Antiviral Activity of Lambda Interferon in Chickens

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Interferons (IFNs) are essential components of the antiviral defense system of vertebrates. In mammals, functional receptors for type III IFN (lambda interferon [IFN- λ]) are found mainly on epithelial cells, and IFN- λ was demonstrated to play a crucial role in limiting viral infections of mucosal surfaces. To determine whether IFN- λ plays a similar role in birds, we produced recombinant chicken IFN- λ (chIFN- λ) and we used the replication-competent retroviral RCAS vector system to generate mosaic-transgenic chicken embryos that constitutively express chIFN- λ . We could demonstrate that chIFN- λ markedly inhibited replication of various virus strains, including highly pathogenic influenza A viruses, *in ovo* and *in vivo*, as well as in epithelium-rich tissue and cell culture systems. In contrast, chicken fibroblasts responded poorly to chIFN- λ . When applied *in vivo* to 3-week-old chickens, recombinant chIFN- λ strongly induced the IFN-responsive Mx gene in epithelium-rich organs, such as lungs, tracheas, and intestinal tracts. Correspondingly, these organs were found to express high transcript levels of the putative chIFN- λ receptor alpha chain (chIL28RA) gene. Transfection of chicken fibroblasts with a chIL28RA expression construct rendered these cells responsive to chIFN- λ treatment, indicating that receptor expression determines cell type specificity of IFN- λ action in chickens. Surprisingly, mosaic-transgenic chickens perished soon after hatching, demonstrating a detrimental effect of constitutive chIFN- λ expression. Our data highlight fundamental similarities between the IFN- λ systems of mammals and birds and suggest that type III IFN might play a role in defending mucosal surfaces against viral intruders in most if not all vertebrates.

Interferons (IFNs) play a central role in the defense of vertebrates against viral infections (1). Virus-induced IFNs represent a complex mixture of type I and type III IFNs. Type I IFNs include alpha interferon (IFN- α), IFN- β , and various other minor IFN species which all signal through a common cell surface receptor that is present on a broad range of cell types (2, 3). Type III IFNs in humans include IFN- λ 1, IFN- λ 2, and IFN- λ 3 (also named interleukin-29 [IL-29], IL-28A, and IL-28B, respectively) which signal through a heterodimeric cell surface receptor composed of the IFN- λ -specific IL28R α chain and the IL10R β chain (4, 5). In mammals, the IL28R α chain is preferentially expressed by epithelial cells (6), while IL10R β appears to be ubiquitously expressed (7). Consequently, IFN- λ appears to protect mainly epithelial cells of the respiratory and the gastrointestinal tract from viral infections (8, 9). Ligand recognition by either type I or type III IFN receptors results in swift activation of the JAK-STAT pathway, which in turn leads to phosphorylation of STAT1 and STAT2 and to the formation of active transcription IFN-stimulated gene factor 3 (ISGF3). A large body of data suggest that type I and type III IFNs upregulate virtually identical sets of IFN-stimulated genes (ISGs) which code for antiviral proteins such as Mx or 2',5'-oligoadenylate synthetase (OAS) (10–12).

The chicken (*Gallus gallus*) has been used as a prototypical species to investigate the avian IFN system. Several IFN- α as well as single IFN- β , IFN- ω , IFN- τ , and IFN- κ genes have been identified, representing the chicken type I IFN system (13, 14). Chicken IFN- α (chIFN- α) administration was shown to significantly delay Rous sarcoma virus-induced tumor formation, exemplifying its *in vivo* antiviral properties (15). Another study demonstrated that chIFN- α treatment ameliorates disease progression following experimental infection of chickens with a highly pathogenic influenza A virus (HPAIV) H5N1 strain (16).

In addition to the type I IFNs, a single gene for chicken IFN- λ (chIFN- λ) has been described, and its product was shown to trigger a moderate antiviral response in the chicken macrophage cell

line HD11 and in primary chicken embryonic fibroblasts (CEF) (17). A more recent report indicated that stimulation of CEFs with recombinant chIFN- λ induces ISGs in a temporal pattern distinct from that of type I IFNs (18). However, it has been unknown to date whether IFN- λ contributes to antiviral protection in infected birds.

To address this issue *in vitro*, *in ovo*, and *in vivo*, we employed recombinant chIFN- λ and took advantage of the replication-competent retroviral gene transfer vector system RCAS (19, 20) to generate chicken embryos overexpressing chIFN- λ . We observed ISG responses preferentially in epithelium-rich organs that strongly express IL28RA transcripts. In birds treated with chIFN- λ , replication of a highly pathogenic influenza virus was delayed, demonstrating that this cytokine plays a decisive role in host resistance.

MATERIALS AND METHODS

Viruses. Influenza A virus strains used in this study are A/WSN/1933 H1N1 (WSN), A/Chicken/United Arab Emirates/R66/2002 H9N2 (R66), A/FPV/Rostock/34 H7N1 (FPV Rostock), and recombinant A/swan/Germany/R65/2006 H5N1 (rR65-wt). In addition, a recombinant virus carrying genome segments HA, NP, NA, M, and NS of the avian strain R65 and segments PB1, PB2, and PA of the mammalian strain

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A/Puerto Rico/8/1934 H1N1 (PR8), designated rR65-PR8(123), was used (16). Furthermore, the velogenic Newcastle disease virus (NDV) strain Herts-33 and the infectious bronchitis virus (IBV) strain Massachusetts 41 (M41; kindly provided by C. Winter, Hannover, Germany) were used. Vesicular stomatitis virus (VSV) strains employed in this study were strain Indiana and a VSV strain expressing green fluorescent protein (VSV-GFP) (21).

Virus stocks were produced in embryonated chicken eggs or Madin-Darby canine kidney (MDCK) cell cultures. Viral titers of influenza, NDV, and VSV strains were determined by immunostaining with True-Blue substrate (KPL, Gaithersburg, MD, USA) on MDCK cells as described previously (16) and are expressed as focus-forming units (FFU). IBV M41 titers were determined as median cell culture infectious doses (CID₅₀) on primary chicken embryo kidney cells.

Cell and organ cultures. Chicken fibroblast DF-1 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; supplemented with 10% fetal calf serum [FCS], penicillin-streptomycin [Pen/Strep], and L-glutamine) at 37°C and split as needed. Chicken lung CLEC-213 cells (kindly provided by P. Quéré [22]) were cultured in DMEM-F12 (supplemented with 10% FCS, Pen/Strep, L-glutamine, and insulin-transferrin-selenium-X [Gibco]) at 37°C. Tracheal organ cultures were harvested from chicken embryos at embryonic day 19 (ED19) as described previously (16). Rings were cultivated individually with 0.6 ml 199 Hanks' medium (supplemented with 0.2% bovine serum albumin, Pen/Strep, and L-glutamine) in 5-ml tubes at 37°C rotating in an overhead shaker. Ciliary activity was controlled at 48 h postpreparation, and tracheal rings with 100% ciliary activity were selected for further experiments.

Viral vectors expressing chIFN- α or chIFN- λ for generating mosaic-transgenic chickens. The coding sequence of chIFN- α was amplified from plasmid pcDNA1-chIFN- α 1 (14). cDNA for chIFN- λ was generated from RNA of virus-infected primary CEFs by reverse transcription (RT)-PCR using specific primers. Two different chIFN- λ mRNA sequences were detectable in our CEF culture, which differed from each other by a single glutamic acid-to-lysine polymorphism at amino acid position 150 (accession numbers [KF680102](#) and [KF680103](#)). All experiments described here were performed with the Glu-150 variant ([KF680102](#)).

Sequences coding for chIFN- α and chIFN- λ were introduced into the RCASBP(A) plasmid (hereinafter referred to as RCAS [19]) via the ClaI restriction site, and correct insertion was verified by sequencing. DF-1 cells were transfected with RCAS constructs using Nanofectin (Peqlab, Erlangen, Germany) by following the manufacturer's instructions. After 16 to 24 h of incubation, the medium was replaced and the cells were cultured for at least 10 days before they were used for virus infection studies or for generating mosaic-transgenic chicken embryos.

Mosaic-transgenic chicken embryos were generated by injecting 10⁶ RCAS-infected DF-1 cells into the allantoic cavity of eggs (Lohmann Tierzucht GmbH, Cuxhaven, Germany) at ED3. This leads to infection of embryonic cells by RCAS particles produced by the injected cells, followed by integration of the viral DNA into the genome of host cells. Retroviral proteins and transgene products were shown to be detectable primarily in blood vessels, heart, and skin of mosaic-transgenic birds generated using RCAS vectors (20). The injected eggs were incubated in an egg incubator and either subjected to virus challenge at ED14, harvested for production of tracheal organ cultures at ED19, or allowed to develop until hatching.

Production of recombinant chIFN- α and chIFN- λ . *E. coli*-derived chIFN- α was produced as previously described, and biological activity was determined using CEC-32 reporter cells expressing firefly luciferase under the control of the chicken Mx promoter (23). For production of recombinant chIFN- λ , the coding sequence of chIFN- λ was amplified from plasmid RCAS-chIFN- λ and cloned into pcDNA3.3 (Invitrogen, Carlsbad, CA, USA). HEK 293 cells were transfected with pcDNA3.3-chIFN- λ using Nanofectin (Peqlab). After selection with G418 (500 μ g/ml), individual cell clones were picked and expanded. The antiviral activity of chIFN- λ in the supernatant was determined using the bioassay described below. For production of highly concentrated chIFN- λ stocks, a

selected cell clone was maintained for 3 days in Opti-MEM containing 0.2% bovine serum albumin (BSA) before the supernatant was harvested, cleared from cellular debris by low-speed centrifugation, and concentrated using Centricon 70 columns (100 kDa; Millipore, Billerica, MA, USA). Aliquots of concentrated supernatant were stored at -80°C. chIFN- λ titers were determined using the VSV-GFP bioassay as described below.

Generation of DF-1 cells stably expressing chIL28RA. To obtain a putative sequence of the chIFN- λ receptor alpha chain (chIL28RA), total RNA was extracted from the lung of a VALO White Leghorn chicken (Lohmann Tierzucht GmbH) before RT was performed using Revertaid (Fermentas, Schwerte, Germany) and oligo(dT). PCR was performed using Phusion polymerase (Fermentas) and primers chIL28RA-F (5'-AGC CTTTGAATTTAACTTTTATCA-3') and chIL28RA-R (5'-TTCAGC ATGCTCTGCATGGAGAA-3'). The gel-purified PCR product was cloned into pcDNA3.3 vector (Invitrogen), and the resulting plasmid was used to transfect DF-1 cells. After G418 selection (500 μ g/ml), individual clones were selected for strong responses to chIFN- λ treatment using the VSV-GFP assay. All experiments described here were performed with DF-1-chIL28RA clone 23.

VSV-GFP bioassay. chIFN- λ -induced protection against infection with VSV-GFP (21) was used to screen for chIFN- λ -responsive cell clones as well as for titration of recombinant chIFN- λ preparations. Briefly, cells seeded in 96-well plates were treated overnight with serial dilutions of IFN preparations diluted in Opti-MEM with 0.2% BSA. Subsequently, cells were infected with VSV-GFP at a multiplicity of infection (MOI) of 0.5. At 24 h postinfection (p.i.), fluorescence was quantified using an Infinite M200 plate reader (Tecan, Crailsheim, Germany), and signal intensities were corrected by subtraction of the background fluorescence of uninfected control wells. Results were expressed as percentage of antiviral activity, which is defined as the percentage of reduction of corrected signal intensities compared to untreated VSV-GFP-infected control wells. chIFN- λ titers were determined on DF-1-chIL28RA clone 23 cells, and one unit was defined as the 50% inhibitory concentration value calculated by nonlinear curve fitting with GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

qRT-PCR analysis. For quantitative RT-PCR (qRT-PCR) analysis, organ samples or pharyngeal swabs were frozen in Trifast (Peqlab) at -70°C immediately after sampling. Organ homogenates were centrifuged at 6,000 $\times$ g for 30 min at 4°C, and supernatants were collected. Samples were then processed through Direct-zol 96 columns (Zymo, Freiburg, Germany) and treated with DNase (Fermentas). RT was performed using Revertaid (Fermentas) with oligo(dT) (for measuring chMx and chIL28RA mRNA) or random hexamers (for measuring viral RNA) with 1 μ g of DNase-treated RNA in a final volume of 20 μ l. One microliter of RT product was used for qPCR analysis using the Sensifast SybrHi-Rox kit (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions. Primers for influenza A virus detection were derived from Fouchier et al. (24), and primers for chMx mRNA amplification were derived from Schusser et al. (25). Primers qPCRmbRA-F (5'-GAAGCCG GATCTGAAGACAA-3') and qPCRmbRA-R (5'-TGATATCTTAAC ACATCCTTCCTCAG-3') were used for detecting chIL28RA mRNA. Chicken β -actin transcripts were amplified using primers chBActin-F (5'-CACAGATCATGTTTGAGACCTT-3') and chBActin-R (5'-CATCACA ATACCAGTGGTACG-3'). qRT-PCR results were calculated as chMx- or chIL28RA-to-actin ratios, by the 2^{- Δ CT} method.

IFN treatment and experimental infection of chickens with influenza A virus. Fertilized specific-pathogen-free White Leghorn chicken eggs were purchased from Lohmann Tierzucht GmbH and allowed to hatch. Chicks were raised in the premises of the University of Freiburg. To measure *in vivo* induction of IFN-stimulated genes, three-week-old chickens were treated with either 5 $\times$ 10⁶ units of recombinant chIFN- λ derived from stably transfected HEK 293 cells (n = 4 birds), 2 $\times$ 10⁶ units of *Escherichia coli*-derived chIFN- α (n = 4), or phosphate-buffered saline (PBS) (n = 3) by combined intravenous (i.v.) and ocular (o.n.) application twice at a 6-h interval. Two hours after the second treatment, all

FIG 1 DF-1 cells transgenically expressing chIFN- λ do not acquire substantial virus resistance. DF-1 cells persistently infected with empty RCAS vector (RCAS-Ø), RCAS expressing chIFN- λ (RCAS-chIFN- λ), or RCAS expressing chIFN- α (RCAS-chIFN- α) were infected with the indicated challenge viruses at an MOI of 0.001, and viral titers in the supernatants were determined at the indicated time points. Results are presented as means of log-transformed viral titers ($\pm$ standard errors of the means [SEM]) from 2 or 3 independent experiments. The origin of the y axis indicates the detection limit of the titration.

animals were euthanized and organ samples were collected for measuring chMx and chIL28RA mRNA levels by qRT-PCR.

In a second experiment, three groups of seven chickens each were similarly treated with either 2×10^6 or 1×10^6 units of HEK 293-derived chIFN- λ or with PBS. At 2 h after the second treatment, all birds were infected oculonasally with 10^6 FFU of influenza A virus strain rR65-PR8(123) H5N1 per bird as described previously (16). Thereafter, treatment was repeated daily with the same dose. Since birds were housed in an isolation unit after infection and since i.v. injection was not feasible under these conditions, the treatment was performed by combined intramuscular (i.m.) and o.n. application. Pharyngeal swabs were collected daily for viral RNA detection by qRT-PCR. Birds were monitored for the presence of symptoms twice daily, and clinical signs were recorded as clinical scores 0 (no clinical signs), 1 (mild clinical signs), 2 (severe clinical signs), or 3 (dead), as described previously (16). At day 5 p.i., all animals were euthanized, and brain samples were collected for titration of viral loads. The infection experiment was conducted in an isolation unit under biosafety level 3 conditions.

All animal experiments were performed in compliance with the German animal protection law (TierSchG). The animals were housed and handled in accordance with good animal practice as defined by FELASA (www.felasa.eu/recommendations) and the national animal welfare body GV-SOLAS (www.gv-solas.de/). All animal experiments were approved by the local animal welfare committees.

Statistical analysis. Viral RNA and mRNA levels and viral titers were log₁₀ transformed for statistical analysis. Pairwise comparisons of an IFN-treated group to an untreated control group were performed by Student's *t* test. Multiple comparisons of more than two groups were performed by one-way analysis of variance (ANOVA) and subsequent Tukey's comparison of means. Survival curves were compared by log rank (Mantel-Cox) test, and Wilcoxon rank-sum test was used for comparison of clinical scores of two different groups. All tests were performed using GraphPad prism (GraphPad software). *P* values of <0.05 were considered to indicate significant differences between groups.

Nucleotide sequence accession numbers. chIFN- λ and chIL28RA mRNA sequences generated during this study were submitted to GenBank under accession numbers [KF680102](#) to [KF680105](#).

RESULTS

Weak antiviral activity of chIFN- λ in fibroblast cell line DF-1.

To assess the antiviral activity of chIFN- λ , we constructed a replication-competent retroviral vector that constitutively expresses chIFN- λ cDNA (RCAS-chIFN- λ). Transfection of chicken DF-1 fibroblasts with this construct resulted in rapid virus spread and stable expression of chIFN- λ (data not shown). At approximately 2 weeks posttransfection, cells were infected with either VSV, in-

fluenza A virus strain WSN (H1N1), or FPV Rostock (H7N1) at a multiplicity of infection (MOI) of 0.001. Cell cultures persistently infected with the empty vector (RCAS-Ø) or with the vector carrying the chIFN- α gene (RCAS-chIFN- α) served as negative and positive controls, respectively. chIFN- α blocked VSV and WSN almost completely, and it strongly reduced titers of FPV Rostock. In contrast, chIFN- λ had only very mild inhibitory effects on these viruses (Fig. 1), in agreement with previous observations of other groups (17).

Embryonated chicken eggs transgenically expressing chIFN- λ exhibit a high degree of virus resistance. To assess the biological properties of chIFN- λ *in ovo*, we generated mosaic-transgenic chicken embryos by inoculating fertilized eggs with RCAS-chIFN- λ or RCAS-Ø at ED3. Generation of chIFN- α mosaic-transgenic embryos was not successful due to the fact that the RCAS-chIFN- α vector was unstable under these conditions (unpublished data). At ED14, the embryos were challenged by inoculating either various influenza A viruses, NDV Herts-33, or IBV M-41 via the allantoic cavity. For all viral strains analyzed, mean titers in allantoic fluids from chIFN- λ -transgenic embryos at 24 or 36 h p.i. were reduced by at least four log₁₀ units compared to those of empty vector-transgenic embryos (Fig. 2). In addition, survival of chIFN- λ -transgenic chicken embryos infected with strain rR65-wt (H5N1) was significantly prolonged ($P < 0.05$; Fig. 3), clearly demonstrating the antiviral potency of chIFN- λ .

chIFN- λ inhibits influenza virus multiplication in embryonic tracheal organ cultures. Since mammalian IFN- λ is known to act predominantly on epithelial cells, we next investigated the protective effect of chIFN- λ on tracheal organ cultures. Tracheal rings originating from chIFN- λ -transgenic or empty vector-transgenic chicken embryos were infected with influenza A strains rR65-wt or WSN. Viral titers in rings from chIFN- λ -transgenic embryos were markedly reduced (Fig. 4).

Constitutive chIFN- λ expression is lethal for newly hatched chickens. To investigate the antiviral activity of chIFN- λ *in vivo*, mosaic-transgenic embryos were allowed to hatch. While no detrimental effect of chIFN- λ expression on embryonic development was observed (data not shown), hatching rates of RCAS-chIFN- λ -transgenic eggs were slightly reduced compared to those of empty vector-transgenic eggs in two independent experiments (Fig. 5A). After hatching, chicks expressing chIFN- λ were less active than control animals (data not shown), lost rather than gained

FIG 2 Embryonated chicken eggs transgenically expressing chIFN- λ exhibit a high degree of virus resistance. Mosaic-transgenic embryos were produced by infecting the eggs with empty RCAS vector (RCAS-Ø) or RCAS expressing chIFN- λ (RCAS-chIFN- λ) at ED3. At ED14, virus challenge experiments were performed by inoculating 10^2 FFU per egg [R66, rR65-wt, rR65-PR8(123)] or 10^3 FFU per egg (WSN, Herts-33, M41) into the allantoic cavity. Allantoic fluids were harvested at 24 h [rR65-wt, rR65-PR8(123), Herts-33] or 36 h (R66, WSN, M41) postinfection. Symbols indicate viral titers of individual eggs. The origin of the y axis indicates the detection limit of the titration.

body weight (Fig. 5C), and died or had to be euthanized within the first 2 weeks of life (Fig. 5B). Necropsy of chicks euthanized at day 4 posthatching revealed markedly higher volumes of residual yolk sacs in chIFN- λ -transgenic chicks (Fig. 5D) than those in control animals, indicating less efficient yolk absorption. No further prominent macroscopic lesions were observed.

Antiviral activity of chIFN- λ depends on the presence of chIL28RA. The antiviral activity of mammalian IFN- λ depends on the expression of its cognate receptor on target cells and more precisely of its alpha subunit, IL28RA, whose expression is restricted mainly to epithelium-rich tissues (6). We hypothesized that restricted expression of IL28RA could account for the lack of chIFN- λ responsiveness in DF-1 cells. Sequences present in GenBank predict a soluble protein for chIL28RA (FJ947119; 26), which appears unlikely to be signaling competent due to the lack of an intracellular domain, as well as putative membrane-bound chIL28RA (FJ947118; XM_417841; XM_004947908). When performing RT-PCR with RNA from chicken lungs, we detected transcripts coding for the predicted soluble isoform (KF680105; data

not shown) as well as for the putative membrane-bound protein consisting of 567 amino acids that comprises potential signal peptide and transmembrane regions (KF680104; Fig. 6A). The membrane-bound protein exhibits substantial homology to mouse and human IL28RA and contains tyrosine residues corresponding to Tyr-343 and Tyr-517 in human IL28RA (Fig. 6A), which were shown to be critical for IFN- λ responsiveness (11).

To demonstrate that the putative chIL28RA confers chIFN- λ responsiveness, we transfected DF-1 cells with plasmid encoding chIL28RA and selected for permanently transfected cell clones. Such clones and parental DF-1 cells were then treated with serial dilutions of recombinant chIFN- λ , control medium, or chIFN- α before challenge with VSV-GFP. chIFN- λ treatment induced good protection in chIL28RA-transfected but not parental DF-1 cells (Fig. 6B). In agreement, qRT-PCR revealed 1,400-fold-elevated chIL28RA transcript levels in stably transfected DF-1 cells (data not shown). These results confirmed the functionality of our chIL28RA sequence and allowed us to use the newly generated cell line for quantifying recombinant chIFN- λ preparations *in vitro*. Interestingly, the stable cell line CLEC-213, which was derived from chicken lung epithelial cells (22), had 4-fold-increased chIL28RA transcript levels compared to those of nontransfected DF-1 cells (data not shown) and was also responsive to chIFN- λ . However, higher cytokine concentrations were required to induce antiviral activity in CLEC-213 cells than in chIL28RA-transfected DF-1 cells (Fig. 6B).

chIL28RA expression in organs correlates with Mx gene transcription in response to chIFN- λ treatment of chickens. Groups of three to four three-week-old chickens were treated with either 5×10^6 units of recombinant chIFN- λ derived from stably transfected HEK 293 cells, 2×10^6 units of *E. coli*-derived chIFN- α , or PBS by combined i.v. and o.n. application twice at a 6-h interval. Two hours after the second treatment, all animals were euthanized and organ samples were collected. Transcription of chIL28RA and the IFN-stimulated chMx gene was determined by qRT-PCR. chIL28RA transcription levels were lowest in the

FIG 3 Influenza virus-induced embryonic death is delayed by RCAS-mediated expression of chIFN- λ . Mosaic-transgenic embryos generated by infection with the indicated RCAS vectors at ED3 were inoculated with 10^2 FFU per egg of influenza A virus strain rR65-wt (H5N1) at ED14. Survival of the embryo was monitored by candling the eggs at the indicated time points. Statistical analysis revealed a significant difference between the two groups ($P < 0.05$; log rank test).

FIG 4 Reduced growth of influenza virus in trachea explanted from mosaic-transgenic embryos expressing chIFN- λ . Tracheal organ cultures were prepared at ED19 from embryos that were made transgenic using empty RCAS vector (RCAS- $\emptyset$) or RCAS vector expressing chIFN- λ (RCAS-chIFN- λ). Vital tracheal rings were infected with influenza A virus strains rR65-wt (10^2 FFU per ring) or WSN (10^3 FFU per ring). Viral loads in the supernatants are presented as means of log-transformed viral titers ($\pm$ SEM) of seven tracheal rings originating from seven individual eggs. The origin of the y axis indicates the detection limit of the titration.

brain, while no marked differences were observed between the other organs tested. IFN treatment did not result in prominent upregulation of IL28RA gene transcription (Fig. 7A). In contrast, transcription of the chMx gene was induced significantly in all organs after treatment of birds with either chIFN- λ or chIFN- α (Fig. 7B). In epithelium-rich organs, such as intestine, lung, trachea, and kidney, chMx was more prominently induced by chIFN- λ than by chIFN- α , while in brain, liver, and heart, the response to chIFN- α was dominant (Fig. 7B).

Treatment of chickens with recombinant chIFN- λ provides partial protection against infection with highly pathogenic H5N1 influenza A virus. Groups of seven birds were treated twice with either a high or a low dose (2×10^6 or 1×10^6 units) of chIFN- λ as described above. At 2 h after the second treatment, all birds were inoculated with 10^6 FFU of influenza A virus strain

rR65-PR8(123) H5N1 per bird by the oculonasal route. After infection, the IFN treatment was repeated daily by combined intramuscular and oculonasal applications. The rR65-PR8(123) challenge virus was demonstrated to cause severe clinical disease in chickens but was sensitive to treatment with *E. coli*-derived chIFN- α (16). In the experiment presented here, infected chickens developed mild to severe clinical signs starting at day 2 postinfection with no significant differences between IFN-treated and control groups ($P > 0.05$; Fig. 8A). Shedding of viral RNA was quantified by qRT-PCR analysis of pharyngeal swabs. At 1 to 3 days postinfection, viral loads were significantly reduced in the group treated with 2×10^6 units of chIFN- λ compared to those of the PBS-treated group ($P < 0.05$), while treatment with 10^6 units of chIFN- λ had only mild effects (Fig. 8B). At the end of the experiment, viral RNA levels in pharyngeal swabs were similar in all

FIG 5 RCAS-mediated expression of chIFN- λ has lethal consequences for chickens. Embryos made transgenic with empty RCAS vector (RCAS- $\emptyset$) or RCAS expressing chIFN- λ (RCAS-chIFN- λ) at ED3 were allowed to hatch. (A) Mean hatching rates ($\pm$ SEM) from two independent experiments. (B) Survival of mosaic-transgenic chicks carrying either RCAS- $\emptyset$ or RCAS-chIFN- λ . (C) Mean body weights per group ($\pm$ SEM) at the indicated times posthatching ($n = 8$). (D) Weight of residual yolk sac of chicks euthanized at day 4 posthatching (same animals as in panel C). Asterisks indicate significant differences to the RCAS- $\emptyset$ group (Student's *t* test, $P < 0.05$).

FIG 6 DF-1 cells expressing cDNA encoding putative chicken IL28RA respond to chIFN-λ and acquire virus resistance. (A) Amino acid sequences of putative chicken IL28RA (GenBank accession number [KF680104](#)), human IL28RA ([NP_734464](#)), and murine IL28RA ([AAH57856](#)) were aligned using MEGA6 software with subsequent manual modification. Conserved positions are shaded in gray. Signal peptides (predicted by Signal P) are shown in bold, and predicted transmembrane regions (predicted by TMHMM server 2.0) are presented in boxes. Arrowheads indicate conserved tyrosine residues shown to be critical for signaling of the human protein (11). (B) Parental DF-1 cells, DF-1 cells stably transfected with a chIL28RA expression construct, and chicken lung CLEC-213 cells were treated overnight with different dilutions of recombinant chIFN-α or chIFN-λ before infection with VSV-GFP at an MOI of 0.5. The concentration of the undiluted chIFN-α preparation was 2×10^5 units/ml (equaling 10^4 units per well). Fluorescence was measured at 24 h postinfection. Results are presented as mean antiviral activity ($\pm$ SEM) from two independent experiments.

three groups. Viral titers in brain samples collected at day 5 postinfection were significantly reduced by about 1 log₁₀ unit ($P < 0.05$) in the group treated with 2×10^6 units of chIFN-λ compared to those of the PBS-treated group (Fig. 8C). These data demonstrate that *in vivo* treatment with high doses of recombinant chIFN-λ provides partial protection against experimental infection with a highly pathogenic influenza A virus.

DISCUSSION

We report here that chIFN-λ is antivirally active in chickens. Our results indicate that, similar to mammalian IFN-λ, chIFN-λ acts

predominantly on epithelial tissues. In fact, chicken fibroblasts are largely refractory to the antiviral action of chIFN-λ, which is in agreement with previously published work (17, 18). In contrast, chIFN-λ inhibited replication of influenza viruses in primary embryonic tracheal organ cultures and in the chicken lung cell line CLEC-213, although the antiviral activity of chIFN-λ was less prominent than that of chIFN-α in these systems. Consistent with these results, treatment with chIFN-λ *in vivo* resulted in strong induction of the interferon-stimulated Mx gene in epithelial-rich tissues, such as intestine, trachea, and lung, while in organs with lower proportions of epithelial cells, the response to chIFN-α was

FIG 7 chIFN-λ treatment induces transcription of the chMx gene *in vivo*. Three-week-old chickens were treated twice at an interval of 6 h with 5×10^6 units of recombinant chIFN-λ or 2×10^6 units of recombinant chIFN-α by combined intramuscular and oculonasal application. PBS-treated birds were used as controls. Two hours after the second treatment, birds were euthanized and mRNA levels for chIL28RA (A) and chMx (B) genes were determined by qRT-PCR. Different lowercase letters within one organ indicate significant differences between treatment groups (one-way ANOVA with subsequent Tukey's comparison of means, $P < 0.05$).

dominant. The responsiveness to chIFN-λ correlated with transcript levels of the chicken homologue of IL28RA, which codes for the ligand-binding molecule in the functional IFN-λ receptor complex of mammals (4). In addition, ectopic expression of chIL28RA rendered DF-1 cells highly responsive to chIFN-λ. Thus, the IFN-λ system of birds seems to resemble the IFN-λ system of mammals with regard to the nonuniform expression of the IL28Rα chain, which translates into a high degree of cell type specificity of the IFN-λ response (6).

Our attempts to transgenically overexpress chIFN-λ yielded strong evidence that this cytokine possesses antiviral activity also *in ovo*. Experiments with embryonated eggs showed that viral titers of a broad range of viruses were markedly reduced in chIFN-λ mosaic-transgenic embryos infected via the allantoic cavity. Furthermore, challenge experiments with highly pathogenic influenza A virus demonstrated that transgenic embryos were partially protected from virus-induced death. These data suggest that chIFN-λ produced by the transgenic embryo is able to trigger the IFN-λ receptor of epithelial cells in the chorioallantoic membrane. However, our work also showed that chIFN-λ can have detrimental effects. Our attempts to produce chIFN-λ mosaic-transgenic chickens were not successful since such birds died within the first 2 weeks after hatching. The only gross lesions observed in chicks euthanized at the age of 4 days were nonadsorbed yolk sacs. Yolk is the nutrition supply for the embryo, and it is engulfed and digested by the yolk sac epithelium during embryogenesis and after hatching. In posthatching development, the yolk may additionally be transported into the intestine via a stalk and taken up by the intestinal epithelium following digestion by intes-

FIG 8 Treatment of three-week-old chickens with purified chIFN-λ partially protects from challenge with H5N1 virus rR65-PR8(123). Chickens were treated daily with the indicated doses of chIFN-λ, with the first dose given 8 h prior to virus challenge. Infection with 10^6 FFU of rR65-PR8(123) per bird was by the oculonasal route. (A) Clinical inspection was done twice daily, and symptoms were scored according to severity. (B) Viral loads in pharyngeal swabs were quantified by qRT-PCR. Results are presented as means of log-transformed values ($\pm$ SEM; $n = 7$). (C) Chickens were euthanized on day 5 postinfection, and viral titers in brains were determined. The origin of the y axes indicates the detection limit of the test. Asterisks indicate significant differences to the untreated control group (Student's *t* test; $P < 0.05$).

tinal enzymes (27, 28). Thereby the volume of the yolk sac decreases with time. Since detrimental effects in chIFN- λ -transgenic chicks appear to occur during or shortly after hatching, it is tempting to speculate that constitutive chIFN- λ expression interferes with one of the latter processes by an unknown mechanism. An alternative explanation may be that chIFN- λ impairs the establishment of the normal gut flora and thereby interferes with nutrient absorption after hatching. It is of interest to note that daily injection of highly concentrated interferon preparations was lethal for newborn mice, with mortality occurring after seven to 14 days of treatment (29).

Additional evidence that chIFN- λ plays a role in the antiviral defense of chickens came from experiments in which 3-week-old chickens were treated with recombinant chIFN- λ before and during infection with highly pathogenic H5N1 influenza A virus. Previous work demonstrated that many H5N1 field isolates are extremely aggressive in chickens and that the high virulence of such viruses cannot be ameliorated by treating the birds with chIFN- α (16). However, the H5N1 reassortant virus rR65-PR8(123), which carries the polymerase genes of a human influenza virus, grows substantially slower in chickens than the rR65-wt parental virus and fails to induce disease if birds are treated with chIFN- α (16). In our current study, we used this reassortant virus to evaluate the protective potential of recombinant chIFN- λ . We found that chIFN- λ treatment was not very effective in preventing rR65-PR8(123)-induced disease, but high doses (2×10^6 units per bird and treatment) of the cytokine clearly delayed viral excretion via the upper respiratory tract and resulted in reduced spread of the virus to the brain. We speculate that a further increase of the dosage might have resulted in a more pronounced antiviral effect. However, sufficiently concentrated chIFN- λ preparations to challenge this hypothesis were not available. Observations in mice revealed that type I IFN contributes most to the protective antiviral response toward influenza A virus infections, whereas IFN- λ plays only a minor role in the lung (8, 30). We speculate that similarly in the chicken respiratory tract, not all mucosal cells are responsive to chIFN- λ . Thus, the treatment can only delay but not completely prevent a highly pathogenic avian influenza virus from passing the epithelial barrier and reaching cell types in other organs that are not responsive to IFN- λ . This view is in agreement with results of a study in cattle, in which adenovirus-mediated overexpression of bovine IFN- λ could dramatically reduce and delay viral replication and expression of foot and mouth disease virus, a virus whose primary replication site is the upper respiratory tract. Interestingly, the antiviral effect was less prominent when the epithelial barrier was passed by intramucosal injection of the virus, compared to intranasal aerosol inoculation (31). More recent work in the mouse model system showed that IFN- λ is of central importance for the control of murine rotaviruses which primarily infect intestinal epithelial cells (9). It will be of great interest to determine in future studies if the same holds for the chicken and if, as predicted from studies in the mouse, IFN- λ might also be able to control the replication of gastrointestinal viruses with a strong epitheliotropism, such as rotaviruses, in birds.

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