JOURNAL OF VIROLOGY, Sept. 2010, p. 9140–9148 Vol. 84, No. 18
0022-538X/10/$12.00 doi:10.1128/JVI.00717-10

Alpha/Beta Interferon (IFN-α/β)-Independent Induction of IFN-λ1 (Interleukin-29) in Response to Hantaan Virus Infection▽

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Received 3 April 2010/Accepted 24 June 2010

Type III interferons ([IFNs]IFN-λ and interleukin-28 and -29 [IL-28/29]) are recently recognized cytokines with innate antiviral effects similar to those of type I IFNs (IFN-α/β). Like IFN-α/β, IFN-λ-expression can be induced by viruses, and it is believed that type I and III IFNs are regulated in the same manner. Hantaviruses are weak IFN-α/β inducers and have surprisingly been shown to activate IFN-α/β-independent IFN-stimulated gene (ISG) expression. Here, we show that in Hantaan virus (HTNV)-infected human epithelial A549 cells, induction of IFN-λ1 preceded induction of MxA and IFN-β by 12 and 24 h, respectively, and IFN-α was not induced at all. Furthermore, induction of IFN-λ1 and MxA was observed in HTNV-infected African green monkey epithelial Vero E6 cells, a cell line that cannot produce type I IFNs, clearly showing that HTNV can induce IFN-λ1 and ISGs in the complete absence of IFN-α/β. In HTNV-infected human fibroblast MRC-5 cells, which lack the IFN-λ receptor, induction of MxA coincided in time with IFN-β-induction. UV-inactivated HTNV did not induce any IFNs or MxA in any cell line, showing that activation of IFN-λ1 is dependent on replicating virus. Induction of both IFN-β and IFN-λ1 in A549 cells after poly(I:C)-stimulation was strongly inhibited in HTNV-infected cells, suggesting that HTNV can inhibit signaling pathways used to simultaneously activate types I and III IFNs. In conclusion, we show that HTNV can cause type I IFN-independent IFN-λ1 induction and IFN-λ1-specific ISG induction. Importantly, the results suggest the existence of specific signaling pathways that induce IFN-λ1 without simultaneous type I IFN induction during virus infection.

Viruses can be recognized in infected cells through their interaction with pattern recognition receptors (PRRs). Cells that recognize viral pathogen-associated molecular patterns via PRRs activate several different signaling pathways, which in turn leads to the induction of type I interferon (IFN-α/β) gene expression (8, 22). RNA viruses are normally recognized by the PRR retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), or Toll-like receptor 3 (TLR3). These PRRs in turn activate IKKε/TANK-binding kinase 1 (TBK1), the IKKα/β/γ complex, and mitogen-activated protein kinase (MAPK), resulting in activation of IFN regulatory factor 3 (IRF3), IRF7, NF-κB, AP-1, and subsequent type I IFN transcription (8, 22, 34). In an autocrine as well as a paracrine fashion, secreted IFN-α/β activates expression of IFN-stimulated genes (ISGs) that, in turn, induce an antiviral state, thereby inhibiting viral replication and dissemination (8, 16, 34).

Type III IFNs (IFN-λ1/interleukin-29 [IL-29], IFN-λ2/IL-28A, and IFN-λ3/IL28B) were recently discovered and shown to have antiviral effects (3, 20, 32). Their biological activities overlap to some extent with those of type I IFNs, and they can induce similar subsets of ISGs as does IFN-α/β (4, 21). Viral infection can trigger both type I and III IFN production (4, 21). Specific induction of IFN-λ has previously not been reported (42), and it is currently believed that activation of type I and type III IFN production is regulated via the same mechanisms (21, 25, 26, 42). However, it was recently shown that the organization of the IFN-λ1 enhancer differs significantly from the IFN-β enhanceosome (41), suggesting that there might be differences in the transcriptional regulation of IFN-λ1 and IFN-β.

Hantaviruses are the causative agents of two severe human diseases: hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas, with a mortality rate of up to 40% depending on the specific hantavirus species (31, 43). Hantaviruses are enveloped negative-stranded RNA viruses with a three-segmented genome (31, 43). Most disease-causing viruses can at least partly avoid and/or inhibit production of IFN-α/β (8, 16, 29), and this is also the case for hantaviruses (2, 11, 14, 28, 39). RIG-I and MDA5 do not recognize Hantaan virus (HTNV) or Sin Nombre virus RNA (11, 28), and this is at least partly due to how hantaviruses replicate their RNA-genome (11). Moreover, it has been reported that the New York-1 hantavirus Gn protein is able to disrupt TBK1-TRAF3 complex formation (2) and that the HTNV nucleocapsid protein can sequester NF-κB in the cytoplasm (39), thereby interfering with the induction of IFN-β expression. Certain hantaviruses have an open reading frame for a possible nonstructural protein, and this protein has been shown to inhibit the production of IFN-β (14). From these reports it is clear that hantaviruses possess several ways of avoiding and inhibiting activation of type I IFN expression, and it is therefore no surprise that activation of type I IFN responses after infection with pathogenic hantaviruses is weak and occurs late after infection (1, 10, 31, 33). Interestingly, it was recently shown that ISGs, such as MxA, are induced in an

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▽ Published ahead of print on 30 June 2010.

IFN-α/β-independent manner after a hantavirus infection (28). However, as MxA is induced by only type I and III IFNs (13), it could be speculated that IFN-λ is involved in type I IFN-independent ISG induction. Here, in an effort to better understand induction of ISGs caused by hantaviruses and possible roles for IFN-λ1 in the activation of innate immune responses, we have investigated if HTNV can induce IFN-λ and type I IFN-independent activation of MxA expression.

We observed strong induction of IFN-λ that occurred prior to induction of MxA and IFN-β in response to HTNV infection in A549 cells. Furthermore, IFN-λ1 and MxA were induced in HTNV-infected Vero E6 cells, a cell line that lacks the capacity to produce type I IFNs, showing that induction of IFN-λ and ISGs by HTNV can occur in the complete absence of type I IFNs. We also show that induction of both IFN-β and IFN-λ1 is inhibited in HTNV-infected cells stimulated with poly(I:C), suggesting that the observed type I IFN-independent IFN-λ1 induction is not caused by a specific inhibition of type I IFN induction. The results indicate that there are specific, and previously unknown, mechanisms that can specifically trigger IFN-λ1 induction. UV-inactivated HTNV did not induce type I or III IFN, suggesting that the PRR responsible for the type I IFN-independent induction of IFN-λ1 recognizes only replicating virus.

MATERIALS AND METHODS

Cells and viruses. Human A549 and MRC-5 cells and African green monkey Vero E6 cells were used in this study. Cells were grown in minimal essential medium ([MEM] A549 and MRC-5 cells) or Dulbecco's modified Eagle's medium ([DMEM] Vero E6 cells) supplemented with 5% (A549 and Vero E6 cells) or 10% (MRC-5 cells) fetal bovine serum (FBS), 100 U of penicillin/ml, and 100 μg of streptomycin/ml (Invitrogen). All reagents used for cell culture were endotoxin free or with the lowest detectable level of endotoxins available. Cells were grown for 2 weeks or longer under these conditions before they were used in experiments to minimize levels of background IFN production. HTNV strain 76-118, grown on Vero E6 cells as described below, was used for infectious experiments. Cells were infected or treated with the same amount of UV-inactivated virus as a control for nonreplicating virus or with medium alone as a negative control. At different time points postinfection (p.i.), supernatants were harvested while cells were lysed with TriPure (Roche Diagnostics) and stored at −70°C until further use. Levels of progeny virus in supernatants were determined as described below.

Propagation and titration of HTNV. The HTNV virus stock was prepared by infecting monolayers of Vero E6 cells in 175-cm^2 flasks with approximately 10,000 focus-forming units (FFU) of HTNV. Four days after infection, medium (approximately 35 ml/flask in a total of three flasks) was discarded, and fresh medium was added to the flasks. Every day for the next 7 days medium was collected, and fresh medium was added to the flasks. The collected supernatants were centrifuged in order to get rid of possible cell debris, and one small aliquot was drawn from each supernatant for subsequent titration and determination of the number of infectious virus particles/ml (FFU/ml). The remaining supernatants were stored in 50-ml Falcon tubes at −70°C. The supernatants with the largest amounts of virus particles/ml were then thawed, pooled, aliquoted, and refrozen. The titer of the virus stock used in the experiments was 2.7×10^6 FFU/ml.

In order to obtain a very high multiplicity of infection (MOI), a portion of the clarified supernatants with high titers was centrifuged for 4 h at 45,000 rpm in a 45T-rotor in an Optima LE-80K Ultracentrifuge (Beckman Coulter). The pellet was resuspended, aliquoted, and refrozen. The titer of the ultracentrifuged HTNV was 3×10^9 FFU/ml. All stocks were kept at −70°C until further use. After being thawed, virus was diluted to working concentrations in DMEM containing 2% FBS, 10 mM HEPES, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Cells in 24-well plates were infected with 200 μl/well of diluted virus.

Virus titrations were carried out as follows. Samples were diluted 10-fold in Hank's balanced salt solution (HBSS) containing 2% FBS, 20 mM HEPES, 100 U of penicillin/ml, and 100 μg of streptomycin/ml and incubated on confluent Vero E6 cells in 24-well plates for 1 h. Cells were then overlaid with 0.5% agarose-medium (Eagles' minimal essential medium [EMEM] containing 0.5% agarose, 5% FBS, 20 mM HEPES, 100 U of penicillin/ml, and 100 μg of streptomycin/ml) and incubated for 6 days at 37°C in 5% CO_2. Foci of infected cells were stained with polyclonal rabbit anti-HTNV serum, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), and were visualized with 3,3′,5,5′-tetramethylbenzidine (Sigma) and counted.

Stimulation of cells. Cells were treated with poly(I:C) (Sigma) and recombinant human IFN-β and IFN-λ1 (Peprotech). Cells were pretreated for 24 h before infection, or infected cells were treated 24 h after infection.

ELISAs and bioassay. Levels of IFNs in supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits specific for IFN-α (Mabtech), IFN-β (Nordic Biosite), IFN-λ1 (R&D Systems), and IFN-λ2 (R&D Systems). The ELISAs were performed according to the manufacturers' instructions.

A bioassay was used to measure levels of bioactive IFNs secreted from the cells. Supernatants to be tested were added to A549 cells, and cells were incubated for 24 h and subsequently infected with green fluorescent protein-expressing Newcastle disease virus (NDV-GFP) (27). Seventeen to 20 h later, the number of cells expressing GFP was determined. As HTNV is known to interfere with subsequent IFN stimulation of cells via inhibition of STAT1 phosphorylation (35), all supernatants were UV inactivated before they were added to the cells. Recombinant human IFN-β and IFN-λ1 were used as controls.

Quantitative RT-PCR. Before cDNA synthesis, purified total cellular RNA was treated with Turbo DNA free (Ambion) to remove any contaminating DNA. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and random primers. The TaqMan minor groove binder (MGB) probe and primers for the MxA gene are described elsewhere (17). For HTNV and African green monkey β-actin, Primer Express software (Applied Biosystems) was used to design primers and probes. The forward primer 5′-GACCGTTGTCCACCA ACATG, reverse primer 5′-GGAAAAAAATGCCCCAAGCT, and probe 5′-T TGTTTATAGCAGGTATTGCT-MGB were used for HTNV, and the forward primer 5′-GCTGCCCTGAGGCTCTCTT, reverse primer 5′-TGATGGAGTT GAAGGTAGTTTCATG, and probe 5′-TTCCTGGGCATGGAGT-MGB were used for African green monkey β-actin. For MxA, a 0.9 μM concentration of each primer and 0.05 μM probe were used. For HTNV and African green monkey β-actin, a 0.9 μM concentration of each primer and 0.2 μM probe were used. Quantitative reverse transcription-PCRs ([RT-PCRs] Q-PCRs) for human β-actin, IFN-α2, -β, -λ1, and -λ2 were performed using commercial TaqMan gene expression assays from Applied Biosystems. All reactions were performed using 2× TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations on a 7900HT sequence detection system (Applied Biosystems). The criterion for activated gene transcription was set at 2-fold or higher induction compared to untreated cells.

Detection of STAT1 phosphorylation by immunofluorescence. Vero E6 cells were grown on coverslips. When 100% confluent, cells were treated with IFN-λ1 or left untreated for 60 min, followed by fixation in ice-cold methanol-acetone (1:1) and blocking in phosphate-buffered saline (PBS) containing 10% horse serum, 1% bovine serum albumin, and 0.02% NaN_3. To permeabilize the nuclei, cells were incubated in 0.5% Nonidet P-40 (Sigma). After a rinse in Tris-buffered saline (TBS), coverslips were incubated with an anti-phosphorylated (Tyr-701) STAT1 rabbit polyclonal antibody (Cell Signaling Technology) and finally a secondary Alexa Fluor 549 goat anti-rabbit IgG (Invitrogen) antibody. Nuclei were stained using 4′,6′-diamidino-2-phenylindole ([DAPI] Sigma-Aldrich).

RESULTS

IFN-λ1 expression is induced prior to MxA and IFN-β during HTNV infection. It has been shown that HFRS/HCPS-causing hantaviruses induce a weak innate immune response in infected cells and that the innate immunity is induced rather late after infection (1, 10, 31, 33). To analyze this more in detail, we infected A549 cells with HTNV, stimulated cells with UV-inactivated HTNV, or incubated cells with medium only. Cells and supernatants were then collected at 6, 12, 24, 48, 72, and 96 h p.i. (hpi) for subsequent analyses.

To analyze the kinetics of innate immune activation after HTNV infection, we determined levels of transcription for the ISG MxA, type I IFNs, and type III IFNs in infected cells compared to uninfected cells (medium controls) at each sam-

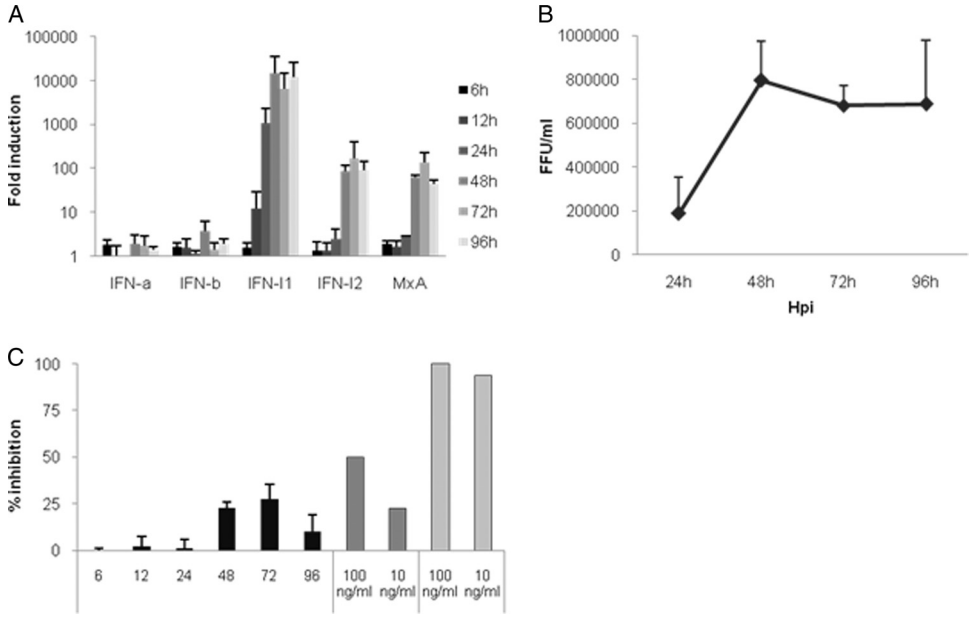

FIG. 1. HTNV induces expression of IFN-λ1 prior to IFN-β and MxA in epithelial A549 cells. A549 cells were infected with HTNV (MOI of 3), and then cellular RNA was analyzed for levels of mRNA by Q-PCR, supernatants were analyzed for progeny virus titers by titration on Vero E6 cells, and total levels of bioactive IFNs in the supernatants were determined by an NDV-GFP assay. (A) mRNA expression of IFNs and MxA in HTNV-infected A549 cells at 6, 12, 24, 48, 72, and 96 h after infection was evaluated by Q-PCR. The data were normalized using β-actin and are presented as relative expression compared to the uninfected control. Error bars represent standard deviations of the means from two independent experiments. (B) Supernatants from infected cells were collected at 24, 48, 72, and 96 h after infection, and viral titers were determined. Error bars represent standard deviations of the means. (C) Supernatants drawn at 6, 12, 24, 48, 72, and 96 h after HTNV infection were UV inactivated and then analyzed for total antiviral capacity using an NDV-GFP assay. Data are presented as percent inhibition of NDV-enhanced GFP replication for supernatants from HTNV-infected compared to noninfected cells collected at the indicated times after HTNV infection. Human recombinant IFN-β and IFN-λ1 were used as controls. Error bars represent standard deviations of the means from one experiment.

pling time point. Increased MxA expression was observed from 24 hpi, with a peak at 72 hpi (Fig. 1A), showing that type I and/or type III IFNs were likely to be produced in response to HTNV. However, increased levels of IFN-α were not observed at any time point after infection (Fig. 1A), and elevated levels of IFN-β were observed only at 48 hpi (Fig. 1A). In contrast, clearly elevated levels of IFN-λ1 mRNA expression were observed from 12 hpi, with a peak at 48 hpi (Fig. 1A). Induction of IFN-λ2 occurred later than for IFN-λ1 and was observed from 24 hpi, with a peak at 72 hpi (Fig. 1A). Cells treated with UV-inactivated HTNV showed no induction of type I or III IFNs or MxA at any time point after infection (data not shown).

Concentrations of progeny virus in supernatants from infected cells increased up to 48 hpi, followed by a slight decrease until 96 hpi (Fig. 1B). As expected, no replicating virus was observed in any of the supernatants from cells treated with UV-inactivated virus or with medium (data not shown).

HTNV infection induces weak antiviral responses. The Q-PCR data showed that MxA was induced before IFN-β, simultaneously with IFN-λ2, but clearly after IFN-λ1 (Fig. 1A), suggesting that secreted IFN-λ1 is the prime inducer of MxA during HTNV infection in A549 cells. We therefore analyzed levels of IFNs in supernatants from infected cells by ELISAs. However, no IFN-α, IFN-β, IFN-λ1, or IFN-λ2 could be detected, suggesting that levels of IFNs were below the detection limits of the ELISAs. A sensitive bioassay based on NDV-GFP (27) replication in A549 cells was therefore performed to analyze total levels of bioactive IFN in the supernatants. Human recombinant IFN-λ1 (rIFN-λ1) and rIFN-β were used as controls; IFN-λ1 clearly inhibited NDV-GFP replication, although not as strongly as IFN-β, showing that this bioassay could detect bioactive IFN-λ and IFN-β (Fig. 1C). Supernatants from HTNV-infected A549 cells collected 6 to 24 hpi showed no evidence of antiviral activity (Fig. 1C). However, an antiviral effect was observed from 48 hpi, with a peak at 72 hpi, showing that low levels of IFNs are being produced during HTNV infection of A549 cells (Fig. 1C).

IFN could not be detected by ELISA, and no antiviral activity could be observed in the bioassay in supernatants from cells stimulated with UV-inactivated HTNV or medium (data not shown).

IFN-λ1 is secreted by HTNV-infected MRC-5 cells. Since A549 cells express CRF2-12, the receptor for IFN-λ (20, 32), it might be that some, if not all, of the secreted IFN-λ is consumed by the cells, thus decreasing the amount of free IFN-λ in supernatants to levels undetectable by ELISA. To determine if HTNV could induce IFN-λ production, we therefore infected MRC-5 cells, a cell line that does not express CRF2-12 (23). Supernatants were collected at 6, 12, 24, 48, 72, and 96 hpi. ELISAs for IFN-α, IFN-β, and IFN-λ2 were negative at all time points for all supernatants. In contrast, IFN-λ1 could be detected in supernatants harvested from 48 hpi and onwards,

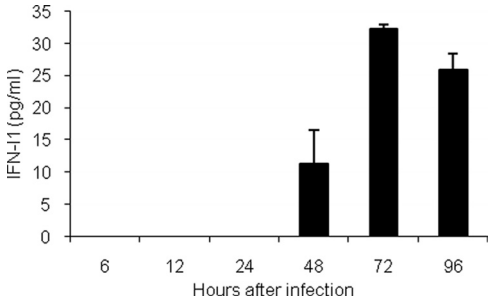

FIG. 2. IFN-λ1 is secreted from HTNV-infected cells. IFN-λ1 levels in supernatants from HTNV-infected (MOI of 1) MRC-5 fibroblasts at 6, 12, 24, 48, 72, and 96 h after infection were measured by ELISA. Error bars represent standard deviations of the means from two independent experiments.

with a peak at 72 hpi (Fig. 2), clearly showing that HTNV is able to induce IFN-λ1-production.

In order to analyze induction of IFNs and MxA in response to HTNV in a cell line that lacks CRF2-12, MRC-5 cells were infected, and the relative quantities of mRNA expression compared to expression in uninfected control cells were determined with Q-PCR. As expected, and in contrast to the results observed for A549 cells, induction of MxA in HTNV-infected MRC-5 cells did not precede the induction of IFN-β; IFN-β, MxA, and IFN-λ1 were all induced from 24 hpi while IFN-λ2 was induced from 48 hpi (Fig. 3A).

The strong antiviral responses observed in infected MRC-5 cells, compared to A549 cells, were reflected by a clear decrease in progeny virus production in HTNV-infected MRC-5 cells: the highest level of progeny virus was detected in supernatants at 48 hpi, and thereafter clearly lower titers were observed at 72 and 96 hpi (Fig. 3B).

HTNV induces IFN-λ1 and MxA independent of IFN-α/β. The finding that HTNV could induce IFN-λ1 without simultaneous or preceding type I IFN induction (Fig. 1A) indicated that IFN-α/β is indispensable for induction of IFN-λ1. However, there is low but constant constitutive expression of IFN-α and IFN-β mRNA in A549 and MRC-5 cells (37), and we therefore wanted to determine if this background production of type I IFNs was needed for the induction of IFN-λ1 and subsequent MxA induction. In order to test if IFN-λ and/or MxA could be induced in a truly IFN-α/β-independent manner, we therefore analyzed induction of these genes in African green monkey epithelial Vero E6 cells that lack the capacity to produce type I IFNs due to a chromosomal deletion (7). Importantly, epithelial cells can normally produce, and respond to, IFN-λ1, and it has been shown that MxA is induced in Vero E6 cells stimulated with recombinant type I IFN (30), showing that Vero E6 cells can respond to IFN stimulation.

Stimulation of Vero E6 cells with recombinant human IFN-λ1 caused phosphorylation and nuclear translocation of STAT1 (Fig. 4A) and induced MxA mRNA expression (Fig. 4B), clearly showing that Vero E6 cells can respond to IFN-λ. As controls for IFN-λ1-induced induction of MxA, we stimulated A549 and MRC-5 cells with IFN-λ1. As expected, we observed MxA induction in A549 but not in MRC-5 cells (data not shown). Furthermore, induction of IFN-λ1 and MxA was observed in poly(I:C)-stimulated Vero E6 cells (Fig. 4C), clearly showing that IFN-λ and MxA can be induced in an IFN-α/β-independent manner in Vero E6 cells.

Next, we investigated if HTNV could induce induction of IFN-λ1 and MxA in Vero E6 cells. No IFN-λ1 or IFN-λ2 in supernatants from HTNV-infected Vero E6 cells at any time point after infection was detected by the specific ELISAs (data not shown). However, expression of both IFN-λ1 and MxA mRNA was induced in HTNV-infected Vero E6 cells (Fig. 4D), which clearly shows that HTNV can induce IFN-λ1 and MxA in the complete absence of type I IFNs.

IFN-λ1 inhibits HTNV progeny virus production in cells lacking type I IFNs. We have previously shown that prestimulation of A549 cells with IFN-λ causes strong antiviral effects on subsequent HTNV infection (35). However, as there is a constitutive background production of type I IFNs in A549 cells, it might be that the observed antiviral effect is dependent on a synergistic effect between the endogenously produced type I IFN and the IFN-λ treatment. To test if the antiviral capacity of IFN-λ was effective in the complete absence of type I IFNs, we therefore stimulated Vero E6 cells with IFN-λ1 for 24 h and then infected the cells with HTNV. A lower level of progeny virus production was observed in supernatants from IFN-λ1-treated Vero E6 cells at 30 hpi than in nontreated cells

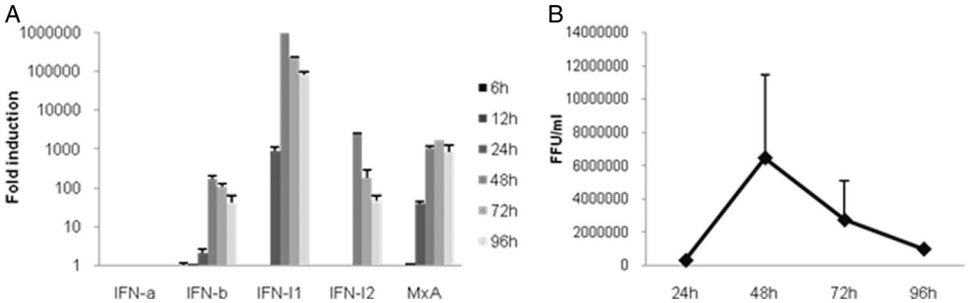

FIG. 3. Induction of MxA coincides in time with induction of IFN-β in HTNV-infected MRC-5 cells. MRC-5 cells were infected with HTNV (MOI of 1); then cellular RNA was analyzed for levels of mRNA by Q-PCR, and supernatants were analyzed for progeny virus titers by titration on Vero E6 cells. (A) mRNA expression of IFNs and MxA in HTNV-infected MRC-5 cells at 6, 12, 24, 48, 72, and 96 h after infection was evaluated by Q-PCR. The data were normalized using β-actin and are presented as relative expression compared to the uninfected control. Error bars represent standard deviations of the means from one representative experiment. (B) Supernatants from infected cells at 24, 48, 72, and 96 h after infection were collected, and viral titers were determined. Error bars represent standard deviations of the means from two independent experiments.

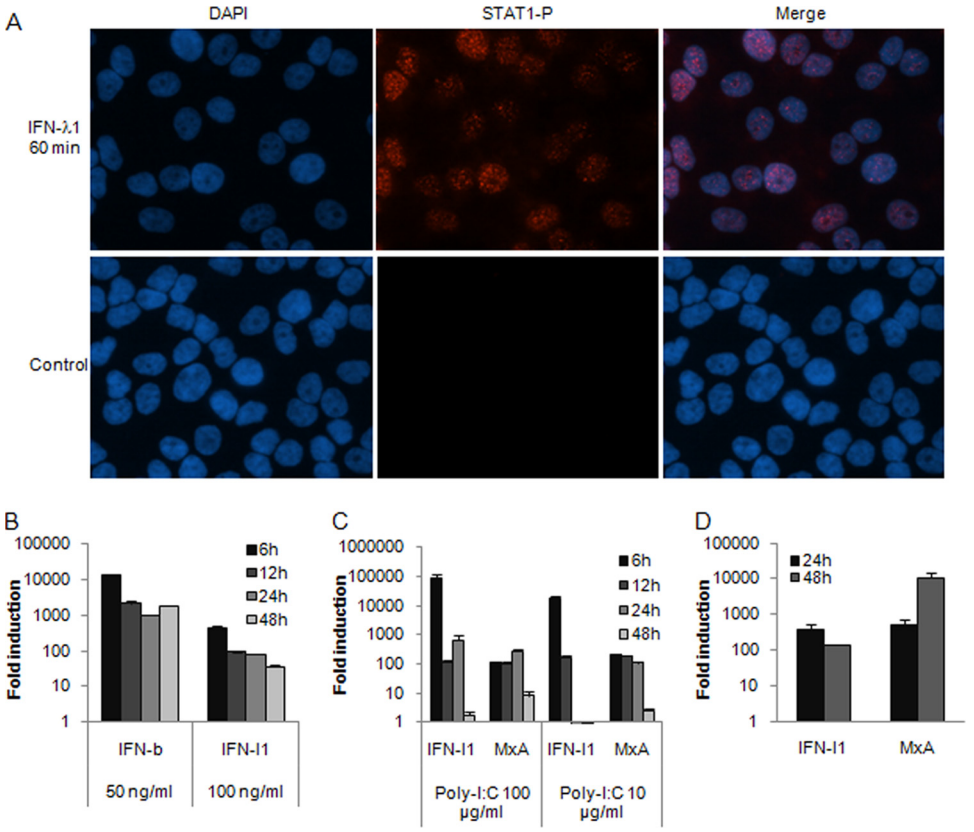

FIG. 4. HTNV induces IFN-λ1 and MxA in the complete absence of type I IFNs. Vero E6 cells, that lack the capacity to produce type I IFNs due to a chromosomal deletion (7) were tested for their capacity to respond to and produce IFN-λ1 and then used to test if HTNV infection can induce IFN-λ1 and MxA in the complete absence of type I IFN production. (A) Nuclear translocation of phosphorylated STAT1 (STAT1-P) 60 min after treatment of Vero E6 cells with IFN-λ1. Glass slides with Vero E6 cells were treated with 100 ng/ml human recombinant IFN-λ1 (upper) or left untreated (lower) for 60 min and then fixed and stained for phosphorylated STAT1 (red) and nuclei (blue). (B) Vero E6 cells were stimulated with 100 ng/ml of human recombinant IFN-λ1 (or 50 ng/ml of IFN-β as a control), and MxA mRNA expression was evaluated by Q-PCR at 6, 12, 24, and 48 h after treatment. The data were normalized using β-actin and are presented as relative expression compared to the unstimulated control. Error bars represent standard deviations of the means from one representative experiment. (C) Vero E6 cells were stimulated with 10 or 100 μg/ml of poly(I:C) or with medium alone as a control. mRNA expression of IFN-λ1 and MxA in the cells at 6, 12, 24, and 48 h after treatment was then determined by Q-PCR. The data were normalized using β-actin and are presented as relative expression compared to the unstimulated control. Error bars represent standard deviations of the means from one representative experiment. (D) mRNA expression of IFNs and MxA in HTNV-infected (MOI of 4) Vero E6 cells was evaluated by Q-PCR at 24 and 48 h after infection. The data were normalized using β-actin and are presented as relative expression compared to the noninfected control. Error bars represent standard deviations of the means from one representative experiment.

(Fig. 5A), clearly showing that IFN-λ has antiviral capacity against HTNV also in cells lacking the ability to produce type I IFNs.

Induction of IFN-λ1 and MxA is dose dependent and correlates to production of progeny virus. In light of the finding that HTNV induces IFN-λ1 and MxA (Fig. 4D) and that IFN-λ1 induced a strong antiviral effect against HTNV (Fig. 5A) in Vero E6 cells, we next determined if induction of IFN-λ and/or MxA was dependent on the dose of infectious viral and if there might be a correlation with inhibition of progeny virus production. Vero E6 cells were infected with HTNV, and then viral titers in supernatants and possible induction of IFN-λ1 and MxA in the cells at different time points after infection were determined.

Interestingly, infection of Vero E6 cells with a low dose of virus resulted in a clearly higher production of progeny viruses from 7 days p.i. than observed in supernatants from cells initially infected with a 1,000-times-higher dose of virus (Fig. 5B). Furthermore, levels of HTNV S RNA in the infected cells did not increase over time from day 4 p.i. in cells infected with a high dose of HTNV while it clearly increased until day 7 p.i. in cells infected with a low dose of HTNV (Fig. 5C).

Induction of IFN-λ1 and MxA in infected compared to uninfected cells was observed already by day 1 after infection when the higher dose of virus was used but not until day 4 after infection when the lower dose was used (Fig. 5D). At day 7 and later times postinfection, the expression levels of IFN-λ1 and MxA had increased to the same levels for both doses used in the study (Fig. 5D). The results show that infection of cells with a high dose of HTNV activates strong antiviral innate immune responses, resulting in lower levels of progeny virus production than in cells initially infected with a low dose of HTNV.

HTNV does not specifically inhibit IFN-β induction. The observation that HTNV induced IFN-λ1 in the absence of the

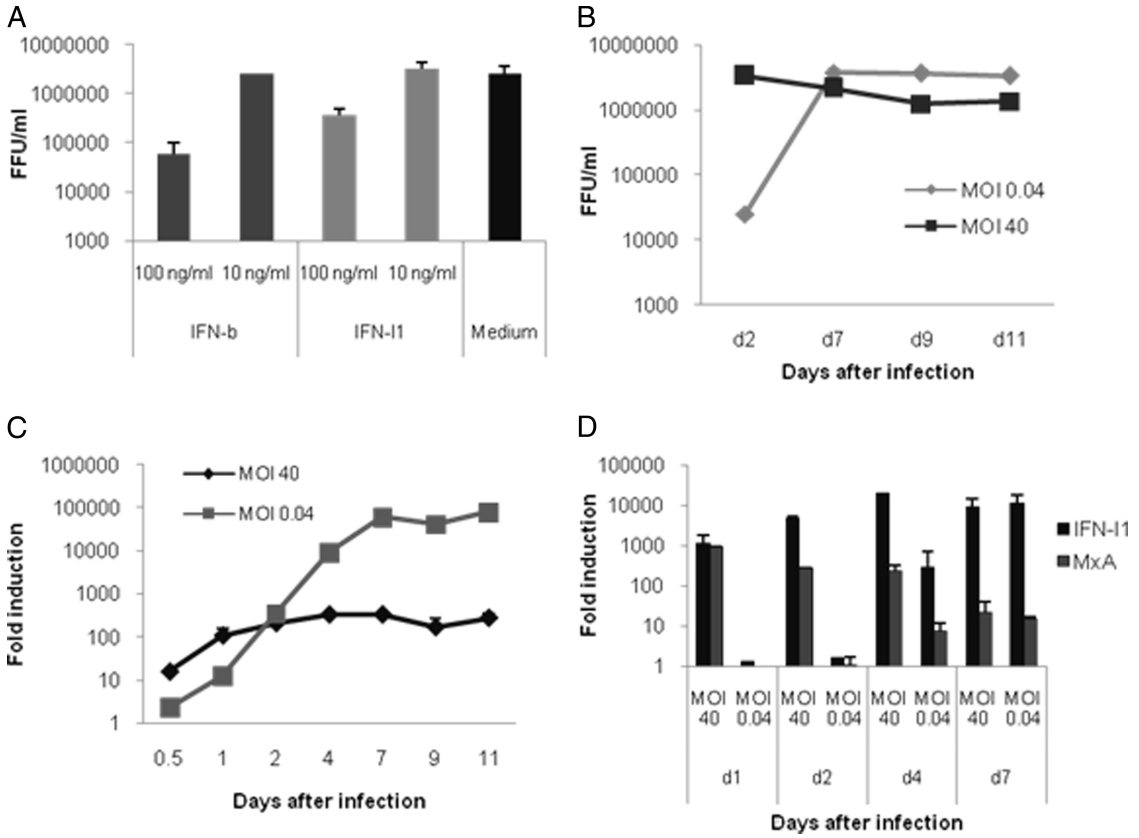

FIG. 5. IFN-λ1 inhibits HTNV replication in Vero E6 cells. The antiviral capacity of IFN-λ1 against HTNV in the complete absence of IFN-α/β was analyzed in Vero E6 cells. Cells were either stimulated with 10 or 100 ng/ml of recombinant IFN-λ1 (or with 10 or 100 ng/ml of IFN-β as control) before infection or infected with high and low doses of HTNV in order to analyze the effect of exogenously added IFN-λ1 and endogenously produced IFN-λ on HTNV-replication. (A) Production of progeny virus in supernatants from Vero E6 cells treated with recombinant human IFN-λ1. Vero E6 cells were stimulated with IFN-λ1 or IFN-β or incubated with only medium as a control for 24 h before infection (MOI of 4). Supernatants from infected cells were then collected at 30 hpi, and viral titers were determined. Error bars represent standard deviations of the means from one representative experiment. (B) Vero E6 cells were infected with high (MOI of 40) or low (MOI of 0.04) doses of HTNV. Supernatants were then collected at 2, 7, 9, and 11 days after infection, and viral titers were determined. Error bars represent standard deviations of the means from two independent experiments. (C) Vero E6 cells were infected with high (MOI of 40) or low (MOI of 0.04) doses of HTNV. HTNV S RNA levels in infected cells were evaluated by Q-PCR at 12 h and at 1, 2, 4, 7, 9, and 11 days after infection. The data were normalized using β-actin and are presented as relative expression compared to levels of HTNV S RNA at 6 h after infection for infection with a high dose or low dose, respectively. Error bars represent standard deviations of the means from one representative experiment. (D) mRNA expression of IFN-λ1 and MxA in Vero E6 cells, infected with a high (MOI of 40) or low (MOI of 0.04) dose of HTNV, at 1, 2, 4, and 7 days after infection was evaluated by Q-PCR. The data were normalized using β-actin and are presented as relative expression compared to the noninfected control. Error bars represent standard deviations of the means from one representative experiment.

induction of type I IFNs in A549 cells (Fig. 1A) suggests either (i) that HTNV activates a signaling pathway that activates both type I and type III IFNs but selectively inhibits type I IFN induction or (ii) that HTNV activates a signaling pathway that selectively activates only IFN-λ1. The New York-1 virus Gn cytoplasmic tail can interact with TRAF3 and inhibit TBK-1-directed IFN responses (1, 2), indicating that known pathways activating type I IFN production during RNA virus infection might be inhibited in hantavirus-infected cells, as activation of IFN-α/β via TLR3, TLR4, TLR7, TLR9, RIG-I, and MDA-5 is dependent on functional TRAF3 (8, 22). In order to determine if the observed IFN type I-independent induction of IFN-λ1 caused by HTNV might be explained by a specific inhibition of type I IFN responses, we therefore stimulated HTNV-infected A549 cells with poly(I:C), which is a TLR3 agonist known to induce both type I and III IFNs (5). Levels of IFN-β and IFN-λ1 mRNA in poly(I:C)-stimulated infected and noninfected cells were compared to levels in untreated infected and uninfected cells, respectively, at 1.5, 3, and 6 hpi.

We observed an almost complete inhibition of IFN-λ1 induction and a 25 to 75% inhibition of IFN-β induction in the poly(I:C)-treated infected cells (Fig. 6). The data show that HTNV strongly inhibits induction of both IFN-β and IFN-λ1 after poly(I:C) stimulation. This suggests that the observed type I IFN-independent IFN-λ1 induction observed during HTNV infection might be caused by HTNV-induced activation of a signaling pathway that specifically induces IFN-λ1.

DISCUSSION

Induction of type I and III IFNs after pathogen recognition is activated by PRRs; RNA viruses can be sensed by TLR3,

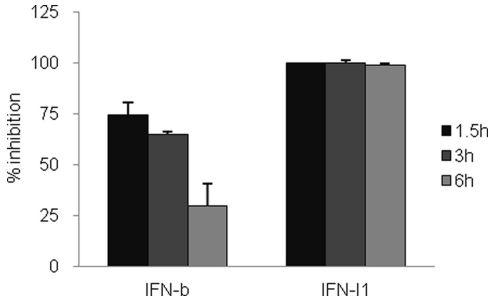

FIG. 6. HTNV inhibits poly(I:C)-mediated induction of IFN-β and IFN-λ1 in A549 cells. Infected (MOI of 5) and noninfected cells were stimulated with poly(I:C) (10 μg/ml) 48 h after HTNV infection or incubated with only medium as a control. Induction of IFN-β and IFN-λ1 mRNA expression at 1.5, 3, and 6 h after poly(I:C) stimulation was determined by Q-PCR. The data were normalized using β-actin, and relative expression in treated compared to nontreated cells was calculated for infected and noninfected cells, respectively. Data are presented as percent inhibition of relative expression for HTNV-infected compared to noninfected cells. Error bars represent standard deviations of the means from one representative experiment.

RIG-I, and MDA5. Hantavirus, belonging to the *Bunyaviridae* family, is a group of negative-stranded RNA viruses that can cause two severe and often fatal human diseases, HFRS and HCPS (31, 43). Hantavirus infection induces weak type I IFN responses and only late after infection (1, 10, 31, 33). HFRS/HCPS-causing hantaviruses are not recognized by RIG-I or MDA5 (11, 28). Whether TLR3 can recognize hantaviruses is under debate; Sin Nombre virus does not activate TLR3 (28), while MxA induction after HTNV infection has been shown to be TLR3 dependent (12). In this article we report that the prototypic hantavirus, HTNV, is a strong inducer of IFN-λ1 and that activation of IFN-λ1 precedes induction of MxA and IFN-β in A549 cells. The finding that IFN-λ can be induced independently of simultaneous IFN-α/β induction suggests the existence of activating pathways that specifically induce IFN-λ1 without simultaneous IFN-α/β induction.

Specific activation of IFN-λ expression has not been reported earlier (42), and type I and III IFNs have been described as being coinduced upon stimulation of cells (21, 25, 26, 42). IFN-λ1 transcription can be activated by IRF3 and IRF7, in a manner resembling that for IFN-β-transcription, whereas IFN-λ2 can be activated by IRF7, thereby resembling the manner by which IFN-α is induced (26). However, it was recently shown that the IFN-λ1 enhanceosome differs from the IFN-β enhanceosome (41), suggesting that IFN-λ1 and IFN-β can be differently regulated. The specific PRR and signaling pathway responsible for the HTNV-induced induction of IFN-λ1 remain to be detected. Recently, Prescott and coworkers reported that hantavirus, indeed, is likely to activate an unknown PRR that in turn can activate type I IFN-independent MxA-induction (28). As hantaviruses induce very poor IFN-α/β responses (1, 10, 31, 33), signaling pathways that induce simultaneous type I and type III IFN responses might not be activated, making it possible to detect specific type III IFN expression during hantavirus infection. In contrast to what is observed for hantaviruses, most virus infections normally induce type I IFN responses rapidly (8, 16, 22, 34). If other viruses specifically induce IFN-λ, they might simultaneously also activate type I IFN production via other mechanisms, thereby making it impossible to distinguish between induction of specific type III IFN responses and induction of simultaneous type I and III IFN responses. This may explain why type I IFN-independent IFN-λexpression has earlier not been detected.

We report that HTNV induces strong MxA responses in Vero E6 cells, a cell line devoid of type I IFN responses but with functional type III IFN responses. This clearly shows that hantaviruses can activate ISGs in the complete absence of type I IFNs. The findings that HTNV induces IFN-λ before type I IFN and MxA, combined with the knowledge that IFN-λ can induce ISGs (3, 4, 20, 21, 33), and that MxA is induced only by type I or III IFN (13) strongly suggest that MxA is induced by IFN-λ1 in a type I IFN-independent manner also in A549 cells. This might also explain the previously reported IFN-α/β-independent production of MxA during hantavirus infection (28). The finding that HTNV can inhibit induction of type I and III IFNs after poly(I:C) treatment shows that hantavirus can block TLR3-activated signaling pathways that induce both type I and III IFNs. This suggests that hantaviruses might be able to efficiently block downstream signaling events that normally cause type I and III IFN production. One possible target important for the signaling pathways used to induce simultaneous type I and III IFNs is the TBK1-TRAF3 complex, shown to be targeted by the New York-1 hantavirus Gn protein (1, 2).

We show that induction of IFN-λ1 in Vero E6 cells after HTNV infection with low as well as high doses of HTNV ultimately correlates with stabilized levels of progeny virus production and HTNV S RNA levels in the infected cells. Interestingly, a similar pattern was observed in A549 cells. Although induction of IFN-λ1 and MxA was observed in A549 cells, titers of progeny virus did not decrease much at late time points after infection. The data suggest that viral replication can be inhibited, albeit weakly, in an established HTNV infection by endogenously produced IFN-λ1 in the complete absence of type I IFNs. In contrast, a clear reduction of progeny virus production was observed over time in MRC-5 cells. IFN-λ is known to be a weaker inducer of antiviral effectors than type I IFNs, and this might explain why we observed a steady state in production of progeny virus rather than reduced titers over time in A549 and Vero E6 cells, whereas we observed a clear reduction in MRC-5 cells. As IFN-β was induced in MRC-5 cells, it seems likely that the induction of type I IFNs is responsible for the decrease in progeny virus production observed in this cell line.

Capillary leakage is a hallmark for all hemorrhagic fevers. It is not known how hantaviruses cause disease in humans, but it is believed that HFRS/HCPS are at least partly immunomediated (31). Already from the first day of symptoms, most HFRS/HCPS patients show highly activated adaptive immune responses (31), as well as hantavirus-specific and strong cytotoxic T-lymphocyte (CTL) responses (18) and decreased regulatory T (Treg)-cell responses (44). Furthermore, elevated serum levels of the cytotoxic granula proteins perforin and granzyme B have been observed in patients (19), and as hantavirus infections *per se* are not cytopathogenic, the capillary damage might be caused by cytotoxic immune cells. Interestingly, it has recently been shown that IFN-λ may enhance adaptive immunity,

suppress Th2 responses, reduce Treg populations, and increase antigen-specific cytolytic degranulation by CTLs (6, 15, 24), clearly showing that IFN-λ possesses other important properties in addition to its ability to induce an antiviral state in cells. The similarities between the effects of IFN-λ on the adaptive immune system and the observed strong adaptive immune responses in HFRS/HCPS patients are interesting, and further studies addressing this issue might increase knowledge of the possible roles for IFN-λ in pathogenesis during diseases as well as in protection against viruses. Recently, single nucleotide polymorphisms (SNPs) near the IFN-λ gene *IL28B* (encoding IFN-λ3) were identified. These SNPs predisposes hepatitis C virus (HCV)-infected individuals to successful combined pegylated IFN-α and ribavirin treatment and to spontaneous clearance (9, 36, 38, 40), underscoring the importance for IFN-λ during viral infections.

In conclusion, we show that HTNV can activate type I IFN-independent induction of IFN-λ1 and that this precedes induction of MxA in A549 cells. Importantly, our findings show that mechanisms leading to production of type III IFNs can be separated from those of type I IFNs during virus infection.

ACKNOWLEDGMENTS

The study was supported by the Åke Wibergs Stiftelse, Jeanssons Stiftelse, Stiftelsen Clas Groschinskys Minnesfond, the Swedish Society of Medicine, the Swedish Society for Medical Research, the Royal Swedish Academy of Sciences, and the Swedish Medical Research Council.

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