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RAPID COMMUNICATION

Defective interferon priming and impaired antiviral responses in a patient with an *IRF7* variant and severe influenza

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Abstract

Influenza infection is common worldwide with many individuals affected each year during epidemics and occasionally pandemics. Previous studies in animal models and a few human cases have established an important role of innate type I and III interferon (IFN) for viral elimination and mounting of antiviral responses. However, genetic and immunological determinants of very severe disseminated influenza virus infection in humans remain incompletely understood. Here, we describe an adult patient with severe influenza virus A (IAV) infection, in whom we identified a rare variant E331V in IFN regulatory factor (IRF)7 by whole-exome sequencing. Examination of patient cells demonstrated a cellular phenotype suggesting functional IRF7 impairment, since priming with IFN was almost abolished and IFN responses to IAV were significantly impaired in patient cells. Moreover, IAV replication was significantly higher in patient cells than in controls. Finally, expression of *IRF7* E331V in HEK293 cells demonstrated significantly reduced activation of both *IFNA7* and *IFNB* promoters in a luciferase reporter gene expression assay compared to *IRF7* wild type. These findings provide further support for the essential role of IRF7 in amplifying antiviral IFN responses to ensure potent and sustained IFN responses during influenza virus infection in humans.

Keywords Influenza virus · IRF7 · Interferon · Innate immunity · Primary immunodeficiency

Introduction

Influenza virus is a common pathogen that usually causes mild respiratory disease [1]. Some cases of influenza infection do, however, lead to severe and fulminating disease with development of acute respiratory distress syndrome and pneumonia in affected individuals [2]. The pathogenesis of these severe cases remains largely unknown, although studies in both mice and humans have suggested an important role of type I and type III interferons (IFNs) in protective immunity against influenza virus [3–6]. Moreover, genome-wide association studies have suggested a role for single-nucleotide polymorphisms (SNPs) in influenza pathogenesis based on their presence in cohorts with severe influenza [7, 8], most notably in interferon transmembrane protein 3 in both the European and Chinese population [9, 10]. IFNs are mainly induced by the transcription factors IFN regulatory factor (IRF)3 and IRF7 upon recognition of influenza virus by upstream pattern recognition receptors [11]. IRF3 is primarily involved in the early response to virus [12], whereas IRF7 is crucial for the induction and amplification of IFNs at later stages of infection, thus driving a positive feedback loop [13]. IRF7 deficiency caused by homozygous loss-of-function variants was recently demonstrated to underlie severe influenza infection in a child [14], underscoring the importance of IRF7 in induction of antiviral responses. Another study reported IRF9 deficiency in a child with severe influenza pneumonitis, leading to impaired responses to type I IFNs and increased viral burden [15]. Finally, we previously reported an adult

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patient heterozygous for a variant in the cytosolic RNA sensor retinoic-acid-inducible-gene (RIG)-I [16]. Surprisingly, among the more than 350 reported primary immunodeficiencies (PIDs), including defects in the innate and the adaptive immune response [17–19], defects in IRF7, IRF9, and RIG-I are the only PIDs that seem to selectively predispose to severe influenza in humans [14–16]. Thus, the induction of and the response to type I and type III IFNs appear to be particularly important for mounting a protective immune response against influenza virus. In this study, we report the identification of a rare variant in IRF7 together with a cellular phenotype suggesting impaired type I IFN production and amplification in an adult patient suffering from severe influenza infection.

Materials and methods

Primary cells

The 45-year-old male Caucasian patient (now referred to as P) described in the present study was one out of 12 patients identified by searching in an extra corporal membrane oxygenation (ECMO) database as well as medical files of patients with a throat swabs positive for influenza A virus (IAV). P was admitted to the Intensive Care Unit (ICU) in 2014 during the swine flu pandemic with severe H1N1 IAV infection. He did not have a medical history suggesting increased frequency or severity of infections and did not have any known co-morbidities predisposing to severe influenza. Blood samples were obtained from P after full recovery and from healthy controls. Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-stabilized whole blood by Ficoll density gradient centrifugation and stored in liquid nitrogen until use. PBMCs were thawed in RPMI media with 10% heat inactivated FBS and 100 U/mL penicillin, 100 µg/mL streptomycin preheated to 37 °C.

DNA purification

Genomic DNA was purified from EDTA stabilized blood using EZ1 DNA Blood 350 µL kit (Cat # 951054, Qiagen).

Whole-exome sequencing (WES)

WES was performed on genomic DNA from the patient using KAPA HTP library preparation and Nimblegen SeqCap EZ MedExome Plus kits and analyzed with Nextseq version 2 chemistry [2 × 150 basepairs] (Ilumina). Single-nucleotide polymorphisms were called relative to hg19.

Bioinformatics

Variant call files were uploaded to Ingenuity variant analysis (IVA) software (Qiagen) and filtered based on rarity (frequency < 0.1%) and predicted deleteriousness with a combined annotation-dependent (CADD) score > 15. Furthermore, variants were filtered according to biological relevance by uploading a gene list based on literature searches on PubMed, ClinVar, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, Online Mendelian Inheritance in Man (OMIM), and biological filters in IVA related to influenza virus, immune response and immunodeficiencies. All identified variants were confirmed by manual inspection of the BAM files.

Sanger sequencing

IRF7 genomic DNA was amplified from P and a healthy control by PCR using Phusion Hot Start II DNA polymerase (F-549S, Thermo Fischer Scientific) and the following primers: IRF7-E331V forward: 5′-CACCAGCTCTGAAGAAGGGG-3′ and IRF7-E331 V reverse: 5′-AGCTGCACGTTCCTATACGG-3′. Confirmation of the variant was done using Sanger sequencing with the IRF7-E331V reverse primer.

Stimulation and infection of cells

PBMCs were seeded at a density of 5×10^5 cells per well and left to rest over night at 37 °C. Subsequently, cells were infected for 6 h with influenza A virus strain A/PR/8/34 (IAV PR8) (Charles River) at an MOI of 0.1, 1 and 3, or for 16 h with IAV PR8 or the clinical isolate influenza A virus strain A/Hansa/Hamburg/05/09 (IAV pdm 09), kindly provided by Peter Stäheli at an MOI of 0.1. For priming, cells were pretreated with 100 U/mL of IFN-β (11410-2, R&D Systems) prior to the infection for 16 h with the two IAV strains.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from cell lysates with NucleoSpin® 96 RNA kit (740709.4, Macherey–Nagel), followed by reverse transcription of RNA into cDNA using the QuantiTect Reverse Transcription kit (205314, Qiagen), according to manufacturer's instructions. mRNA expression levels were quantified with PerfeCTa® qPCR FastMix® II (733-2108, VWR) and the following TaqMan primer/probes (Life Technologies): *TBP*: Hs00427620_m1; *IFNB1*: Hs01077958_s1; *IFNA2*: Hs00265051_s1; *IFNL1*: Hs00601677_g1; *IFIT1(ISG56)*: Hs03027069_s1; and *TNFA*: Hs01113624_g1.

Cloning and sequencing of IRF7 mRNA expression

RNA isolated from PBMCs from P primed with 100 U/mL of IFN-β was reverse transcribed into cDNA with QuantiTect Reverse Transcription kit (205314, Qiagen) and Oligo(dT) primers (Sigma-Aldrich). IRF7 was subsequently amplified by PCR using Phusion Hot Start II DNA polymerase (F-549S, Thermo Fischer Scientific) and the following primers: *IRF7* E331V mRNA forward: 5′-GCTGGACGTGAC CATCATGTA and IRF7 E331V mRNA reverse: 5′-TGA AGTCGAAGATGGGGGTG, followed by insertion into pJET1.2 by blunt-end ligation using CloneJET PCR cloning kit (K1231, Thermo Fischer) according to manufacturer's protocols. The cloned products were transformed into competent DH5α cells and colonies were Sanger sequenced to determine expression ratio of the c.992A or c.992T alleles using the following primer: pJET forward: 5′-CGACTCACT ATAGGGAGAGCGGC-3′.

Western blotting

Western blotting was performed as previously described [20]. The expression of IRF7 and vinculin protein in PBMCs from P and a healthy control as well as in HEK293 cells was measured using a polyclonal rabbit antibody (4920, Cell signaling technology) 1:1000 and a monoclonal mouse antibody (V9131-100UL, Sigma-Aldrich) 1:1000 for vinculin expression. Primary antibodies were visualized using a secondary horseradish peroxidase-coupled anti-rabbit antibody for IRF7 (711-035-152, Jackson ImmunoResearch) and an anti-mouse antibody for vinculin (715-036-150, Jackson ImmunoResearch) both 1:10,000.

Plasmids

IRF7 cDNA encoding isoform b was cloned into pcDNA3.1 using the XhoI and BamHI restriction sites. The E331V variant was then introduced by site-directed mutagenesis using Pfu Ultra III polymerase (600670-51, Agilent) and the following primers: E331V sense: 5′-GGCCCC CGAAGCACC AGGTGCAACC-3′ and E331V antisense 5′-GGTTGCACC TGGTGCTTCGGGGGCC-3′.

Luciferase reporter assay

IRF3 deficient HEK293T cells were generated as previously described [20]. Cells were then seeded at a concentration of 4×10^5 cells per well in Dulbecco's modified Eagle's medium (DMEM) with 100 U/mL penicillin, 100 µg/mL streptomycin and 5 ug/mL plasmocin and 10% FBS and cultured for 24 h. Cells were subsequently transfected with 250 ng of pEF-BOS N-FLAG *TBKI* vector together with empty vector or 250 ng of pcDNA3.1 encoding *IRF7* WT or the *IRF7* E331V variant. In addition, cells were co-transfected with 970 ng of Firefly luciferase reporter plasmid, under the control of the *IFNB* or *IFNA7* promoter and 30 ng of Renilla luciferase reporter plasmid under the control of the *ACTB* promoter. All transfections were supplemented with empty pcDNA3.1 plasmids for a total of 2 µg of DNA/well and lysed 24 h later with Passive Lysis Buffer (E1910, Promega). Firefly luciferase and Renilla luciferase activities were measured using the Dual Glo reporter assay (E1910, Promega) according to manufacturer's protocol.

IAV replication in monocyte-derived macrophages

Monocyte-derived macrophages (MDMs) were differentiated from PBMCs from P and two healthy controls. PBMCs were seeded at a concentration of 7.5×10^5 cells/well in RPMI with 10% HI-FBS, 10% human AB serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 15 ng/ml human macrophage colony-stimulating factor (M-CSF) (300-25, Peprotech). The medium was changed to DMEM containing 100 U/mL penicillin, 100 µg/mL streptomycin, 10% human AB serum and 15 ng/mL M-CSF on day four and seven. On day nine, cells were washed twice and infected with an MOI of 0.1 of influenza A virus strain A/PR/8/34 (IAV PR8) (Charles River) diluted in DMEM with 100 U/mL penicillin, 100 µg/mL streptomycin, 1% bovine serum albumin (BSA) pH 7.4 and 0.5 ug/mL TPCK-trypsin. Medium was removed 2 h post-infection and cells were washed twice and incubated in DMEM with 100 U/mL penicillin, 100 µg/mL streptomycin, 1% bovine serum albumin (BSA) pH 7.4 and 0.5 ug/mL TPCK-trypsin. Supernatants were harvested after 48 and 72 h and titrated with TCID50 assay on MDCK cells incubated for 72 h. MDCK cells were stained with crystal violet and virus titers determined based on cell death.

Statistics

Experiments were performed either in biological duplicates or triplicates and most experiments were repeated two or three times. Statistical significance was calculated using the non-parametric Mann–Whitney rank sum test: $*p < 0.05$; $**p < 0.01$.

Results

Identification of a rare variant in IRF7 in a patient with severe influenza infection

In the present study, we performed WES on 12 adult patients previously hospitalized at the ICU with severe H1N1 IAV

infection during the swine flu pandemic in 2009/10. In P, we identified a rare variant (frequency = 0.014% in gnomAD European subpopulation) in the transcription factor *IRF7*, which is heterozygous in the patient (Fig. 1a). The variant (c.992A>T) is located within the inhibitory domain of IRF7 (Fig. 1b) and causes an amino acid substitution of glutamic acid (E) to valine (V) at position 331 (IRF7 isoform b). The presence of the variant was confirmed by Sanger sequencing (Fig. 1c). The E331V variant has a CADD score of 29.3, which is above the MSC for *IRF7* of 3.13, indicating that the variant may be deleterious to the function of the protein [21]. Furthermore, the variant is predicted by SIFT and PolyPhen-2 to be damaging or probably damaging, respectively (Fig. 1a). Moreover, the E331 residue is well conserved throughout evolution (Fig. 1d). Several other variants were detected by WES, however, none of these were

a

	E331V
Transcript variant	c.992A>T
Transcript ID	NM_004029.3
Protein variant	p.E331V
gnomAD Frequency	0.014%
CADD-score	29.3
MSC	3.13
SIFT prediction	Damaging
PolyPhen-2	Probably Damaging

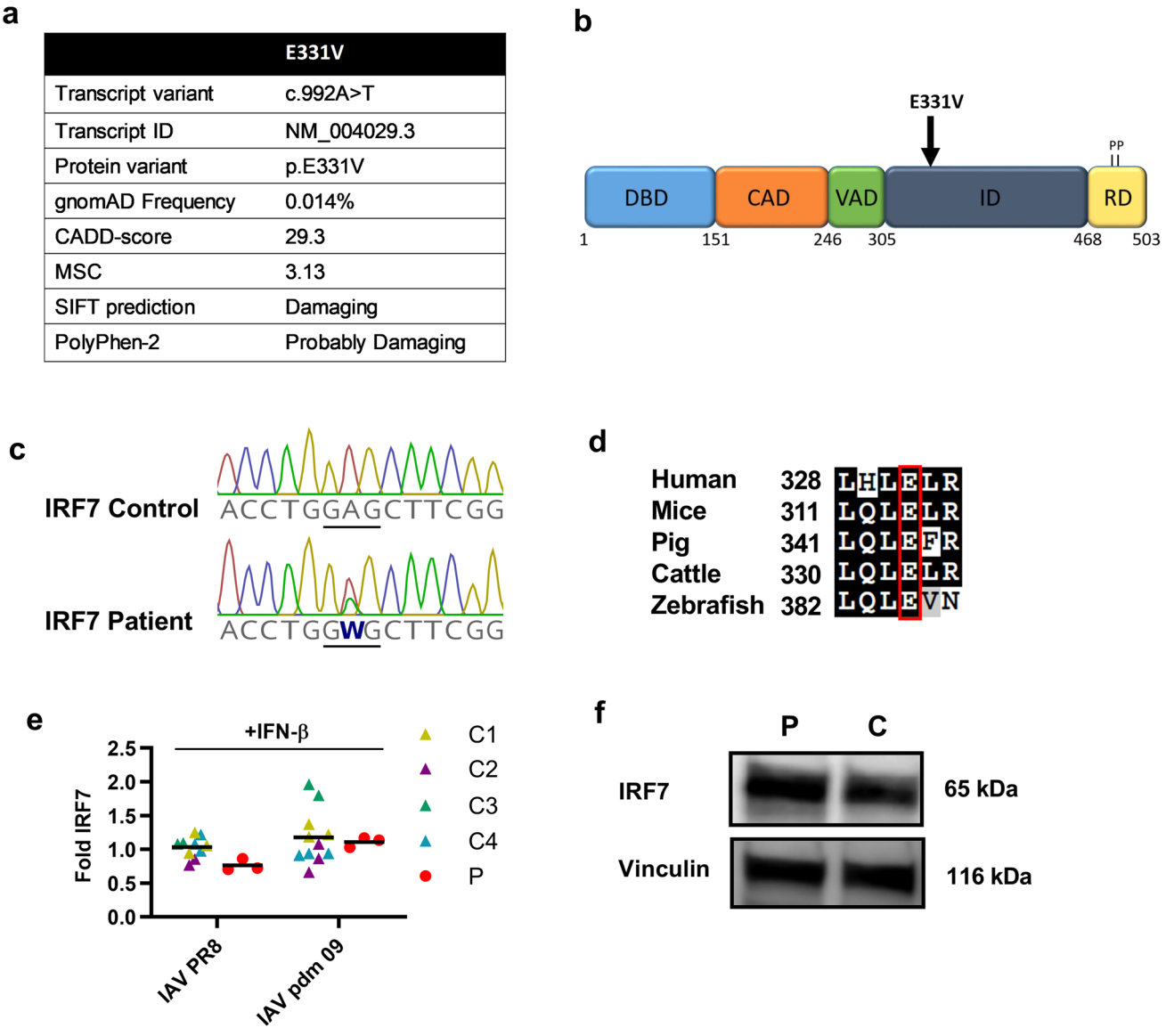


Fig. 1 Identification of an *IRF7* E331V variant in a patient with severe influenza. **a** Specifications, frequency, and bioinformatics predictions of the *IRF7* E331V variant identified in P by whole-exome sequencing (WES). **b** IRF7 protein structure with the localization of the E331V variant indicated. *DBD* DNA-binding domain, *CAD* constitutive activation domain, *VAD* virus-activated domain, *ID* inhibitory domain, *RD* signal response domain. **c** Sanger sequencing confirming the presence of the *IRF7* E331V variants in P. **d** Protein alignment of IRF7 in five different species demonstrating evolutionary conservation. **e** IRF7 mRNA expression in PBMCs from P and four healthy controls following pretreatment with 100 U/mL of IFN-β for 18 h and subsequent infection for 16 h with an MOI of 0.1 of IAV PR8 or a clinical isolate of IAV H1N1 from the 2009 pandemic (IAV pdm 09). IRF7 mRNA was measured by RT-qPCR and fold induction was calculated by dividing IRF7 expression in primed samples with unprimed. **f** Western blotting for IRF7 protein expression on lysates from patient and control PBMCs. Vinculin was used as loading control

consistent with severe influenza infection or results achieved by functional experiments on patient cells. A full list of variants with a frequency < 0.1% and a CADD score > 15 identified in P by WES is available in Supplementary Table 1. An additional rare variant, P115L *IRF7,* was identified by a specific search in the *IRF7* gene (Supplementary Table 2), but this variant was not predicted to be damaging. The identified *IRF7* variants did not impact on IRF7 expression, as *IRF7* mRNA and protein levels measured in P by RT-qPCR (Fig. 1e) and Western blotting (Fig. 1f), respectively, was comparable in P and healthy control PBMCs. Finally, we investigated expression of *IRF7* wild type (WT) and *IRF7* c.992T mRNA (corresponding to *IRF7* E331V) by sequencing *IRF7* mRNA from P's PBMCs after priming with IFN-β, and found equal expression of the *IRF7* variant and WT allele, excluding uneven allele expression (Supplementary Fig. 1).

Impaired priming of patient cells by IFN in response to IAV infection

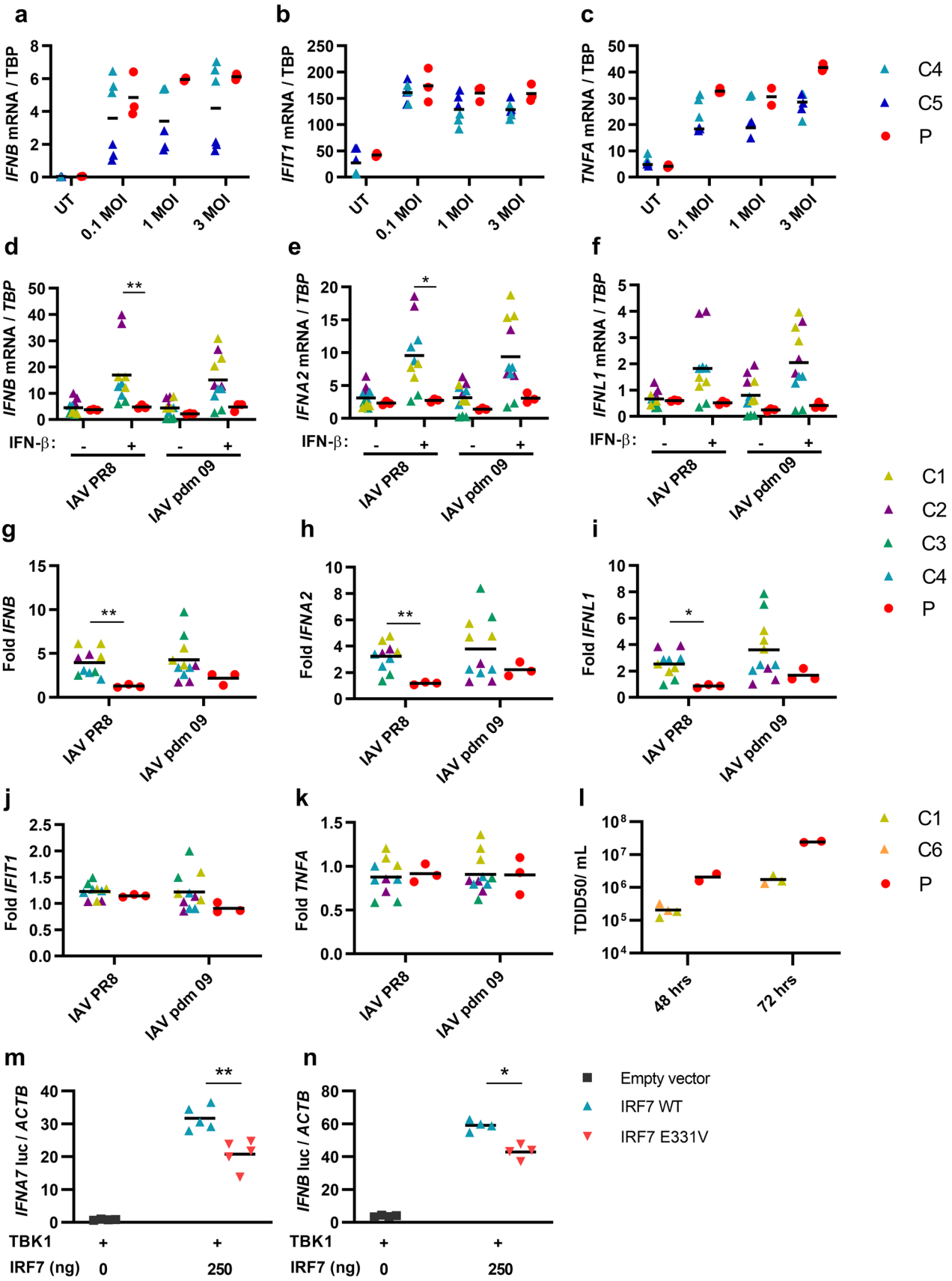
To investigate a potential functional impact of the identified E331V *IRF7* variant, we next examined the response of patient cells to IFN priming and IAV infection by RT-qPCR. We found that PBMCs from P produced similar levels of *IFNB*, the IFN-stimulated gene (ISG) *IFIT1* and the proinflammatory cytokine *TNFA* mRNA, as compared to two healthy controls 6 h after infection with IAV PR8 (Fig. 2a–c). However, as IRF7 is only induced secondary to the first wave of IFN-β production [13], IRF3 would be responsible for most of the IFN induction during this first wave of early IFN response [12]. We, therefore, pretreated/primed PBMCs from P and four healthy controls with IFN-β for 18 h before infection with two different strains of IAV for 16 h. We found that patient cells exhibited markedly reduced expression of *IFNB*, *IFNA2* and *IFNL1* mRNA as compared to healthy controls (Fig. 2d–f), and this impairment was statistically significant for *IFNB* and *IFNA2* induction for the IAV strain PR8. Priming in patient cells after infection with the IAV PR8 strain was significantly impaired when measuring fold induction of *IFNB*, *IFNA2* and *IFNL1* of primed relative to unprimed cells (Fig. 2g–i). In contrast, *IFIT1* mRNA levels following priming and infection with IAV were similar between patient and controls (Fig. 2j) as expected, since *IFIT1* is induced directly by IFN-β priming independently of IRF7 [22]. Likewise, when we measured induction of *TNFA*, not predicted to undergo priming by IFN-β pretreatment, there was no difference between P and controls (Fig. 2k).

Next, we investigated IAV replication and found at least a tenfold higher titer of IAV at 48 and 72 h post-infection in MDMs from P compared to two controls, demonstrating impaired control of IAV replication in patient MDMs (Fig. 2l). To directly examine the functional impact of the *IRF7* E331V variant on *IFN* promoter activation, we expressed cDNA containing the *IRF7* WT or *IRF7* E331V variant in HEK293T cells and measured the ability of the cells to activate transcription of the *IFNA7* and *IFNB* promoters following activation by expression of the upstream kinase *TBK1*. Expression of the *IRF7* E331V variant lead to a significantly reduced induction of the *IFNA7* promoter at transfection with 250 ng of *IRF7* E331V when compared to expression of *IRF7* WT (Fig. 2m). A reduction in *IFNB* promoter activation was also observed for the *IRF7* E331V variant compared to *IRF7* WT, although not as prominent as for the *IFNA7* promoter (Fig. 2n). Equal expression of IRF7 in the cells transfected with *IRF7* WT and *IRF7* E331V IRF7 was confirmed by western blotting for IRF7 (Supplementary Fig. 2).

Discussion

The transcription factor IRF7 is a master regulator of type I and type III IFNs in response to a wide range of pathogens, including influenza virus [12, 13, 23]. In the present study, we report the identification of a rare and potentially deleterious variant in *IRF7* in an adult patient presenting with severe influenza infection with the H1N1 IAV during the swine flu pandemic. The IRF7 variant is located within the inhibitory domain of IRF7, which contains many hydrophobic residues that keep IRF7 in a closed and inactive form in the absence of activating stimuli [24, 25]. Thus, it may be hypothesized that this auto-inhibition is strengthened by the identified variant, which changes a negatively charged glutamic acid residue to a hydrophobic valine, leading to decreased activation of IRF7 upon stimulation.

IRF7 expression is induced by IFN in most cell types, in which it exerts a crucial role at later stages of infection by amplifying the induction of type I IFN, particularly IFN-α2, α5, α6 and α8, and type III IFNs, thus ensuring generation of a potent IFN response [13, 26]. Consistent with this mechanisms, cells from P produced normal levels of *IFNB* after 6 h of infection with IAV, a time point where IRF3 is predicted to represent the major transcription factor responsible for IFN-β induction [12, 27]. Intriguingly, after pretreatment/priming with IFN-β, cells from P produced markedly reduced levels of type I and type III IFNs upon IAV infection compared to healthy controls. Moreover, priming of cells from P with IFN-β did not increase IFN expression compared to non-primed cells, consistent with an impaired positive feedback loop due to defective IRF7 activity. This was in sharp contrast to the situation in cells from healthy controls, which did produce increased levels of *IFNB, IFNA2* and *IFNL1* upon priming, demonstrating the integrity of the IFN amplification loop. Importantly, we


a
IFNB mRNA / TBP
UT
0.1 MOI
1 MOI
3 MOI
b
IFIT1 mRNA / TBP
c
TNFA mRNA / TBP
C4
C5
P
d
IFNB mRNA / TBP
IFN-β:
IAV PR8
IAV pdm 09
e
IFNA2 mRNA / TBP
f
IFNL1 mRNA / TBP
C1
C2
C3
C4
P
g
Fold IFNB
h
Fold IFNA2
i
Fold IFNL1
j
Fold IFIT1
k
Fold TNFA
l
TDID50/ mL
48 hrs
72 hrs
C1
C6
P
m
IFNA7 luc / ACTB
TBK1
IRF7 (ng)
n
IFNB luc / ACTB
Empty vector
IRF7 WT
IRF7 E331V

◂**Fig. 2** Impaired antiviral responses to IAV in patient cells and reduced activity of *IRF7* E331V in a luciferase-based IFN promoter reporter assay. **a**–**c** PBMCs from P and healthy controls were infected for 6 h with an MOI of 0.1, 1 or 3 of IAV PR/8/34 (IAV PR8), or (**d**–**k**) pretreated with 100 U/mL of IFN-β for 18 h followed by infection with an MOI of 0.1 of IAV PR8 or a clinical isolate of IAV H1N1 from the 2009 pandemic (IAV pdm 09) for 16 h. Cells were lysed and total RNA was purified followed by RT-qPCR measuring the induction of *IFNB* (**a**, **d** and **g**), *IFIT*1 (**b** and **j**), *TNFA* (**c** and **k**), *IFNA2* (**e** and **h**) or *IFNL1* (**f** and **i**). Fold induction depicted in **g**–**k** was calculated by dividing primed with the mean of the unprimed samples for both IAV strains. Data are presented as dots with black lines as grand mean. The experiment was run in biological triplicates and performed twice with similar results. **l** IAV replication was measured after infection of MDMs from P and two healthy controls for 48 and 72 h with an MOI of 0.1 of IAV PR8 using the TCID50 assay. The experiment was performed in biological duplicates; however, only one measurement for C6 72 h exists due to a technical error. **m, n** IRF3-deficient HEK293T cells were transfected with 250 ng *TBK1*, and 250 ng of *IRF7* WT or *IRF7* E331V together with an *IFNA7* (**m**) or an *IFNB* (**n**) promoter reporter plasmid. Cells were lysed 24 h later and Firefly luciferase levels were normalized to Renilla luciferase. The experiment was performed twice either in biological duplicates or triplicates and results were pooled. Statistical analysis was conducted using non-parametric Mann–Whitney rank sum test: $*p<0.05$; $**p<0.01$

found markedly higher IAV replication in patient MDMs, reflecting impaired antiviral control. Finally, to directly evaluate the functional impact of the *IRF7* E331V variant harbored by the patient, we expressed the *IRF7* WT and the *IRF7* E331V variant, which revealed significantly reduced activation of both the *IFNA7*- and *IFNB* promoters in cells expressing the variant, therefore demonstrating reduced transcription factor activity of the identified variant.

Ciancanelli et al. previously described IRF7 deficiency in a child with life-threatening influenza and found completely abolished production of type I and type III IFNs and a significant inability to control IAV replication in patient cells [14]. This phenotype is more severe than what we observed in the present study, however, the previously described case of IRF7 deficiency was caused by compound heterozygous null variants [14], whereas the patient described in the present study was heterozygous for a variant in *IRF7*, and with equal expression of the *IRF7* WT and *IRF7* E331V variant after IFN-β priming, thus providing a possible explanation for the milder phenotype observed in the patient presented here.

In conclusion, we here describe an adult patient with severe influenza virus infection, in whom we identified a rare variant in the transcription factor IRF7 and a cellular phenotype suggesting functional IRF7 impairment, since IFN priming was almost abolished and IFN responses to IAV were significantly impaired in patient cells. These findings lend further support to an essential role of IRF7 in amplifying antiviral IFN responses and also provide additional evidence for the central role of type I IFN in antiviral immunity to influenza virus in humans.

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Author contributions TM conceived the idea; MMT, SEJ, THM, HHG, and RH planned and interpreted the experiments. SEJ, MS, and JG included the patients in the study. MMT, SEJ, and HHG performed the experiments. SEJ and MC performed WES analysis. MMT, SEJ, and THM wrote the first draft of the manuscript, and THM wrote the final version, while all authors read, commented, and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest None of the authors have any conflicts of interest to declare.

Ethical approval The study was approved by the Danish Agency for Data Managements and the Regional Ethics Committee (# 1-10-72-251-15). The patient provided informed consent, and the patient and healthy controls were included according to the Helsinki Declaration.

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