The following primers were used to amplify the three IFN- λ genes from human genomic DNA: ifnl-1_F, ifnl-2_F, ifnl-5_F, ifnl-6_F, ifnl-3_R and ifnl-4_R. A first round of amplification was performed with either ifnl-1_F and ifnl-3_R primers or ifnl-2_F and ifnl-4_R primers followed by the second round with either ifnl-5_F and ifnl-3_R primers or ifnl-6_F and ifnl-4_R primers and the corresponding PCR products of the first round as templates.

The resulting PCR products were cloned into the *Bam*HI and *Eco*RI restriction sites of the pEF-SPFL vector³³, generating plasmids pEF-FL-IFN- λ 1gene, pEF-FL-IFN- λ 2gene and pEF-FL-IFN- λ 3gene. Because plasmid pEF-SPFL encodes the signal peptide derived from the human IFN- γ R2 chain followed by the FLAG epitope, this placed the IFN- λ reading frames in the frame of the FLAG epitope (FL-IFN- λ sgene). Pro23 (Fig. 3a) was pre-

dicted to be the first amino acid of each of the mature proteins. After transfection of these plasmids into COS-1 cells, total RNA was isolated from the transfectants, converted to cDNA and amplified with the same primers used to obtain the genomic fragments, to obtain IFN- λ 1, IFN- λ 2 and IFN- λ 3 cDNA fragments, which were subsequently cloned into plasmid pEF-SPFL as before, resulting in plasmids pEF-FL-IFN- λ 1, pEF-FL-IFN- λ 2 and pEF-FL-IFN- λ 3. These plasmids encode IFN- λ molecules tagged at their N terminus with the FLAG epitope (FL-IFN- λ).

The PCR product obtained with primers ifnl-5_r and ifnl-7_r, with the use of plasmid pEF-FL-IFN- λ 1 as a template, was digested with *Bam*HI and *Eco*RI and cloned into corresponding sites of the pEF-SPFL vector, resulting in the plasmid pEF-FL-IFN- λ 1-P. This plasmid encodes FL-IFN- λ tagged at its C terminus with the Arg-Arg-Ala-Ser-Val-Ala sequence encoded within the primer (FL-IFN- λ -P), the consensus amino acid sequence recognizable by the catalytic subunit of the cAMP-dependent protein kinase³⁴, allowing the IFN- λ 1 protein to be phosphorylated. The entire IFN- λ 1 cDNA was also cloned by RT-PCR with primers ifnl-1_r and ifnl-3_r, and RNA from HT29 cells infected with VSV into the pDEF3 vector resulting in the plasmid pEF-IFN- λ 1, which encodes IFN- λ 1 with its own signal peptide.

The PCR product obtained with primers ifnl-8_r and ifnl-3_r and plasmid pEF-FL-IFN- λ 1 as a template was digested with *Sca*I and *Eco*RI and cloned into *Xmn*I and *Eco*RI sites of the pMAL-p2x vector (New England Biolabs, Beverly, MA), resulting in plasmid pMAL-IFN- λ 1. This plasmid encodes a maltose-binding protein-IFN- λ 1 fusion protein (MBP-IFN- λ 1), which is transported to the periplasm of *E. coli* cells. The fusion protein was purified from the periplasm by the cold osmotic shock method according to the manufacturer's protocol. Protein concentration was determined against protein standards on a Coomassie blue-stained gel.

The nucleotide sequences of the modified regions of all constructs were verified in their entirety by DNA sequencing.

Cells, transfection and flow cytometry. The 16-9 hamster-human somatic cell hybrid line, the Chinese hamster ovary cell (CHO-K1) hybrid containing a translocation of the long arm of human chromosome 6 encoding human *IFNGR1* and a transfected human *HLA-B*27*⁵⁵, was from S. Pestka. The cells were maintained in Ham's F12 cell culture medium (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (FBS, Sigma). COS-1 cells, an SV40-transformed fibroblast-like simian CV-1 cell line, epitheloid carcinoma HeLa S3 cells, lung carcinoma A549 cells, keratinocyte HaCaT cells and hepatoma HuH7 cells were maintained in DMEM medium (Sigma) with 10% heat-inactivated FBS. Colorectal adenocarcinoma HT29 cells were maintained in RPMI medium (Sigma) with 10% heat-inactivated FBS. Cells were transfected as previously described^{21,33}. COS-1 cell supernatants were collected at 72 h and used as a source of the expressed proteins.

To detect changes in MHC class I antigen (HLA-B7) expression, cells were treated with COS-1 cell supernatants (100 μ l) or purified recombinant proteins (100 ng/ml of human IFN- α 2 and 100 ng/ml of *E. coli*-produced IFN- λ 1) for 72 h and their MHC class I expression analyzed by flow cytometry. Cell surface expression of the HLA-B7 antigen was detected by treatment with the mouse W6/32 HLA monoclonal antibody (mAb)³⁶, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Ligand binding was performed by flow cytometry as described³³.

EMSA, immunoblotting and northern blotting. Cells were treated with various ligands for 15 min at 37 °C and used for EMSA experiments to detect activation of STAT1, STAT2, STAT3 and STAT5 as previously described²¹. EMSAs were performed either with a 22-bp sequence containing a STAT1 α binding site corresponding to the GAS element in the promoter region of human *IRF1*, with a 27-bp DNA probe containing a consensus ISRE sequence or with a 38-bp DNA probe corresponding to the ISRE sequence present in the proximal promoter region of the gene encoding ISG-15 as described^{24,29,37,38}. A549 cells used for preparing cellular lysates to be tested with the ISRE probe were first pretreated with human IFN- γ 18 h before treatment with human IFN- α 2 or IFN- λ 1. CRF2-4 antibody was purchased from R&D Systems (Minneapolis, MN). STAT1 antibody was from Santa Cruz Biotechnology.

Three days after transfection, conditioned media from COS-1 cells transiently transfected with expression plasmids were collected. FLAG-tagged proteins in the conditioned media (10 μ l) were identified by immunoblotting with FLAG epitope-specific M2 mAb (Sigma) as described³³.

To evaluate STAT activation by immunoblotting, cells were treated with various ligands for 15 min at 37 °C. STAT activation was measured by immunoprecipitating specific STAT proteins from whole cell lysates with STAT1, STAT2, STAT3 or STAT5 antibodies (Santa Cruz Biotechnology), resolving the immunoprecipitates on SDS-PAGE and then blotting with the phosphotyrosine-specific antibodies pY-STAT1 and pY-STAT3 (Cell Signaling Technology Inc., Beverly, MA), pY-STAT2 (Upstate USA Inc., Lake Placid, NY) and pY-STAT5 (Zymed Laboratories, San Francisco, CA).

Northern blotting was performed as described^{25,39} with two blots (catalog number 7757-1, Clontech and catalog number D2802-50, Invitrogen) with a CRF2-12 probe corresponding to the coding region of the CRF2-12 cDNA.

Cross-linking. The FL-IFN- λ 1-P protein was transiently expressed in COS-1 cells and purified from conditioned media by immunoaffinity chromatography with the anti-FLAG M1 gel (Sigma) according to the manufacturer's suggested protocols. FL-IFN- λ 1-P was labeled with [³²P]ATP and used for cross-linking to cells as described^{34,37,40,41}.

Virus infection, antiviral protection and RT-PCR. The following viruses were used in the study: SV (an RNA alphavirus), DV (or DEN2, an RNA flavivirus), VSV (an RNA rhabdovirus, Indiana strain) and EMCV (an RNA picornavirus, BeAn 8386 strain). Conditioned media from virus-infected cells was collected 48 h after infection and used for EMSA or viral killing curve assay. Virus-infected or uninfected cells were disrupted and the RNA was isolated according to protocols suggested by the manufacturer (TRIzol Reagent, Invitrogen or RNeasy method, Qiagen, Valencia, CA). RNA samples were used for nested RT-PCR with primers specific for the three IFN- λ species. The first round was performed with the primers ifnl-1 and ifnl-3 or ifnl-2 and ifn-4 and the second round with the primers ifnl-5 and ifnl-3 or ifnl-6 and ifnl-4 with GeneAmp (RT-PCR) kit reagents (Perkin Elmer, Wellesley, MA). The PCR products were analyzed in a 1.0% agarose gel either stained with ethidium bromide or used for Southern blotting with a probe corresponding to the coding region of the IFN- λ 1 or IFN- λ 2 cDNA. Expression of genes encoding 2',5'-oligoadenylate synthetase (OAS) and MxA protein in response to human IFN- α 2 (10 ng/ml) and *E. coli*-produced IFN- λ 1 (10 ng/ml) was determined by RT-PCR as described^{22,43}. RNA samples were also used for RT-PCR with β -actin specific primers as described³³.

Antiviral assays were performed essentially as described⁴⁴. An equal number of cells was plated in all wells of 96-well microtiter plate and treated with two-fold serial dilutions of indicated ligands (human IFN- α 2 or *E. coli*-produced IFN- λ 1). Twenty-four hours later the cells were challenged with VSV and then further incubated until controls showed full killing by virus (1–2 days). Cells not killed were visualized by staining with crystal violet.

Primers. 10R-11_r: 5'-CCGGATCCTCTTGCTCAGACGCTC-3'; 10R-Nhe_r - 5'-ATCGC TAGCCAGTTGGTACACGGTGAATAC-3'; R11-2_r: 5'-TTTGGAAAGAAACAATGTTCTAGGTC-3'; R11-3_r: 5'-CTGAGGGTACCAATGCAGACTTTCACAATG-3'; R11-6_r: 5'-GGGAATTCATGAGATCCAGGCCCTGAGGAGTTC-3'; R11-7_r: 5'-CTTACCTGG GCCCTCCGC-3'; R12-1_r: 5'-GCCGCGCAGGAAGGCCATGGCCG-3'; R12-2_r: 5'-GCCGGTACCATGGCGGGCCGAGCGTGG-3'; R12-12_r: 5'-TCTAAGCCACCTGCTTCTTGCTG-3'; R12-19_r: 5'-CTGGTACGCCCTGGTGTGCCATCCG-3'; R12-7_r: 5'-GGGTCTAGATCACCTGGCCATGTAATGCCCA-3'; R12-8_r: 5'-AGGCAGCAGCAGC ATCAGATTCGG-3'; R12-9_r: 5'-AGGGCTAGCCAGTTGGCTTCTGGACCTCC-3'; R12-16_r: 5'-CATCCTCTCTCTTCGCTCTG-3'; ifnl-1_r: 5'-GCGGTACCATGGCTG CAGCTTGGACCTGGG-3'; ifnl-2_r: 5'-GCGGTACCATGACTGGGGACTGCACGCCA GTG-3'; ifnl-5_r: 5'-GCGGATCTGTCCCACTTCCAAGCCACC-3'; ifnl-6_r: 5'-GCG GATCTGTGCGCAGGCTCCGCGGGGCT-3'; ifnl-8_r: 5'-GATAGTACTTCCAAGCC ACCACA-3'; ifnl-3_r: 5'-CGGAATTCAGGTGGACTCAGGGTGGTGA-3'; ifnl-4_r: 5'-CGGAATTCAGACACACAGGTCCTCCACTGGCAACACA-3'; ifnl-7_r: 5'-GCGAATTC A TGCCAGGATGCCCGCAATTGAGGTGGACTCAGGGTGGG-3'.

Data analysis. Alignment was generated by the program PILEUP of the Wisconsin Package, version 9.1, Genetics Computer Group (Madison, WI). The CLUSTAL X program was used to generate the phylogenetic tree.

Web addresses. The GenomeScan-predicted peptide Hs1_4548_30_10_1 can be found on the NCBI website at <http://www.ncbi.nlm.nih.gov>.

Accession numbers. The Genbank accession numbers for cDNA and the deduced amino acid sequences were as follows. Human IFN- λ 1 (AY184372), human IFN- λ 2 (AY184373), human IFN- λ 3 (AY184374), mouse IFN- λ (AY184375), human IFN- λ R1 (CRF2-12) (AF439325) and mouse IFN- λ R1 (CRF2-12) (AY184376). The Genbank accession number for the protein that demonstrated a limited similarity to members of both the type I IFN and IL-10 families is AX355968. The UniGene designation of the uncharacterized gene used to clone and characterize CRF2-12 is Hs.105866.

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Competing interests statement

The authors declare that they have no competing financial interests.

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