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An Important Role for Type III Interferon (IFN-λ/IL-28) in TLR-Induced Antiviral Activity[1]

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Type III IFNs (IFN-λ/IL-28/29) are cytokines with type I IFN-like antiviral activities, which remain poorly characterized. We herein show that most cell types expressed both types I and III IFNs after TLR stimulation or virus infection, whereas the ability of cells to respond to IFN-λ was restricted to a narrow subset of cells, including plasmacytoid dendritic cells and epithelial cells. To examine the role of type III IFN in antiviral defense, we generated IL-28Rα-deficient mice. These mice were indistinguishable from wild-type mice with respect to clearance of a panel of different viruses, whereas mice lacking the type I IFN receptor ($IFNAR^{-/-}$) were significantly impaired. However, the strong antiviral activity evoked by treatment of mice with TLR3 or TLR9 agonists was significantly reduced in both $IL\text{-}28RA^{-/-}$ and $IFNAR^{-/-}$ mice. The type I IFN receptor system has been shown to mediate positive feedback on IFN-αβ expression, and we found that the type I IFN receptor system also mediates positive feedback on IFN-λ expression, whereas IL-28Rα signaling does not provide feedback on either type I or type III IFN expression in vivo. Finally, using bone-marrow chimeric mice we showed that TLR-activated antiviral defense requires expression of IL-28Rα only on nonhemopoietic cells. In this compartment, epithelial cells responded to IFN-λ and directly restricted virus replication. Our data suggest type III IFN to target a specific subset of cells and to contribute to the antiviral response evoked by TLRs. ***The Journal of Immunology,*** **2008, 180: 2474–2485.**

Interferons are produced as part of the immediate host response to virus infections and are essential for defense against viruses (1–3). Type I IFNs (or IFN-αβ), which were discovered 50 years ago (4), exert antiviral activity by targeting both the innate and adaptive immune system. IFN-αβ activate NK cells for cytotoxic activity (5), and they also confer an antiviral state upon adjacent noninfected cells (6–8). Moreover, IFN-αβ up-regulate expression of MHC class I (MHC-I)[3] on most cells (and costimulatory molecules on APCs), enhance cross-presentation of exogenous Ag in MHC-I, and promote T cell expansion (9–12). Type II IFN (IFN-γ) also has direct antiviral activity (13–15), but it is particularly known for its immunoregulatory function cytokine (16).

Recently, a novel group of cytokines was discovered by two independent groups and named IFN-λ, IL-28/29 (17, 18), or type III IFN. There are three members of this group, IFN-λ1–3 or IL-28A/B and IL-29, and they signal through a receptor complex consisting of IL-10Rβ and the IL-28Rα chains. Although neither the cytokines nor the receptors display significant sequence similarity to the type I IFN system, they both signal through the JAK1/TYK2 tyrosine kinases and the transcription factor complex IFN-stimulated gene factor 3 consisting of STAT1, STAT2, and IFN regulatory factor (IRF)-9 (17–20). Consequently, type III IFNs trigger a type I IFN-like gene expression profile (19–21), which has been shown to have antiviral activity in vitro (3, 17–19, 21). In vivo, a vaccinia virus engineered to express IFN-λ2 has been reported to be attenuated (22), and we have shown that local administration of the cytokine through the vaginal route protects against subsequent HSV-2 infection (3). However, the role of endogenous type III IFN in host defense against virus infections remains unresolved, although it has been reported that asthmatic patients have higher viral load than do healthy controls after infection with rhinovirus, and this is associated with reduced induction of IFN-λs in bronchial epithelial cells and alveolar macrophages (23).

Studies in cell lines and primary cells suggest that type III IFN, like type I IFN, is expressed in response to viruses and TLR ligands (3, 17, 18, 24), and that most cell types are capable of mediating the response if they receive the proper stimuli. This idea was strengthened recently by a report showing that the promoter regions of the genes encoding types I and III IFN share many similarities, and that these IFNs indeed are induced through common signaling pathways (25).

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[3] Abbreviations used in this paper: MHC-I, MHC class I; BM, bone marrow; cDC, conventional dendritic cell; DC, dendritic cell; EMCV, encephalomyocarditis virus; ES, embryonic stem; IAV, influenza A virus; IFNAR, IFN-αβ receptor; IL-28R, IL-28 receptor; IRES, internal ribosome entry site; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; LCMV, lymphocytic choriomeningitis virus; MAVS, mitochondrial antiviral signaling protein; MEF, mouse embryonic fibroblast; OAS, 2′-5′ oligoadenylate synthetase; pDC, plasmacytoid dendritic cell; p.i., postinfection; TLR, Toll-like receptor; VSV, vesicular stomatitis virus; WT, wild-type.

Because the biological activity of a cytokine is mediated through binding to a specific receptor, information on which cell types respond to a specific cytokine provides important clues as to its biological functions. In contrast to IFN-$\alpha\beta$, which are able to stimulate nearly all nucleated cells, there is now knowledge on a range of cell types that do not respond to IFN-λ (17–19, 26). This suggests that type III IFNs act in a more cell-type specific manner to mediate their biological functions. However, the responsiveness of primary cells to IFN-λ has not been investigated systematically.

In addition to a potential role in antiviral defense, it has also been suggested that IFN-λ exhibits antitumor activity in different murine models (26–28). Although this also involves induction of tumor apoptosis (28), it seems primarily to be mediated by host immune cells, with NK cells being ascribed particularly important roles (27, 28). Moreover, IFN-λ seems to have immunomodulatory functions, because IFN-λ-stimulated human monocyte-derived dendritic cells (DCs) induce proliferation of FOXP3-expressing suppressor T cells with contact-dependent suppressive activity on T cell proliferation (29). This phenomenon may explain why IFN-λ has suppressive activity on CD4 T cells induced by respiratory syncytial virus (30).

In this work we have generated IL-28RA$^{-/-}$ mice and investigated the role of IFN-λ in antiviral defense in vivo. We report that IFN-λ is dispensable for defense against a number of viruses, but it is essential for optimal TLR-activated antiviral defense in a physiologically relevant model of mucosal infection with HSV-2. This antiviral activity of IFN-λ was largely mediated through induction of an antiviral state in epithelial cells. Thus, viruses and TLR ligands induce production of IFN-λ at major portals of entry into the organism, and they activate antiviral activity in cells targeted by viruses.

Materials and Methods

Mice, viruses, cell culture medium, and reagents

The mice used for this study were female C57BL/6, 129Sv (Taconic M&B), IFNAR$^{-/-}$ (B&K Universal), TLR9$^{-/-}$ (Oriental Yeast Co.), and IL-28RA $^{-/-}$ mice. IL-28RA$^{-/-}$ mice were generated as described below. The viruses used were HSV-2 (strains MS and 333), encephalomyocarditis virus (EMCV, FA strain), lymphocytic choriomeningitis virus (LCMV; Armstrong strain), influenza A virus (IAV, rSC35M strain (31)), vesicular stomatitis virus (VSV; Indiana strain), and Sendai virus (Cantrell strain). The growth media used were RPMI 1640, MEM, Iscoves (all BioWhittaker), and CnT-19 and CnT-02 (both CELLnTEC). RPMI and MEM were supplemented with antibiotics (penicillin (200 IU/ml) and streptomycin (200 μg/ml)) and LPS-free FCS (BioWhittaker) at the indicated concentrations. MEM was furthermore supplemented with 2% glutamine, 0.5% nystatin, 10% $NaHCO_3$, and 0.1% garamycin. RPMI 1640 was also supplemented with 1% glutamine and 1% HEPES (BioWhittaker). CnT-19 and CnT-02 were supplemented with penicillin and streptomycin and supplements (CELLnTEC). Recombinant IFN-α and IFN-λ were obtained from R&D Systems. The TLR ligands used were: Pam_3CSK_4 (TLR2), polyinosinic-polycytidylic acid (polyIC) (TLR3), and ODN1826 (TLR9), all from Invivogen.

Generation of IL-28RA$^{-/-}$ mice

IL-28RA$^{-/-}$ mice were generated through a research agreement with Ozgene. A targeting vector that deleted the entire coding region of IL-28RA (including the 7 coding exons, 6 intervening introns, and the first 219-bp of the 3′ untranslated region of the mouse *IL-28RA* gene) was constructed by replacing these sequences with an internal ribosome entry site (IRES)-*LacZ*/MC1-Neo reporter gene/selection cassette (5127 bp). The targeting construct was electroporated into C57BL/6-derived Bruce4 (C57BL/6J strain) embryonic stem (ES) cells. To verify correct targeting of the locus, genomic DNA from ES cells was analyzed by Southern blot. The 5′-end analysis was done using *Kpn*I digestion, and the 3′-end analysis was done using *Xba*I digestion. Correctly targeted ES cells were injected into blastocysts, and animals that transmitted the mutant allele in their germline were obtained. The resulting chimeric mice were bred to C57BL/6J. Heterozygous littermates were bred to generate homozygotes, heterozygotes, and wild-type mice. For genotyping, a PCR-based method was developed. Primers that anneal and amplify a 338-bp region in the 3′ UTR were used to identify the WT gene (5′-TGGCACTTTGTCGTTGGGATTGAG-3′, 5′-AAGGGGAAGCTGAGGTTGAGAGGT-3′). Disruption of the *IL-28RA* gene was identified with primers that anneal to the neomycin resistance gene (5′-TGGCGATGCCTGCTTGCCGAATA-3′) and the 3′ UTR of the *IL-28RA* gene amplifying a 572-bp fragment. PCR was performed in a thermocycler using the following conditions: 1 cycle of 94°C, 1 min; 35 cycles of 94°C, 15 s, 58°C, 30 s, and 68°C, 4 s; and 1 cycle of 68°C, 3 min. IL-28RA$^{-/-}$ mice were born at Mendelian frequencies and showed no overt phenotype.

Generation of bone-marrow (BM) chimeric mice

Recipient mice were lethally irradiated with 9.5 Gy of total-body irradiation. BM was obtained from the femur and tibia of donor mice and collected in sterile RPMI. The cells were subsequently washed twice and resuspended in sterile RPMI. One donor was used to reconstitute 1.5 recipients. Irradiated recipients were reconstituted with donor cells by means of tail vein injection. The transplanted mice were treated with antibiotics and maintained in a clean facility. The mice were used for experiments upon complete reconstitution (>4 wk). Reconstitution was confirmed by measuring IL-28RA by PCR in genomic DNA harvested from splenic lymphocytes.

Treatment of mice in vivo

For vaginal HSV-2 infection, 8–9-wk old mice were pretreated by s.c. injection of 2 mg Depo-Provera (Pfizer). Five days later, mice were anesthetized with Isofluran (Baxter) and inoculated i.vag. with 20 μl of a lethal dose (6.7×10^4 PFU) of HSV-2 strain 333 suspended in Iscoves modified medium. The mice were placed on their backs and maintained under anesthetics for at least 10 min. Twenty-four hours (CpG and polyIC) or 6 h (IFN-λ) before vaginal HSV-2 infection, mice were anesthetized with isofluran and received 25 μg of CpG ODN 1826 or 100 μg of polyIC i.vag. in 20 μl PBS or 5 μg of recombinant carrier-free IFN-λ in 20 μl PBS. Vaginal fluids were collected at the indicated days postinfection (p.i.) by pipetting 2×40 μl of Iscoves into and out of the vagina 12–15 times. The two washes were pooled. Genitally infected mice were examined daily and scored for vaginal inflammation, neurological illness, and death. The severity of disease was graded using the following scores: 0, healthy; 1, genital erythema; 2, moderate genital inflammation; 3, purulent genital lesion and/or generally bad condition; and 4, hindlimb paralysis, mice were sacrificed. For systemic infections, 4-wk-old (HSV-2 and EMCV) or 8-wk-old (LCMV and VSV) female mice were infected i.p. with 1×10^6 and 1×10^2 PFU of HSV-2 (MS) and EMCV, respectively, or i.v. with 1×10^3 and 1×10^6 PFU of LCMV and VSV, respectively. The animals were sacrificed on the indicated time points p.i., and viral load was determined in infected organs (HSV-2, liver, spleen, and brain; EMCV, heart and brain; LCMV, spleen; VSV, spleen). For infection with IAV, animals were anesthetized by i.p. injection of a mixture of ketamine (100 μg/g body weight) and xylazine (5 μg/g body weight) and infected intranasally with 200 PFU of rSC35M in 50 μl of PBS containing 0.3% BSA.

Depletion of plasmacytoid dendritic cells (pDCs) in vivo

For in vivo depletion of pDCs, mice received 500 μg of anti-mPDCA-1 (Miltenyi Biotec) or rat IgG2b control Ab through the tail vein. Twenty-four hours later the mice were used for experiments as described below. The protocol led to ~80% depletion of pDCs as determined by isolation of splenic pDCs by autoMACS (Miltenyi Biotec) 24 h after treatment (data not shown).

Purification and differentiation of primary cells

For isolation of splenocytes, spleens were harvested and kept in RPMI 1640 supplemented with 10% FCS. Spleens were homogenized manually with a glass homogenizer in RPMI supplemented with 10% FCS. Successively, the suspension was passed through a 70-μm cell strainer and the cells were reconstituted to a concentration of 10×10^6/ml in PBS. Erythrocytes were removed using Lympholyte-M (CL5035; Cederlane Laboratories) following the instructions of the manufacturer. The cells were washed, counted, seated in 6-well plates, and left overnight before stimulation as indicated. To isolate splenic pDJs, conventional DCs (cDC), T cells, and B cells, a single-cell suspension was prepared from the spleen. For DCs, spleens were harvested, injected with collagenase D (Sigma-Aldrich) at 1 mg/ml for digestion, and then cut into small pieces and incubated at 37°C for 30 min. For T and B cells, spleens were harvested into RPMI 10% FCS and manually homogenized. The entire material was passed through a 70-μm (DCs) or 40-μm (T and B cells) cell strainer. The

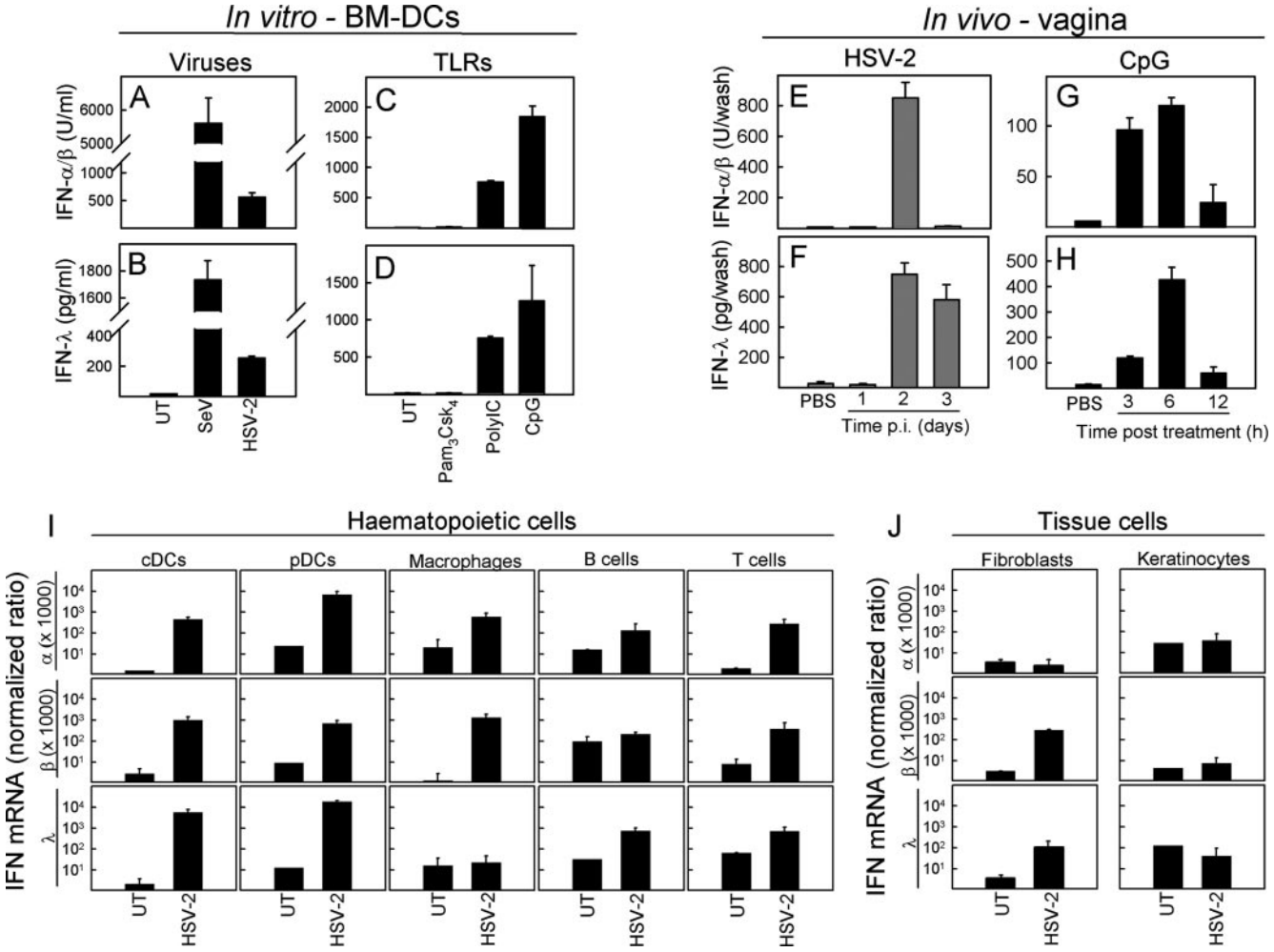

FIGURE 1. Expression of types I and III IFNs in response to viruses and TLR ligands. *A–D*, BM DCs were generated from C57BL/6 mice and cultured. The cells were treated as indicated in the following doses: Sendai virus (MOI 1) and HSV-2 (3×10^6 PFU/ml), Pam_3Csk_4 (200 ng/ml), polyIC (25 μg/ml), and GpC (ODN1826) (1 μM). Supernatants were harvested 18 h posttreatment, and types I and III IFNs were measured by bioassay and ELISA, respectively. *E–H*, 129Sv mice were infected i.vag. with 6.7×10^4 PFU of HSV-2, and C57BL/6 mice were treated with 25 μg of ODN1826. Vaginal washes were collected at the indicated time points posttreatment, and types I and III IFNs were measured by bioassay and ELISA, respectively ($n = 5$). The data in *A–H* are shown as mean values ± SD. *I* and *J*, Hemopoietic and tissue cells were isolated, cultured, and infected with HSV-2 at MOI 1. Four hours postinfection, total RNA was harvested, and expression of IFN-α, IFN-β, and IFN-λ was determined by real-time PCR. The data are shown as normalized ratio relative to β-actin levels.

specific cell populations were isolated using MACS technology and the appropriate kits (Miltenyi Biotec). The cells were subsequently washed, seeded in 6-well plates, and stimulated as indicated. The purity of the cell populations, which was determined by flow cytometry, was: cDCs, 90%; pDCs, 73%; B cells, 93%; and T cells, 94% (data not shown). The Abs used for flow cytometry were: pDCs, anti-mPDCA-1-FITC (130–091-96; Miltenyi Biotec), isotype, rat FITC-IgG2b; cDCs, FITC-CD11c (557400; BD Pharmingen) isotype, Armenian hamster FITC-IgG1*,λ1; B cells, FITC-CD19 (557398; BD Pharmingen), isotype, rat (LEW) FITC-IgG2a,κ; T cells, FITC-CD3e (553061; BD Pharmingen) isotype Armenian hamster FITC-IgG1*κ. For isolation of epithelial cells, vaginas were harvested and incubated in CnT-19 with 5 U/ml dispase (10 mg/ml) (Invitrogen, catalog no. 17105) for 12–14 h at 4ºC. Subsequently, the endothelium was separated from the lamina propria using sterile forceps, cut into small pieces, and incubated with protease (Invitrogen TrypLE, catalog no. 12563) for 10–15 min at 37ºC. CnT-19 was added (2.5 volumes), and the cells were centrifuged for 5 min at $250 \times g$. The cells were suspended in CnT-19, seeded in 6-well plates, and left to settle before further treatment. Keratinocytes were obtained as described by others (32). BM-derived DCs were generated as described earlier (33). Briefly, femur and tibia were removed from mice, and BM cells were flushed from the bone shafts and cultured in Petri dishes at a concentration of 2×10^5 cells/ml in culture medium containing 40 ng/ml recombinant murine GM-CSF (R&D Systems). Fresh medium was added after 3, 6, and 8 days of culture. On days 6 and 8, half of the medium was replaced with fresh medium. After 10 days of culture, nonadherent cells were harvested, washed, resuspended in culture medium containing 20 ng/ml GM-CSF, and further cultured in cell culture Petri dishes (Nunc). Nonadherent cells were harvested after 11 days of culture for flow cytometry or infection with infectious or UV-inactivated virus. The cells were >80% $CD11c^+$ as determined by flow cytometry (data not shown). Mouse embryonic fibroblasts (MEFs) from C57BL/6 and mitochondrial antiviral signaling protein $(MAVS)^{-/-}$ mice were kindly provided by Zhijian Chen (34). Peritoneal macrophages were harvested by lavage of the peritoneal cavity with cold PBS (pH 7.4) supplemented with 5% FCS and 0.4% heparin.

Virus plaque assay

Organs were isolated and immediately put on dry ice and stored at −80ºC until use, at which point they were thawed and homogenized in the appropriate solutions. The homogenates were pelleted by centrifugation at $1600 \times g$ for 30 min, and supernatants were used for plaque assay. In the case of vaginal washes, these were thawed and used directly for plaque titration. To determine viral titers for HSV-2, EMCV, LCMV, and IAV, previously published protocols were used (3, 14, 31). To determine organ virus titers of VSV, serial 10-fold dilutions of the cleared organ suspension (10% (v/w) spleen suspension in PBS containing 1% FCS) were prepared in PBS with 1% FCS. One milliliter of each dilution was added in duplicate to monolayers of L929 cells in Petri dishes plated 48 h earlier. After incubation for 90 min at 37ºC in 5% CO_2, the medium was aspirated, and the monolayers were overlaid with a mixture of 2.5 ml 1% agarose and 2.5 ml

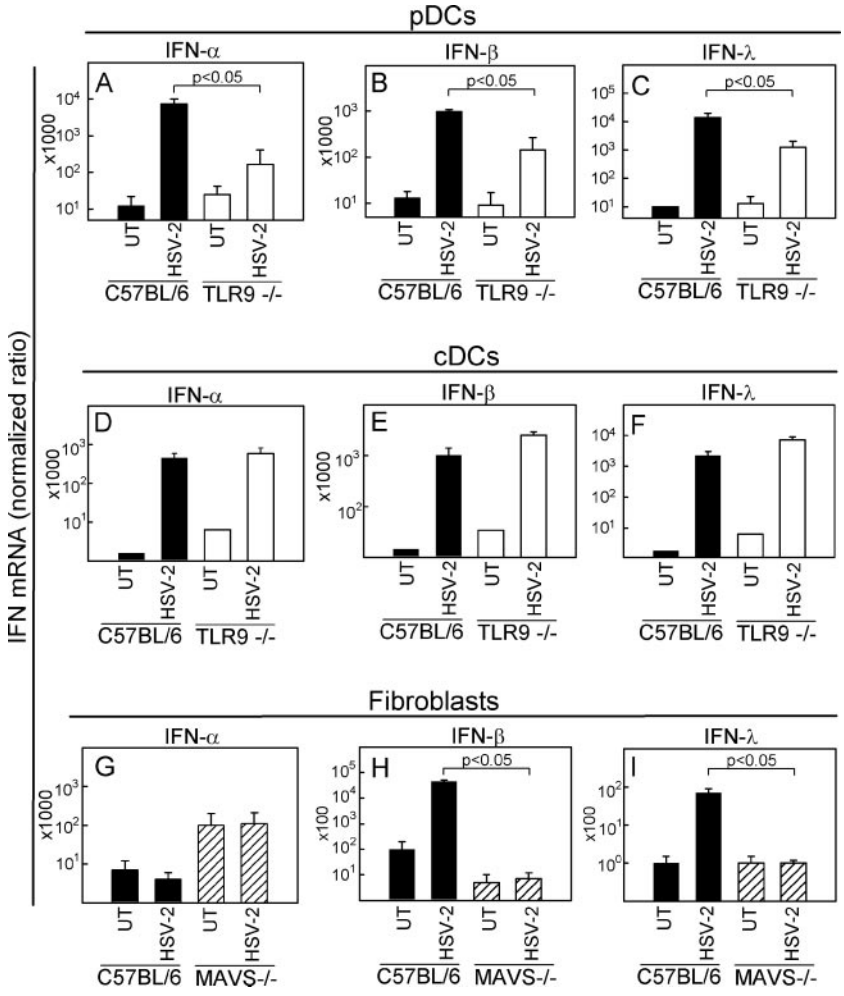

FIGURE 2. Pattern recognition receptors involved in triggering type III IFN expression in response to HSV. pDCs (*A–C*) or cDCs (*D–F*) were isolated from the spleens of C57BL/6 or TLR9$^{-/-}$ mice, and fibroblasts were generated from C57BL/6 or MAVS$^{-/-}$ mice (*G–I*). The cells were cultured and left untreated or infected with 6×10^6 PFU/ml HSV-2 (MOI). Four hours postinfection, total RNA was harvested, and expression of IFN-α, IFN-β, and IFN-λ was determined by real-time PCR. The data are shown as normalized ratio relative to β-actin levels.

MEM. Monolayers were then incubated for 24 h at 37°C in 5% CO_2 before staining with a mixture of 1 ml of 1% agarose and 1 ml of 2 × F11 containing 1% neutral red. After further 24 h of incubation, the numbers of PFU were counted.

Quantitative RT-PCR

Total RNA was extracted with TRIzol (Invitrogen) according to the recommendations of the manufacturer. Briefly, cells were lysed in TRIzol, and chloroform was added, followed by phase separation by centrifugation. RNA was precipitated with isopropanol and pelleted by centrifugation. Pellets were washed with 80% ethanol and re-dissolved in RNase-free water. For cDNA generation, RNA was subjected to reverse transcription with oligo(dT) as primer and Expand reverse transcriptase (both from Roche). Before qRT-PCR, RNA was treated with DNase I (Ambion) to remove any contaminating DNA, the absence of which was confirmed in control experiments in which the reverse transcriptase enzyme was omitted (data not shown). The cDNA was amplified by PCR using the following primers: IFN-α, forward: 5′-CGGTGATGAGCTACTGGC-3′, reverse: 5′-TTTG TACCAGGAGTGTCAAGG-3′; IFN-β, forward: 5′-GGTGGAATGAGAC TATTGTTG-3′/5′-CACTGGGTGGAATGAGACTAT-3′, reverse: 5′-AGG ACATCTCCCACGTC-3′/5′-GACATCTCCCACGTCAATC-3′; IFN-λ, forward: 5′-AGCTGCAGGTCCAAGAGCG-3′, reverse: 5′-GGTGGTCAG GGCTGAGTCATT-3′; 2′-5′ oligoadenylate synthetase (OAS) 1, forward: 5′-TGTCCTGGGTCATGTTAATAC-3′, reverse: 5′-CCGTGAAGCAGGTAG AGA-3′; IRF-7, forward: 5′-CCCAGACTGCCTGTGTAGACG-3′, reverse: 5′-CCAGTCTCCAAACAGCACTCG-3′; β-actin, forward: 5′-TAGCACC ATGAAGATCAAGAT-3′, reverse: 5′-CCGATCCACACAGAGTACTT-3′. Products were measured using SYBR Green I (Qiagen).

IFN bioassay

IFN-$\alpha\beta$ bioactivity was measured by using a L929-cell based bioassay. L929 cells (2×10^4 cells/well in 100 μl) in MEM with 5% FCS were incubated overnight at 37°C in successive twofold dilutions of samples

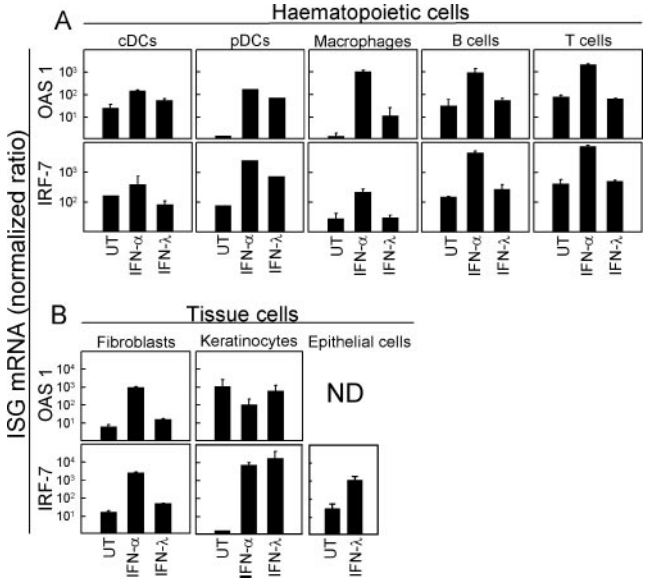

FIGURE 3. Cellular responsiveness to recombinant types I and III IFNs. Cells of (*A*) the hemopoietic or (*B*) the nonhemopoietic compartments were cultured and stimulated with 100 ng/ml IFN-α or IFN-λ2 for 4 h. Total RNA was harvested, and expression of OAS1 and IRF-7 was determined by quantitative RT-PCR. The data were normalized against β-actin and are shown as normalized ratio ×1000.

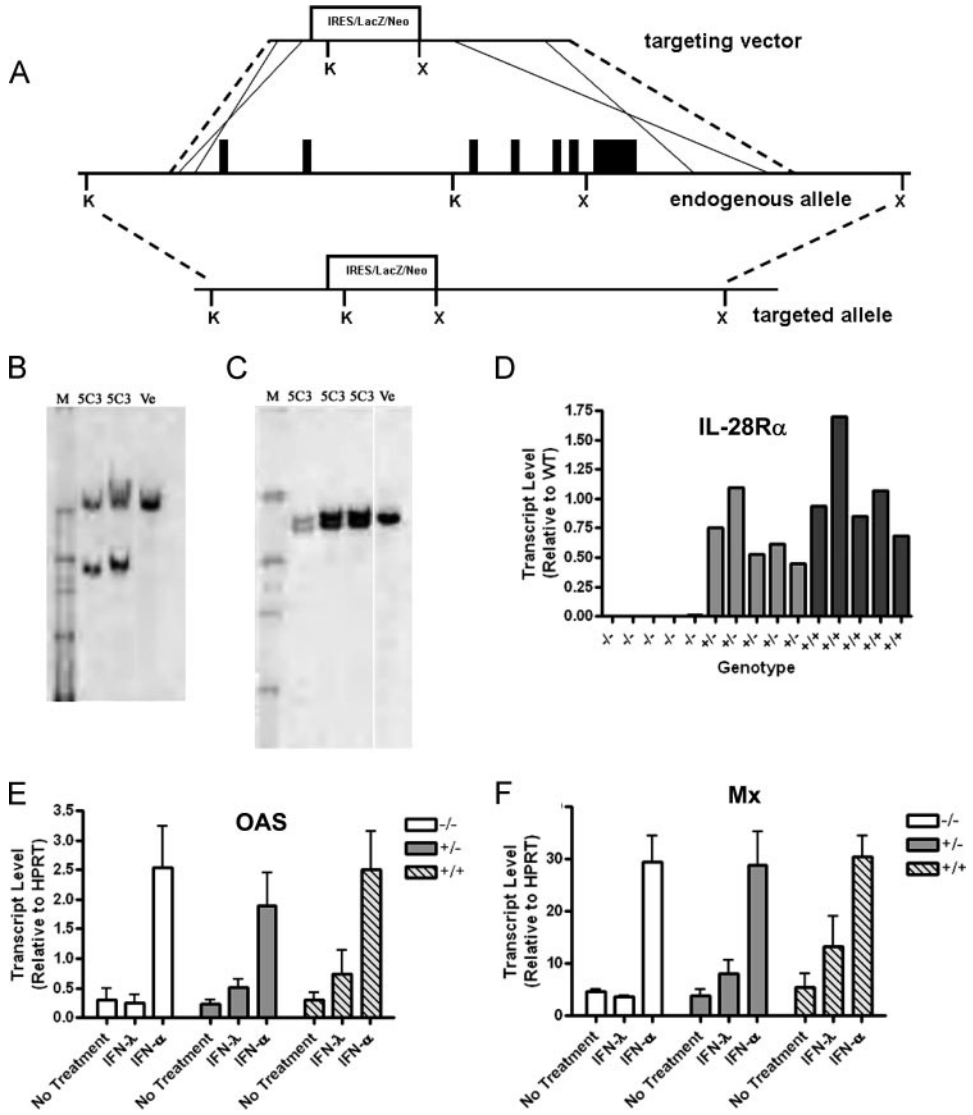

FIGURE 4. Generation of IL-28RA$^{-/-}$ mice. *A*, The relevant section of the endogenous IL-28RA allele (*middle*), the targeting vector (*top*), and the correctly targeted allele (*bottom*) are depicted. IL-28RA exons are represented by black rectangles in the endogenous allele. The open rectangle in the targeting vector and correctly targeted allele indicates the selectable IRES-*LacZ*/MC1-Neo reporter gene/selection cassette. *Kpn*I (K) and *Xba*I (X) restriction sites used for assessment of 5′ and 3′ homologous recombination are shown. *B* and *C*, Southern blot analysis of *Kpn*I- (*B*) or *Xba*I-digested (*C*) genomic DNA from correctly targeted ES cells (clone 5C3) or nontargeted genomic DNA (Ve) probed with 5′ (*B*) or 3′ (*C*) probes. *Kpn*I digestion creates a WT band of ~18.9 kb and a KO band of ~7.5 kb, whereas *Xba*I digestion generates a WT band of ~15.1 kb and a KO band of ~13.5 kb. Clone 5C3 is correctly targeted at both ends. *D*, Splenic expression of IL-28Rα RNA from IL-28RA$^{-/-}$, IL-28RA$^{+/-}$, and IL-28RA$^{+/+}$ mice was determined by real-time RT-PCR. Relative IL-28Rα RNA expression was calculated using the 2^{-ddCt} method with the average WT receptor expression as the calibrator. Genotype is indicated as follows: +/+, WT; +/−, heterozygous; −/−, homozygous IL-28RA$^{-/-}$. Five individual mice from each genotype were analyzed. *E* and *F*, Mouse splenocyte cultures from IL-28RA$^{-/-}$, IL-28RA$^{+/-}$, and IL-28RA$^{+/+}$ mice were treated with 100 ng/ml murine IFN-λ, 100 U/ml murine IFN-α, or left untreated for ~16 h before RNA isolation. Known antiviral genes OAS (*E*) and Mx (*F*) were analyzed using a real-time RT-PCR assay (normalized to hypoxanthine phosphoribosyltransferase (HPRT)). ISG transcript level relative to HPRT level is shown on the *y*-axis. Treatment condition used is shown on the *x*-axis. Genotype is indicated as follows: +/+, WT (striped bar); +/−, heterozygous (solid bar); −/−, homozygous IL-28RA$^{-/-}$ (open bar). The average transcript level and SD ($n = 5$) are shown.

(UV-inactivated for 6 min) or murine IFN-αβ as a standard. Twenty-four hours later, VSV (VSV/V10) was added to the wells, and the cells were incubated for 3 days. The dilution mediating 50% protection was defined as 1 U of IFN-αβ/ml. High levels of IFN-λ or IFN-γ did not interfere with the assay.

ELISA cytokine measurements

IFN-λ was detected by ELISA. For detection, MaxiSorp plates (Nunc) were coated overnight at room temperature with 1 μg/ml rat anti-mouse IL-28 (R&D Systems) diluted in PBS. After blocking for 1 h at room temperature with PBS containing 1% (w/v) of BSA (Sigma-Aldrich), samples and standard dilutions were added to the wells, and the plates were incubated for 2 h at room temperature. Subsequently, the wells were incubated for 2 h at room temperature with biotinylated rat anti-mouse IL-28 detection-antibody (R&D Systems) at a concentration of 250 ng/ml in PBS with 1% (w/v) of BSA. For development, HRP-conjugated streptavidin, diluted 1:200 in PBS with 1% (w/v) of BSA, was added and incubated for 20 min at room temperature, after which 100 μl of substrate buffer (R&D Systems) was added to the wells. The color reaction was stopped with 5% (v/v) H_2SO_4. Between each step the plates were washed three times with PBS containing 0.05% (v/v) Tween 20. The detection limit of the ELISA was 15 pg/ml.

Statistical analysis

The data are presented as means ± SD. The statistical significance was estimated with the Wilcoxon rank sum test (p values of <0.05 were considered to be statistically significant).

Results

Virus infections and selected TLR ligands induce expression of type III IFN

Viral infections and specific TLR ligands trigger expression of type I IFN (3, 17, 18, 24, 35). To directly compare expression of types I and III IFNs, we generated BM-derived DCs from C57BL/6 mice, which were infected with viruses or treated with TLR ligands. The levels of types I and III IFNs were measured in the culture supernatants. As expected, Sendai virus potently induced expression of IFN-$\alpha\beta$ and also stimulated a strong IFN-λ response (Fig. 1, *A* and *B*). HSV-2 also stimulated expression of both types of IFNs, although to a lesser extent. The TLR2 ligand Pam_3Csk_4 was not able to induce expression of either IFN-$\alpha\beta$ or IFN-λ, but agonists for TLR3 and TLR9 both stimulated production of types I and III IFNs (Fig. 1, *C* and *D*). To look for IFN-λ expression in vivo, we challenged C57BL/6 mice with HSV-2 (6.7×10^4 or 6.7×10^5 PFU) through the vaginal route and looked for cytokine expression on consecutive days. At both viral doses, only very little type I or III IFN was detected in vaginal washes (data not shown). By contrast, when 129Sv mice were challenged with the virus, they responded with strong production of both types I and III IFN on day 2, and IFN-λ was also present on day 3 p.i. (Fig. 1, *E* and *F*). Mice receiving CpG produced both types of IFN, which occurred through a more rapid kinetics relative to what was seen during viral infection (Fig. 1, *G* and *H*).

To examine the cellular source of IFN-λ, we isolated cells of both the hemopoietic compartment (splenic cDCs, pDCs, T cells, B cells, and peritoneal macrophages) and the nonhemopoietic compartment (fibroblasts and keratinocytes) and infected these with HSV, which is able to gain entry into most cell types. RNA was harvested, and IFN-α, IFN-β, and IFN-λ expressions were determined by real-time PCR. Expression of type I and III IFNs followed very similar patterns and was induced to varying degrees in most cell types after infection, with pDCs and cDCs being the two most prominent sources of both types of cytokines (Fig. 1, *I* and *J*).

At the level of IFN induction, it has been reported that types I and III IFNs are induced through common pathways (25). Therefore, we isolated pDCs and cDCs from C57BL/6 and $TLR9^{-/-}$ mice and looked for expression of IFNs. Although expression of types I and III IFNs was largely dependent on TLR9 in pDCs, this response was totally independent of this receptor in cDCs (Fig. 2, *A–F*). In fibroblasts, we found that the previously observed dependency on the MAVS pathway for IFN-β was also seen for IFN-λ (Fig. 2, *G–I*). Thus, type I and type III IFNs are expressed during virus infection and in response to selected TLR ligands, and they are induced through common pathways.

Type III IFN targets only a subset of cell types

It has been suggested previously that not all cell types are responsive to IFN-λ (17–19, 26), which is in contrast to IFN-$\alpha\beta$, which is able to stimulate nearly all nucleated cells. To address this question systematically we isolated cells of both the hemopoietic compartment (splenic cDCs, pDCs, T cells, B cells, and peritoneal macrophages) and the nonhemopoietic compartment (fibroblasts, keratinocytes, and epithelial cells) and examined the ability of IFN-α and IFN-λ to induce expression of the two IFN-stimulated genes (ISGs), OAS1 and IRF-7. Among the hemopoietic cells analyzed, all responded to IFN-α treatment by inducing expression of the ISGs, but only the pDCs evoked a significant response to IFN-λ treatment (Fig. 3*A*). In the nonhemopoietic compartment, we found that keratinocytes and vaginal epithelial cells were responsive to IFN-λ, whereas fibroblasts did not induce expression of ISGs in response to IFN-λ treatment (Fig. 3*B*) Thus, type III IFN is able to stimulate both tissue cells at important portals of entry, and also pDCs, which are specialized IFN-producing cells thought to play important roles in antiviral defense (37, 38).

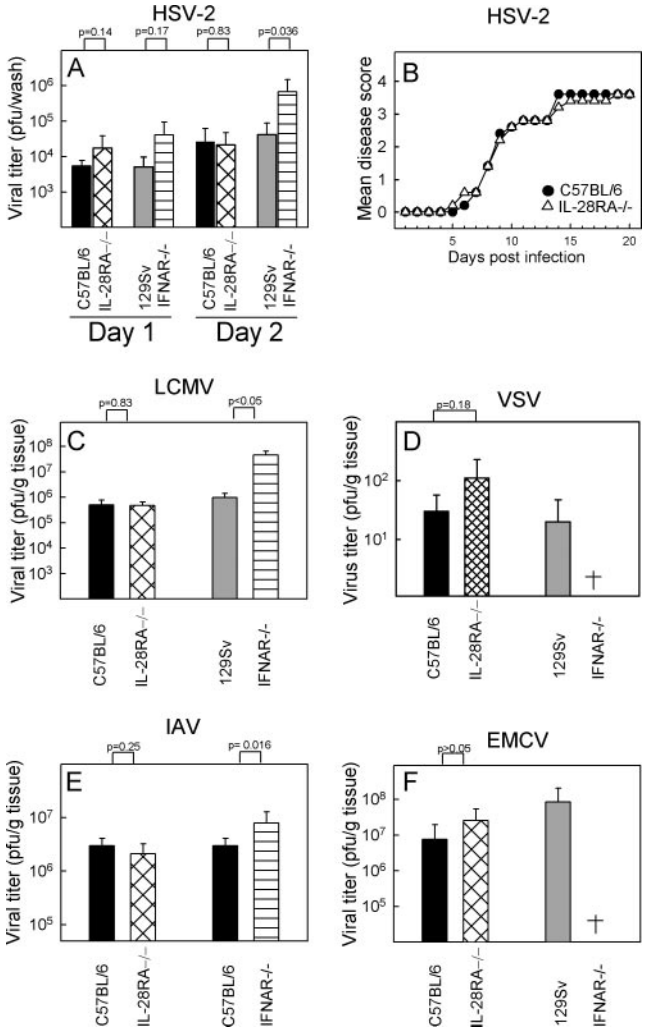

FIGURE 5. Role of IL-28Rα in defense against virus infections. IL-$28RA^{-/-}$, $IFNAR^{-/-}$, and the respective WT littermates were infected with (*A* and *B*) 6.7×10^4 PFU of HSV-2, i.vag.; (*C*) 1×10^3 PFU of LCMV, i.v.; (D) 1×10^6 PFU of VSV, i.v.; (*E*) 2×10^2 PFU of IAV, intranasal route; (*F*) 1×10^2 PFU of EMCV, i.p. *A*, At the indicated time points p.i., vaginal washes were collected and viral load was determined by plaque assay ($n = 6$). *B*, The mice were followed for 20 days and scored clinically. Data are shown as mean disease score. *C*, Three days after infection with LCMV, the mice were sacrificed and viral load in the spleen was determined ($n = 5$). *D*, Three days after infection with VSV, the mice were sacrificed and viral load in the spleen was determined. ($n = 5$). *E*, Lungs were isolated from mice infected with IAV for 3 days, and the levels of infectious virus was determined ($n = 5$). *F*, Three days p.i, the mice were sacrificed and brains were isolated and EMCV levels in organ homogenates were determined by end-point titration ($n = 4–6$). The virus titers are shown as mean values ± SD (†, animals were sacrificed before termination of the experiment).

Generation of IL-28RA$^{-/-}$ mice

To determine the functions of endogenous IFN-λ–IFN-λR interactions, IL-$28RA^{-/-}$ mice were generated by targeted gene disruption. The seven exons containing the entire coding region of IL-28RA were replaced by an IRES-*LacZ*/MC1-Neo reporter

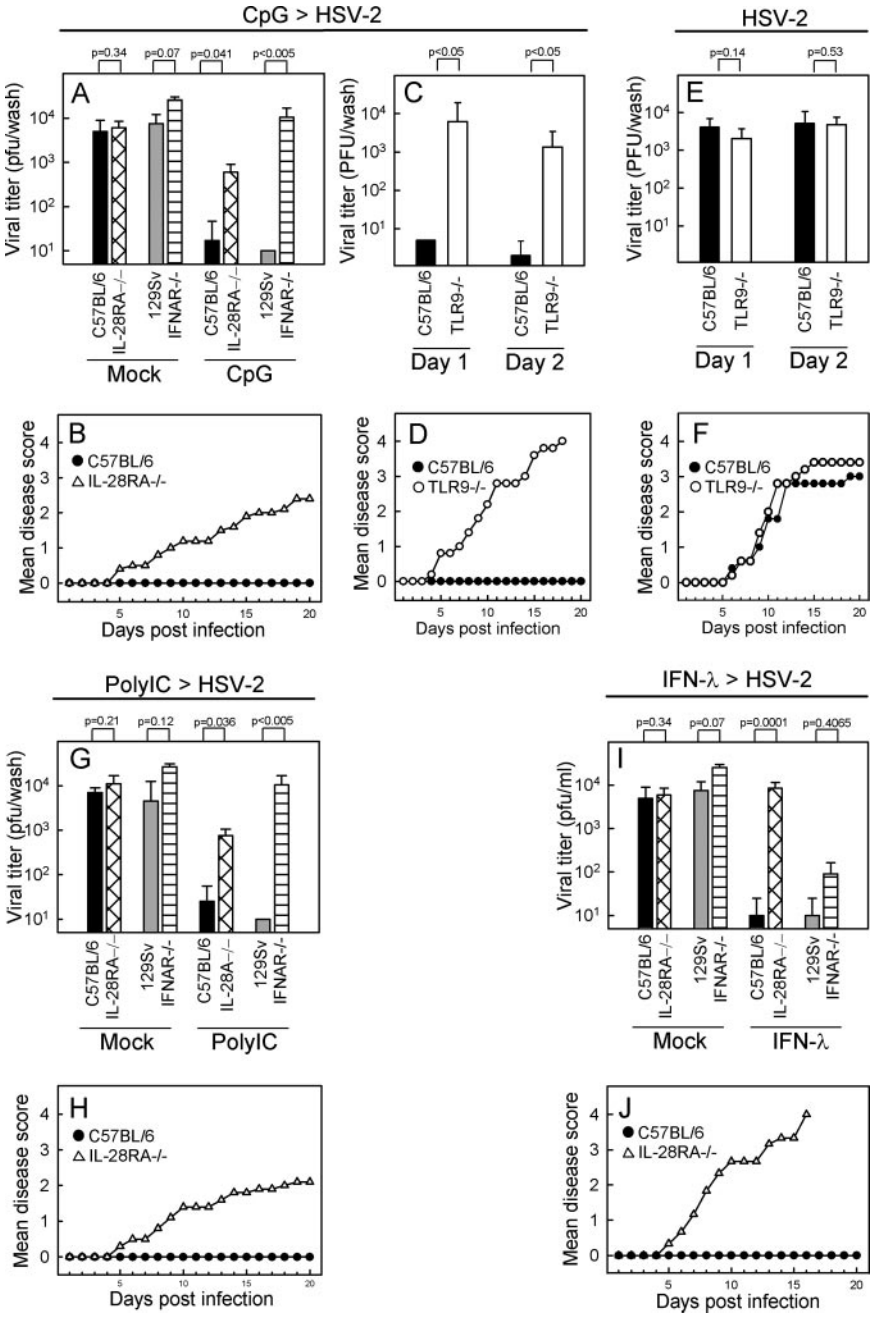

FIGURE 6. Role of IL-28Rα in TLR-activated antiviral defense. IL-28RA$^{-/-}$, IFNAR$^{-/-}$, and the respective WT littermates were treated with 25 μg of ODN1826 (*A–D*), 100 μg of polyIC (*G* and *H*), or 5 μg of IFN-λ2 (*I* and *J*) 24 (*A–D* and *G* and *H*) and 6 h (*I* and *J*), respectively, before i.vag. infection with HSV-2 (*A*, *C–G*, *I*, and *J*: 6.7×10^4 PFU; *B* and *H*: 2.0×10^5 pfu). *A*, *C*, *E*, *G*, and *I*, Vaginal washes were collected on day 1 p.i. (in *C* and *E* also on day 2), and viral load was determined by plaque assay. The virus titers are shown as mean values ± SD (*B*, *D*, *F*, *H*, and *I*). The mice were followed for 20 days and scored clinically. Data are shown as mean disease scores (*A* and *B*: $n = 10$; *C* and *D*: $n = 5$; *E* and *F*: $n = 5$; *G* and *H*: $n = 12$; *I* and *J*: $n = 10$).

gene/selection cassette (Fig. 4*A*). Correct homologous recombination in the transfected ES cells was confirmed by Southern blot analysis (Fig. 4, *B* and *C*). Correctly targeted ES cells were injected into blastocysts, and animals that transmitted the mutant allele in their germline were obtained. IL-28RA$^{-/-}$ mice were born at Mendelian frequencies; they were viable and appeared outwardly normal. No obvious differences in size, behavior, or reproductive ability were seen. The absence of IL-28Rα gene expression (Fig. 4*D*) and IFN-λ-mediated induction of antiviral genes (Fig. 4, *E* and *F*) in the IL-28RA$^{-/-}$ mice was confirmed by real-time RT-PCR, demonstrating that a null allele had been generated.

IL-28Rα plays no essential role in viral-activated host defense

To evaluate the role of the type III IFN system in defense against viral infections, we used different models and compared C57BL/6 and IL-28RA$^{-/-}$ mice with respect to viral load at the sites of infection. In the genital herpes model, the mice were infected vaginally with HSV-2, and viral titers in vaginal washes collected on subsequent days were determined. In the wild-type (WT) mice, the virus was detectable on day 1 p.i. and peaked on day 3, after which it decreased (Fig. 5*A* and data not shown). We were unable to detect any difference in the viral titers in samples obtained from IL-28RA$^{-/-}$ and C57BL/6 on any of the time points examined (Fig. 5*A* and data not shown). These data were supported by the observation that C57BL/6 and IL-28RA$^{-/-}$ mice developed signs of disease with the same kinetics and to the same extent (Fig. 5*B*). Infecting the mice with 10 times more virus also did not lead to differences in viral load in vaginal washes (day 1, C57BL/6: $2.1 \times 10^4 \pm 2.7 \times 10^4$ PFU/wash, IL-28RA$^{-/-}$: $3.7 \times 10^4 \pm 6.7 \times 10^4$ PFU/wash, $p = 0.35$, $n = 9–10$). Similar findings were obtained for vaginal washes collected on days 2 and 3 (data not shown). When examining the type I IFN system, we found that on day 1 p.i. there was no significant difference between IFNAR$^{-/-}$ and 129Sv mice; however, from day 2 p.i. there was a significant difference

between the titers in the vaginal washes of IFNAR$^{-/-}$ mice vs 129Sv mice (Fig. 5*A*). Infection of mice with HSV-2 through the intraperitoneal route also did not reveal any essential role for IFN-λ in host defense against this virus (data not shown).

In addition to the DNA virus HSV-2, we also examined the role of the type III IFN system in defense against the RNA viruses LCMV (arenavirus, −ssRNA) (Fig. 5*C*), VSV (rhabdovirus, −ssRNA) (Fig. 5*D*), IAV (orthomyxovirus, segmented −ssRNA) (Fig. 5*E*), and EMCV (picornavirus, +ssRNA) (Fig. 5*F*). For all viruses tested, we found that IL-28Rα deficiency did not affect the ability to combat the virus, whereas lack of responsiveness to type I IFN significantly reduced the antiviral response. Thus, the type III IFN system seems not to be essential for early virus-activated host defense. By contrast, type I IFN has an important role in this process.

IL-28Rα is essential for TLR-activated antiviral defense

In addition to virus infection, stimulation through certain TLRs also triggers production of IFN-λ (24, 35) (Fig. 1, *C* and *D*). Therefore, we wanted to examine whether signaling through IL-28Rα contributed to the antiviral response evoked by TLR stimulation. To this end we used the murine model for genital herpes, in which local administration of TLR3 and TLR9 ligands induces a strong antiviral response (38–41). The mice were treated with the ligand for 24 h before vaginal infection with HSV-2, and viral load in vaginal washes collected 24 h p.i. was determined. As expected, WT mice receiving CpG ODN (which worked strictly through TLR9, Fig. 6, *C* and *D*) were highly resistant to the infection (Fig. 6, *A* and *B*). By contrast, we found elevated levels of virus in the washes isolated from the IL-28RA$^{-/-}$ mice (Fig. 6*A*). Although this difference between WT and IL-28RA$^{-/-}$ mice was no longer significant on day 2 p.i. (data not shown), the IL-28RA$^{-/-}$ mice did develop signs of disease to a higher extent than did the WT mice (Fig. 6*B*). Similar findings were obtained when the mice were treated with the TLR3 agonist polyIC before HSV-2 infection (Fig. 6, *G* and *H*). HSV-2 is known to be recognized by pDCs through TLR9 (43) (Fig. 2, *A–C*), but this receptor played no role in control of the virus or disease progression when the mice were infected without prior TLR stimulation (Fig. 6, *E* and *F*).

To test whether recombinant IFN-λ could protect against genital herpes, and to examine for a possible cross-talk between the type I and type III IFN systems, we treated mice with IFN-λ 6 h before infection and looked for virus in vaginal washes at the indicated time points p.i. (Fig. 6*I*). The antiviral activity of IFN-λ was abrogated in mice lacking IL-28Rα (Fig. 6, *I* and *J*), whereas this cytokine was able to mediate strong antiviral protection in mice lacking IFNAR. All together, local administration of TLR3 and TLR9 at the vaginal epithelial surface induces an antiviral response, which is dependent on both type I and type III IFNs.

IFN-λ does not mediate positive feedback on production of type I or type III IFN

To address the mechanistic basis for the antiviral function of type III IFN, we first examined whether this cytokine was able to increase IFN production through a positive feedback mechanism. Type I IFN augments its own production at least partly through induction of IRF-7 (43). Because we found that IFN-λ did induce expression of IRF-7 in responsive cell types, we were interested in examining the role of this cytokine in the positive feedback mechanism. Splenocytes were isolated from IFNAR$^{-/-}$ (Fig. 7, *A–C*) or IL-28RA$^{-/-}$ mice (Fig. 7, *D–F*) and the respective WT littermates, and they were infected with Sendai virus in vitro for the indicated amounts of time. Total RNA was isolated and IFN expression was measured by real-time PCR. As expected, we found

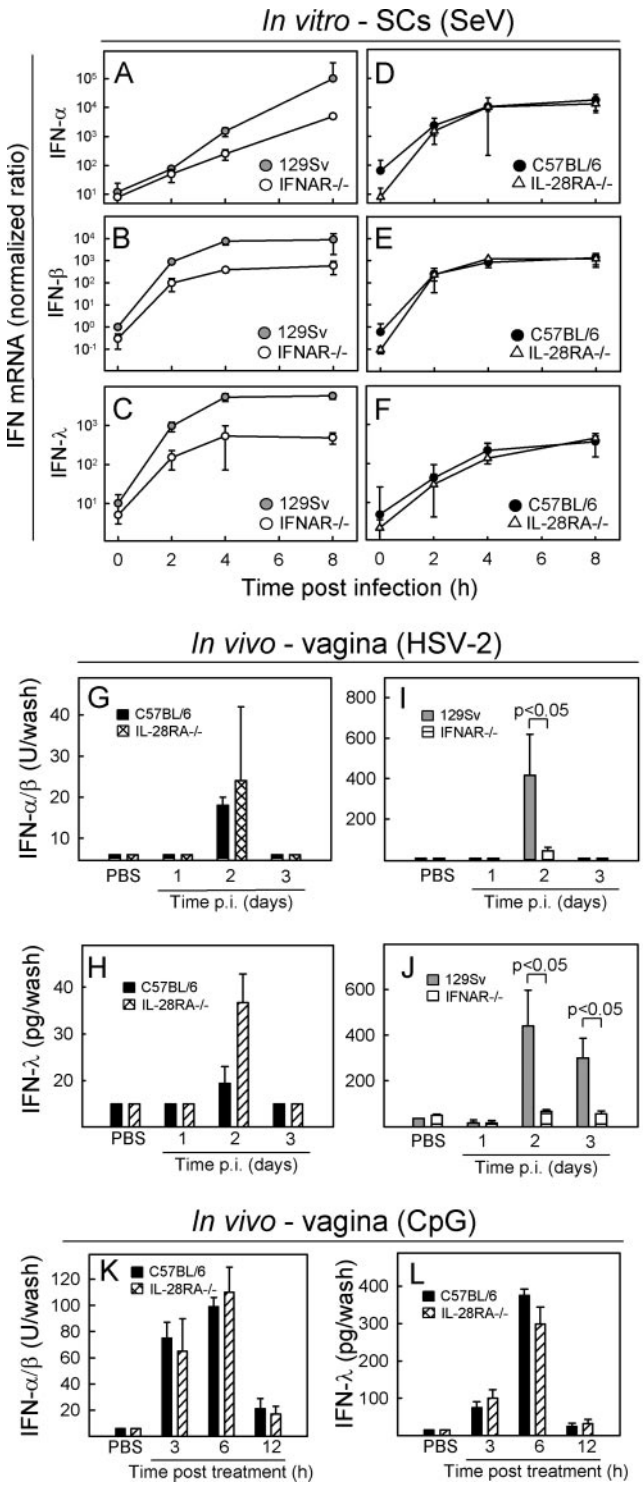

FIGURE 7. Feedback by IL-28Rα and IFNAR signaling on expression of types I and III IFN. *A–F*, Spleen cells were harvested from (*A–C*) 129Sv and IFNAR$^{-/-}$ mice and (*D–F*) C57BL/6 and IL-28Rα$^{-/-}$ mice, and placed in culture before infection with Sendai virus. Total RNA was harvested at the indicated time points, and expression of IFN-α, IFN-β, and IFN-λ was determined by real-time PCR. The data are shown as normalized ratio relative to β-actin levels × 1000. *G–J*, C57BL/6 and IL-28RA$^{-/-}$ mice (*G* and *H*) and 129Sv and IFNAR$^{-/-}$ mice (*I* and *J*) were infected i.vag. with 6.7×10^4 PFU of HSV-2. *K* and *L*, C57BL/6 and IL-28RA$^{-/-}$ mice were treated with 25 μg of ODN1826 before i.vag. infection with 6.7×10^4 PFU of HSV-2. Vaginal washes were collected at the indicated time points after treatment, and types I and III IFNs were measured by bioassay and ELISA, respectively ($n = 5$). The data are shown as mean values ± SD.

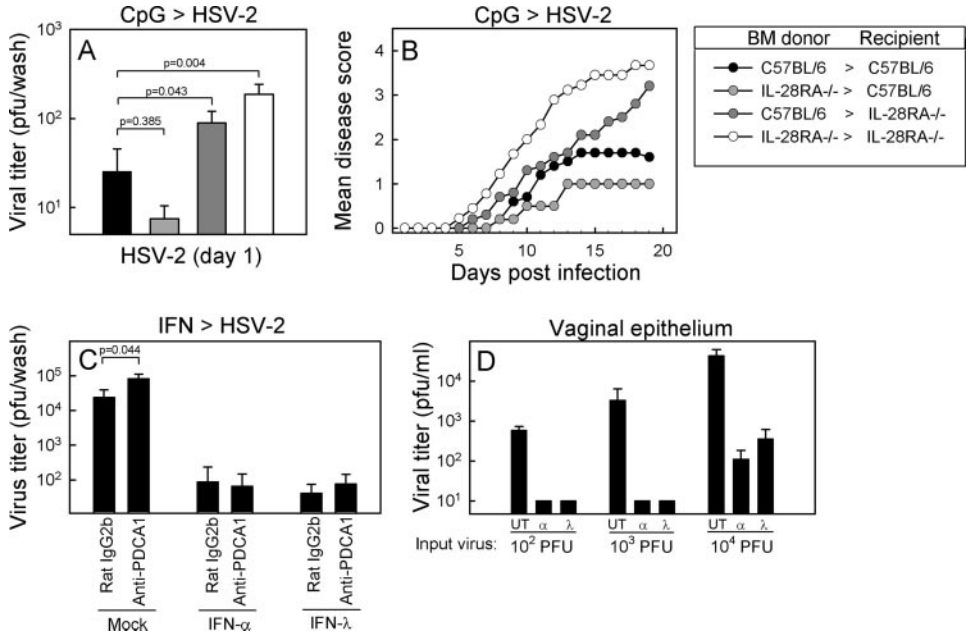

FIGURE 8. Role of hemopoietic vs nonhemopoietic cells in IL-28Rα-dependent antiviral defense. *A* and *B*, C57BL/6-IL-28RA$^{-/-}$ chimeric mice were infected i.vag. with 6.7×10^4 PFU of HSV-2. *A*, On day 1 p.i., vaginal washes were collected and viral load was determined by plaque assay. *B*, The mice were followed for 20 days and scored clinically. Data are shown as mean disease score ($n = 10–17$). *C*, C57BL/6 mice were treated with anti-plasmacytoid DC Ag-1 (anti-PDCA1) or a control Ab 1 day before treatment with 5 μg of IFN-α or IFN-λ2. Six hours later the mice were infected with 6.7×10^4 PFU of HSV-2 (i.vag.). On day 1 p.i., vaginal washes were collected and viral load was determined by plaque assay ($n = 8$). *D*, Vaginal epithelium was isolated from C57BL/6 mice and cultured overnight in the presence of control medium alone or 100 ng/ml IFN-α or IFN-λ2. The cells were infected with the indicated amounts of HSV-2 and left for 48 h, at which point infectious virus in the culture supernatants was quantified by plaque assay. The data are shown as means of triplicate cultures ± SD.

that expression of type I IFNs was significantly compromised in IFNAR$^{-/-}$ mice compared with 129Sv mice (Fig. 7, *A* and *B*). Interestingly, virus-induced expression of IFN-λ was also significantly lower in the IFNAR$^{-/-}$ mice, suggesting that type I IFN mediated positive feedback on type III IFN expression (Fig. 7*C*). By contrast, when we compared splenocytes from C57BL/6 and IL-28RA$^{-/-}$ mice, we found no apparent difference with respect to virus-induced expression of type I or III IFNs (Fig. 7, *D–F*).

To examine the same phenomenon in vivo, we looked for production of IFN-αβ and IFN-λ in vaginal washes from WT and KO mice infected with HSV-2 or treated with CpG ODN (Fig. 7, *G–L*). In C57BL/6 mice, HSV-2 was a very weak inducer of IFN expression, but this response was either not affected or elevated in IL-28RA$^{-/-}$ mice (Fig. 7, *G* and *H*). For mice receiving CpG, we also found that IL-28Rα deficiency did not affect expression of either type I or type III IFN (Fig. 7, *K* and L). The 129Sv mice mounted strong IFN responses (αβ and λ) to vaginal HSV infection, and this was totally abrogated in IFNAR$^{-/-}$ mice (Fig. 7, *I* and *J*).

To examine whether the IFN-λs were capable of stimulating the IFN positive feedback mechanism at all, we treated vaginal epithelial cells overnight with IFN-α or IFN-λ, washed off the cytokines, infected the cells with viruses, and finally harvested supernatants for measurement of types I and III IFNs. Under these experimental conditions IFN-λ was able to augment virus-induced production of both types I and III IFNs (data not shown). Thus, unlike the type I IFN system, type III IFNs are not required for stimulating the IFN positive feedback mechanism, although the cytokine is in possession of this activity. Our experiments also show that, like production of type I IFNs, type III IFNs are regulated through the IFN-αβ-activated positive feedback loop.

IFN-λ stimulates antiviral state in epithelial cells

Only few cell types are responsive to IFN-λ, including pDCs and epithelial cells (Fig. 2). It has been shown that HSV-2 replication occurs exclusively in epithelial cells during vaginal infection in mice (44), and that pDCs are essential for triggering antiviral activity by CpG in this model (41). Therefore, we wanted to examine whether expression of IL-28Rα on cells from the hemopoietic vs the nonhemopoietic compartment contributed to antiviral defense. In particular, we were interested in the role of IL-28Rα on pDCs and epithelial cells. First, we generated BM chimeric mice from C57BL/6 and IL-28RA$^{-/-}$ mice and evaluated how these mice controlled HSV-2 infection after prior stimulation with GpC. As expected, and confirming the finding in Fig. 6*A*, WT mice reconstituted with WT BM controlled the infection significantly better than did IL-28RA$^{-/-}$ mice reconstituted with IL-28RA$^{-/-}$ BM (Fig. 8*A*). The vaginal washes from IL-28RA$^{-/-}$ mice reconstituted with WT BM contained levels of virus that were statistically indistinguishable from the levels in washes obtained from IL-28RA$^{-/-}$ mice reconstituted with IL-28Rα$^{-/-}$ BM, and likewise the viral load in WT mice reconstituted with IL-28Rα$^{-/-}$ BM was statistically indistinguishable from the load in WT mice reconstituted with WT BM. The viral titers correlated largely with the disease scores obtained by following the animals for 20 days p.i. (Fig. 8*B*).

The data shown above suggest that hemopoietic cells were dispensable for the antiviral activity of IFN-λ in this model. To look further into the potential role of pDCs in IFN-λ-mediated antiviral response, we depleted this cell type from C57BL/6 mice and subsequently examined how this affected the antiviral activity of IFN-α and IFN-λ. We observed that depletion of pDCs led to a small, but significant, elevation of the viral load in vaginal washes

on day 1 p.i. (Fig. 8*C*). However, mice receiving either type of IFN displayed very low viral load in vaginal washes, and this was independent of whether pDCs had been depleted (Fig. 8*C*). Thus, although pDCs are responsive to IFN-λ, this cell type seems not to contribute to the antiviral activity of this cytokine in the model used in this study.

Finally, because viral replication occurs exclusively in vaginal epithelial cells during genital HSV-2 infection (44), and because this cell type does respond to IFN-λ treatment, we wanted to examine whether type III IFNs could induce an antiviral state in these cells. We isolated vaginal epithelium, which was placed in culture and either left untreated or treated with IFN-α or IFN-λ overnight. The cells were infected with increasing amounts of HSV-2 and left for 2 days, at which point viral load in the culture supernatants was determined. We found that the virus was able to replicate to a moderate extent, and this was strongly inhibited by both IFN-α and IFN-λ (Fig. 8*D*, $p < 0.05$ for all doses of virus used). All together, cells of the nonhemopoietic compartment are responsible for the antiviral activity of IFN-λ in the genital herpes model, and this seems primarily to be mediated by induction of an antiviral state in vaginal epithelial cells.

Discussion

IFN-λ, also known as IL-28/29 or type III IFN, is a class of cytokines that activate a type I IFN-like program (19–21) and possess antiviral activity (3, 17, 18, 22). We herein describe having generated mice lacking the type III IFN-specific receptor chain IL-28Rα, and show that although IFN-λ is indeed an antiviral cytokine in vivo, it is not required for control of a number of viruses. By contrast, type III IFN plays an important role in mediating antiviral protection by ligands for TLR3 and TLR9, and it seems to work by targeting the nonhemopoietic compartment through induction of an antiviral state in tissue cells in which the virus normally propagates.

We found that types I and III IFNs were produced by most cell types in response to HSV-2 infection in vitro, particularly pDCs and cDCs. After infection in vivo, much less IFN could be detected in the vaginal washes from mice of the same genetic background, a finding that may be due to a lower number of viruses available per cell in vivo as compared with in vitro, or, alternatively, due to trafficking of the high-producing DCs to the lymph nodes (44). Since both viruses and selected TLR ligands induce expression of types I and III IFNs in roughly the same ratios (24, 35), we cannot explain why IFN-λ is essential only for TLR-mediated antiviral protection, and also why the type I IFN system does not protect against infection in IL-28RA$^{-/-}$ mice receiving TLR3 and TLR9 agonists. One possible explanation could be that the IFNs serve slightly different functions in virus- vs TLR-mediated antiviral defense. A report has shown that IFN-λ induced a steady increase of expression of a subset of ISGs, whereas the same genes were induced by IFN-α with a more rapid and transient kinetics (21). Therefore, it is possible that IFN-λ induces a slower but more sustained response, which is important for TLR-mediated antiviral protection where the IFN-inducing pathogen-associated molecular pattern is given only once and rapidly cleared. By contrast, in the case of many virus infections, the IFN-inducing pathogen-associated molecular patterns are present throughout the infection (45), in which case continuing local IFN-αβ production may sustain the antiviral response, and hence not reveal the antiviral activity of IFN-λ.

The present finding that IFN-λ was essential for antiviral protection evoked by stimulation through TLR3 and TLR9, but not for control of a number of virus infections, raises the question if IFN-λ functions as an antiviral cytokine during natural infections. Strong evidence in favor of this was recently provided by Kotenko and colleagues, who found that poxviruses encode an IFN-λ antagonist (46). So why do we observe such a strong dependency on IFN-αβ but not on IFN-λ in virus-activated host defense? One possibility is that IFN-λ is a slow-acting back-up system that is normally not essential for mounting sufficient antiviral activity. Such a system could become important during infection with viruses that rapidly close down production of IFNs by host cells, in which case a slow-acting innate defense system would serve an important function by prolonging the IFN-mediated antiviral activity at the site of infection. Another possibility as to why the observed phenotype in IFNAR$^{-/-}$ mice was much more dramatic than in IL-28Rα$^{-/-}$ mice may relate to the genetic background of the mice. WT 129Sv mice produce much more IFN than do WT C57BL/6 mice, and activities dependent on type I IFN in 129Sv mice may not show the same dependency in other mouse strains. In support of this idea, it has recently been reported that TLR9$^{-/-}$ mice on a mixed 129Sv × C57 background are impaired with respect to clearance of genital herpes through a mechanism dependent on pDC-derived type I IFN (36); in the present study, however, we found that TLR9$^{-/-}$ mice on a C57BL/6 background were indistinguishable from WT mice in the same model. Therefore, to fully evaluate the relative contributions of the types I and III IFN systems in antiviral defense, and to look for possible redundancy in this system, it will be necessary to study the IFNAR$^{-/-}$ and IL-28Rα$^{-/-}$ mice on the same genetic background and to generate IFNAR/IL-28Rα double-deficient mice.

Although direct antiviral activity is undoubtedly one of the main functions of type I IFNs, they also have several immunomodulatory activities on both the innate and adaptive immune system (5–12). Accumulating evidence now suggests that IFN-λ can affect the host response in a broader manner. For instance, treatment with IFN-λ1 induces expression of a subset of inflammatory cytokines in human monocytes and macrophages (47, 48). Others have reported that human blood monocytes gain responsiveness to IFN-λ during differentiation to DCs, and that these DCs induce proliferation of regulatory T cells (29) and modulation of the Th1/Th2 response (49). In a different study it was shown that intrahepatic CD3$^-$DX5$^+$ NK cells express the IFN-λ receptor chains and contribute to the antitumor activities of IFN-λ (28). Therefore, as with type I IFN, type III IFNs possess immunomodulatory activities, which suggests that the IFN-λs can stimulate the antiviral host response through different mechanisms, and also that this class of cytokines has functions beyond antiviral defense, as already suggested (26–28).

Previous work on type III IFNs has indicated that not all cell types are responsive to this cytokine (17–19, 26), which is in contrast to the ubiquitous expression of the type I IFN receptor system. In this study we isolated a number of hemopoietic and tissue cell types and examined them for expression of ISGs in response to stimulation with IFN-α or IFN-λ. We confirmed the broad expression pattern of the IFNAR system, and found that among the cell types examined, that pDCs were the only hemopoietic cell type that responded to IFN-λ. Others have reported that human monocytes, blood monocyte-derived DCs, and murine intrahepatic CD3$^-$DX5$^+$ NK cells respond to IFN-λ or express the receptor chains (28, 29, 47, 49). Therefore, we conclude that only few cell types of the hemopoietic compartment are responsive to IFN-λ, although we cannot exclude that expression of the type III IFN receptor system may be induced by cell differentiation and activation in a broader spectrum of cells. With respect to tissue cells, we found that keratinocytes and epithelial cells, but not fibroblasts, express ISGs in response to IFN-λ treatment, and that such treatment induces a very robust antiviral state in epithelial cells. Our

data thus support the hypothesis that IFN-λ is an antiviral cytokine that targets only a subset of cell types, many of which are located at important portals of entry into the body that constitute primary sites of virus replication.

Concerning the mechanism through which IFN-λ contributes to antiviral protection, we first examined whether type III IFN was able to exert a positive feedback on production of IFN-$\alpha\beta$ and IFN-λ. Type I IFN is known to potently augment its own expression, at least partly through induction of IRF-7 (43). We found that IL-28Rα was not required for mediating positive feedback on IFN production in spleen cells and in the vagina, whereas IFNAR was very important for this process. However, treatment of vaginal epithelial cells with IFN-λ did enhance the IFN response during subsequent virus infection. This may suggest that IFN-λ is capable of mediating the IFN positive feedback mechanism in some cell populations, but that all necessary stimulation may be provided by type I IFN upon viral infection or TLR stimulation, hence leaving the contribution from the IFN-λs undetectable. Interestingly, we also found that the type I IFN system feeds back not only on its own expression but also an expression of type III IFN. This is in line with a recent report that types I and III IFNs are regulated through common pathways (25).

We next examined through which cell types IFN-λ was mediating its antiviral activity. It has been suggested that pDCs are important for protection against genital herpes (36), and that HSV-2 replicates exclusively in vaginal epithelial cells in the model used in this study (44). We found that pDCs and epithelial cells responded to IFN-λ, whereas few other cell types did. Using BM-reconstituted chimeric mice we found that CpG-mediated antiviral protection relied on responsiveness to type III IFNs only for cells of the nonhemopoietic compartment. Moreover, depletion of pDCs did not affect the antiviral activity of IFN-λ in vivo. This in contrast to the mechanism through which type I IFN mediates antiviral protection in the same model, where responsiveness to IFN-$\alpha\beta$ is required in both hemopoietic and nonhemopoietic cells for complete antiviral protection following CpG ODN treatment (41). When we examined the ability of IFN-λ to induce an antiviral state in vaginal cells we found that treatment of cells with this cytokine strongly inhibited replication of HSV-2. Thus, our data strongly suggest that induction of an antiviral state in vaginal epithelial cells is the main mechanism through which IFN-λ mediates antiviral protection in the model used in this study.

In conclusion, we report that IFN-λ is essential for TLR-activated antiviral protection in a physiologically relevant model of mucosal infection with HSV-2. This antiviral activity of IFN-λ was largely mediated by inducing an antiviral state in epithelial cells. Thus, IFN-λ mediates antiviral activity at major portals of entry into the organism by stimulating cells targeted by viruses.

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Disclosures

Zhi Chen, Harald Haugen, and Kevin M. Klucher are current or former employees of ZymoGenetics, and as such receive salary and shares of ZymoGenetics stock.

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