

Murine interferon lambdas (type III interferons) exhibit potent antiviral activity *in vivo* in a poxvirus infection model

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Human interferon lambdas (IFN- λ s) (type III IFNs) exhibit antiviral activity *in vitro* by binding to a receptor complex distinct from that used by type I and type II IFNs, and subsequent signalling through the Janus kinase signal transducers and activators of transcription (STAT) pathway. However, evidence for a function of type III IFNs during virus infection *in vivo* is lacking. Here, the expression of murine IFN- λ s by recombinant vaccinia virus (VACV) is described and these proteins are shown to have potent antiviral activity *in vivo*. VACV expressing murine IFN- λ 2 (vIFN- λ 2) and IFN- λ 3 (vIFN- λ 3) showed normal growth in tissue culture and expressed *N*-glycosylated IFN- λ in infected cell extracts and culture supernatants. The role that murine IFN- λ s play during virus infection was assessed in two different mouse models. vIFN- λ 2 and vIFN- λ 3 were avirulent for mice infected intranasally and induced no signs of illness or weight loss, in contrast to control viruses. Attenuation of vIFN- λ 2 was associated with increases in lymphocytes in bronchial alveolar lavages and CD4⁺ T cells in total-lung lymphocyte preparations. In addition, vIFN- λ 2 was cleared more rapidly from infected lungs and, in contrast to control viruses, did not disseminate to the brain. Expression of IFN- λ 2 also attenuated VACV in an intradermal-infection model, characterized by a delay in lesion onset and reduced lesion size. Thus, by characterizing murine IFN- λ s within a mouse infection model, the potent antiviral and immunostimulatory activity of IFN- λ s in response to poxvirus infection has been demonstrated.

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INTRODUCTION

The type III interferon (IFN) family consists of IFN- λ 1, - λ 2 and - λ 3 [also called interleukin (IL)29, IL28A and IL28B] and these IFNs are the most recent additions to the class II cytokine-receptor ligand family (Kotenko *et al.*, 2003;

Sheppard *et al.*, 2003), which also includes IL10 and the IL10-related cytokines, and type I and type II IFNs (Kotenko & Langer, 2004). Type I IFNs, which include IFN- α/β , are released early from virus-infected cells and are crucial to induction of antiviral protection and subsequent development of adaptive immune responses. Following receptor binding, type I IFNs activate the Janus kinase signal transducers and activators of transcription (STAT) signalling pathway through the formation of the IFN-stimulated gene factor 3 (ISGF3) transcription complex, which comprises STAT1, STAT2 and IFN regulatory factor 9 (Pestka, 1997). Once activated, this complex translocates to the nucleus, where it binds to IFN-stimulated response elements (ISRE), regulating expression of numerous genes that are required for induction of antiviral immunity (Darnell *et al.*, 1994).

Human IFN- λ s were identified based on limited sequence similarity to members of the class II cytokine-receptor ligand family and are secreted proteins that bind to a unique

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The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AY869695 (129/Sv mouse IFN- λ 2) and AY869696 (IFN- λ 3).

An amino acid alignment of mouse IFN- λ 2 and IFN- λ 3 with the mouse type I and type II IFNs is available as supplementary material in JGV Online.

heterodimeric receptor (IFN- λ R), composed of CRF2-12 (also designated IFN- λ R1) and CRF2-4 (also designated IL10R2). Despite binding to a unique receptor, IFN- λ s share many functional characteristics with IFN- α/β . Both families of IFNs are induced by virus infection or double-stranded RNA, signal via the Janus kinase (Jak)-STAT signal-transduction pathway, activate ISRE-regulated gene expression and upregulate major histocompatibility complex (MHC) class I antigen expression. In addition, cells treated with IFN- λ s are resistant to the cytopathic effect induced by virus infection (Kotenko *et al.*, 2003; Sheppard *et al.*, 2003). Further studies have elucidated the biochemical events at the IFN- λ receptor that are required for signal transduction in response to ligand binding. Like the type I IFN receptor, phosphorylation of specific tyrosine residues within the cytoplasmic domain of the IFN- λ receptor is necessary for triggering STAT activation and signal transduction (Dumoutier *et al.*, 2004).

The IFN- λ s are a distinct family of class II cytokine-receptor ligands that, *in vitro*, exhibit biological characteristics similar to those of IFN- α/β . However, little is known about the activity of these molecules within the host during virus infection, and how they may affect induction of antiviral protection and development of adaptive immune responses *in vivo*. This was addressed here by cloning murine IFN- λ 2 and - λ 3 and constructing vaccinia viruses (VACVs) expressing these cDNAs. By using these viruses (vIFN- λ 2 and vIFN- λ 3) and control VACVs lacking the IFN- λ cDNAs, we demonstrated potent antiviral activity of IFN- λ s in two mouse models. Analysis of the cellular immune responses in mice infected intranasally indicated that attenuation was associated with a greater influx of lymphocytes into the lung, reduced IFN- γ levels and reduced virus titres. In a localized dermal model, expression of IFN- λ 2 delayed the formation of lesions and reduced the maximum lesion size. These data demonstrate the potent antiviral and immunostimulatory activity of IFN- λ s *in vivo*.

METHODS

Cells and viruses. Monkey BS-C-1 and CV-1, murine PAM212 and L929 and rabbit RK-13 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g glucose l^{-1} , 2 mM L-glutamine, 50 U penicillin ml^{-1} , 50 μ g streptomycin sulphate ml^{-1} and 10% fetal bovine serum (FBS) (all from Invitrogen). The Western Reserve (WR) strain of VACV, lacking gene *B8R* encoding the IFN- γ receptor (v Δ B8R) (Symons *et al.*, 2002), was propagated in BS-C-1 cells in DMEM/2.5% FBS.

Plasmid construction. Mouse 129/Sv strain genomic DNA and primers 5'-CCGGTACCATGCTCCTCCTGCTGTGCCTCTGC-3' (mifnl-2F), 5'-CCGGATCCTGTCCCCAGGGCCACCAGGC-3' (mifnl-6F), 5'-GAGGAATTCAGGTCAGACACACTGGTCTCC-3' (mifnl-4R) and 5'-CGGAATTCAGACACACAGGTCCCCACTGGCAACACA-3' (ifnl-4R) were used to amplify mouse IFN- λ 2 and IFN- λ 3 genes. PCR products obtained with primers mifnl-2F and mifnl-4R were cloned into vector pDEF3 (Goldman *et al.*, 1996) by using *KpnI* and *EcoRI*, generating plasmids pEF-mIFN- λ 2gene and pEF-mIFN- λ 3gene. cDNAs encoding mature mIFN- λ s (Asp20 was predicted to be the first amino acid of the mature proteins; Fig. 1)

were obtained subsequently by RT-PCR, using mRNAs from COS-1 cells transfected with plasmids pEF-mIFN- λ 2/3gene as template and mifnl-6F and ifnl-4R primers. The fragments were cloned into the *BamHI* and *EcoRI* sites of vector pEF-SPFL (Kotenko *et al.*, 2000), generating plasmids pEF-FL-mIFN- λ 2 and pEF-FL-mIFN- λ 3. Because plasmid pEF-SPFL encodes a signal peptide followed by the FLAG epitope, this abutted the IFN- λ -coding region in frame with the FLAG epitope. Therefore, these plasmids encode mIFN- λ s tagged at their N terminus with the FLAG epitope (FL-mIFN- λ 2/3). The nucleotide sequences of all constructs were verified by DNA sequencing.

FLAG-tagged murine IFN- λ 2 and IFN- λ 3 were amplified by PCR from pEF-FL-mIFN- λ 2 and pEF-FL-mIFN- λ 3, respectively, using primers 5'-AATTGTCGACCATGCGACCGACGC-3' and 5'-AATTATCGATTCAGACACACTGGTCTC-3'. Amplified products were digested with *SalI* and *Clal* (restriction sites underlined) and cloned into vector p Δ B8R (Symons *et al.*, 2002) that had been digested with *XhoI* and *Clal*, generating plasmids p Δ B8R-SEL-IFN λ 2 and p Δ B8R-SEL-IFN λ 3.

Generation of recombinant VACVs expressing IFN- λ s.

Recombinant VACV expressing murine IFN- λ 2 or IFN- λ 3 under the control of a strong synthetic early and late promoter (pSEL) from the B8R locus were constructed by transient dominant selection using the *Ecogpt* selectable marker (Boyle & Coupar, 1988; Falkner & Moss, 1990). CV-1 cells were infected with v Δ B8R at 0.05 p.f.u. per cell and transfected with p Δ B8R-SEL-IFN λ 2 or p Δ B8R-SEL-IFN λ 3. Intermediate virus was plaque-purified in BS-C-1 cells in the presence of 25 μ g mycophenolic acid (MPA) ml^{-1} . Upon withdrawal of MPA, viruses resolved to vIFN- λ 2, vIFN- λ 3 or parental v Δ B8R-2 or v Δ B8R-3, and were plaque-purified and analysed by PCR using primers B7Rfwd 5'-TATTGATATGTATACCATTGACTCGTC-3' and B9Rrev 5'-GAATCCATTGCCCTTCAGTTATC-3'. Revertant viruses, vRev- λ 2 or vRev- λ 3, were also obtained by transient dominant selection by infecting CV-1 cells with vIFN- λ 2 or vIFN- λ 3, respectively, and transfecting with p Δ B8R. Intracellular virus was prepared by centrifugation through a 36% (w/v) sucrose cushion as described previously (Wilcock & Smith, 1994).

Immunoblotting. RK-13 cells were infected at 10 p.f.u. per cell or mock-infected with medium alone, washed and overlaid with FBS-free DMEM. At 24 h post-infection (p.i.), supernatants and cells were prepared as described previously before resolution by SDS-PAGE (12% gel) under reducing conditions (Bartlett *et al.*, 2004). Proteins were transferred to nitrocellulose and probed with mouse anti-FLAG mAb (Sigma-Aldrich) and bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich) and visualized with the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences).

ISRE-luciferase assays. PAM212 cells were transfected with 0.5 μ g pSV- β -gal (Promega), containing the β -galactosidase (β -gal) gene, and 1 μ g of either pISRE-luc or pCIS-CK (Stratagene). At 18 h p.i., the medium was replaced with DMEM/10% FBS containing either recombinant murine IFN- λ 2 (Peprtech) or supernatant fluid taken from vIFN- λ 2-, vIFN- λ 3- or v Δ B8R-infected cells. After 7 h, cells were harvested into reporter lysis buffer (Promega) and analysed for luciferase and β -gal activity. Luciferase activity was measured by using the luciferase-assay system as instructed by the manufacturer (Promega). β -gal activity was measured by incubation with chlorophenol red β -D-galactopyranoside (CPRG) reagent (Roche) at 37 °C for 30 min and measuring A_{570} . β -gal activity was used to normalize transfection efficiency and pCIS-CK was used to control for background luciferase activity.

Mouse infection models. The virulence of VACVs was determined in female, 6–8-week-old BALB/c mice. Mice were anaesthetized and infected intranasally with 5×10^3 p.f.u. virus in 20 μ l PBS,

or intradermally with 10^6 p.f.u. virus in 10 μ l PBS. Mice infected intranasally were weighed daily and assessed for signs of illness as described previously (Alcami & Smith, 1992). For intradermal infections, the sizes of lesions were measured daily with a micrometer (Tschärke & Smith, 1999). On the indicated days p.i., mice were sacrificed and lavaged as described by Hussell *et al.* (1997). Bronchial alveolar lavage (BAL) samples were centrifuged at 1500 g to obtain BAL cells that were enumerated by using a haemocytometer and trypan blue exclusion. Cell-free BAL fluid was assayed for IFN- γ by using a mouse IFN- γ immunoassay Quantikine kit (R&D Systems). Virus titres in lung and brain homogenates were determined as described previously (Bartlett *et al.*, 2004). Leukocytes were obtained from lung homogenates by enzymic digestion, lysis of erythrocytes and centrifugation through 20% Percoll (Sigma-Aldrich) as described by Lindell *et al.* (2001). Leukocytes were resuspended in 1.0 ml RPMI/5% FBS and live cells were enumerated by using a haemocytometer and trypan blue exclusion.

Flow-cytometric analysis of lung lymphocytes. Purified lung leukocytes were blocked with 10% normal rat serum, 0.5 μ g Fc block (BD Biosciences) in FACS buffer (PBS containing 0.1% BSA and 0.1% sodium azide) on ice for 20 min. CD4⁺ [H129.19—phycoerythrin (PE)] and CD8⁺ (53-6.7-PECy5) lymphocytes were identified by their characteristic size (forward scatter) and granularity (side scatter) and by CD3⁺ (17A2—fluorescein isothiocyanate) staining as described previously (Reading & Smith, 2003). After staining, cells were washed twice with FACS buffer and then fixed with 1% paraformaldehyde in PBS. Samples were analysed on a Becton Dickinson FACSCalibur flow cytometer, collecting data on at least 50 000 gated lymphocytes from each sample.

Alignment and phylogenetic comparison. Programs MULTALIGN (<http://prodes.toulouse.inra.fr/multalin/multalin.html>); gap penalties: gap weight 2, gap length weight 1) and TREE TOP (http://www.genebee.msu.su/services/phtree_reduced.html) were used to create the amino acid alignment of mouse IFNs and the phylogenetic tree, respectively. References and additional information are available from the websites.

RESULTS

Cloning and expression of murine IFN- λ s

Following the discovery of human IFN- λ proteins, the sequence of the mouse genome was searched to identify mouse IFN- λ orthologues. Although there are three genes encoding closely related but distinct human IFN- λ s (IFN- λ 1, IFN- λ 2 and IFN- λ 3), the search revealed the existence of only two intact mouse genes, representing mouse IFN- λ 2 and IFN- λ 3 gene orthologues. The mouse IFN- λ 1 gene orthologue contains a stop codon in the first exon and is therefore predicted not to encode an intact protein. Mouse IFN- λ 2 and IFN- λ 3 are very similar to each other (98% similarity and 96.9% identity) and are clearly related to other mouse IFNs (Fig. 1) and to human IFN- λ s (59–60% amino acid identity). Both mouse cytokines are active on human and mouse cells (data not shown) and, like their human counterparts, are able to upregulate MHC class I antigen expression and induce an antiviral state in responsive cells (data not shown).

To characterize the IFN- λ s and examine their function *in vivo* during a virus infection, murine IFN- λ 2 and IFN- λ 3 cDNAs were expressed from the VACV strain WR

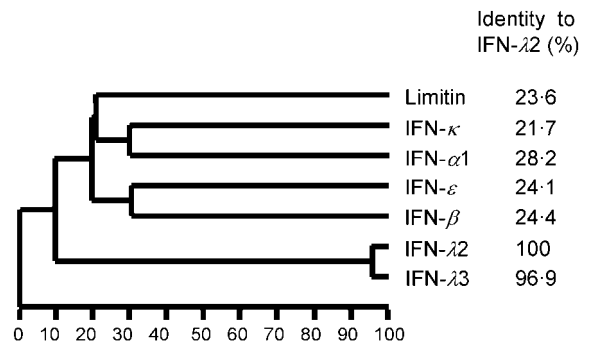


Fig. 1. Phylogenetic tree showing the relationships of murine IFN- λ 2 (GenBank accession no. AY869695) and IFN- λ 3 (AY869696) with the mouse type I IFNs limitin (AY220466), IFN- α 1 (AY226993), IFN- β (X14029), IFN- κ (F547990) and IFN- ϵ (AY190044), and type II IFN, IFN- γ (NM_008337). The sequences were aligned as described in Methods and the alignment (see Supplementary Figure in JGV Online) was used to create the tree shown (Methods). Bootstrap values are 100% for each branch-point of the tree. The x-axis scale represents amino acid identity (%) between individual IFNs and/or IFN subgroups. Amino acid identity (%) of all IFNs to IFN- λ 2 is also shown.

lacking gene *B8R* encoding the VACV IFN- γ receptor v Δ B8R (Symons *et al.*, 2002), forming viruses vIFN- λ 2 and vIFN- λ 3. Each IFN- λ cDNA was engineered to encode an N-terminal FLAG tag downstream of a signal peptide to enable detection of the recombinant IFN- λ proteins. Matched revertant viruses (vRev- λ 2 and vRev- λ 3) from which the IFN- λ genes had been removed were also constructed. To characterize the IFN- λ s, BS-C-1 cells were infected with each virus and FLAG-tagged proteins in the cells and supernatants were detected by immunoblotting (Fig. 2). The predicted size of secreted, FLAG-tagged IFN- λ 2 and IFN- λ 3 (lacking the signal peptide) was 21 kDa. However, SDS-PAGE analysis of supernatant proteins revealed that IFN- λ 2 migrated as a broad band (30–36 kDa), whereas IFN- λ 3 consisted of two distinct bands, a major band of 22–25 kDa and a minor band of 35 kDa (Fig. 2a). IFN- λ 2 and IFN- λ 3 each contain a potential site for addition of N-linked carbohydrate, suggesting that the mature proteins might be glycosylated. For vIFN- λ 3, the potential glycosylation site is suboptimal and this may explain why only a fraction of the protein was glycosylated. The glycosylated status of the secreted proteins was confirmed by digestion with peptide N-glycosidase F (PNGase F) (Fig. 2b) and by synthesis of the proteins in the presence of tunicamycin (Fig. 2c). In the former case, the 35 kDa forms were converted into smaller proteins of 25 kDa, and in the latter case, the majority of each protein produced migrated as the smaller 25 kDa form. Collectively, these data indicate that the IFN- λ 2 and IFN- λ 3 proteins are secreted proteins with N-linked glycans.

To assess whether murine IFN- λ 2 and IFN- λ 3 proteins are monomeric or oligomeric, supernatants from vIFN- λ -infected cells were concentrated tenfold and analysed by

size-exclusion chromatography (Fig. 2d). For both IFN- λ 2 and IFN- λ 3, the majority of the proteins eluted within the range 30–36 kDa, indicating that the proteins were monomeric under physiological conditions.

Growth kinetics of IFN- λ -expressing VACV *in vitro*

Pretreatment of cells with human IFN- λ 1 or IFN- λ 3 protected cells expressing the IFN- λ receptor (IFN- λ R) from virus-induced cytopathic effect (Kotenko *et al.*, 2003; Sheppard *et al.*, 2003). It was therefore possible that replication of an IFN- λ -expressing VACV in mouse cells expressing IFN- λ R might be restricted because of an antiviral response. To investigate this, PAM212 or BS-C-1 cells, which do or do not express murine IFN- λ R,

respectively, were infected with vIFN- λ 2 or v Δ B8R-2 at 0.1 (Fig. 3a) or 10 (data not shown) p.f.u. per cell. The growth of vIFN- λ 2 and v Δ B8R-2 was indistinguishable under either condition, indicating that expression of IFN- λ 2 did not retard VACV growth under the conditions tested.

ISRE-regulated gene expression is induced by recombinant VACV-expressed murine IFN- λ

Like type I IFNs, human IFN- λ s signal through the ISGF3 transcriptional complex, resulting in transcriptional upregulation of ISRE-controlled genes involved in induction of antiviral activity (Kotenko *et al.*, 2003). To determine whether PAM212 cells are responsive to murine IFN- λ s, we measured activation of ISRE-regulated gene expression after transfection of these cells with an ISRE-luciferase reporter gene and incubation with murine IFN- λ 2 produced by *Escherichia coli* or vIFN- λ 2. IFN- λ 2 from both sources and IFN- λ 3 from vIFN- λ 3-infected cells induced luciferase production above that of controls (v Δ B8R-infected cell supernatants or mock-treated cells) (Fig. 3b and data not shown), confirming the biological activity of these virus-expressed proteins *in vitro*.

Expression of IFN- λ attenuates VACV in a murine intranasal model

Intranasal infection of BALB/c mice with VACV strain WR causes a systemic infection characterized by pneumonia and virus dissemination to other organs (Reading & Smith, 2003). To assess the outcome of expression of murine IFN- λ in this infection model, groups of mice were infected intranasally with either vIFN- λ 2 (Fig. 4a), vIFN- λ 3 (Fig. 4b) or the matched control viruses and were monitored for change in body weight and signs of illness as described previously (Alcami & Smith, 1992). Mice infected with either the parent or revertant viruses lost weight rapidly after day 4 p.i., whereas mice infected with vIFN- λ 2 or vIFN- λ 3 lost no weight and showed no signs of illness, like mock-infected

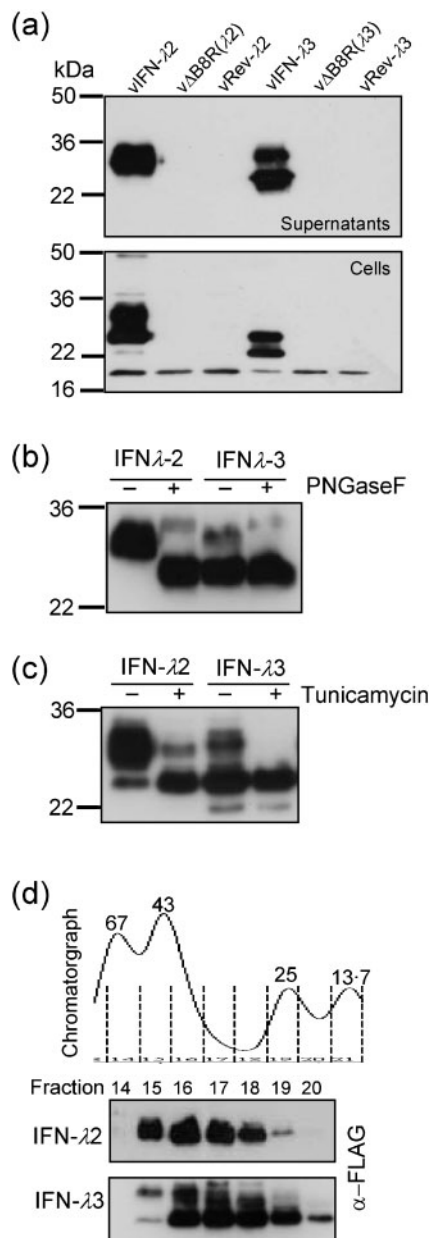


Fig. 2. Expression of murine IFN- λ s by vIFN- λ 2- or vIFN- λ 3-infected cells. (a) BS-C-1 cells were infected with vIFN- λ 2 or vIFN- λ 3 or with the control parental or revertant virus. At 18 h p.i., FLAG-tagged proteins in supernatants and cell extracts were resolved by SDS-PAGE and detected by immunoblotting using an α -FLAG mAb. (b, c) Murine IFN- λ proteins are *N*-glycosylated. (b) Proteins in the supernatants of infected cells were incubated with PNGase F (Roche) to remove *N*-linked carbohydrate and analysed as in (a). Protein in the supernatant from 5×10^5 infected cells was incubated with 12 U PNGase F for 1 h at 37 °C. (c) L929 cells were infected with the indicated virus in the presence or absence of 1 μ g tunicamycin ml⁻¹ (Sigma). At 18 h p.i., proteins in supernatants (concentrated fivefold) and cell extracts were analysed as in (a). (d) Gel filtration. Supernatants from vIFN- λ -infected cells were mixed with molecular mass marker proteins (BSA, 67 kDa; hen-egg ovalbumin, 43 kDa; chymotrypsin, 25 kDa; ribonuclease A, 13.7 kDa) and fractionated by size-exclusion chromatography. FLAG-tagged IFN- λ protein present in fractions 14–20 was analysed as in (a) and aligned with the protein mass markers.

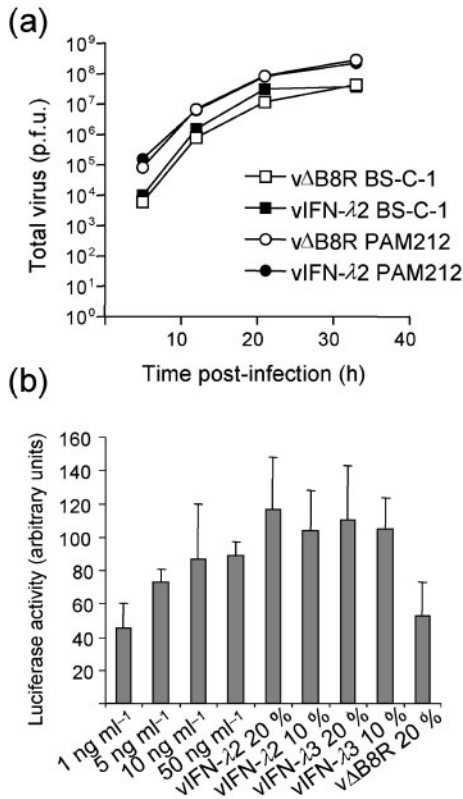


Fig. 3. Analysis of recombinant murine IFN-λs *in vitro*. (a) Monkey BS-C-1 or mouse PAM212 fibroblasts were infected with vIFN-λ2 at 0.1 p.f.u. per cell. Cells were harvested at the indicated times p.i. and infectious virus associated with cells was measured by plaque assay. (b) Mouse PAM212 cells were transfected with pSV-β-gal and pSRE-luc. At 24 h post-transfection, cells were treated with 1, 5, 10 or 50 ng ml⁻¹ murine IFN-λ2 protein from *E. coli* dissolved in serum-free DMEM. Alternatively, cells were treated with serum-free conditioned supernatants from vΔB8R-, vIFN-λ2- or vIFN-λ3-infected cells (diluted to 10 or 20% of overlay volume). Luciferase activity was monitored after 4 h. Results are expressed as the luciferase activity (arbitrary units), normalized by using β-gal expression as an internal control.

controls (Fig. 4a and b). This showed that expression of IFN-λ2 or IFN-λ3 rendered VACV avirulent.

Virus titres were measured in lungs and brains at different times p.i. (Fig. 4c). At day 2 p.i., there was no significant difference between the groups. However, by days 5 and 7 p.i., there was significantly less virus in vIFN-λ2-infected lungs than in controls ($P < 0.05$) and, strikingly, at no time investigated was any virus detected in brains of vIFN-λ2-infected animals, unlike controls.

Attenuation of vIFN-λ2 is associated with increased lymphocyte recruitment

IFNs have direct antiviral activity and are important for the adaptive immune responses (Malmgaard, 2004), and

cellular immunity is important in recovery to orthopox-virus infection (Lane *et al.*, 1969). To assess whether expression of IFN-λ2 affected cellular infiltration into the infected lungs, lung lymphocytes were prepared from infected mice and total T lymphocytes (CD3⁺), CD4⁺ and CD8⁺ T cells were analysed (Fig. 5a). On days 2 and 5 p.i., no significant differences were observed in the relative numbers of CD3⁺, CD4⁺ or CD8⁺ T lymphocytes for each infected group. However, by day 7 p.i., significantly more CD3⁺ and CD4⁺ T lymphocytes were present in the lungs of vIFN-λ2-infected mice than in those of controls, whilst no difference in the relative proportion of CD8⁺ T cells was observed.

The numbers of cells present in lung washes obtained from infected mice at various times p.i. were also determined for each group (Fig. 5b) and, by day 7, there were significantly more cells in BALs from vIFN-λ2-infected mice than controls. Despite this, at day 7 p.i., there was significantly less IFN-γ in BAL fluid from vIFN-λ2-infected mice compared with controls (Fig. 5c).

Expression of IFN-λ2 attenuates VACV in an intradermal model

Several human tissues, including lung and skin, express mRNA for IFN-λR (Kotenko *et al.*, 2003). Given this, the virulence of vIFN-λ2 was also tested in a mouse dermal model in which virus is injected intradermally into the mouse ear pinnae and causes a mild, localized infection without signs of systemic illness or virus dissemination to other organs (Tscharke & Smith, 1999). In this model, infection with vIFN-λ2 caused a delay in lesion formation and a smaller peak lesion size than for controls (Fig. 6).

DISCUSSION

Production of IFN is an important innate response to virus infection. This has been illustrated in several ways, including the direct antiviral activity of IFN in cell culture, the reduced ability of transgenic mice with defects in IFNs, IFN receptors or intracellular signalling components to resist virus infection, and the encoding of numerous proteins that combat the action of host IFNs by viruses (Pestka *et al.*, 2004). After the discovery of IFN in 1957, type I (IFN-α/β) and type II (IFN-γ) IFNs have been shown to have direct antiviral activity and to promote the adaptive immune response. IFN-γ in particular is a potent stimulator of the Th1 immune response that is important for clearance of virus infections.

Recently, a third group of IFNs was discovered. Originally, these were called IFN-λs, or IL28A, IL28B and IL29, but they have now been designated type III IFNs by the Nomenclature Committee of the International Society for Interferon and Cytokine Research. Based on *in vitro* studies in human cells, human IFN-λs possess intrinsic antiviral activity and induce an antiviral state in several cell types against encephalomyocarditis virus (EMCV) and vesicular

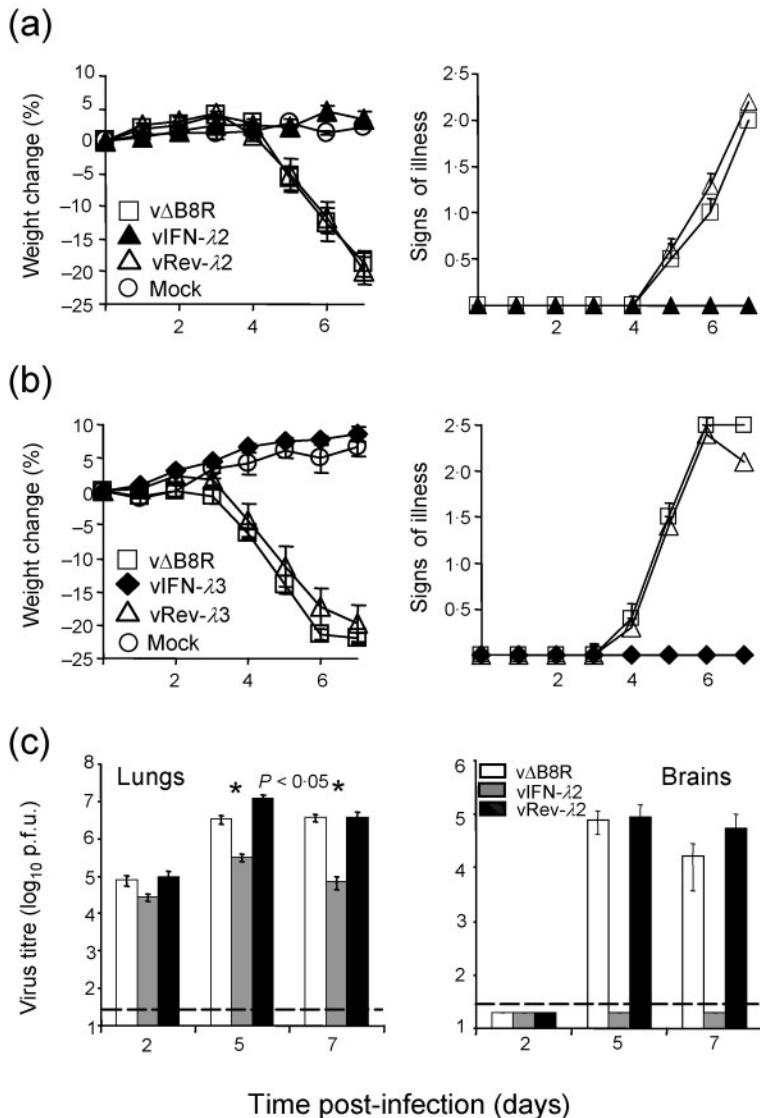


Fig. 4. Intranasal infection of mice with recombinant VACVs. Groups of five mice were mock-infected or infected intranasally with 5×10^3 p.f.u. of either (a) vIFN-λ2, (b) vIFN-λ3 or control viruses and the percentage weight change or signs of illness of the animals were recorded daily. The mean weight of each group of animals on each day is expressed as the percentage of the mean weights of same group on day zero. Signs of illness were assigned a scale of 0–4 as defined previously (Alcami & Smith, 1992). Mean values (\pm SEM) for each group are shown. (c) At the indicated days p.i., lungs and brains were assayed for infectious virus by plaque assay. Asterisks denote days on which the titre of virus in the lungs of vIFN-λ2-infected mice was significantly different from viral titres in lungs of both control groups. Mean values (\pm SEM) for groups are shown and the dashed line represents the detection limit of the assay.

stomatitis virus (VSV) (Kotenko *et al.*, 2003). IFN-λs are produced by various cells in response to viral infection and upregulate MHC class I antigen expression, thereby providing more efficient presentation of viral antigens for immune recognition (Kotenko *et al.*, 2003). However, to date, the role of IFN-λs in defence against virus infection *in vivo* has not been investigated.

One method of studying the function of cytokines, chemokines or IFNs has been to express these from recombinant poxviruses and study the outcome on virus infection in animal models (Ramshaw *et al.*, 1992) and this approach was utilized here. For the parent virus, we used a strain of VACV WR that was engineered to lack the gene encoding the viral IFN-γR (Symons *et al.*, 2002). This was selected as a safety feature because the VACV IFN-γR does not neutralize mouse IFN-γ (Alcami & Smith, 1995) and loss of this gene does not affect virus virulence in mouse models (Symons *et al.*, 2002). However, the viral IFN-γR does bind and

inhibit human IFN-γ and, therefore, the virus would be predicted to be less virulent in man. Recombinant VACVs expressing IFN-λ2 or -λ3 were constructed and shown to replicate normally in cell culture (Fig. 3), but to be attenuated dramatically in an intranasal-infection model (Fig. 4). Unlike animals infected by control viruses, animals infected by vIFN-λ2 or vIFN-λ3 appeared entirely normal and lost no weight. Moreover, virus titres in lungs were reduced and the dissemination of virus to the brain was blocked completely. This attenuated phenotype was accompanied by enhanced numbers of CD4⁺ T cells in lungs and enhanced numbers of lymphocytes in BAL at 7 days p.i. (Fig. 5). At this late time p.i., there was a reduced level of IFN-γ in the BAL fluid (Fig. 5) and this may reflect the reduced virus titres as the virus infection was cleared. In the intradermal-infection model, the attenuation was less dramatic, but there was a delay in the rate at which lesions developed and the peak lesion size was reduced (Fig. 6). This suggests that, although type III IFNs are produced by many

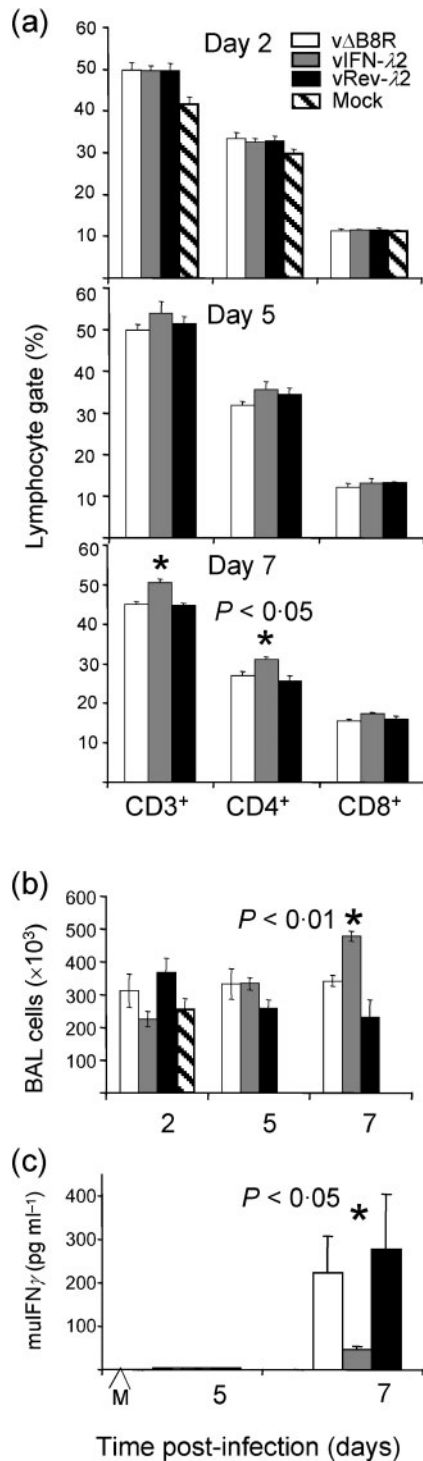


Fig. 5. Analysis of the cellular immune responses in the lungs of virus-infected mice. Groups ($n=5$) of mice were infected intranasally with 5×10^3 p.f.u. of the indicated virus. On days 2, 5 and 7 p.i., animals were sacrificed and BALs and lungs were harvested. (a) Cells from lung homogenates were stained for lymphocyte surface markers CD3, CD4 and CD8 and the results shown represent the percentage of positive cells in the lymphocyte gate for each group. (b) Viable cells in BALs were enumerated by trypan blue exclusion. (c) IFN- γ in BAL fluid was determined by ELISA. For all parts, data represent the mean (\pm SEM) and the asterisks indicate where results for vIFN- λ were significantly different from those for both control groups. The level of IFN- γ from mock-infected animals is indicated by M and an arrowhead.

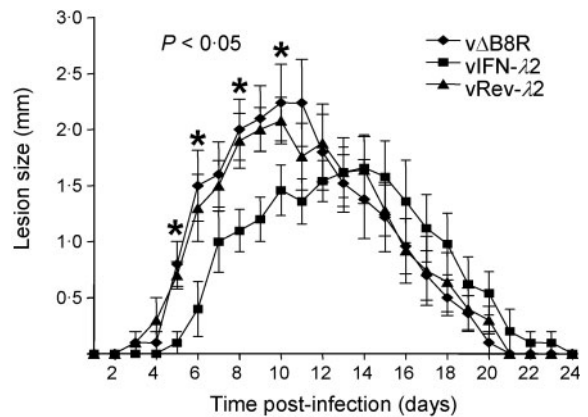


Fig. 6. Intradermal infection of mice with vIFN- $\lambda 2$. Groups of mice ($n=5$) were infected by intradermal injection into the ear pinna with 10^6 p.f.u. of the indicated virus. The lesion size was determined daily and data shown are the mean lesion sizes for each group of animals. Asterisks indicate days on which the mean lesion size for vIFN- $\lambda 2$ -infected animals was significantly different from both control groups.

interesting to determine whether viruses possess specific strategies to interfere with type III IFNs, as they do for type I and type II IFNs. These data also suggest a potential therapeutic role for type III IFNs.

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different cells, their impact may be greater after infection of the respiratory tract, which normally leads to systemic infection. There are several other examples where the outcome of infection with VACVs engineered to lack specific immunomodulators has been different in intranasal and intradermal models (Tscharke *et al.*, 2002).

Overall, these data indicate that IFN- λ s are important mediators of the antiviral response *in vivo* and it will be

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