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Kinetics of interferon-λ and receptor expression in response to *in vitro* respiratory viral infection

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Summary. – The major protective immune response against viruses is the production of type I and III interferons (IFNs). IFNs induce the expression of hundreds of IFN-stimulated genes (ISGs) that block viral replication and further viral spread. In this report, we analyzed the expression of IFNs and some ISGs (MxA, PKR, OAS-1, IFIT-1, RIG-1, MDA5, SOCS-1) in alveolar epithelial cells (A549) in response to infection with influenza A viruses (A/California/07/09 (H1N1pdm); A/Texas/50/12 (H3N2)); influenza B virus (B/Phuket/3073/13); adenovirus type 5 and 6; or respiratory syncytial virus (strain A2). Influenza B virus had the ability to most rapidly induce IFNs and ISGs as well as to stimulate excessive IFN-α, IFN-β and IFN-λ secretion. It seems curious that IAV H1N1pdm did not induce IFN-λ secretion, but enhanced type I IFN and interleukin (IL)-6 production. We emphasized the importance of the negative regulation of virus-triggered signaling and cellular IFN response. We showed a decrease in *IFNLR1* mRNA in the case of IBV infection. The attenuation of SOCS-1 expression in IAV H1N1pdm can be considered as the inability of the system to restore the immune status. Presumably, the lack of negative feedback loop regulation of proinflammatory immune response may be a factor contributing to the particular pathogenicity of several strains of influenza.

Keywords: lambda interferons; MxA; influenza; respiratory syncytial virus; A549 cells

Introduction

Components of the innate immune response are activated in response to viral infection (Cole and Ho, 2017). The most important components of the innate immune response are type I and III interferons (IFNs) (Cole and Ho, 2017). IFNs induce the activation of defense mechanisms and prepare cells for possible viral invasion. While the antiviral properties of type I IFNs have been widely studied (Randall and Goodbourn, 2008), much less is known about the features of type III IFNs (IFN-λ). In humans, four IFN-λ subtypes have been found: IFN-λ_1 (IL-29); IFN-λ_2 (IL-28A); IFN-λ_3 (IL-28B); and IFN-λ_4. IFN-λ are encoded by interferon lambda 1-4 genes (*IFNL1-4)*. Among IFN-λ_{1-3}, there is high conservation of amino acid sequence (Miknis *et al*., 2010). The actions of IFN-λ on the cell are carried out by binding to the heterodimeric receptor (IFNλR). IFN-λ functions significantly overlap with those of type I IFNs and induce the expression of analogous interferon-stimulated genes (ISGs) (Crotta *et al*., 2013). Expression of both type I and type III IFNs is induced by the activation of the two most important cytosolic sensors, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). RIG-I and MDA5 appear to differentially stimulate IFNs in response to different virus-derived structures, with RIG-I generally responding most potently to 5' di and tri-phosphate double-stranded RNAs (dsRNA);

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Abbreviations: AdV = adenovirus; IAV = influenza A virus; IBV = influenza B virus; IFN(s) = interferon(s); IFNλR = interferon-λ receptor; hour(s) post-infection = hpi; IL = interleukin; ISG(s) = interferon stimulated gene(s); RLR = RIG-I-like receptor(s); RSV = respiratory syncytial virus

MDA5 preferentially associates with long dsRNA (Brisse and Ly, 2019).

Despite obvious similarities in mechanisms of induction and downstream signaling, there are obviously some differences in the functioning of type I and type III IFNs. Presumably, type I IFNs have the potential to induce inflammation in addition to antiviral function, while type III IFNs promote the production of antiviral ISGs without inducing inflammation (Sun Y *et al*., 2018).

The selectivity of type III IFNs is due to peculiarities of receptor subunit expression. IFN-λ actions are carried out through the heterodimeric IFN-λ receptor (IFNλR), consisting of the IFNλR1 and IL10R2 subunits. The IL10R2 subunit is also part of the receptor complexes for IL-10, IL-22, and IL-26; it is expressed in cells of various tissues (Miknis *et al*., 2010). Expression of the IFNλR1 subunit demonstrates a more limited cellular distribution and is present in epithelial cells (Sommereyns *et al*., 2008), keratinocytes (Zahn *et al*., 2011) differentiated dendritic cells (Yin *et al*., 2012; Zhang *et al*., 2013) and hepatocytes (Dickensheets *et al*., 2013).

Consequently, the mucous membranes of the respiratory and gastrointestinal tracts are tissues that are mainly targeted by IFN-λ (Sommereyns *et al*., 2008). This tissue specificity correlates with IFN-λ antiviral activity, which manifests itself mainly in relation to viruses with high tropism for epithelial tissues (Hermant and Michiels, 2014; Lozhkov *et al*., 2020). This class of viruses includes respiratory viruses such as influenza A and B virus (IAV, IBV), respiratory syncytial virus (RSV), and some types of adenovirus (AdV).

IFN-α/β and IFN-λ action stimulates the expression of hundreds of ISGs. Myxovirus resistance proteins (MxA) are localized in the cytoplasm and exhibit pronounced antiviral activity against a wide range of viral pathogens (Haller, 2015). Mx proteins are GTPases; they are required to inhibit IAV replication by blocking the transport of viral nucleocapsid complexes into the cell nucleus (Haller, 2015). 2'-5'-oligoadenylate synthetase (OAS) promotes the oligomerization of ATP by forming 2'5'-phosphodiester bonds. 2'5'-oligoadenylate activates RNase L, which is responsible for the degradation of both viral and host mRNA (Randall and Goodbourn, 2008). One of the protein kinase R (PKR) substrates is eIF2a (Eukaryotic translational initiation factor 2a). This kinase action leads to the inhibition of translation initiation and activation of autophagy. PKR also participates in various antiviral mechanisms (induction of apoptosis, cell cycle arrest) (Randall and Goodbourn, 2008). The Suppressor of Cytokine Signaling 1 (SOCS-1) is a negative regulator of IFN-dependent signaling cascades. SOCS-1 directly interacts with the kinase inhibitory region of JAK1 and attenuates its activity, which leads to the suppression of JAK/STAT-dependent signaling. Also, SOCS-1 is able to direct JAK1 to proteasomal degradation (Wei and others 2014). Interferon-induced protein with tetratricopeptide repeats 1 (IFIT-1) is able to inhibit the initiation of protein synthesis at several stages. It has been shown that IFIT-1 is able to interact with eukaryotic translation initiation factor 3 subunit e (eIF3e) to block translation. Retinoic acid-inducible gene I (RIG-1) and melanoma differentiation-associated protein 5 (MDA5) are RNA helicases that detect viral RNA and induce IFNs expression (Randall and Goodbourn, 2008).

It is well known that respiratory viruses induce IFNs and ISGs production. However, a vast majority of research is focused on features of one or several viral strains; however, matching the data from unrelated research that was carried out in different cell lines should be approached with caution. The number of works that are devoted to a direct comparison of the kinetics of IFNs expression stimulated by a wide panel of respiratory viruses is limited. In the present research paper, we evaluated the dynamics of *IFNL* expression using A549 cells infected with RNA viruses (IAV, IBV, RSV) and a DNA virus (AdV). AdV and RNA viruses are quite different in pathogenesis, thus we observed distinct *IFNL* expression.

Materials and Methods

Viruses. All viral strains were obtained from the Virus and Cell Culture Collection of the Smorodintsev Research Institute of Influenza (St. Petersburg, Russia). Influenza viruses were grown in 11-day-old embryonated eggs, purified by sucrose gradient, and stored at -80°C. The infectivity values of the viral stocks in MDCK cells were: 3.2×10^7 $TCID_{50}$/ml for A/California/07/09 ((A)H1N1pdm09); 3.2×10^7 $TCID_{50}$/ml for A/Texas/50/12 ((A)H3N2); and 3.2×10^5 $TCID_{50}$/ml for B/Phuket/3073/13 (Yamagata lineage). Adenovirus working stocks were generated by infecting A549 cells at a multiplicity of infection (MOI) of 0.001 for 72 h. The supernatant was then clarified by centrifugation, aliquoted, and stored at –80°C. The infectivity of the viral stocks in A549 cells was 3.2×10^6 $TCID_{50}$/ml for both serotype 5 (AdV-5) and serotype 6 (AdV-6). The RSV A2 strain was grown in HEp-2 cells. The infectivity of the RSV stocks in HEp-2 cells was 3.2×10^7 $TCID_{50}$/ml.

Infection of cells. The A549 (CCL-185) cell line was obtained from the American Type Culture Collection (ATCC, USA). A549 cells (human type II alveolar epithelial line) were cultured in F12K medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA). For infection, cells were seeded onto 12-well plates (Thermo Scientific Nunc, USA) at 5×10^5 cells per well. Around 100% confluent monolayers were washed with DPBS (Gibco, USA) and infected at a MOI of 1. After 60 min

of adsorption at 37°C, the virus-containing inoculum was removed, and 1 ml of fresh medium was added. Every plate contained at least three replicates of uninfected cells. The infected cells and non-infected controls were incubated at 37°C (5% CO_2 with humidification) and harvested at 4, 8, and 24 h after infection.

Primer and probe design. Primers and fluorescent oligonucleotide probes, containing fluorescent reporter dyes at the

Table 1. Primers and probes

Target	Position	Sequence 5'–3'
poly IFNA **NM_024013.3*** NM_000605.4; NM_021068.4 NM_002169.3; NM_021002.2 NM_021057.2; NM_002170.4 NM_002171.2; NM_006900.4 NM_002172.3; NM_002173.3 NM_021268.2; NM_002175.2	qH_IFNA_F294	AGCCATCTCTGTCCTCCATGAG
	qH_IFNA_R580	TTTCTGCTCTGACAACCTCCC
IFNB **NM_002176.4**	qH_IFNB1_F227	TGCCTCAAGGACAGGATGAACT
	qH-IFNB1_R405	TTAGCCAGGAGGTTCTCAACAATAG
	qH_IFNB1_O285	(HEX)-TCCAGAAGGAGGACGCCGCATTGAC-(BHQ1)
IFNL1 **NM_172140.2**	qH_IFNL1_F192	GTCACTCAAGCTGAAAAACTGGA
	qH_IFNL1_R580	GGTTCCCATCGGCCACATATT
	qH_IFNL1_O277	(FAM)-TTCTCCAGGTGAGGGAGCGCC-(BHQ1)
IFNL2/3 NM_172138.2* NM_172139.4	qH_IFNL2/3_F347	GAGGGCCAAAGATGCCTTAGAA
	qH_IFNL2/3_R518	AGTGTCAGCGGTGGCCT
	qH_IFNL2/3_O453	(HEX)-GCAGCTGCAGGTGAGGGAGC-(BHQ1)
IL10R2 **NM_000628.5**	qH_IL10R2_F691	CGGAACAAAGCTGGGGAATG
	qH_IL10R2_R911	CCCAAAAACTCTTTCAGGTGCT
	qH_IL10R2_O833	(TAMRA)-CTCGGCTGCTTCGCCTTGCT-(BHQ2)
IFNLR1 NM_173065.3	qH_IFNLR1_F3481	AACAAACTACGGAGCTGGGG
	qH_IFNLR1_R3593	AGTTACCTCCACGAAGCAGC
	qH_IFNLR1_O3531	(JOE)-AAATTCCCAGACCTGTACCGATGTTCT-(BHQ1)
IFIT-1 **NM_001548.5*** NM_001270927.2; NM_001270928.2 NM_001270929.2; NM_001270930.2	qH_IFIT-1_F1376	AAACTTCGGAGAAAGGCATTAGAT
	qH_IFIT-1_R1540	TGAAATGAAATGTGAAAGTGGCTG
	qH_IFIT-1_O1479	(HEX)-CCTGAGACTGGCTGCTGACTTTGAGAAC-(BHQ1)
MxA **NM_001144925.2*** NM_002462.5; NM_001178046.3	qH_MxA_F2345	GAGACAATCGTGAAACAGCAAATCA
	qH_MxA_R2449	TATCGAAACTCTGTGAAAGCAAGC
	qH_MxA_O2373	(HEX)- CACTGGAAGAGCCGGCTGGGATATG-(BHQ1)
RIG-1 **NM_014314.4*** NM_001385907.1; NM_001385910.1 NM_001385912.1; NM_001385913.1 NM_001385914.1	qH_RIG1_F1940	GAGCACTTGTGGACGCTTTA
	qH_RIG1_R2053	ATACACTTCTGTGCCGGGAG
	qH_RIG1_O2002	(ROX)-CCTGGCATATTGACTGGACGTGGC-(BHQ2)
MDA5 **NM_022168.4**	qH_IFIH-1_F2667	AAACCCATGACACAGAATGAACA
	qH_IFIH-1_R2864	TGTGAGCAACCAGGACGTAG
	qH_IFIH-1_O2744	(Cy5.5)-CACAGTGGCAGAAGAAGGTCTGGA-(BHQ3)
SOCS-1 **NM_003745.2**	qH_SOCS-1_F1030	CCTGGTTGTTGTAGCAGCTTA
	qH_SOCS-1_R1132	CCTGGTTTGTGCAAAGATACTG
	qH_SOCS-1_O1093	(ROX)-CCTGGTTGTTGTAGCAGCTTA-(BHQ2)
GAPDH NM_002046.7*; NM_001289745.3 NM_001289746.2; NM_001357943.2	qH_GAPDH_F23	CAGTCAGCCGCATCTTCTTTTGCGTCG
	qH_GAPDH_R152	CAGAGTTAAAAGCAGCCCTGGTGACCAG
	qH_GAPDH_78	(FAM)-TGGGGAAGGTGAAGGTCGGAGTCAACGG ATTTGGTC-(BHQ1)

* = primers and probe are specified in the sequence of this mRNA-transcript.

5'-end and a quencher at the 3'-end (Table 1), were commercially synthesized and HPLC-purified (Evrogen, Russia).

RNA isolation. Total RNA was isolated from A549 cells using TRIZol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentrations and integrity were analyzed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

Reverse transcription reaction. Two micrograms of total RNA were treated by DNase (Promega) and then directly reverse transcribed using oligo-dT_{16} primers and MMLV reverse transcriptase (Promega). Complementary DNA synthesis was carried out at 42°C for 60 min; products were stored at -20°C until use.

PCR analysis. Real-time PCR assays were performed using the CFX96 Real-Time PCR System (Bio-Rad, USA). Evaluation of *IFNL*1, *IFNL*2-3, *IL10RB*, *IFNλR1, MxA, IFIT-1, RIG-1, MDA5, IFNB, SOCS-1, and GAPDH* genes expression was performed in 25 µl reaction solutions containing 12.5 µl BioMaster HS-qPCR mix (2x) (BioLabMix, Russia), 2 µl cDNA (synthesized from 1 µg of total RNA). The reaction mixtures contained 500 nM of each primer and 200 nM of each probe. Conditions of the two-step PCR were 95°C for 5 min, followed by 40 amplification cycles (95°C for 15 s, 61°C for 30 s). For viral RNA gene expression analyses, the CDC Influenza A/B Typing Kit (# FluIVD03-1, Centers for Disease Control and Prevention, USA) and the Real-time RT-PCR Assay for RSV (Centers for Disease Control and Prevention, USA) were used. Relative expression values of genes of interest were calculated via ΔΔCt method using GAPDH as a housekeeping gene.

ELISA. Human IFN-$\lambda_{1/3}$, IL-6, and IL-10 concentrations in cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits or antibodies. Lambda IFN levels were evaluated by human IL-29/IL-28B (IFN-lambda 1/3) DuoSet ELISA (DY1598B, R&D Systems, USA). IL-6 was measured by human IL-6 DuoSet ELISA (DY206, R&D Systems, USA). Measurement of IL-10 was performed using Rat Anti-Human IL-10 Capture Antibodies (554705, BD Biosciences, USA); Biotin Anti-Human and Viral IL-10 Detection Antibodies (554499, BD Biosciences, USA); and Recombinant Human IL-10 (1064-IL-010, R&D Systems, USA). Viral loads were evaluated using in-house antibodies (Plotnikova *et al.*, 2020).

Statistical data processing. Data processing was carried out in Microsoft Excel. GraphPad Prism was used to evaluate the statistical significance of differences.

Results

Respiratory viruses induce IFN genes expression with different kinetics in A549 cells

To assess the impact of respiratory viruses on IFN expression, A549 cells were infected with RNA viruses (IBV, IAV H1N1pdm09, IAV H3N2, RSV) and a DNA virus (AdV serotype 5 and 6) at the same MOI and were incubated 4, 8, and 24 hours post-infection (hpi).

The replicative cycle of influenza is about 8 h. The production of mRNA and proteins of RSV reaches its peak at 15–20 hpi, while the AdV life cycle is a bit longer. Cells were infected without trypsin to exclude the possibility of infection with viral offspring. Thus, IFNs and ISGs production refer to a single replicative cycle of any virus. At first, we showed that the viral genomes are capable of effectively replicating in the A549 cell (Supplementary Fig. S1a). Hence, all investigated viruses exhibited the ability to replicate and to form new viral particles in A549 cells (Supplementary Fig. S1b). Also, it should be noted that infection with IBV is associated with a cytopathic effect (destruction of cells monolayer), while infection with other viruses did not result in any visible changes.

Changes in IFN expression were virus-specific. The results obtained by RT-PCR revealed that IBV is the fastest inducer of type I and type III IFNs compared to other viruses (Fig. 1). In response to IBV infection, the expression *IFNB* and *IFNL* mRNA increased more than 1000- and 10000-fold already at 4 hpi, and then it had been maintained at a stable high level at 8 hpi and 24 hpi. It is noteworthy that AdV infection of both types led to the suppression of IFN mRNA. At 24 hpi all viruses induced excessive *IFNL2/3* and *IFNA* levels augmentation, while the expression of *IFNL1* and *IFNB* was elevated only in RNA viruses.

Surprisingly, the IFN-λ level was significantly increased only in the RSV and IBV cell culture supernatants (Fig. 2a). The levels of IFN-α and IFN-β (Fig. 2bc) were increased in response to influenza virus infection (both IAV and IBV).

Despite IFNs being the primary, universal activators of the innate immune response, we have noted that the production of type I and type III IFN in response to infection is virus-specific.

IBV is a faster and stronger ISGs inducer than other RNA viruses

We also evaluated changes in the expression of several ISGs in response to viral infection. In both virus-infected and uninfected cells, IFNs induce the expression of MxA, which makes MxA an excellent marker for detecting the activation of an IFN-dependent response (Haller *et al.*, 2015).

We evaluated *MxA* expression kinetics at 4, 8, and 24 hpi (Fig. 3a). In general, *MxA* expression at 24 hpi was elevated upon infection with all viruses. However, the *MxA* mRNA level in AdV-infected cells was significantly lower than

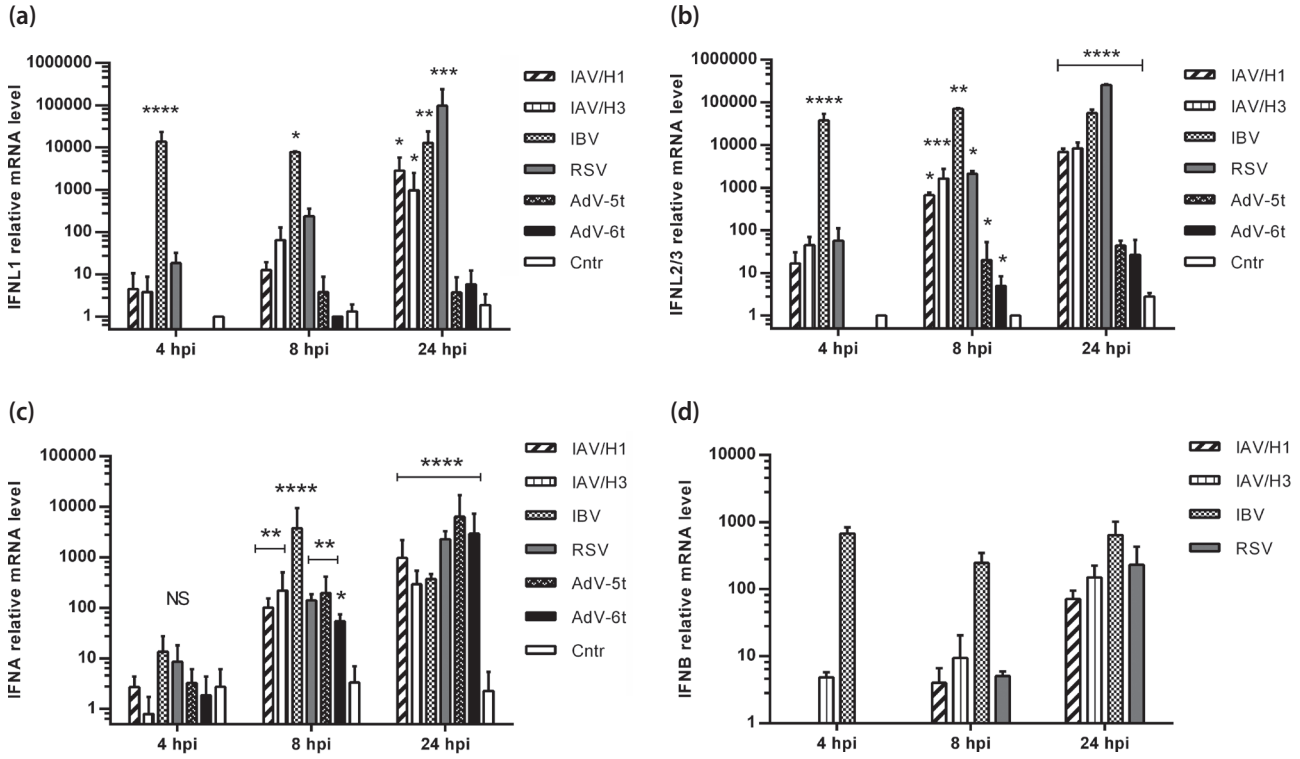

Fig. 1

Respiratory viruses stimulate IFNs expression with different kinetics

Expression levels of *IFNL1* **(a)**; *IFNL2/3* **(b)**; *IFNA* **(c)**; and *IFNB* **(d)** were measured at 4, 8, and 24 hpi. Gene expression was analyzed via ΔΔCt method (relative to GAPDH). Statistical significance (p-value) was determined by ordinary two-way ANOVA, followed by a pairwise Dunnett's multiple comparisons test: **** — Adjusted p-value < 0.0001; *** — < 0.001; ** — < 0.01; * — < 0.05 compared to Cntr. Cntr – intact cells that were cultured in the same conditions and were not infected (instead, sterile medium F12K was added). At least three biological replicates were used for each experimental data point. Data are represented as mean ± SD.

in those with RNA virus infections. At 4 hpi, an increase in *MxA* expression (about two orders of magnitude) was noted in cells infected with IBV. By 8 hpi, IBV-induced *MxA* expression had reached its maximum and was also significantly increased compared to all other groups. For all viruses with the exception of the IBV, *MxA* expression dynamics correlated with viral genome replication (Supplementary Table S1).

The expression of interferon-induced protein with tetratricopeptide repeats 1 (*IFIT-1*) at 24 hpi looked similar to *MxA* (Fig. 3b). However, AdV did not induce *IFIT-1* expression. Therefore, it can be assumed that an increase *IFIT-1* expression was largely synchronized with changes in *IFNL1* and *IFNB* mRNAs levels.

We also evaluated the expression of *OAS-1* and *PKR* (Fig. 3cd). In general, we noted time-delayed *OAS-1* and *PKR* mRNA induction. At 4 hpi the level of *OAS-1* and *PKR* mRNA did not change compared to the control (not shown), and at 8 hpi it was augmented in the case of IBV. By 24 hpi, the expression of these genes increased in case of all RNA viruses.

The levels of IL-6 and IL-10 in cell culture supernatants were analyzed (Supplementary Fig. S2). IL-6 and IL-10 were significantly increased in case of IBV and (A)H1N-1pdm09. The IL-6 level for RSV and (A)H3N2 also exceeded control values. Thus, secretion of IL-6 and IL-10 generally correlated with type I IFN.

Expression of IFNλR subunits and the cytosolic sensors MDA5 and RIG-1 is associated with the ability to induce ISGs

Possible regulation of IFN-λ-dependent signaling activation by variation in IFNλR subunit expression was evaluated. The expression levels of *IFNλR1* and *IL10R2* were assessed (Fig. 4). A significant decrease in the expression of *IFNλR1* subunit (more than 5-fold compared to the control) was noted one day after IBV infection (Fig. 4). Presumably, the decrease in *IFNλR1* level was associated with a rapid increase in *IFNL* and ISGs expression. There were no significant changes in the expression levels of *IL10R2*, a nonspecific IFNλR subunit.

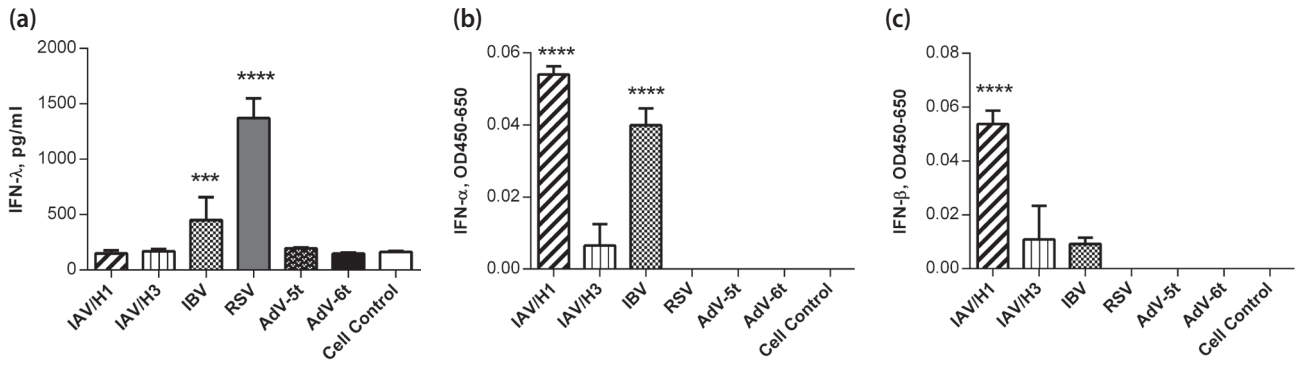

Fig. 2

IAV H1 stimulates IFN-α/β, but not IFN-λ secretion

Levels of IFN-λ **(a)**; IFN-α **(b)**; and IFN-β **(c)** were measured in cells supernatants by ELISA at 24 hpi. Statistical significance (p-value) was determined by ordinary one-way ANOVA, followed by a pairwise Dunnett's multiple comparisons test: **** — Adjusted p-value < 0.0001; *** — < 0.001 compared to Cntr. Cntr – intact cells that were cultured in the same conditions and were not infected (instead, sterile medium F12K was added). At least three biological replicates were used for each experimental data point. Data are represented as mean ± SD.

MDA5 and *RIG-1* expression is induced by autocrine or paracrine action of IFNs, so that an increase in the expression of these genes can be considered as a positive feedback loop that could further augment IFNs production. Already by 4 hpi, IBV infection led to an increase in the expression of both *RIG*-1 and *MDA5* (Fig. 5a,b). *RIG*-1 expression was enhanced in response to infection with IBV, A/H3N2, and RSV at 24 hpi. Nevertheless, we noted that

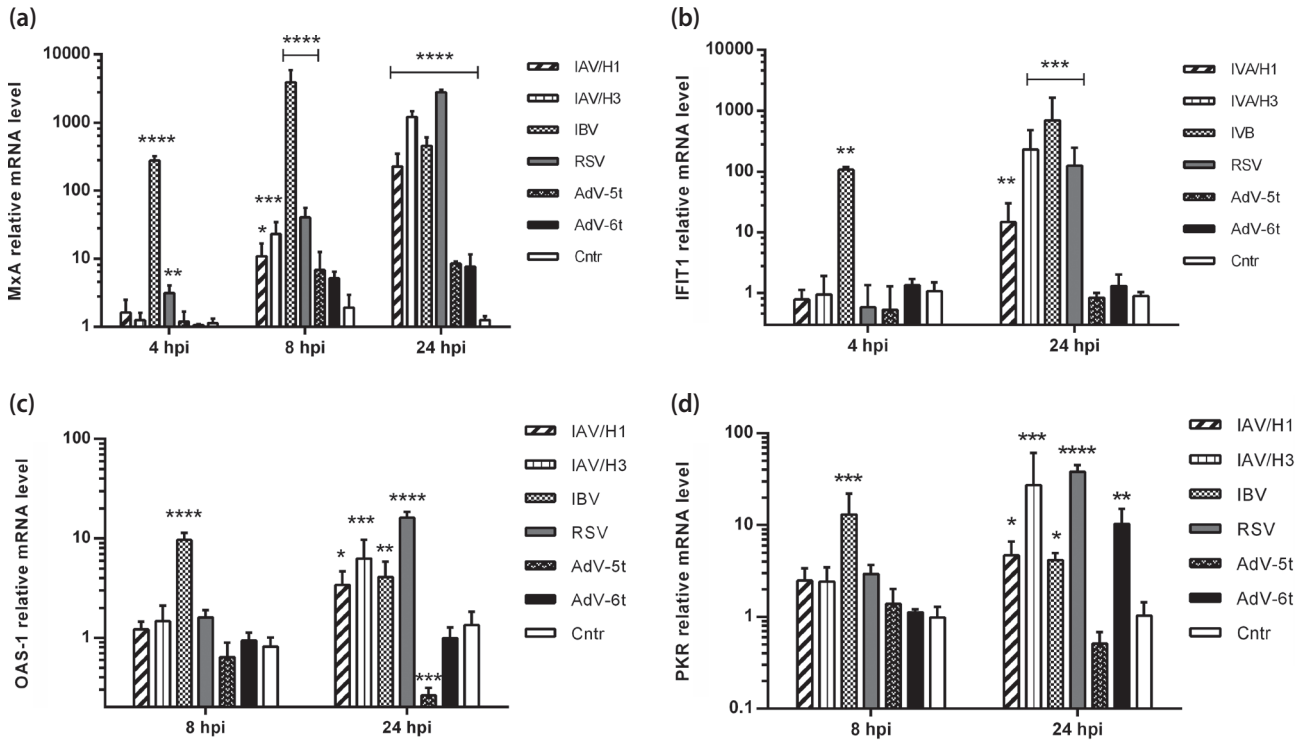

Fig. 3

Respiratory viruses induce the expression of canonical ISGs: MxA (a); IFIT1 (b); OAS-1 (c); and PKR (d)

Gene expression was analyzed via ΔΔCt method (relative to GAPDH). Statistical significance (p-value) was determined by ordinary two-way ANOVA, followed by a pairwise Dunnett's multiple comparisons test: **** — Adjusted p-value < 0.0001; *** — < 0.001; ** — < 0.01; * — < 0.05 compared to Cntr. Cntr – intact cells that were cultured in the same conditions and were not infected (instead, sterile medium F12K was added). At least three biological replicates were used for each experimental data point. Data are represented as mean ± SD.

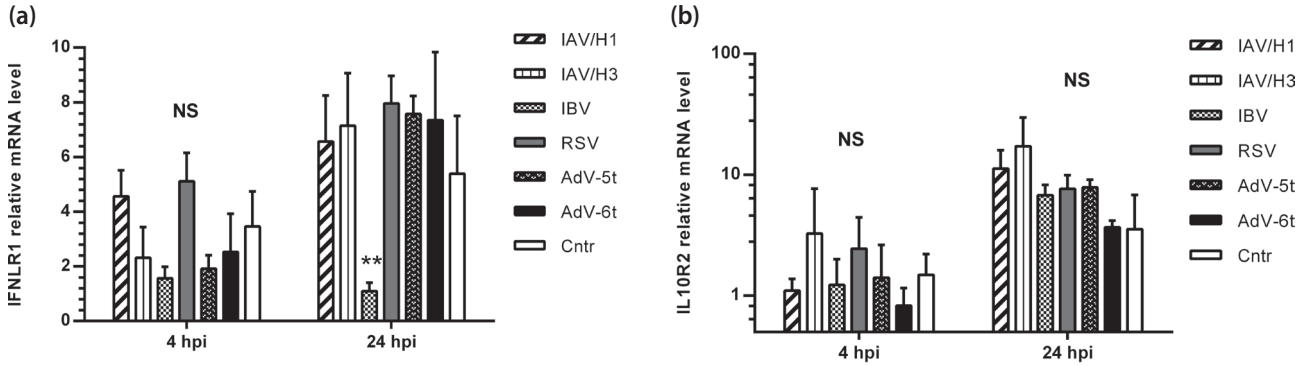

Fig. 4

IBV lowers IFNLR1 expression at 24 hpi

The expression of specific IFNLR1 **(a)**; and non-specific IL10RB **(b)** IFN-λ receptor subunits were assessed by RT-PCR. Gene expression was analyzed via ΔΔCt method (relative to GAPDH). Statistical significance (p-value) was determined by ordinary two-way ANOVA, followed by a pairwise Dunnett's multiple comparisons test: ** — Adjusted p-value < 0.01 compared to Cntr. Cntr – intact cells that were cultured in the same conditions and were not infected (instead, sterile medium F12K was added). At least three biological replicates were used for each experimental data point. Data are represented as mean ± SD.

RNA-sensors expression decreased from 4 hpi to 24 hpi in the case of IBV. In addition, an increase in *MDA5* expression was observed for all RNA viruses, except IAV/H1.

We also measured the expression of *SOCS*-1 (Fig. 5c), which takes part in a negative feedback loop to attenuate cytokine signaling. By 4 hpi, we did not find any significant changes in *SOCS-1* expression. By 24 hpi, *SOCS*-1 expression was significantly increased in cells infected with IAV/H3, IBV and RSV. Interestingly, IAV/H1 did not cause an increase in the expression of SOCS-1 or RNA-sensors (RIG-1 and MDA5).

Discussion

In our previous work (Plotnikova *et al.*, 2021) we showed that IFN-λ1 exhibits antiviral activity against various RNA viruses (IAV, SARS-CoV-2, CHIKV). IFNs are a major

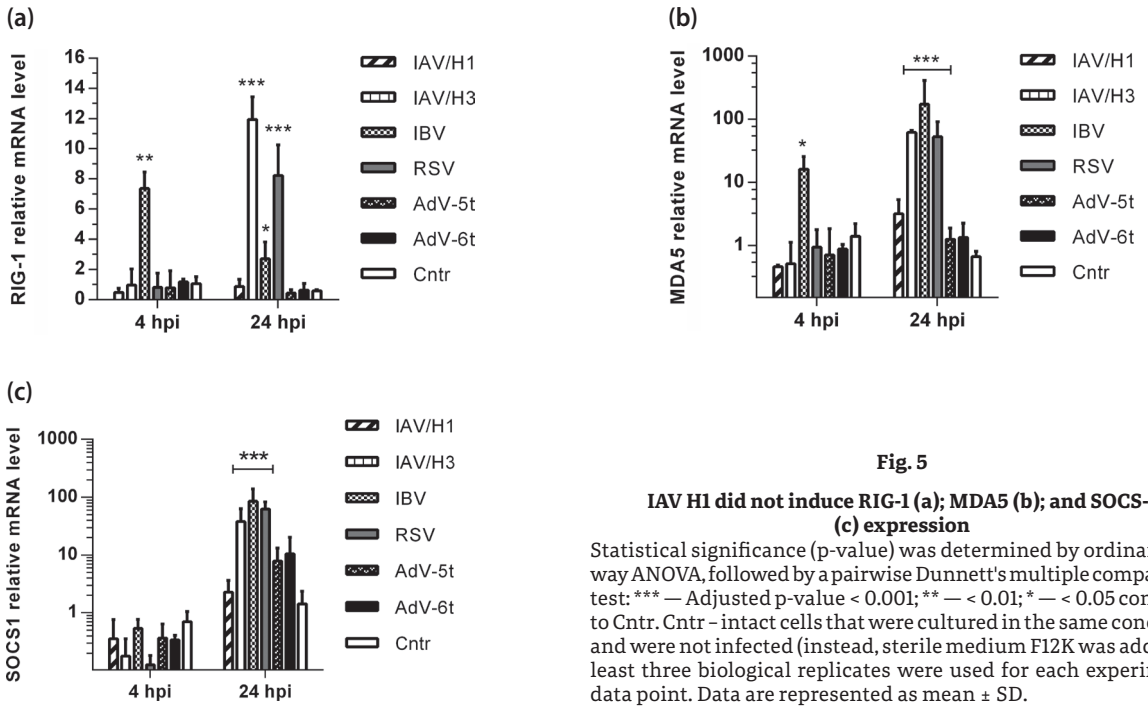

Fig. 5

IAV H1 did not induce RIG-1 (a); MDA5 (b); and SOCS-1 (c) expression

Statistical significance (p-value) was determined by ordinary two-way ANOVA, followed by a pairwise Dunnett's multiple comparisons test: *** — Adjusted p-value < 0.001; ** — < 0.01; * — < 0.05 compared to Cntr. Cntr – intact cells that were cultured in the same conditions and were not infected (instead, sterile medium F12K was added). At least three biological replicates were used for each experimental data point. Data are represented as mean ± SD.

component of innate defense against viruses. The production of endogenous IFN-λ by epithelial cells is a natural defense mechanism that limits the growth and spread of RNA viruses. In this work, we assessed the dynamics of *IFNL* and the expression of several ISGs in response to the infection of A549 cells with respiratory viruses (H1pdm09, H3, IBV, RSV, AdV types 5 and 6).

It is known that the induction of IFNs, proinflammatory cytokines, and chemokines is associated with strain pathogenicity (Cole and Ho, 2017). When studying the IFN status of A549 cells, we showed that infection with RNA viruses (especially IBV) led to a significant increase in *IFNL* and *IFNB* mRNA levels, meanwhile, AdV infection elicited only a weak increase of *IFNL* and *IFNB* mRNA. It is noteworthy that in the early stages of AdV infection, IFN mRNA was completely suppressed. In our work, we used AdVs (types 5 and 6), which belong to the serotype C and exhibit high tropism for respiratory epithelial cells. It has been shown that AdV E1B 55K plays a major role in the repression of ISGs transcription in the early stage of adenoviral infection (Chahal *et al*., 2012; Hidalgo *et al*., 2019). At 24 hpi, the pattern of *IFNA* was similar to *IFNL2/3* and the pattern of *IFNB* was similar to *IFNL1*. So, neither AdV strain upregulated *IFNB* and *IFNL1* mRNA levels.

It is noteworthy that 24 hours after infection, the production of type I and III IFNs was stimulated only by RNA viruses. However, despite a significant increase in mRNA level, IAV did not induce IFN-λ production at the protein level. A statistically significant increase of IFN-λ secretion was observed only in the case of RSV and IBV infections. Suppression of IFN-λ-dependent signaling is one of the strategies used by the IAV to escape the host immune response (Wei *et al*., 2014). Weak IFN-λ secretion upon IAV infection may be associated with the activity of the viral proteins PA-X or NS1 (Chaimayo *et al*., 2018).

Only infection with IBV led to the production of both type I and type III IFNs. According to Sun's assumptions (Sun *et al*., 2018), type I IFNs have the potential to induce inflammation in addition to the antiviral response, while lambda IFNs promote antiviral ISGs expression without excessive inflammation. In our study, IBV infection was associated with a cytopathic effect and led to increases in the pro-inflammatory factors IFN-α, IFN-β, IFN-λ, IL-6, and IL-10. It should be noted that although IL-10 itself cannot be attributed to mediators that promote inflammation, it can be a marker of uncontrolled immunopathology (Guo and Thomas, 2017).

It has been shown that IAV infection induces *IFNL* expression mainly through the RIG-I-dependent pathway (Wei *et al*., 2014). Induction of IFN expression occurs already in response to IBV penetration into the cell; and RIG-I cytosolic RNA sensors play a key role in virus recognition (Mäkelä *et al*., 2015). Assuming that both viral genome replication and the production of IFNs can lead to a change in the expression of cytosolic sensors by positive feedback mechanisms (RIG-I and MDA5 are also ISGs), we evaluated the expression of both RLRs at an early stage (4 hpi) and a late stage (24 hpi) of infection. Infection with RNA viruses resulted in an increase in *MDA5* and *RIG-1* expression by 24 hpi, with the exception of IAV/H1. It has been observed that IBV induces early expression of *IFNL1* and *IFNB* mRNA (as early as 2 hpi) in monocyte-derived dendritic cells. IAV causes noticeable activation of these genes much later (only starting from 8–12 hpi) (Strengell *et al*., 2012; Sun Y *et al*., 2018).

Type I and III IFNs can up-regulate SOCS proteins, which negatively regulate IFN signaling by inhibiting the JAK-STAT signaling pathway (Schneider *et al*., 2014). Here, we found that *SOCS-1* expression was elevated in IAV H3N2, IBV, or RSV-infected cells 24 hpi. In general, these observations are consistent with the changes in RNA-sensors expression. Importantly, *SOCS-1* mRNA level was not elevated at 4 hpi following IBV infection, whereas expression of other ISGs (*MxA, IFIT1, RIG-I, MDA5, OAS-1, PKR*) and *IFNL* was clearly elevated at this timepoint. A similar pattern is observed for IAV/H1 at later stage of infection. It has been shown that the physiological role of SOCS1 proteins is to prevent tissue damage caused by the potent pro-inflammatory effects of type I IFNs (Blumer *et al*., 2017).

It seems curious that infection with IAV/H1 (H1N1 pdm09) did not increase *SOCS-1, MDA5*, and *RIG-1* expression, while we observed that upregulation of *MxA, OAS-1, IFIT-1, PKR* was correlated with reproduction of viral genome. It is well known that SOCS can suppress IFN-mediated antiviral immunity, promoting virus infection and replication (Huang *et al*., 2020). Also, it was shown that the non-structural protein 1 of RSV inhibits the antiviral IFN-mediated response by inducing the up-regulation of SOCS-1 and SOCS-3 (Zheng *et al*., 2015). However, the key role of SOCS is the negative feedback regulation of cytokine production, and violation of this function ultimately results in the disturbance of homeostasis restoration. We assume that SOCS-1 suppression plays a prominent role in the pathogenesis of highly pathogenic IAV strains that is associated with excessive cytokine and chemokine secretion. Along these lines, attenuation of *SOCS-1* expression can serve as a marker indicating an increased potential of IAV/H1 to cause hypercytokinemia. This remains to be further determined. In the future, we intend to study this issue in more detail by evaluating the expression, production and phosphorylation of IRFs during infection of A549 cells with an IAV strain with deletions in *NS1* gene.

Modulation of IFN signaling can be accomplished by alteration of receptor subunit expression (Stanifer *et al*.,

2019). For instance, it was discovered that in nasopharyngeal swabs of children with a severe course of rhinovirus, *IFNLR1* expression was elevated compared to samples of children infected with RSV (Pierangeli *et al.*, 2018). Evaluation of *IFNLR* subunit expression showed that only with IBV was there a slight decrease in *IFNLR1* mRNA level, while non-specific subunit *IL10R2* mRNA level did not change (Fig. 5). At the moment, there is not much information available regarding molecular mechanisms of the negative regulation of *IFNLR1* expression (Stanifer *et al.*, 2019). In any case, decreased *IFNLR1* expression appears to be a natural compensatory mechanism realized in response to excessive activation of IFNλR-mediated signaling.

Conclusion

In the present study we emphasized the importance of the negative regulation of virus-triggered signaling and cellular IFN response. We showed a decrease in IFNLR1 mRNA in the case of IBV infection, in which very rapid and early activation of IFNs and ISGs occurred. It's curious that IAV did not induce IFN-λ secretion, but enhanced type I IFN and IL-6. The attenuation of SOCS-1 expression in H1N1pdm can be considered as the inability of the system to restore the immune status.

Supplementary information is available in the online version of the paper.

References

Blumer T, Coto-Llerena M, Duong FHT, Heim MH (2017): SOCS1 is an inducible negative regulator of interferon λ (IFN-λ)-induced gene expression in vivo. J. Biol. Chem. 292, 17928–17938. https://doi.org/10.1074/jbc.M117.788877

Brisse M, Ly H (2019): Comparative Structure and Function Analysis of the RIG-I-Like Receptors: RIG-I and MDA5. Front. Immunol. 10, 1586. https://doi.org/10.3389/fimmu.2019.01586

Chaimayo C, Dunagan M, Hayashi T, Santoso N, Takimoto T (2018): Specificity and functional interplay between influenza virus PA-X and NS1 shutoff activity. PLoS Pathog. 14(11), e1007465. https://doi.org/10.1371/journal.ppat.1007465

Chahal JS, Qi J, Flint SJ (2012): The human adenovirus type 5 E1B 55 kDa protein obstructs inhibition of viral replication by type I interferon in normal human cells. PLoS Pathog. 8, e1002853. https://doi.org/10.1371/journal.ppat.1002853

Cole SL, Ho LP (2017): Contribution of innate immune cells to pathogenesis of severe influenza virus infection. Clin. Sci. (London) 131, 269–283. https://doi.org/10.1042/CS20160484

Crotta S, Davidson S, Mahlakoiv T, Desmet SJ, Buckwalter RM, Albert ML, Staeheli P, Wack A (2013): Type I and type III interferons drive redundant amplification loops to induce a transcriptional signature in influenza-infected airway epithelia. PLoS Pathog. 9, e1003773. https://doi.org/10.1371/journal.ppat.1003773

Dickensheets H, Sheikh F, Park O, Gao B, Donnelly RP (2013): Interferon-lambda (IFN-λ) induces signal transduction and gene expression in human hepatocytes, but not in lymphocytes or monocytes. J. Leukoc. Biol. 93, 377–385. https://doi.org/10.1189/jlb.0812395

Guo XJ, Thomas PG (2017): New fronts emerge in the influenza cytokine storm. Semin. Immunopathol. 39, 541–550. https://doi.org/10.1007/s00281-017-0636-y

Haller O, Staeheli P, Schwemmle M, Kochs G (2015): Mx GTPases: dynamin-like antiviral machines of innate immunity. Trends Microbiol. 23, 154–163. https://doi.org/10.1016/j.tim.2014.12.003

Hidalgo P, Ip WH, Dobner T, Gonzalez RA (2019): The biology of the adenovirus E1B 55K protein. FEBS Lett. 593(24), 3504–3517. https://doi.org/10.1002/1873-3468.13694

Hermant P, Michiels T (2014): Interferon-λ in the context of viral infections: production, response and therapeutic implications. J. Innate Immun. 6, 563–574. https://doi.org/10.1159/000360084

Huang S, Liu K, Cheng A, Wang M, Cui M, Huang J, Zhu D, Chen S, Liu M, Zhao X, Wu Y, Yang Q, Zhang S, Ou X, Mao S, Gao Q, Yu Y, Tian B, Liu Y, Zhang L, Yin Z, Jing B, Chen X, Jia R (2020): SOCS proteins participate in the regulation of innate immune response caused by viruses. Front. Immunol. 11, 558341. https://doi.org/10.3389/fimmu.2020.558341

Lozhkov AA, Klotchenko SA, Ramsay ES, Moshkoff HD, Moshkoff DA, Vasin AV, Salvato MS (2020): The Key Roles of Interferon Lambda in Human Molecular Defense against Respiratory Viral Infections. Pathogens 9, 989. https://doi.org/10.3390/pathogens9120989

Mäkelä SM, Österlund P, Westenius V, Latvala S, Diamond MS, Gale M, Julkunen I (2015): RIG-I Signaling Is Essential for Influenza B Virus-Induced Rapid Interferon Gene Expression. J. Virol. 89, 12014–12025. https://doi.org/10.1128/JVI.01576-15

Miknis ZJ, Magracheva E, Li W, Zdanov A, Kotenko SV, Wlodawer A (2010): Crystal structure of human interferon-λ1 in complex with its high-affinity receptor interferon-λR1. J. Mol. Biol. 404, 650–664. https://doi.org/10.1016/j.jmb.2010.09.068

Pierangeli A, Statzu M, Nenna R, Santinelli L, Petrarca L, Frassanito A, Gentile M, Antonelli G, Midulla F, Scagnolari C (2018): Interferon lambda receptor 1 (IFNL1R) transcript is highly expressed in rhinovirus bronchiolitis and correlates with disease severity. J. Clin. Virol. 102, 101–109. https://doi.org/10.1016/j.jcv.2018.03.003

Plotnikova MA, Klotchenko SA, Lebedev KI, Lozhkov AA, Taraskin AS, Gyulikhandanova NE, Ramsay ES, Vasin AV (2020): Antibody microarray immunoassay for screening and differential diagnosis of upper respira-

tory tract viral pathogens. J. Immunol. Methods. 478, 112712. https://doi.org/10.1016/j.jim.2019.112712

Plotnikova M, Lozhkov A, Romanovskaya-Romanko E, Baranovskaya I, Sergeeva M, Kaa K, Klotchenko S, Vasin A (2021): IFN-λ1 Displays Various Levels of Antiviral Activity In Vitro in a Select Panel of RNA Viruses. Viruses 13, 1602. https://doi.org/10.3390/v13081602

Randall RE, Goodbourn S (2008): Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. 89 (Pt 1), 1–47. https://doi.org/10.1099/vir.0.83391-0

Schneider WM, Chevillotte MD, Rice CM (2014): Interferon-stimulated genes: A complex web of host defenses. Annu. Rev. Immunol. 32, 513–545. https://doi.org/10.1146/annurev-immunol-032713-120231

Sommereyns C, Paul S, Staeheli P, Michiels T (2008): IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. PLoS Pathog. 4, e1000017. https://doi.org/10.1371/journal.ppat.1000017

Stanifer ML, Pervolaraki K, Boulant S (2019): Differential regulation of type I and type III interferon signaling. Int. J. Mol. Sci. 20, 1445. https://doi.org/10.3390/ijms20061445

Strengell M, Peter Sarin L, Poranen MM, Fagerlund R, Melén K, Julkunen I (2012): Incoming Influenza A Virus Evades Early Host Recognition, while Influenza B Virus Induces Interferon Expression Directly upon Entry. J. Virol. 86, 11183–11193. https://doi.org/10.1128/JVI.01050-12

Sun Y, Jiang J, Tien P, Liu W, Li J (2018): IFN-λ: A new spotlight in innate immunity against influenza virus infection. Protein Cell. 9, 832–837. https://doi.org/10.1007/s13238-017-0503-6

Wei H, Wang S, Chen Q, Chen Y, Chi X, Zhang L, Huang S, Gao GF, Chen JL (2014): Suppression of Interferon Lambda Signaling by SOCS-1 Results in Their Excessive Production during Influenza Virus Infection. PLoS Pathog. 10, e1003845. https://doi.org/10.1371/journal.ppat.1003845

Yin Z, Dai J, Deng J, Sheikh F, Natalia M, Shih T, Lewis-Antes A, Amrute SB, Garrigues U, Doyle S, Donnelly RP, Kotenko SV, Fitzgerald-Bocarsly P (2012): Type III IFNs Are Produced by and Stimulate Human Plasmacytoid Dendritic Cells. J. Immunol. 189, 2735–2745. https://doi.org/10.4049/jimmunol.1102038

Zahn S, Rehkämper C, Kümmerer BM, Ferring-Schmidt S, Bieber T, Tüting T, Wenzel J (2011): Evidence for a pathophysiological role of keratinocyte-derived type III interferon (IFNλ) in cutaneous lupus erythematosus. J. Invest. Dermatol. 131, 133–140. https://doi.org/10.1038/jid.2010.244

Zhang S, Kodys K, Li K, Szabo G (2013): Human type 2 myeloid dendritic cells produce interferon-λ and amplify interferon-α in response to hepatitis C virus infection. Gastroenterology. 144, 414–425. https://doi.org/10.1053/j.gastro.2012.10.034

Zheng J, Yang P, Tang Y, Pan Z, Zhao D (2015): Respiratory syncytial virus nonstructural proteins upregulate SOCS1 and SOCS3 in the different manner from endogenous IFN signaling.J. Immunol. Res. 2015, 738547. https://doi.org/10.1155/2015/738547

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SUPPLEMENTARY INFORMATION

Kinetics of interferon-λ and receptor expression in response to *in vitro* respiratory viral infection

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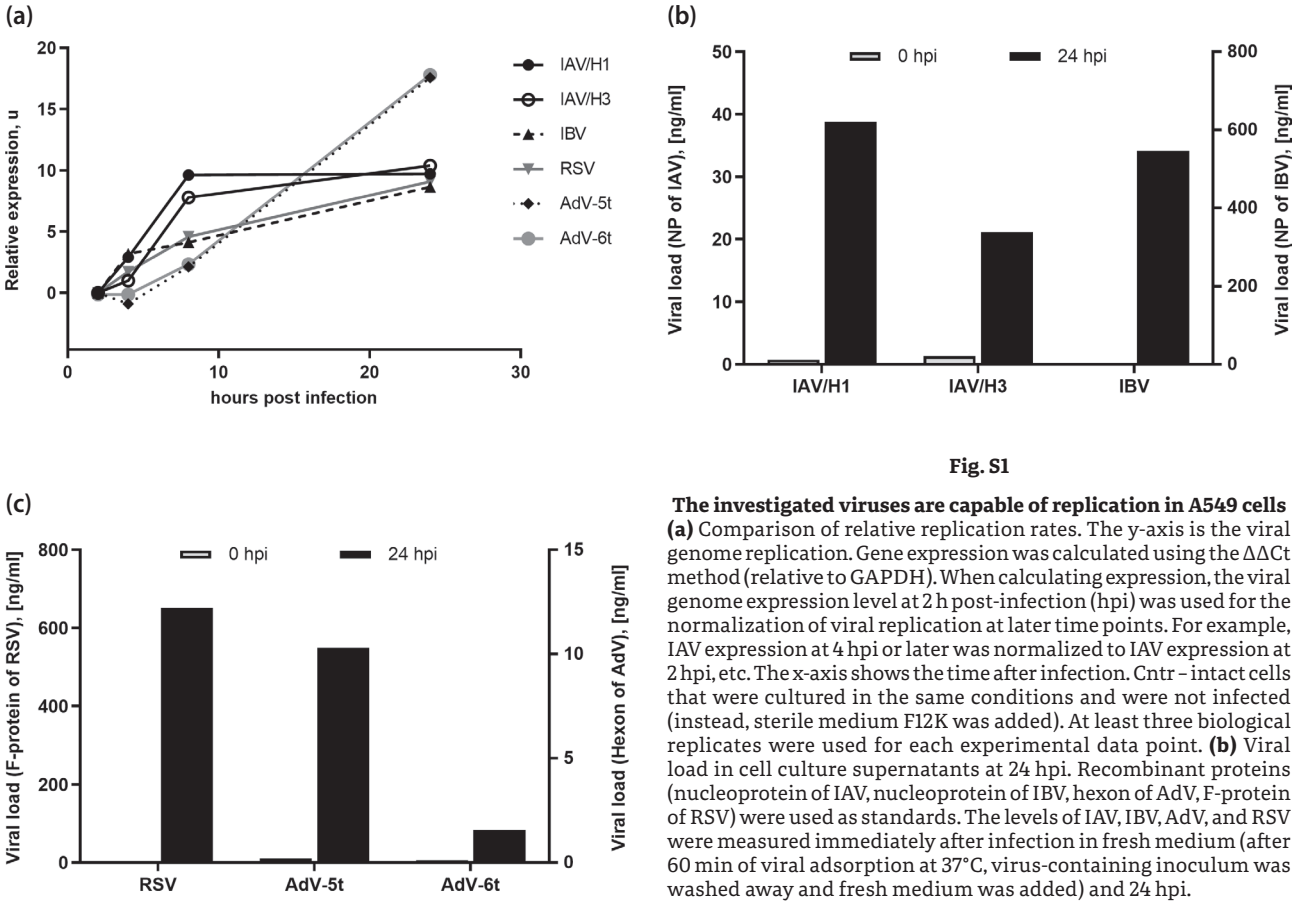

Fig. S1

The investigated viruses are capable of replication in A549 cells **(a)** Comparison of relative replication rates. The y-axis is the viral genome replication. Gene expression was calculated using the ΔΔCt method (relative to GAPDH). When calculating expression, the viral genome expression level at 2 h post-infection (hpi) was used for the normalization of viral replication at later time points. For example, IAV expression at 4 hpi or later was normalized to IAV expression at 2 hpi, etc. The x-axis shows the time after infection. Cntr – intact cells that were cultured in the same conditions and were not infected (instead, sterile medium F12K was added). At least three biological replicates were used for each experimental data point. **(b)** Viral load in cell culture supernatants at 24 hpi. Recombinant proteins (nucleoprotein of IAV, nucleoprotein of IBV, hexon of AdV, F-protein of RSV) were used as standards. The levels of IAV, IBV, AdV, and RSV were measured immediately after infection in fresh medium (after 60 min of viral adsorption at 37°C, virus-containing inoculum was washed away and fresh medium was added) and 24 hpi.

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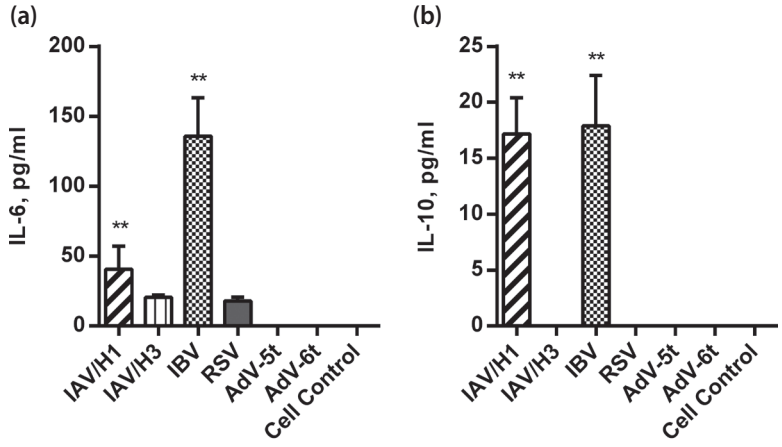

Fig. S2

IAV H1 and IBV stimulate cytokine secretion

IL-6 **(a)** and IL-10 **(b)** levels were measured by ELISA in cell supernatants at 24 hpi. Statistical significance (p-value) was determined by ordinary one-way ANOVA, followed by a pairwise Dunnett's multiple comparisons test: ** — Adjusted p-value < 0.01 compared to Cntr. Cntr – intact cells that were cultured in the same conditions and were not infected (instead, sterile medium F12K was added). At least three biological replicates were used for each experimental data point. Data are represented as mean ± SD.

Table S1. Correlation between the accumulation of viral mRNA and the expression of IFNL or MxA upon infection of A549 cells with various respiratory viruses

Virus		*MxA*	*IFNL1*	*IFNL2/3*
H1	r	**0,7**	0,4762	**0,85**
	P-value summary (two-tailed)	* 0,0433	ns 0,2431	** 0,0061
H3	r	**0,9667**	0,8619	**1**
	P-value summary (two-tailed)	*** 0,0002	** 0,0047	**** < 0,0001
IBV	r	0,5476	-0,1667	0,119
	P-value summary (two-tailed)	0,171	ns 0,7033	ns 0,793
RSV	r	**0,8833**	**0,8167**	**0,9167**
	P-value summary (two-tailed)	** 0,0031	* 0,0108	** 0,0013
AdV	r	**0.75**	-0.4	0.8
	P-value summary (two-tailed)	* 0.0255	ns 0.75	ns 0.3333
AdV	r	**0.9**	0.5	0.6
	P-value summary (two-tailed)	** 0.002	ns >0.9999	ns 0.4167