Sendai virus pathogenesis in mice is prevented by 

Iif2 and exacerbated by interferon

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The Type I/III interferon (IFN) system has major roles in regulating viral pathogenesis, usually ameliorating pathogenesis by impairing virus replication through the antiviral actions of one or more IFN-induced proteins. Ifit2 is one such protein which can be induced by IFN or virus infection and is responsible for protecting mice from neuropathogenesis caused by vesicular stomatitis virus. Here, we show that Ifit2 also protects mice from pathogenesis caused by the respirovirus, Sendai virus (SeV). Mice lacking Ifit2 (Ifit2-/-) suffered severe weight loss and succumbed to intranasal infection with SeV strain 52, at a dose that killed only few wild-type mice. Viral RNA was detectable only in lungs and SeV titers were higher in Ifit2-/- mice, compared to wild-type mice. Similar infiltration of immune cells was found in the lungs of both mouse lines, corresponding to similar levels of many induced cytokines and chemokines. In contrast, IFN-β and IFN-λ3 expression were considerably higher in the lungs of Ifit2-/- mice. Surprisingly, type I IFN receptor knock-out (IFNAR-/-) mice were less susceptible to SeV than Ifit2-/- mice, although their pulmonary virus titers were similarly high. To test the intriguing possibility that type I IFN-action enhances pathogenesis in the context of elevated SeV replication in lungs, we generated Ifit2/IFNAR-/- double knock-out mice. These mice were less susceptible to SeV than Ifit2-/- mice, although viral titers in their lungs were even higher. Our results indicate that high SeV replication in the lungs of infected Ifit2-/- mice cooperates with elevated IFN-β induction to cause disease.
The IFN system is an innate defense against virus infections. It is triggered quickly in infected cells, which then secrete IFN. Via their cell surface receptors on surrounding cells, they induce transcription of numerous IFN-stimulated genes (ISG), which in turn protect these cells by inhibiting virus life cycles. Hence, IFNs are commonly considered as beneficial during virus infections. Here, we report two key findings. Firstly, lack of a single ISG in mice, Ifit2, resulted in high mortality after SeV infection of the respiratory tract, following higher virus loads and higher IFN production in Ifit2-/- lungs. Secondly, mortality of Ifit2-/- mice was reduced when mice also lacked the type I IFN receptor, while SeV loads in lungs were still high. This indicates that type I IFN exacerbates pathogenesis in the SeV model, and that limitation of both viral replication and IFN production is needed for effective prevention of disease.
INTRODUCTION

The interferon (IFN) system has a major regulatory role in viral pathogenesis. The synthesis of both type I and type III IFNs is induced upon virus infection of cells; the secreted IFNs in turn, acting through different receptors in uninfected cells, induce the expression of IFN-stimulated genes (ISG), some of which block virus replication (1-3). Thus, the IFN system is viewed primarily as a component of the host’s innate immune defense against virus infection. In addition to the innate antiviral function of type I IFNs, they modulate the adaptive immune responses as well (4, 5). Consequently, in virus-infected mice, there are major IFN-dependent changes in the levels of other cytokines and activated immune cells. Hence, the ultimate pathology of the infected mouse is determined not only by its cell-intrinsic antiviral effects, but by a combination of several diverse effects of the IFN system.

We have been studying the contribution of the IFN system to the host defense against Sendai virus (SeV), a respirovirus of the Paramyxoviridae. The Cantell strain of SeV strongly activates the cytoplasmic RIG-I-like receptor (RLR) pathway in infected cultured cells and induces the synthesis of type I IFN and the ISGs (6). The above effect is triggered by viral defective interfering (DI) particles which are abundant in most laboratory stocks of SeV-Cantell (7). In contrast, the SeV 52 strain, which produces fewer DI particles, is a relatively poor inducer of IFN (8). In mice, SeV52, but not SeV Cantell, causes robust respiratory tract infection and resultant pathogenesis (8). Using both in vitro and in vivo systems, we established the important transcription factor IRF-3 as a major player in the host protection against SeV (9, 10). A part of this protective effect of the RLR-activated IRF-3 is provided by its traditional function as a transcription factor that induces IFN and ISGs (10, 11). But, another independent role of IRF-3 is to promote apoptosis of the SeV-infected cells. In the absence of IRF-3, SeV-infected cells in
culture become persistently infected, an effect that can be prevented by the pro-apoptotic
property, not the transcriptional function, of IRF-3 (9, 10). Thus, both the ISG induction and the
pro-apoptotic effects of IRF-3 provide anti-SeV innate immune protection.

The antiviral actions of ISGs, which number in hundreds, are primarily directed against a diverse
spectrum of viruses (1, 2). We and others have been identifying the specific antiviral functions of
different ISGs and our recent focus has been on the Ifit genes, which are strongly induced by
type I IFNs (12). However, they can also be induced by virus infection in the absence of IFNs;
any signaling pathway trigged by different pattern recognition receptors capable of activating
IRF-3 or IRF-7, such as TLR3, TLR4, TLR7, TLR9, RLRs or cGAS/STING, causes induction of
these genes (13, 14). Thus, in cells infected with an RNA virus that activates the RLR pathway,
an Ifit gene will be directly induced in the infected cell via activated IRF-3 and secondarily
induced by the IFN concomitantly secreted by the infected cell. Contributions of the two
pathways to Ifit induction can be distinguished in IFNAR-/- mice which are insensitive to type I
IFNs, because they do not express one subunit of the type I IFN-receptor. The Ifit proteins, 4 in
human and 3 in mouse, comprised of multiple degenerate tetratricopeptide repeats, are distinct
but similar in structure and different Ifits have different biochemical and antiviral properties (12,
15). They do not have any enzymatic activity but they exert their functional effects through
binding to specific cellular or viral RNAs and proteins. For example, Ifit1 and Ifit2 can inhibit
initiation of translation by binding either to the initiation factor eIF3, or to the 5’-ends of mRNAs
(16-21). Mouse Ifit1 can recognize mRNAs that are devoid of 2’-O-methylation in their caps and
also binds to RNAs carrying triphosphates at their 5’-ends (20-22). However, no RNA-binding
property of mouse Ifit2 has been reported.
The generation of different *Ifit* knock-out mouse lines has opened the way to evaluate the roles of these proteins in pathogenesis caused by different viruses (23, 24). Using *Ifit2-/-* mice, we have shown that Ifit2 prevents neuropathogenesis in mice caused by the rhabdovirus, vesicular stomatitis virus (VSV) (24, 25) and the coronavirus, mouse hepatitis virus (MHV) (26). The protective effect of Ifit2 is cell type-specific and virus-specific. Moreover, Ifit2 does not appreciably inhibit VSV replication in cell cultures indicating that viral pathogenesis is a complex process in which the efficiency of virus replication is an important, but not the exclusive, factor (24). Here, we report that Ifit2 also protects mice from pulmonary pathogenesis caused by intranasal infection with SeV. Our data indicate that both higher loads of SeV present in the lungs as well as pro-pathogenic actions of virus-induced type I IFN contribute to enhanced morbidity and mortality in infected *Ifit2-/-* mice.
MATERIALS AND METHODS

Mice

All mice were of C57BL/6 background, between 8-12 weeks in age, and mixed sexes. Wild-type mice were purchased from Taconic Farms, Inc. *Ifit2*−/− and *Ifit1*−/− mice were previously described (24, 25). *IFNAR*−/− mice were a gift from Kaja Murali-Krishna (Emory University, Atlanta, GA). Homozygous double knock-out mice (*Ifit2*−/−, *IFNAR*−/−) were generated by crossing single knock-out mice. All animal procedures were approved by the Cleveland Clinic Institutional Animal Care and Use Committee (IACUC).

Viruses and infections

Sendai virus (52 strain) seed stock was purchased from ATCC (catalog #VR-105) and grown in chicken embryos by Charles River Laboratories, delivered as sterile allantoic fluid containing propagated infectious virus. For all infections, 1.2x10⁵ pfu of virus (medium dose) were intranasally administered, unless stated otherwise (0.34x10⁵ = low dose and 4.9x10⁵ pfu = high dose); 35 µl of endotoxin-free PBS containing SeV were slowly instilled into the nostrils of isoflurane-anesthetized mice. PBS-only was used as control. Mice were monitored daily for weight loss and symptoms of pathogenesis, including hunching, lethargy, and respiratory distress.

Bronchioalveolar lavage

Mice were anesthetized with pentobarbital (150 mg/kg) and cardiac perfusion was performed with 10 ml of PBS to remove blood. Using a 24G feeding needle (Fine Science Tools), a bronchioalveolar lavage (BAL) was performed by inflating lungs with 700 µl of sterile saline and collecting the fluid back for cell counting.
Quantitative RT-PCR

Mice were anesthetized with pentobarbital (150 mg/kg) and blood was removed from organs by cardiac perfusion with 10 ml of PBS. Organs were collected and snap-frozen in liquid nitrogen. RNA was extracted using TRIzol reagent (Invitrogen) followed by DNase I treatment (DNAfree, Applied Biosystems/Ambion) and reverse transcription with random hexamers (ImProm-II, Promega) were performed according to manufacturer’s instructions. 0.5-1 ng of cRNA was used in 384 well-format realtime PCRs in a Roche LightCycler 480 II using Applied Biosystem’s PowerSYBR PCR mix. PCR primers for murine Ifit2, Ifit1, IFN-β, and 18S rRNA have been published (24, 27). SeV primers targeted the P gene sequence (forward: 5’-

CAAAAGTGAGGCGAAGGAGAA-3’ and reverse: 5’-CGCCCAGATCTGTGATACAGA-3’). Primers for murine Ly6G (28) and IFN-λ3 (29) were published previously. Primers for F4/80 (=Emr1) (forward: 5’-GAGACGTGTTGCTGACATG-3’ and reverse: 5’-

AGGATCTGAAAAGTTGCAAGA-3’), Cd4 (forward: 5’-TCCTTCCCACCTCAACTTG-3’ and reverse: 5’-

TCAAGACGCTTCTTCTAGT-3’ and reverse: 5’-ACCGTGCGAGACTAGATA-3’) were from Cornelia Bergmann, Lerner Research Institute, Cleveland, Ohio. Analysis was done with Roche LightCycler software and GraphPad Prism 5.02 was used to graph RNA expression as

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2^{\Delta\text{CP of 18S rRNA} / 2^{\Delta\text{CP of target RNA}}}; \text{CP} = \text{threshold crossing point.}
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Cytokine ELISA

Mice were anesthetized with pentobarbital (150 mg/kg) and blood was removed from organs by cardiac perfusion with 10 ml of PBS. Lungs were collected and snap-frozen in liquid nitrogen, weighed and homogenized in pestle tubes (Kimble/Kontes) in 1 ml of PBS and spun down at
10,000 x g. The supernatant was used for enzyme-linked immunosorbent assays (ELISA) with either the Verikine mouse IFN-β ELISA kit (PBL, Piscataway, NJ), or a multiplex ELISA to detect IL-1α, IL-1β, IL-6, MCP-1, IFN-γ, TNFα, MIP-1α, GM-CSF and RANTES via Quansys Biosciences’ (Logan, UT) Q-Plex Array™ kit.

**Virus quantification**

Mice were anesthetized with pentobarbital (150 mg/kg) and blood was removed from organs after cardiac perfusion with 10 ml of PBS. Organs were collected and snap-frozen in liquid nitrogen. Lungs were weighed and pestle/tube-homogenized (Kimble/Kontes) in 1 ml of PBS. Virus was titered by plaque assay. Briefly, virus containing samples were incubated with trypsin for 30 minutes at 37°C, waterbath-sonicated at low power, and titered on LLC-MK2 cells (ATCC) followed by an agar overlay. After 3 days, plaques were visualized by removal of the agar overlay and incubation with guinea pig red blood cells (Colorado Serum Company, Denver, CO).

**Statistical analysis**

Statistical significance of mouse survival differences was calculated by Mantel-Cox log rank test. To assess significance of differences of gene expressions or virus titers, an unpaired t-test was used. Asterisks indicate significance levels: * p<0.05, ** p<0.005, *** p<0.0005. All calculations were performed using GraphPad Prism 5.02 software.
RESULTS

*Ifit2*-/- mice are more susceptible to SeV pathogenesis

To compare the susceptibilities of wt and *Ifit2*-/- mice to intranasal infection with SeV, we tested different doses of virus inoculum: low (0.34x10^5 pfu / mouse), medium (1.2x10^5 pfu / mouse) and high (4.9x10^5 pfu / mouse) (Fig 1A, B, C). As the dose of the virus increased, more and more wild-type (wt) mice died; however, at every dose tested, the *Ifit2*-/- mice were more susceptible. We chose the medium dose, which produced the most marked differential between the wt and *Ifit2*-/- mortality, to study the mechanisms underlying the pathogenesis. At this dose, *Ifit2*-/- mice lost body weight at a much higher rate than wt mice (Fig 1D). The maximum weight loss was at 7 days post infection (dpi) for both strains, at which point 10 out of 11 *Ifit2*-/- mice but only 2 out of 13 wt mice were sacrificed because of excessive weight loss. All of the surviving wt mice started gaining weight after 7 dpi, recovering normal weights by 14 dpi; the lone surviving *Ifit2*-/- mouse recovered weight at the same rate. No sex-specific differences were observed. These results demonstrated that Ifit2, an IFN-induced protein, can protect mice from pathogenesis caused by SeV infection. The above conclusion suggested that *IFNAR*-/- mice, which do not express one subunit of the type I IFN receptor and hence cannot respond to type I IFN, would be equally or more susceptible to SeV pathogenesis, compared to the *Ifit2*-/- mice. However, contrary to this expectation, they were much less sensitive (Fig 1E).

Higher viral loads in lungs of *Ifit2*-/- mice

With the above observations in mind, we set out to identify the mechanism behind the higher susceptibility of the *Ifit2*-/- mice. SeV is known to be pneumotropic in mice (30), with efficient virus replication exclusively in lungs. Indeed, we detected viral RNA only in the lungs of the infected mice; no virus replication was detectable in the blood, brain, kidney, liver or heart,
neither in wt nor Ifit2-/- mice. Importantly, at 5 dpi, the viral RNA level was ~2.5-fold higher in
the lungs of Ifit2-/- mice, compared to wt mice (Fig 2A). Further analyses showed that the viral
RNA level peaked at 5 dpi and then declined. The IFNAR-/- mice had similarly elevated levels of
viral RNA, compared to wt (Fig 2B). We also quantified infectious virus particles in lungs and
confirmed that Ifit2-/- and IFNAR-/- mice had produced comparable amounts of virus at 5 dpi,
about 4-fold more than wt mice (Fig 2C). These observations suggested that a higher viral load
in lungs might be the cause of pathogenesis in Ifit2-/- mice. However, with similarly high viral
loads, the IFNAR-/- mice were not as susceptible (see Fig 1E), suggesting that other factors, such
as IFN action, might have contributed to pathogenesis.

Expression of virus-inducible type I and III interferon mRNAs in lungs

Virus infection causes, either directly or indirectly, induction of many cellular genes. To identify
any possible difference between the wt and Ifit2-/- mice in their ability to support infection-
induced gene expression in the lung, we measured the mRNA levels of Ifit2 and Ifit1, as
representative genes that can be induced directly by the activation of the RLR signaling pathway
by SeV infection or by IFN produced by the infected cells. Ifit1 mRNA was induced in the lungs
of wt and Ifit2-/- mice in a time dependent fashion (Fig 3A) and as expected, Ifit2 mRNA was
induced in the wt, but not the Ifit2-/- mice (Fig 3B). The induction of these genes was not
mediated by type I IFN, because they were also induced in IFNAR-/- mice. However, when we
examined the levels of type I and III IFN mRNAs, there was significant difference between the
two mouse lines. There were no detectable IFN-α mRNAs in the lungs of any mouse (using
primers to detect six IFN-α subtypes, data not shown); however, both IFN-β mRNA (Fig 3C)
and IFN-λ3 (type III IFN) mRNA (Fig 3D) were induced by SeV much more in Ifit2-/- mice than
in wt mice, with levels peaking at 2 dpi. Similarly high induction of IFN-β and IFN-λ3 mRNAs was also observed in IFNAR-/- mice, who however cannot respond to IFN-β.

SeV-induced cytokine and chemokine production in lungs

We confirmed our observation of more IFN-β mRNA induction in Ifit2-/- and IFNAR-/- mice by measuring the levels of IFN-β protein in lungs (Fig 4A). More IFN-β was produced in the lungs of infected Ifit2-/- and IFNAR-/- mice compared to wt mice. Virus infection also causes the synthesis of other cytokines and chemokines, by the infected or bystander cells. To examine whether there were differences in these responses, we determined the levels of a number of cytokines and chemokines in the lungs of SeV-infected wt and Ifit2-/- mice. No significant difference in the induced levels of IL-6, MCP-1, IFN-γ, RANTES (Fig 4B), and IL-1α, IL-1β, TNFα, MIP-1α or GM-CSF (Fig 4C) was found between the two mouse lines. The IFNAR-/- mice also had similar levels of the above cytokines and chemokines, except that the levels of IL-1α and RANTES were higher in these mice.

Immune cell infiltration of SeV-infected lungs

To examine whether the lungs of Ifit2-/- mice and wt mice showed differences in immune cell infiltration during the course of infection, we first determined the total cell numbers in bronchio-alveolar lavages (BAL). There was no difference in overall cellular infiltration between the two mouse lines; as expected, numbers increased with time after infection (Fig 5A). Next we determined the identities of the infiltrating cells by quantifying the accumulation of mRNAs of cell-type-specific markers in lungs. We found similar kinetics of appearance and accumulation for macrophages (F4/80) and granulocytes (Ly6G) early after infection, as well as CD4 and CD8 T cells late after infection (7 dpi) in both wt and Ifit2-/- lungs (Fig 5B).
Pro-pathogenic role of type I IFN in SeV pathogenesis

Our analyses, presented above, showed that compared to wt mice, Ifit2-/- mice were more susceptible to SeV (Fig 1), their lung virus titers were higher (Fig 2) and they induced more type I and type III IFNs (Fig 3). These three parameters are interdependent because virus infection induces the two types of IFNs which, in turn, inhibit virus replication. To determine which of these three elements contributed to the observed pathogenesis in Ifit2-/- mice, we compared two different doses of SeV inoculum. A higher dose of virus was more lethal for wt mice (Fig 6A).

Surprisingly, the higher dose of inoculum did not yield increased virus loads in the lungs of the wt mice; similar virus levels were attained at both medium and high doses during the course of infection (Fig 6C), and the level was lower than that in the Ifit2-/- mice (Fig 6C). In contrast, much more IFN-β was induced in the lungs of the wt mice infected with the high dose of virus (Fig 6D). This suggested that a lower level of pulmonary virus load combined with a high local IFN-β expression may be sufficient to kill mice. To further test the possibility of type I IFN escalating pathogenesis, we used IFNAR-/- mice, in which type III IFN, but not type I IFN, can signal. Increasing the SeV inoculum dose did not increase lethality in the IFNAR-/- mice (Fig 6B), although their lungs had as high a level of SeV as Ifit2-/- mice (Fig 6C), suggesting that high virus replication was lethal only when elevated levels of type I IFN could signal via IFNAR. To strengthen our hypothesis that both viral load and IFN-β level in the lungs of the infected mice contribute to pathogenesis, we generated the Ifit2-/-, IFNAR-/- double knock-out mice (DKO) and compared their responses to infection with those of single knock-out mice. At both medium and high doses of virus infection, the Ifit2-/- mice were more susceptible than, not only the wt and the IFNAR-/- mice, but also the DKO mice (Fig 7A). DKO mice expressed high levels of IFN-β (Fig 7B), which however could not function in absence of IFNAR; high levels of
IFN-λ3 were also expressed in these mice (Fig 7C). Moreover, virus titers in DKO mice were the highest overall, significantly higher than those in wt, Ifit2/-/- or IFNAR/-/- mice infected with medium or high doses of SeV (Fig 7D). These results provided further support for the notion that both high virus loads and the action of type I IFN, but not type III IFN, contributed to the observed pathogenesis in SeV-infected mice.
DISCUSSION

Paramyxoviruses and the interferon system interact at multiple levels and hence, the pathogenic outcome of infection is determined by the balance among many positive and negative effects. The most common animal model for studying SeV pathogenesis uses intranasal infection with the SeV 52 strain, which is fatal even for wt mice, at a high dose. Both innate and adaptive immunity contribute to the host defense, but although the type I IFN system is a major component of the former, it is not required for developing an adaptive immune response against SeV in this mouse model (8). Most of the information regarding the nature of interactions between SeV and the IFN system has been acquired from studies done by infecting cells in culture with the Cantell strain of SeV, which is not pathogenic in mice (7, 8). SeV induces IFN synthesis strongly by activating the RLR pathway but it also encodes the V and C proteins which block the induction (31, 32). Type I IFN, in turn, blocks SeV replication through the action of one or more of the numerous ISGs which are induced in IFN-treated cells. Many of these ISGs, including Ifit2, can also be induced without any IFN involvement, by the IRF-3 transcription factor activated in SeV-infected cells (27). IRF-3 has an additional role in SeV-infected cells; it triggers apoptosis of the infected cell by binding to the pro-apoptotic protein Bax and translocating it to mitochondria (10). Both ISG action and the apoptotic response determine the extent of virus replication and the fate of the infected cell. In the absence of IRF-3, SeV establishes persistent infection in various cell types (33, 34). Until now, it was not known how SeV replication is controlled by ISGs in vivo; this study identified Ifit2 as a major anti-SeV ISG in infected mice. Ifit2−/− mice are susceptible to neuropathogenesis caused by VSV, a rhabdovirus, and MHV, a coronavirus, but not by the picornavirus EMCV (24, 26). Thus, Ifit2 action is virus-specific.
VSV infection spreads into many organs in IFNAR-/- mice, but not in wt mice, indicating that various ISGs protect these organs. However, in Ifit2-/- mice, VSV replicated efficiently only in neurons; other tissues were as protected as in wt mice (24). Thus, Ifit2 action is also cell-type-specific. The current study shows that Ifit2 also protects mice from pulmonary infection by SeV. However, its absence did not promote infection of other tissues (Fig 2A) indicating that other organs are protected, not by Ifit2, but by different means. It should also be noted that Ifit2’s protective role was observed in a relatively narrow range of inoculum dose. High dose infection (4.9x10⁵ pfu) of wt mice increased their mortality to a level seen with low dose (0.34x10⁵ pfu) infection of Ifit2-/- mice (compare Figs 1A and 1C). Thus, Ifit2 made a significant difference in the disease outcome only when the inoculum was neither too low nor too high.

Investigation of the host response in lungs of SeV-infected wt and Ifit2-/- mice showed that several responses were similar in both lines of mice. Several cytokines and chemokines were induced to similar levels (Fig 4), as were the magnitude and the composition of the infiltrating cell population, which contributes to cell-mediated immunity. We did not measure the virus-specific humoral antibody response because a previous report showed that it is not elicited in the time frame of our experiments (8). There was little difference in lung pathology, as determined by histology of lung sections, and the extent of apoptosis, as determined by TUNEL assay and active Caspase 3 immunohistochemistry (data not shown). One significant difference between the wt and the Ifit2-/- infected mice was in the pulmonary virus load, being about 4-fold higher in the Ifit2-/- mice (Fig 2). This observation demonstrated Ifit2’s conventional antiviral role in our mouse model. This is in contrast to similar intranasal infection of Ifit2-/- mice with VSV, where no enhanced viral replication was observed in the lungs although the virus titer was higher in the
brain. These observations suggest that Ifit2’s antiviral effects are specific for both the infecting 

virus and the target tissue.

The other significant difference between the two SeV-infected mouse lines was in the levels of 

the induction of IFN-β and IFN-λ3 mRNAs. More of both cytokines were induced in the infected 

Ifit2−/− mice as compared to infected wt mice (Fig 3, Fig 4A). Both IFNs can be induced by virus 

infection and both can impart antiviral effects by inducing ISGs (3). To determine whether either 

of them contributes to the observed pathogenesis, we used IFNAR−/− mice. In the VSV model, 

these mice were much more susceptible than Ifit2−/− mice, showing virus replication in many 

organs, including brain, liver and lungs, and dying before neuropathy was observable (24); 

similarly, they were more susceptible to MHV infection (26). In contrast, in the current study, we 

observed that the susceptibility of the IFNAR−/− mice to SeV pathogenesis was definitely lower 

than that of Ifit2−/− mice but not as low as that of the wt mice (Fig 1B and 1E). However, like 

Ifit2−/− mice, infected IFNAR−/− mice expressed higher levels of IFN-β (Fig 3C, 4A) and IFN-λ3 
(Fig 3D) mRNAs; they also had similarly high virus loads (Fig 2C). Because IFNAR−/− mice can 
respond to IFN-λ but not IFN-β, our observations suggested that the magnitude of SeV-mediated 
pathogenesis was determined by both high virus loads and high levels of type I IFN.

The above hypothesis was tested using a higher dose of virus to infect the mice, which increased 
the mortality of the wt mice, but not IFNAR−/− mice (Fig 6A, 6B). The low virus yield in the 
infected wt mice did not change by increasing the virus inoculum dose (Fig 6C), but the levels of 
IFN-β was much higher in wt mice infected with the high dose of virus; this IFN-β induction was 
even higher than that in Ifit2−/− mice infected with the medium dose (Fig 6D). It appears that in 
Ifit2−/− mice, infected with medium or high doses of virus, the high levels of both virus load and 
IFN-β together were causative for causing maximal pathogenesis. The wt mice, on the other
hand, infected with a high dose of SeV, were only partially susceptible because, although they expressed high IFN-β levels, their viral loads were low. Conversely, the lower susceptibility of the IFNAR-/- mice (Fig 6B) was not due to low virus loads (Fig 6C) but because of their inability to respond to IFN-β. We sought more genetic evidence in support of our hypothesis by testing SeV pathogenicity in a new mouse line missing both Ifit2 and IFNAR proteins (Fig 7). In these DKO mice, both IFN-β (Fig 7B) and IFN-λ3 (Fig 7C) were strongly induced and the virus yield was very high (Fig 7D). In spite of these properties, they were slightly, but significantly, less susceptible than the Ifit2-/- mice (Fig 7A); they were however more susceptible than wt mice (Fig 7A) and IFNAR-/- mice (compare Figs 7A and 6B). Higher virus loads in lungs after SeV infection, as seen in Ifit2-/-, IFNAR-/- and DKO mice, were probably not the direct driver of pathogenesis, but they resulted in more pronounced production of pro-pathogenic IFNs. This role of type I IFN was revealed whenever IFNAR was absent (IFNAR-/- and DKO mice) or when the amounts of produced IFN-β were limited (wt mice), leading to reduced pathogenesis and mortality. On the other hand, a role of the virus load in pathogenesis, without an involvement of induced type I IFN, is apparent in the higher pathogenesis in IFNAR-/- mice, compared to wt mice (Figs 1E and 2C). Our results are consistent with the conclusion that Ifit2 protects mice from pulmonary infection by SeV, but virus-induced IFN-β promotes the resultant pathogenesis. Although type I IFN is viewed primarily as an antiviral cytokine, its pro-pathogenic role, in certain models, has been noted before (35). Two groups recently reported that type I IFN signaling contributes to disease progression in mice persistently infected with the arenavirus LCMV. Blocking type I IFN action enabled virus clearance and restoration of a normal state of immune activation (36, 37). In another study, Davidson et al. reported pro-pathogenic activity of type I IFN in mice infected with influenza virus; IFNAR-/- mice were less susceptible to
pathogenesis caused by influenza virus infection (35). The pro-pathogenic role of type I IFN has also been noted in mice infected with non-viral pathogens. In mice and patients infected with *Mycobacterium tuberculosis*, type I IFN augments pathogenesis (38). Our results, reported here, add a new example to the above list. In wt mice, this negative effect of IFN is not apparent because the protective effect of the ISG *Ifit2* limits SeV replication; only by manipulating the viral dose and the genetic background of the mice, the negative effect of type I IFN was uncovered. The mechanism of this effect remains to be determined.

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REFERENCES


FIGURE LEGENDS

Figure 1: Ifit2 protects against morbidity and mortality after SeV infection.
Survival of wt and Ifit2-/− mice after SeV infection with A, low dose (0.34x10^5 pfu), B, medium
dose (1.2x10^7 pfu) or C, high dose (4.9x10^5 pfu). D, weight loss of wt and Ifit2-/− mice infected
with medium SeV dose; number of surviving mice is indicated on the right. E, survival of
IFNAR-/− and wt mice infected with medium SeV dose. Asterisks indicate statistical significance.

Figure 2: Ifit2 and type I IFN limit SeV replication in lungs.
A, SeV RNA levels in different organs from wt and Ifit2-/− mice (n=3) at 5 d p.i., measured by
real-time RT-PCR. B, SeV RNA in lungs of wt, Ifit2-/− and IFNAR-/− mice (n=4-8) at different
times after infection. C, infectious SeV titers in lungs of wt, Ifit2-/− and IFNAR-/− mice (n=3-5)
at different times after infection. Asterisks indicate statistical significance. ND, not detected.

Figure 3: Increased type I and III interferon induction in Ifit2-/− and IFNAR-/− lungs after
SeV infection. (A) Ifit1, (B) Ifit2, (C) IFN-β and (D) IFN-λ3 mRNA expression in lungs of wt, Ifit2-/− and
IFNAR-/− mice (n=3-7) after SeV infection, measured by real-time RT-PCR. Asterisks indicate
statistical significance; n.s., not significant; ND, not detected.

Figure 4: Elevated production of IFN-β, but not of other cytokines, in Ifit2-/− lungs after
SeV infection. A, IFN-β protein levels in wt, Ifit2-/− and IFNAR-/− lung homogenates after SeV
infection, measured by ELISA (n=3-5). B and C, cytokine and chemokine protein levels in wt,
Ifit2-/- and IFNAR-/- lung homogenates at 5 days after SeV infection, measured by multiplex ELISA (n=3). Asterisks indicate statistical significance.

Figure 5: Similar infiltration by immune cells in wt and Ifit2-/- lungs after SeV infection.
A, cell numbers in bronchio-alveolar lavage fluid of wt and Ifit2-/- mice at different times after SeV infection (n=3). B, accumulation of immune cell marker mRNAs in wt and Ifit2-/- whole lung tissue (n=4) after SeV infection, measured by real-time RT-PCR. No statistically significant differences between wt and Ifit2-/- were found in A or B.

Figure 6: High SeV dose increases pathogenesis and IFN-β induction but not subsequent virus replication in wt mice.
Survival of (A) wt and (B) IFNAR-/- mice after infection with medium or high SeV dose. C, infectious SeV titers in lungs of wt, Ifit2-/- and IFNAR-/- mice (n=3-5) at 5 days after infection with medium or high SeV dose. D, IFN-β mRNA expression in lungs of wt, Ifit2-/- and IFNAR-/- mice (n=4-6) at 2 days after SeV infection (medium or high dose), measured by real-time RT-PCR. Experiments in A and B share mice with figure 1B, 1C and 1E. Medium dose infection in C and D are shared with figure 2C and 3C, respectively. Asterisks indicate statistical significance; n.s., not significant.

Figure 7: Type I IFN exacerbates SeV pathogenesis in absence of Ifit2.
A, survival of wt, Ifit2-/- and DKO (Ifit2-/-, IFNAR-/- double KO) mice after infection with low, medium or high SeV dose. (B) IFN-β and (C) IFN-λ3 mRNA expression in lungs of wt and DKO mice (n=5) 2 days after SeV infection (medium or high dose), measured by real-time RT-PCR.
RT-PCR. D, infectious SeV titers in lungs of wt, Ifit2-/-, IFNAR-/- and DKO mice (n=3-5) at 5 days after infection with medium or high SeV dose. Experiments in A share mice with figure 1A, B, C. Panel D is an extension of figure 6C. Asterisks indicate statistical significance; n.s., not significant.