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Both Type I and Type III Interferons Are Required to Restrict Measles Virus Growth in Lung Epithelial Cells

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Compliance with Ethical Standards

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Abstract

Measles virus (MeV) first infects immune cells in the respiratory tract of a human host, spread to lymphoid organs throughout the body, and finally enter and grow in respiratory epithelial cells before being released and transmitted to the next host. Thus, efficient growth in respiratory epithelial cells is important for the person-to-person transmission of MeV. Upon viral entry, host cells detect viral nucleic acids and produce interferons (IFNs) to control viral growth. Type I (IFN-α/β) and type III (IFN-λ) IFNs have largely common induction and signaling mechanisms and stimulate expression of similar target genes, but utilize distinct receptors. To determine relative contributions of type I and type III IFNs to the control of MeV growth in epithelial cells, we examined MeV growth in the human lung epithelial cell line H358 and its mutants lacking either type I or type III IFN

receptor. Our results revealed that both type I and type III IFNs are required to restrict MeV growth in H358 cells. Furthermore, our results showed that the induction of type III as well as type I IFNs was increased in the absence of the MeV nonstructural V protein.

Introduction

Measles is a febrile infectious disease with high morbidity and mortality. Although safe and effective attenuated live vaccine is available, measles still claims about 100,000 lives per year largely in developing countries [6]. Measles virus (MeV), the causative agent, belongs to the Genus *Morbillivirus* within the Family *Paramyxoviridae*, and has a single-stranded negative-sense RNA genome with six genes encoding the nucleocapsid, phospho (P)-, matrix, fusion, hemagglutinin, and large proteins, respectively [11]. The P gene encodes additional proteins, the V and C proteins, by a process of RNA editing and by an alternative translation initiation in a different reading frame, respectively.

The cellular receptors for MeV are the signaling lymphocyte activation molecule (SLAM) on immune cells and nectin 4 on epithelial cells [23, 25, 41]. MeV is airborne, and first infects $SLAM^+$ immune cells such as macrophages and dendritic cells in the respiratory tract of the host [19]. These infected immune cells are carried to draining lymph nodes where more $SLAM^+$ immune cells are infected. Infected immune cells then enter blood vessels, migrate to epithelia, and transmit the virus to nectin 4^+ respiratory epithelial cells from the basolateral surface [9, 21]. Finally, the virus is released from the apical surface of respiratory epithelial cells, and transmitted to the next host, repeating the infectious cycle [20, 23, 39]. Thus, efficient growth in respiratory epithelial cells is important for the person-to-person transmission of MeV.

Upon infection with RNA viruses, host cells detect viral nucleic acids through retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and Toll-like receptors. Ligand engagement of these receptors causes the activation of downstream adapter proteins, specific kinases and transcription factors, resulting in the induction of type I and type III interferons (IFNs) [1, 16, 18]. Secreted IFNs bind to the IFN receptors on the surface of adjacent cells and activate the JAK/STAT signaling pathway, which stimulates expression of IFN-stimulated genes (ISGs) encoding the proteins with antiviral activities. Although type I IFNs (IFN-α and -β) and type III IFNs (IFN-λ)

are induced through similar mechanisms [1, 18, 28], their receptors, composed of heterodimers, are different. The type I IFN receptor is comprised of IFNAR1 and IFNAR2, whereas the type III IFN receptor subunits are IFNLR1 (IL-28RA) and IL-10R2 [17, 34]. Almost all cells express the type I IFN receptor and IL-10R2, whereas IFNLR1 is expressed only on epithelial cells, hepatocytes and immune cells [1, 18, 34]. It is thought that type III IFNs are critical in localized control of viral infections at epithelial surfaces, while type I IFNs are important for control of systemic infections [10, 22, 24]. Although humans have four genes encoding type III IFNs (IFNλ1-4), IFNλ4 is functional in some populations, but not in others [12, 31].

Previous studies with MeV have shown that RIG-I and melanoma differentiation-associated gene 5, members of RLRs, contribute to the induction of type I IFNs in MeV-infected epithelial cells [14, 15, 30]. Epithelial cells are reported to produce type III IFNs upon MeV infection [2, 26]. However, it is unknown whether type III IFNs indeed function in MeV-infected epithelial cells, and whether type I and type III IFNs are redundant, complementary or dually essential in controlling MeV infection in epithelial cells. In this study, we examined MeV growth in a human lung epithelial cell line and its mutants lacking either type I or type III IFN receptor to answer these questions.

Materials and methods

Cells and virus

H358 cells [3], a lung adenocarcinoma cell line, were maintained in RPMI-1640 (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (FBS) (Sigma), 50 U/ml of Penicillin, 50 μg/ml of Streptomycin (Gibco), and 5 μg/ml Plasmocin Prophylactic (InvivoGen). Vero cells stably expressing human SLAM (Vero/hSLAM) [27] were maintained in Dulbecco's Modified Eagle Medium (Wako Pure Chemical Industries) supplemented with 7.5% FBS, 50 U/ml of Penicillin, 50 μg/ml of Streptomycin, and 5 μg/ml Plasmocin Prophylactic.

A recombinant MeV, based on a clinical isolate [37], expressing enhanced green fluorescent protein (EGFP) (IC323-EGFP) [13] and its derivative IC323-EGFP-ΔV [14], which lacks expression of the V protein, were used in this study. Virus stocks were prepared and titrated on Vero/hSLAM cells. To minimize the production of

defective interfering (DI) particles, virus stocks were passaged only once after the plasmid-mediated production of the recombinant viruses.

Reagents

Human IFNλ1 (PeproTech) and universal IFNα (PBL) were dissolved in phosphate buffered saline containing 0.1% bovine serum albumin (BSA), aliquoted, and kept at -80 °C until use. Poly (I:C), the synthesized double-stranded RNA polyinosinic-polycytidylic acid, was purchased from GE Healthcare. Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific) was used for poly (I:C) transfection.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNAs were extracted using TRIzol reagent (Invitrogen), treated with RQ1 DNase (Promega), reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega) and random primer. qPCR was carried out using SYBR Premix Ex Taq II (TaKaRa) and LightCycler (Roche) as described previously [38]. The primer sets used were 5'-CTCCTGGCTAATGTCTATCA-3' and 5'-GCAGAATCCTCCCATAATAT-3' for human IFNβ mRNA, 5'-CTTGGATTGCCCATTTTGCG-3' and 5'-CATGGCTAAATCGCAACTGC-3' for human IFNλ1 mRNA, 5'-CCTGACGCTGAAGGTTCTGG-3' and 5'-ATATGGTGCAGGGTGTGAAGG-3' for human IFNλ2/3 mRNA, 5'-CTGGAACGGTGAAGGTGACA-3' and 5'-AAGGGACTTCCTGTAACAATGCA-3' for human β-actin mRNA, respectively. The amounts of individual mRNAs were normalized by that of actin mRNA and shown as relative values. Statistical significances of some data sets were analyzed by two-tailed student′s t-test and a *p*-value less than 0.05 was considered as statistically significant.

Western blot analysis

Whole cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore). The blot was blocked with 5% skim milk or BSA in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.4, 137.5 mM NaCl) containing 0.05% or 0.1% Tween 20 (TBS-T) for

1 h at room temperature. We selected appropriate concentrations of Tween20 and blocking reagent depending on the antibodies used. Then, the membrane was incubated overnight with primary antibodies at 4 °C, washed by TBS-T for 5 min three times, and incubated with appropriate secondary antibodies for 1 h at room temperature. After washing with TBS-T another three times, the membrane was treated with Chemi-Lumi One Super reagent (nakalai tesque) and chemiluminescence was detected using VersaDoc 5000 imager (Bio-Rad). Rabbit polyclonal antibody (pAb) against STAT1 (Santa Cruz Biotechnology), rabbit pAb against STAT2 (Santa Cruz Biotechnology), rabbit pAb against phosphorylated STAT2 (Tyr690) (Cell Signaling Technology), rabbit monoclonal antibody (mAb) against phosphorylated STAT1 (Tyr701) (58D6 ; Cell Signaling Technology), mouse mAb against actin (C-2 ; Santa Cruz Biotechnology) or β-actin (Clone BA3R ; BioVision), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (GE health care), and HRP-conjugated anti-mouse IgG (Jackson Immuno Research) were used.

Enzyme-linked immunosorbent assay (ELISA)

H358 cells were infected with IC323-EGFP or IC323-EGFP-ΔV at a multiplicity of infection (MOI) of 0.1 for 1 h, and then cultured with fresh medium. At 24, 48, 72 h after infection, small amounts of culture supernatant were collected and kept at -80 °C until determination of IFN concentrations using ELISA kits. LEGEND MAX ELISA Kit with Pre-coated Plates Human IL-29 (IFN-λ1) (BioLegend) and DIY Human Interferon Lambda 2/3 (IL-28A/B) ELISA kit (PBL) were used for determination of IFN concentrations, by following the manufactures′ instructions except the usage of 0.5 M sulfuric acid in place of the stop solution provided in the kits.

Gene knockout (KO) by the CRISPR/Cas9 system

Gene targeting plasmids were generated by inserting annealed primer dimers into the *BbsI* restriction site in the pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (Addgene). The sequences introduced were 5′-TCGTGTAGGGTTCAACCGCA-3′ for STAT1, 5′-GACCCTAGTGCTCGTCGCCG-3′ for IFNAR1-1 [43], 5′-ACAGGAGCGATGAGTCTGTC-3′ for IFNAR1-2 [7], 5′-TCGCGCCACCGTCTACGGGT-3′ for IFNLR1-1, 5′-ACTGAACGTGTAGATGGTTC-3′ for IFNLR1-2, and

5′-GCACTACCAGAGCTAACTCA-3′ as scramble control, respectively. Each targeting plasmid was transfected into H358 cells along with pBAsi-hU6 Pur empty plasmid (TaKaRa) to select puromycin resistant clones. Two days after transfection, the cells were transferred onto 10 cm dish and selected under 1μg/ml puromycin. KO of the target gene was confirmed by western blot analysis.

Growth kinetics of virus

To evaluate the effect of IFNλ1 on virus growth, H358 cells were infected with IC323-EGFP at an MOI of 0.3 for 1 h. The supernatant was removed and replaced with fresh medium containing various concentrations of IFNλ1. At 48 h after infection, both cells and supernatants were harvested. H358 cells lacking STAT1, IFNAR1 or IFNLR1, or scramble control cells were infected with IC323-EGFP at an MOI of 0.1 for 1 h, and then cultured with fresh medium. At 24, 48 and 72 h after infection, both cells and supernatants were harvested. Virus titers in the collected samples were determined on Vero/hSLAM cells by plaque titration. Statistical significances were analyzed by two-tailed student′s t-test and a *p*-value less than 0.05 was considered as statistically significant.

Results

IFNλ1 treatment inhibited MeV growth in a lung epithelial cell line

To study the role of type III IFNs in MeV infection of epithelial cells, we used a human lung epithelial cell line H358, which has been shown to express the MeV epithelial receptor nectin 4 [23, 25]. When H358 cells are transfected with poly (I:C), mRNAs for all of IFNβ, IFNλ1 and IFNλ2/3 were induced, peaking at 12 h post transfection, as revealed by RT-qPCR (Fig. 1a). Since IFNλ2 and IFNλ3 genes are highly homologous, their mRNAs were measured together, not individually. Examination with RT-qPCR also revealed that H358 cells express mRNAs for the type III IFN receptor subunits, IFNLR1 and IL-10R2 (data not shown). Furthermore, IFNλ1 stimulation led to phosphorylation of STAT1 and STAT2, two members of the STAT family involved in the type III IFN signaling, in H358 cells (Fig. 1b). These results indicate that type III IFNs are operative in H358 cells, justifying the use of this cell line to study the role of type III IFNs in MeV infection. We then infected H358 cells with IC323-EGFP, a recombinant MeV harboring the gene encoding the

enhanced green fluorescent protein [13]. At 1 h post infection (p.i.), the culture medium was replaced with that containing IFNλ1, and the incubation was continued. Cells and culture supernatants were harvested at 48 h p.i. and MeV titers were determined by the plaque assay. IFNλ1 treatment was found to inhibit MeV growth by 85%, compared with the untreated control (Fig. 1c). Thus, type III IFN can inhibit MeV growth in human epithelial cells.

Type III IFNs were induced in H358 cells after MeV infection

To examine whether MeV infection does induce the production of type III IFNs in epithelial cells, we infected H358 cells with IC323-EGFP (described as wild-type (wt) virus in this subsection) or its V protein-deficient mutant (ΔV) [14], and examined productions of type I and III IFNs at mRNA and protein levels. The MeV V protein has been shown to inhibit the induction of type I IFNs by blocking the activities of MDA5 and RIG-I [4, 8, 14, 33]. Accordingly, ΔV MeV induces larger amounts of type I IFNs compared with the parental virus [14]. In wt virus infected-H358 cells, the amount of IFNβ mRNA was increased as reported previously [14], and those of IFNλ1 and IFNλ2/3 mRNAs were also increased 70-fold and 3.6-fold at 48 h p.i., respectively, compared with uninfected control cells (Fig. 2a). Examination with ELISA detected the productions of IFNλ1 and IFNλ2/3 at 72 h p.i., but almost none at 24 and 48 h p.i. (Fig. 2b). The expression levels of IFNβ, IFNλ1 and IFNλ2/3 mRNA in ΔV virus-infected H358 cells at 48 h. p.i. were 17, 6 and 8 times higher, respectively, than those in wt virus-infected cells (Fig. 2a). Upon infection with ΔV virus, IFNλ1 and IFNλ2/3 were detected even at 48 h p.i., and the amounts of IFNλ1 and IFNλ2/3 at 72 h p.i. were 5.9 and 7.6 times larger, respectively, than those in wt virus-infected cells (Fig. 2b).

Generation of type I or type III IFN receptor KO H358 cells

In order to determine relative contributions of type I and type III IFNs to the control of MeV growth in H358 cells, we generated the type I IFN receptor subunit IFNAR1 KO or type III IFN receptor subunit IFNLR1 KO H358 cells as well as STAT1 KO H358 cells, by using the CRISPR/Cas9 system [5, 32]. STAT1 is a common signal transduction molecule of type I and type III IFN receptors [1, 17, 18]. STAT1 KO was verified by western blot analysis (Fig. 3a). Further, no phosphorylated STAT1 was detected in the KO cells treated with human IFNα or IFNλ1 (Fig. 3a). We

produced two clones each of IFNAR1 KO and IFNLR1 KO H358 cells by using different target sequences to reduce the possibility of off-target effects. The clones were screened for successful targeting by genotyping, and then examined for the KO phenotype. STAT1 molecules in IFNAR1 KO clones were phosphorylated after treatment with IFNλ1, but not with IFNα, whereas those in IFNLR1 KO clones were phosphorylated after treatment with IFNα, but not with IFNλ1 (Fig. 3b). The results functionally confirmed the absence of targeted IFN receptors in these KO cells.

MeV growth in type I or type III IFN receptor KO cells

We compared MeV growth in these type I or type III IFN receptor KO H358 cells with that in control cells obtained using scramble sequence. The cells were infected with IC323-EGFP at an MOI of 0.1, and virus titers (combined titers of cell-associated and cell-free viruses) were determined at 24, 48 and 72 h p.i. As expected, MeV grew better in STAT1 KO cells deficient in both type I and type III IFN signaling pathways than in control cells (Fig. 4a). In IFNAR1 KO cells (IFNAR1 KO1 and KO2 clones), virus titers were 4~32 times and 14~21 times higher at 48 and 72 h p.i., respectively, compared with those in control cells (Fig. 4b). Similarly, virus titers in IFNLR1 KO cells (IFNLR1 KO1 and KO2 clones) were 13~34 times and 14~17 times higher at 48 and 72 h p.i., respectively, than those in control cells (Fig. 4c). In all cases, the differences in virus titers were much smaller at 24 h p.i., compared with those at later time points. The results indicate that MeV growth is enhanced in epithelial cells in the absence of either type I or type III IFN signaling.

Discussion

The production of type III IFNs was detected in MeV-infected H358 cells, consistent with previous studies with human primary nasal epithelial cells [2, 26] and human lung adenocarcinoma cell line A549 [2, 26], indicating that MeV generally induces type III IFNs in human respiratory epithelial cells. Most viruses have evolved strategies to counteract IFN activities. The MeV C protein has been shown to suppress the production of DI particles, which are a potent type I IFN inducer [29, 35]. This function of the C protein is expected to restrict the production of type I IFNs in MeV-infected cells. The MeV V protein is also known to suppress the induction of type I IFNs and interfere with type I IFN signaling by inhibiting STAT1 and STAT2 phosphorylation [40]. Since type I and type III IFNs largely share

common induction and signaling mechanisms [1, 18, 28], the type III IFN pathway may also be antagonized by these viral proteins. In fact, our results showed that the induction of type III as well as type I IFNs was greatly increased in the absence of the MeV V protein. The activities of the MeV C and V proteins may at least partly explain the finding that *in vivo* productions of type I and type III IFN are limited after MeV infection of macaques and human patients [36, 42, 44].

Although type I and type III IFNs are thought to be analogous to each other in terms of the induction, signaling and target genes, some differences have been noted between them. For example, mouse intestinal epithelial cells express high levels of type III IFN receptor, but only very low levels of type I IFN receptor, and accordingly type III IFNs, but not type I IFNs, act to inhibit reovirus growth in mouse intestinal epithelial cells [22]. In influenza virus infection of mice, type III IFNs are first produced at respiratory epithelia to suppress initial viral spread. If infection progresses, type I IFNs are produced to enhance viral resistance, yet cause immunopathology due to the productions of pro-inflammatory cytokines, because neutrophils, which respond to both type I and type III IFNs, upregulate antimicrobial functions but exhibit pro-inflammatory activation only to type I IFNs [10].

It is unknown whether type I and type III IFNs have distinct functions in MeV infection of human epithelial cells. In our experiments, higher titers of MeV were obtained in IFNAR1 KO or IFNLR1 KO epithelial cells, compared with control cells. Furthermore, the titers obtained in IFNAR1 KO or IFNLR1 KO epithelial cells were comparable with the titer in STAT1 KO epithelial cells. How is this observation explained? One possible explanation would be that either type I or type III IFNs alone cannot produce enough amounts of ISGs to restrict MeV growth in epithelial cells. Another, though not mutually exclusive, explanation may be that type I and type III IFNs produce some different sets of ISGs [1, 18, 28], which, in addition to common ISGs, are required to restrict MeV growth in epithelial cells. Thus, type I and type III IFNs are not redundant, but may be additive and/or dually essential to restrict MeV growth.

It should be noted that while the production of type III IFNs was hardly detected in H358 cells infected with MeV (IC323-EGFP) at 48 h p.i. (Fig. 2b), MeV growth was apparently enhanced in IFNLR1 KO H358 cells at the same time point (Fig. 4c). Since type III IFN mRNAs were already induced at 48 h p.i. (Fig. 2a), a small amount

of type III IFNs then produced by infected cells may be sufficient to act on neighboring cells to suppress MeV growth.

In this study, we used the lung adenocarcinoma cell line H358, because it expresses the MeV epithelial receptor nectin 4, produces type III IFNs and is amenable to genetic manipulations (e.g. gene KO). H358 has also been widely used as a model for respiratory epithelial cells. However, the present results should be extended, in future studies, to primary differentiated respiratory epithelial cells grown on air-liquid interface to simulate *in vivo* conditions better.

Conflict of interests

The authors declare no conflict of interest.

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Figure legends

Fig. 1 Inhibition of MeV growth in H358 cells by IFNλ1 treatment. (a) Levels of IFNβ, IFNλ1 and IFNλ2/3 mRNAs in H358 cells transfected with poly (I:C) were measured by RT-qPCR at indicated time points after transfection. Ctl, untreated control cells; +, cells transfected with poly (I:C); -, cells transfected with transfection reagent only. The values of cells transfected with poly (I:C) at 12 h post transfection as normalized by β-actin mRNA levels are set to 100%. Data indicate the mean ± standard deviation (SD) of triplicate samples. (b) Phosphorylation of STAT1 and STAT2 in H358 cells treated for 1 h with indicated concentrations of human IFNλ1 was examined by western blot analysis. (c) H358 cells were infected with IC323-EGFP at an MOI of 0.3, and at 1 h p.i. culture media were changed to those containing indicated concentrations of human IFNλ1. Cells and culture supernatant were harvested at 48 h p.i. and virus titers were determined. Data indicate the mean ± SD of triplicate samples. Data are representative of the results from three independent experiments.

Fig.2 Induction of type III IFNs in MeV-infected H358 cells. H358 cells were infected at an MOI of 0.1 with IC323-EGFP (wt) or its V protein-deficient mutant (ΔV). (a) Levels of IFNβ, IFNλ1 and IFNλ2/3 mRNAs at 48 h p.i. were measured by RT-qPCR. The values of cells infected with wt virus as normalized by β-actin mRNA

levels are set to 100%. (b) Levels of IFNλ1 and IFNλ2/3 proteins at indicated time points p.i. were determined by ELISA. Ctl, uninfected cells. Data indicate the mean ± SD of triplicate samples. Data are representative of the results from at least two independent experiments.

Fig.3 Generation of H358 cells lacking STAT1, IFNAR1 or IFNLR1. (a) STAT1 KO H358 cells were treated with 1000 units/ml of IFNα or 100 ng/ml of IFNλ1 for 1 h, and expression and phosphorylation of STAT1 were determined by western blot analysis. Scramble, control cells obtained using scramble sequence. (b) IFNAR1 or IFNLR1 KO H358 cells were treated with 1000 units/ml of IFNα or 100 ng/ml of IFNλ1 for 1 h, and phosphorylation of STAT1 was examined by western blot analysis. -, Untreated with IFN.

Fig. 4 MeV growth in STAT1, IFNAR1 or IFNLR1 KO cells. STAT1 KO (a), IFNAR1 KO (b) or IFNLR1 KO (c) H358 cells were infected at an MOI of 0.1 with IC323-EGFP. Scramble control cells were also infected. Cells and culture supernatant were harvested at indicated time points, and virus titers were determined. Data indicate the mean ± SD of triplicate samples. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Data are representative of the results from at least two independent experiments.

Fig. 1

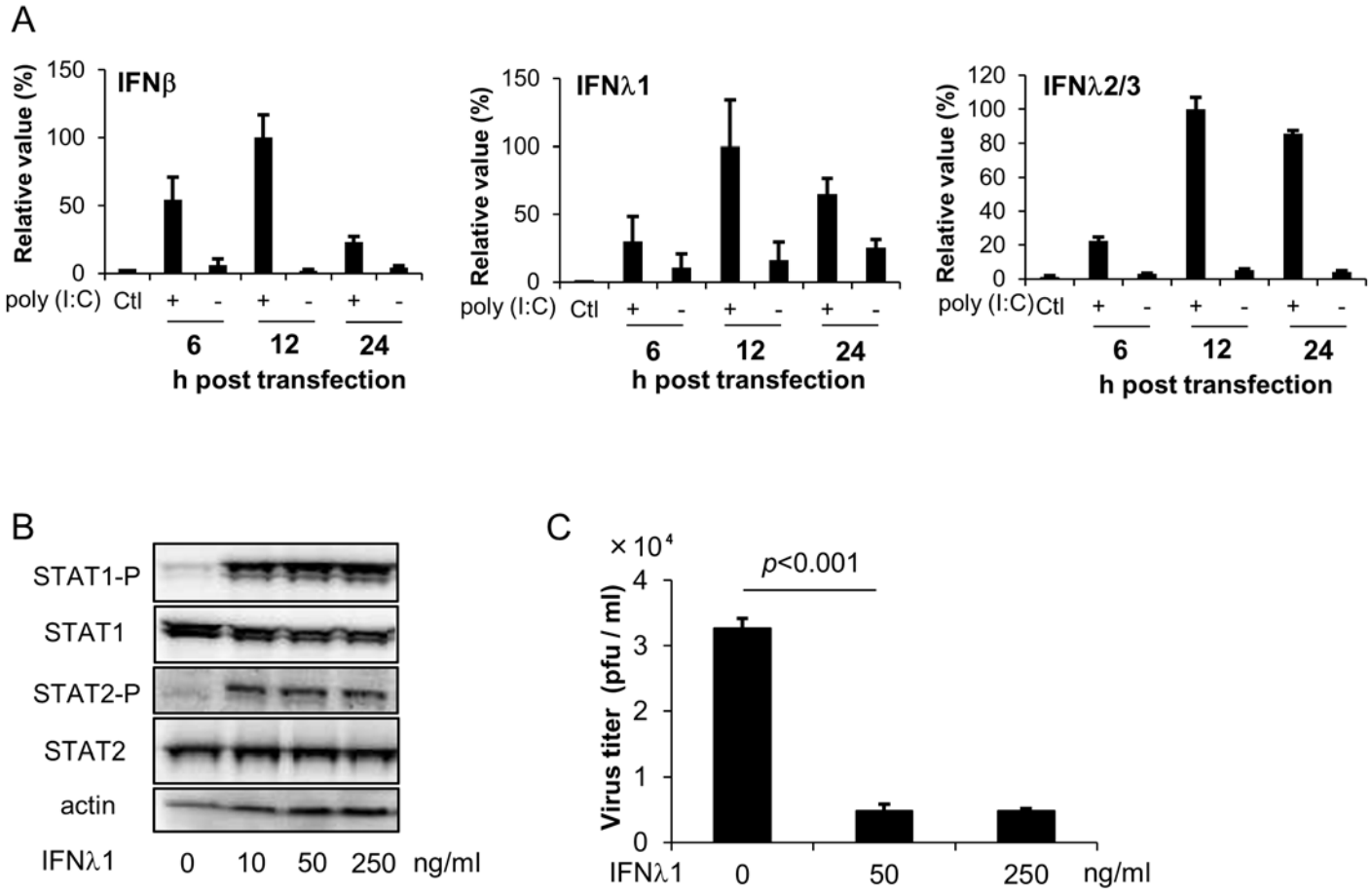

A
IFNβ
IFNλ1
IFNλ2/3
Relative value (%)
poly (I:C) Ctl + - + - + -
6 12 24
h post transfection
B
STAT1-P
STAT1
STAT2-P
STAT2
actin
IFNλ1 0 10 50 250 ng/ml
C
× 10⁴
p<0.001
Virus titer (pfu / ml)
IFNλ1 0 50 250 ng/ml

Fig. 2

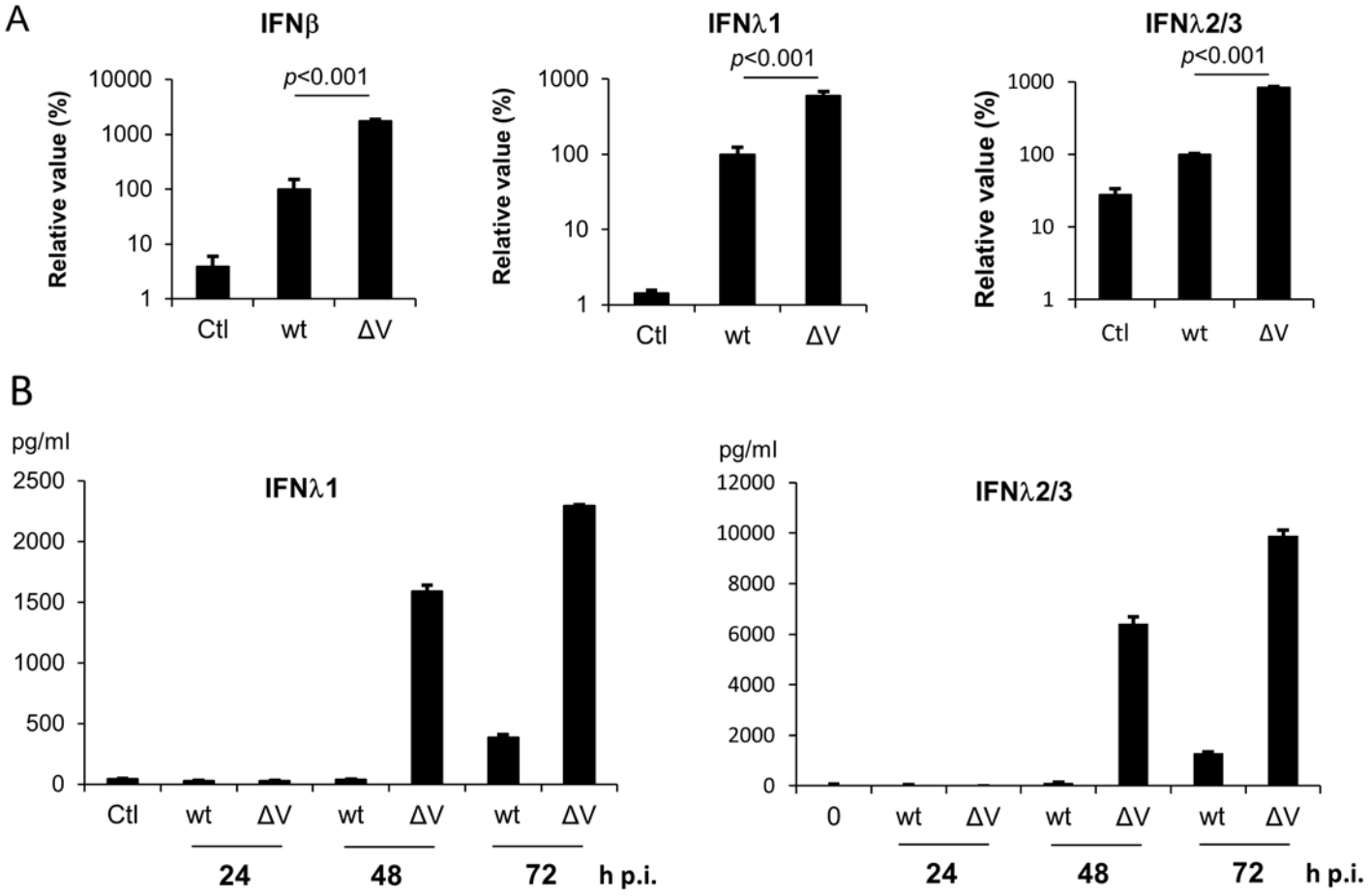

A
IFNβ
IFNλ1
IFNλ2/3
p<0.001
Relative value (%)
10000
1000
100
10
1
Ctl
wt
ΔV
B
pg/ml
2500
2000
1500
1000
500
0
12000
10000
8000
6000
4000
2000
0
24
48
72
h p.i.

Fig. 3

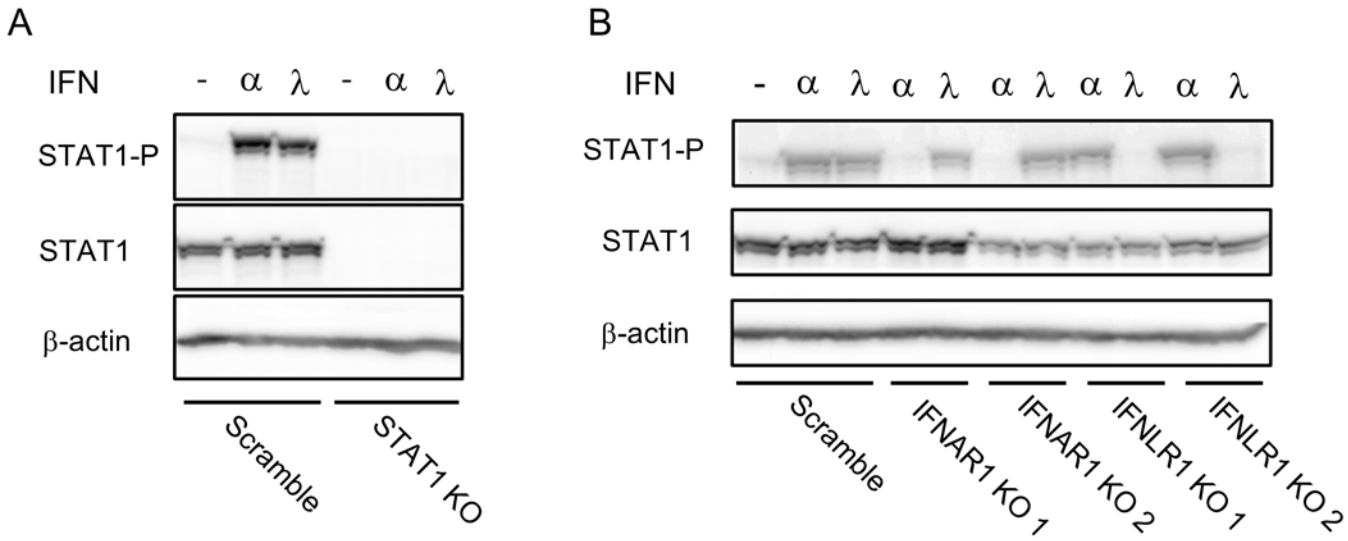
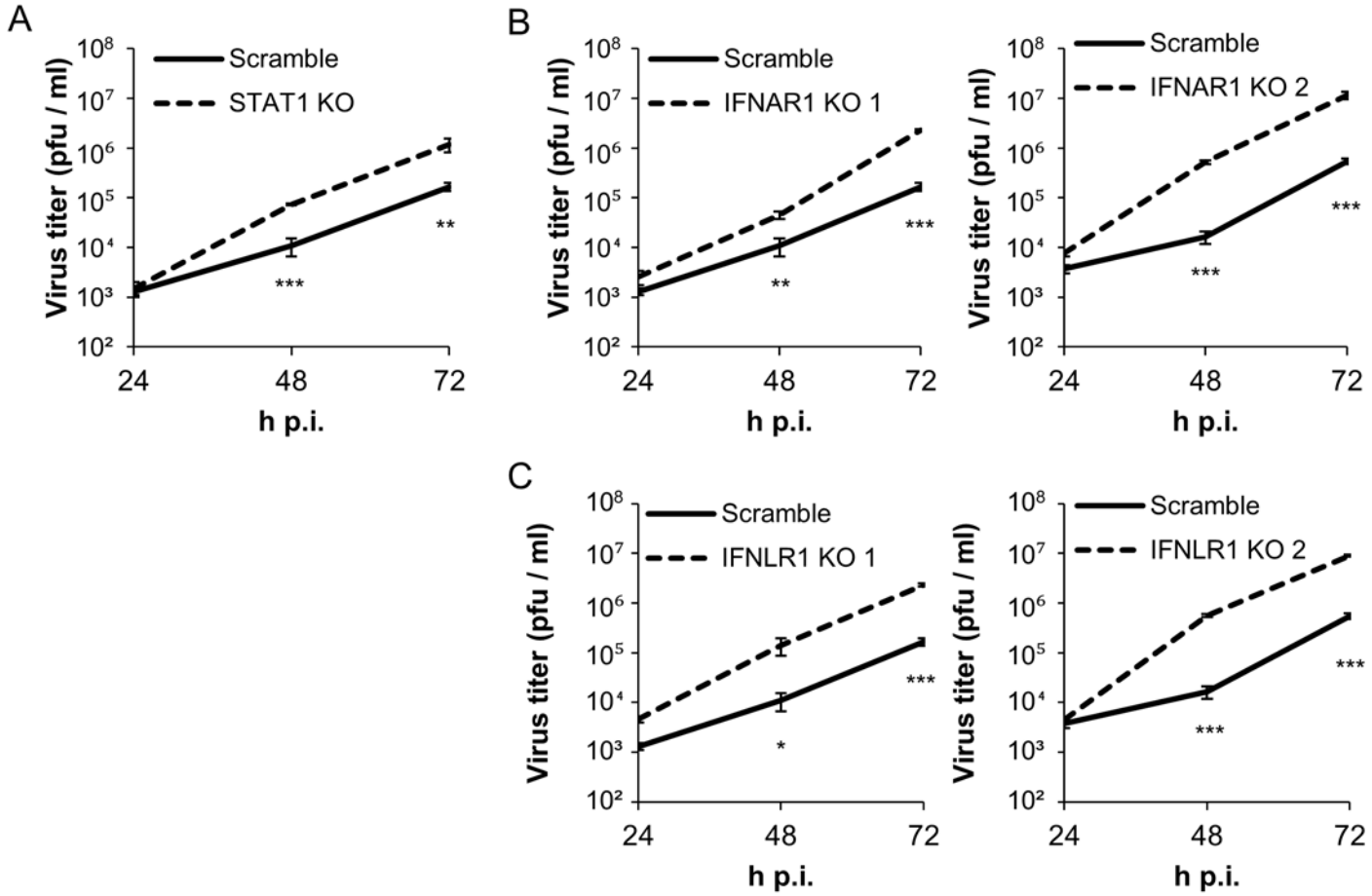

A
IFN
- α λ - α λ
STAT1-P
STAT1
β-actin
Scramble
STAT1 KO
B
IFN
- α λ α λ α λ α λ α λ
STAT1-P
STAT1
β-actin
Scramble
IFNAR1 KO 1
IFNAR1 KO 2
IFNLR1 KO 1
IFNLR1 KO 2

Fig. 4


A
Scramble
STAT1 KO
Virus titer (pfu / ml)
h p.i.
B
Scramble
IFNAR1 KO 1
IFNAR1 KO 2
C
IFNLR1 KO 1
IFNLR1 KO 2