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NEMO is a key component of NF-κB- and IRF-3-dependent TLR3-mediated immunity to herpes simplex virus

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Abstract

Background—Children with germline mutations in *TLR3*, *UNC93B1*, *TRAF3* and *STAT1* are prone to herpes simplex virus-1 (HSV-1) encephalitis (HSE), owing to impaired TLR3-triggered, UNC-93B-dependent, interferon (IFN)-α/β and/or -λ-mediated STAT1-dependent immunity.

Objective—We explore here the molecular basis of the pathogenesis of HSE in a child with a hypomorphic mutation in *NEMO*, which encodes the regulatory subunit of the IκB kinase (IKK) complex.

Methods—The TLR3 signaling pathway was investigated in the patient's fibroblasts by analyses of IFN-β, -λ, and IL-6 mRNA and protein levels, by quantitative PCR and ELISA, respectively, upon TLR3 stimulation (TLR3 agonists or TLR3-dependent viruses). NF-κB activation was assessed by EMSA and IRF-3 dimerization on native gels after stimulation with a TLR3 agonist.

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Results—The patient's fibroblasts displayed impaired responses to TLR3 stimulation in terms of IFN-β, -λ, and IL-6 production, owing to impaired activation of both NF-κB and IRF-3. Moreover, vesicular stomatitis virus (VSV), a potent IFN-inducer in human fibroblasts, and HSV-1, induced only low levels of IFN-β and -λ in the patient's fibroblasts, resulting in enhanced viral replication and cell death, as reported for UNC-93B-deficient fibroblasts.

Conclusion—HSE may occur in patients carrying *NEMO* mutations, due to the impairment of NF-κB- and IRF-3-dependent-TLR3-mediated antiviral IFN production.

Keywords

NEMO; immunodeficiency; Toll-like receptor 3; herpes simplex encephalitis

INTRODUCTION

In humans, loss-of-function mutations in the gene encoding NF-κB essential modulator (NEMO) cause X-linked dominant incontinentia pigmenti (IP) in girls and death *in utero* in boys [1-3]. Hypomorphic mutations in *NEMO* are associated with X-linked recessive (XR) anhidrotic ectodermal dysplasia (EDA) with immunodeficiency (ID) (XREDA-ID) [1, 2, 4-13]. The typical EDA phenotype results from the impaired development of skin appendages. However, the developmental phenotype depends on the mutation, as some boys have a much more severe clinical presentation, with osteopetrosis and lymphedema (XR-OL-EDA-ID) [5, 7, 11], whereas others display no overt developmental defect [10, 14-16]. A hallmark of NEMO defects is a delayed or poor clinical and biological inflammatory response. In addition, most patients have no detectable serum antibody response to glycan antigens, despite infection with and/or vaccination against encapsulated bacteria [17]. The infectious phenotype varies considerably between patients. More than half of all known patients with XR-EDA-ID have suffered from clinical disease caused by pyogenic bacteria, such as *Streptococcus pneumoniae*, and mycobacteria, such as *Mycobacterium avium* [1, 4-8, 12, 14, 18-22]. Fungi, such as *Pneumocystis jiroveci*, have also caused disease in a few patients [5, 7, 18, 19]. Finally, viral infections have been diagnosed in some patients [5, 6, 19], including herpes simplex virus-1 (HSV-1) encephalitis (HSE) in a child with a pure ID without EDA [14, 15].

This patient carries a frameshift insertion in codon 37 of *NEMO* exon 2 (*110_111insC*), creating the most upstream known premature stop codon in *NEMO* [14, 15]. This mutation has been shown to be hypomorphic, due to the re-initiation of translation at amino acid 38[15]. The residual level of the truncated NEMO protein produced was sufficient for normal fetal and skin appendage development, but insufficient for the development of optimal protective immunity to infection. From the age of 15 months, the patient suffered from disseminated *M. avium* disease with adenitis, osteomyelitis and dermatitis, and from bronchiectasis caused by pyogenic bacteria, such as *S. pneumoniae*. Immunological examinations revealed defects of the IL-12-IFN-γ circuit in peripheral blood mononuclear cells (PBMCs), possibly accounting for mycobacterial disease [2324, 25]. Impaired cellular responses to TNF-α and LPS in whole blood and to IL-1Rs in fibroblasts[26-30] probably accounted for pyogenic bacterial diseases. At the age of 12 years, while receiving prednisone, the patient developed HSE, which was fatal despite acyclovir treatment. To our knowledge, this boy is the only child with a *NEMO* mutation and HSE reported to date [14, 15].

HSE is a rare complication of HSV-1 infection affecting about 2 to 4/1,000,000 individuals per year [31, 32]. The pathogenesis of this devastating illness has long remained a mystery. The disease is limited to the central nervous system (CNS) and patients with known classical primary immunodeficiencies are not prone to HSE [31]. Autosomal recessive (AR) STAT-1

deficiency was the first genetic etiology of HSE [33] to be identified, in a child with unusual clinical features, combining mycobacterial disease, reflecting an impaired response to IFN-γ, and HSE, probably reflecting an impaired response to IFN-α/β and/or IFN-λ [34]. Three genetic etiologies of isolated HSE in otherwise healthy children have been reported, in the form of AR UNC-93B [35], autosomal dominant (AD) TLR3 [36], and AD TRAF3 [37] deficiencies. The fibroblasts of these patients displayed impaired induction of IFN-β and -λ in response to TLR3 stimulation. These data are consistent with the lack of HSE in IRAK-4- and MyD88-deficient patients, whose blood cells do not produce IFNs in response to TLR7, TLR8 or TLR9 [22, 27-29, 38]. The TLR3-dependent induction of IFN-α/β and -λ is crucial for protective immunity to primary infection with HSV-1 in the CNS, in at least some children [39, 40]. We therefore investigated the molecular pathogenesis of HSE in the child bearing the *110_111insC* mutation in *NEMO*, by investigating the response of the TLR3-IFN pathway to TLR3 agonists and TLR3-dependent viruses.

METHODS

Case report

The clinical features of this patient have been reported elsewhere [14, 15]. Our study was conducted in accordance with the Helsinki Declaration, with informed consent obtained from each patient or the patient's family. The experiments described were conducted in Paris and New York, in accordance with local regulations and with the approval of the CCPPRB of Necker-Enfants Malades Hospital, Paris, France and of the IRB of Rockefeller University.

TLR3 agonists, viral infection and ELISA

We used a synthetic analog of dsRNA (polyinosinepolycytidylic acid, poly(I:C), Amersham), a nonspecific TLR3 agonist, at various concentrations (1 to 50 μg/ml), and IPH31 (an optimized polyA:U dsRNA, specific agonist of TLR3, provided by Innate-Pharma) with or without Lipofectamine™ 2000. For viral stimulation, we used the dsDNA virus herpes simplex virus-1 (HSV-1, strain KOS-1, multiplicity of infection (MOI) = 1) and the ss(-)RNA virus vesicular stomatitis virus (VSV, strain Indiana, MOI = 1). Primary fibroblasts and SV-40-transformed fibroblasts (SV40-fibroblasts) from patients, healthy and negative controls ($NEMO^{-/-}$ IP cells derived from a female fetus with a completely skewed pattern of X inactivation expressing the *NEMO* allele bearing a deletion of exons 4-10 [41], and $UNC\text{-}93B^{-/-}$ cells derived from an HSE patient carrying the homozygous mutation *1034del4* [35]) were grown in 24-well plates (10^5 cells/well) with DMEM (Invitrogen) supplemented with 10% FBS. After 24 hours of stimulation with the TLR3 agonists or viruses, supernatants were harvested and ELISA was carried out to assess the production of IFN-β (TFB, Fujirebio, Inc.), IL-6 (Sanquin), and IFN-λ, as previously described [35].

IRF-7 western blotting and signal transduction studies in fibroblasts

Immunoblotting for IRF-7 was carried out with a monoclonal antibody directed against the N-terminus of human IRF-7 (sc-744472, Santa Cruz Biotechnology). An antibody against tubulin (sc-23948, Santa Cruz Biotechnology) was used to control for protein loading, for each sample. DNA-binding activity was assessed by electrophoretic mobility assay (EMSA) and IRF-3 dimerization was assessed by western blotting, as previously described[15, 35].

Cell viability and mortality assays

Assays of cell viability and mortality after viral infection were performed as previously described [33]. Briefly, SV40-fibroblasts were left untreated or were treated with IFN-α2b (10^5 IU/ml) for 18 hours before infection with various titers of HSV-1 or VSV for 72 and 24

hours, respectively. Viability was assessed by evaluating the reduction of resazurin, according to the manufacturer's protocol (Sigma-Aldrich). Cell mortality was assessed by measuring the amount of LDH released into the medium, according to the manufacturer's protocol (Roche).

RESULTS

Impaired TLR3 responses in the patient's fibroblasts

Dermal fibroblasts naturally and selectively express TLR3 at their cell membrane and can secrete both IFN-β and IFN-λ constitutively and upon appropriate stimulation, such as that mediated by TLR3 [36, 37]. An impaired TLR3 response in fibroblasts is the only phenotype consistently associated with HSE [35-37] . In addition, the response of fibroblasts to poly(I:C) is TLR3-dependent, and their response to the neurotropic viruses VSV and HSV-1 is, at least largely, TLR3-dependent [35-37] (and unpublished data). We thus investigated the TLR3 pathway in fibroblasts from the patient bearing the hypomorphic *110-111insC NEMO* mutation (P), using NEMO-deficient fetal (IP) fibroblasts [41] and UNC-93B-deficient (UNC-93B$^{-/-}$) [35, 36] fibroblasts as controls. The induction of IFN-β and -λ by the TLR3 agonists poly(I:C) and IPH31 was impaired in P primary (Supplementary Figure 1) and SV40-fibroblasts (Fig. 1A), as shown by comparison with healthy control cells. IL-6 production in response to poly(I:C) and IPH31 was also weak in P SV40-fibroblasts (Fig. 1A). NEMO IP and UNC-93B$^{-/-}$ fibroblasts, used as negative controls, did not respond to these agonists (Fig. 1A). The response to intracellular delivery of poly(I:C) or IPH31, by transfection in the presence of Lipofectamine, was normal in P and UNC-93B$^{-/-}$ cells. All subsequent *in vitro* studies were performed with poly(I:C), the most potent known activator of TLR3 in cultured fibroblasts. We assessed the poly(I:C) dose-dependent production of IFN-β, -λ, and IL-6 in all SV40-fibroblast cell lines. The P SV40-fibroblasts displayed only a residual response, whereas NEMO IP and UNC-93B$^{-/-}$ SV40-fibroblasts did not respond at all (Fig. 1B). In addition, the induction of IFN-β, IFN-λ and IL-6 mRNA in response to poly(I:C), as assessed by RT-qPCR, was severely impaired in P SV40-fibroblasts at all time points, as shown by comparison with the healthy control, and was completely abolished in NEMO IP and UNC-93B$^{-/-}$ cells (Fig. 1C). In conclusion, the patient's cells displayed impaired, but not abolished production of IFN-β, IFNCλ and IL-6 in response to TLR3 agonists, suggesting that the NEMO allele is hypomorphic for this cellular phenotype.

Impaired TLR3-dependent IRF-3 and NF-κB activation in the patient's SV40-fibroblasts

Stimulation with poly(I:C) or with dsRNA associated with viral infections activates IKK-related kinases, resulting in the phosphorylation and nuclear translocation of the transcription factors NF-κB and IRF-3, and, at later stages, of IRF-7 [43]. Following activation by kinase-mediated phosphorylation, IRF-3 dimerizes and accumulates in the nucleus, where it regulates the expression of IFN genes [42]. In P SV40-fibroblasts, IRF-3 dimerization in response to poly(I:C) was strongly impaired in a time-course analysis, whereas it was abolished in NEMO IP and UNC-93B $^{-/-}$ SV40-fibroblasts (Fig. 2A). Nuclear factor-kappa B (NF-κB) complex DNA-binding activity, which plays a crucial role in the regulation of immune and inflammatory responses, was also impaired in P SV40-fibroblasts treated with poly(I:C) and IL-1β, whereas NEMO IP and UNC-93B$^{-/-}$ SV40-fibroblasts did not respond at all to poly(I:C) stimulation (Fig. 2B). In mouse embryonic fibroblasts (MEFs), constitutive IRF-3 activation controls IFN-β production, and IRF-7 is required not only for IFN-α production, but also for a full IFN signature [43]. It is therefore the major regulator of type I IFN immune responses [44]. We thus measured total IRF-7 protein levels after poly(I:C) stimulation for 16 hours, in P and control SV40-fibroblasts. Despite the variability of baseline protein levels in unstimulated cells, healthy control and P cells displayed similar increases in IRF-7 protein levels at 16 hours, whereas NEMO IP SV40-fibroblasts showed

no increase upon stimulation (Fig. 2C). Thus, the *110-111insC NEMO* mutation impaired the activation of NF-κB and IRF-3, but not that of IRF-7, in response to the TLR3 stimulation of SV40-fibroblasts.

Impaired control of TLR3-dependent viruses in the patient's SV40-fibroblasts

The production of IFN-β and IFN-λ in response to HSV-1 and VSV, a rodent neurotropic virus that is both cytopathic and a potent IFN inducer in human fibroblasts, was weaker in P than in control SV40-fibroblasts (Fig. 3A). NEMO IP and UNC-93B$^{-/-}$ SV40-fibroblasts displayed no IFN response to these two viruses. These results are consistent with the partial nature of the defect observed in P cells upon TLR3 stimulation. Mortality rates following VSV infection were higher in P SV40-fibroblasts than in those from the healthy control (Fig. 3B left panel). Similar results were obtained for HSV-1 infection (Fig. 3C left panel). Moreover, complementation of the cell death induced upon viral infection following treatment with exogenous recombinant IFN-α2b was observed for all patients' cells except with STAT1 deficient cells (Fig. 3B and 3C right panels). Thus, SV40-fibroblasts from the patient carrying the *110_111insC NEMO* mutation displayed impaired IFN induction in response to TLR3 stimulation, resulting in higher levels of virus-induced cell death compared to controls' SV40-fibroblasts. These findings therefore provide a possible molecular basis for the pathogenesis of HSE in this patient.

DISCUSSION

We know, from the mutations conferring predisposition to HSE in human *TLR3*, *UNC93B1*, *TRAF3*, and *STAT1*, that the TLR3-IFN pathway is critical for protective immunity to HSV-1 primary infection in the CNS, at least in some children. By inference from the fibroblastic phenotype, the pathogenesis of HSE observed in patients with defects in the TLR3-IFN pathway involves impaired TLR3-dependent IFN-α/-β- or -λ-mediated anti-HSV-1 immunity, resulting in enhanced HSV-1 replication, and virus-induced cell death in the CNS [35-37]. In this study, we elucidated the molecular pathogenesis of HSE in a child carrying the *110_111insC* mutation in *NEMO*. The P SV40-fibroblasts displayed severe impairment of IFN -β and -λ production, confirming the requirement of the TLR3-IFN signaling pathway for protective immunity to HSE in childhood. We also observed high levels of cell death in NEMO *110_111insC* cells following viral infection *in vitro*, with a phenotype intermediate between cells from healthy controls and from patients with HSE and complete deficiencies of TLR3, UNC-93B, or STAT1 [33, 35, 36]. Moreover, cell viability was restored by prior treatment with recombinant IFN-α2b, as observed in NEMO- and UNC-93B-deficient cells but not STAT1-deficient cells, demonstrating that the cell mortality rate resulted from decreased IFN production rather than impaired IFN responses. It is unclear whether HSE in these patients results from the impaired production of IFN-α/β, IFN-λ, or both. The identification of patients with mutations in a single gene encoding an anti-viral IFN or its receptor would provide an answer to this question [45, 46]. Nevertheless, the impairment of the TLR3-IFN pathway itself, as documented in dermal fibroblasts in this report, is at least sufficient to account for HSE in the patient studied here.

Our findings also show that a *NEMO* mutation can impair not only the activation of NF-κB, but also that of IRF-3. Indeed embryonic fibroblasts from an IP fetus with complete NEMO deficiency [41] displayed abolition of the induction of both pathways upon TLR3 activation, suggesting an interconnection. This is consistent with recent studies implicating NEMO in the recruitment of kinases for the phosphorylation of the IRF-3 and IRF-7 transcription factors [42]. Signaling to IRF-3 occurs through two divergent members of the IKK family, IKK-ε and TBK1, which can also directly phosphorylate IRF-7 [47, 48]. It has been reported that a NEMO-interacting protein known as TANK (TRAF family member-associated NF-κB activator) is involved in regulating NF-κB activity via an association with IKK-ε and

TBK1 [49]. The patient's cells displayed impaired activation of both NF-κB and IRF-3 in response to TLR3 stimulation, accounting for the defect in IFN production in response to poly(I:C) stimulation. The low level of IRF-3 activation in cells lacking NEMO or carrying the *110_111insC NEMO* mutation may result from impaired direct or indirect interactions of NEMO with TBK1 and IKK-ε [42, 49]. Unlike NEMO IP SV40-fibroblasts, in which no IRF-7 protein induction was detected in response to poly(I:C), the P SV40-fibroblasts displayed normal IRF-7 protein induction in response to poly(I:C) stimulation. This may be consistent with a partial NEMO defect due to translation re-initiation [15], impairing the activation of IRF-3 and, to a lesser extent, NF-κB, but not the induction of IRF-7, which, independently or in concert, contributes to the residual IFN production in P SV40-fibroblasts. Altogether, these data suggest that NEMO is a key component in the signaling pathway governing immunity to HSV-1 in the CNS: TLR3 – UNC-93B – TRAF3 - NEMO - IRF-3/NF-κB - IFN.

Another question of clinical relevance is whether children carrying other mutations in *NEMO* may also be prone to HSE [1-10, 12, 14, 15, 19, 21, 24, 50-65]. We assessed the response to TLR3 (poly(I:C)) stimulation in 10 SV40-fibroblastic cell lines carrying various *NEMO* mutations (Supplementary Table 1). In all cell lines, we found that the induction of IFN-β and IFN-λ was impaired to some degree (Supplementary Figure 2A). We also assessed IRF-3 dimerization in the NEMO-mutated SV40-fibroblast cell lines from patients P1 to P8. Impaired dimerization was observed in the cells of all these patients, and the degree of impairment was similar in most cases to that observed for NEMO IP or the patient under study (Supplementary Figure 2B). Based on these results, patients bearing the *NEMO* mutations tested, and possibly patients bearing other hypomorphic *NEMO* mutations, are potentially vulnerable to HSE. Our data suggest that even a partial NEMO defect may impair protective immunity to HSV-1 in the CNS in the course of primary infection. Nevertheless, some of these patients have been infected with HSV-1 without developing HSE. This incomplete clinical penetrance is not entirely surprising, as it has already been reported for TLR3 and UNC-93B defects [35, 36, 66]. Many factors may affect clinical penetrance, including age at infection with HSV-1, the viral inoculum and human modifier genes [67]. It remains unclear which patients with mutations in *NEMO* actually display clear genetic susceptibility to HSE. The fibroblastic TLR3 response may be used to predict the clinical risk of HSE, taking into account the incomplete clinical penetrance of all known HSE-predisposing genetic defects, including mutations in *NEMO*. Of particular clinical value, our findings suggest that HSE is unlikely to occur in a patient carrying a mutation in *NEMO* but with a normal TLR3 response.

Clinical implications

Mutations in the NF-κB essential modulator (NEMO) affect the activation of both NF-κB and IRF-3 in response to TLR3 stimulation and may therefore increase susceptibility to HSE.

Capsule Summary

Mutations in the NF-κB essential modulator (NEMO) affect the activation of both NF-κB and IRF-3 in response to TLR3 stimulation and may therefore increase susceptibility to herpes simplex encephalitis in children.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

EDA-ID	Anhidrotic ectodermal dysplasia with immunodeficiency
IKK	Inhibitor of nuclear factor-κB kinase complex
NEMO	Nuclear factor-κB essential modulator
NF-κB	Nuclear factor-κB
IRF-3/7	Interferon regulatory factor 3/7
TLR	Toll-like receptor
HSE	Herpes simplex encephalitis
HSV-1	Herpes simplex virus-1
VSV	Vesicular stomatitis virus

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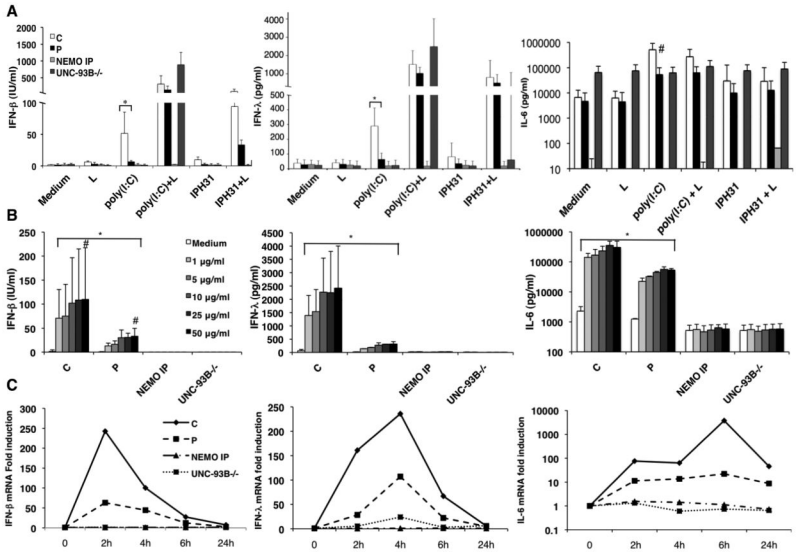

Figure 1. Induction of IFN-β, IFN-λ and IL-6 in response to TLR3 agonists

(A) Production of IFN-β, IFN-λ and IL-6 by SV40-fibroblasts from a healthy control (C), the patient (P), a patient with complete NEMO deficiency (NEMO IP) and a patient with UNC-93B deficiency (UNC-93B$^{-/-}$), unstimulated or stimulated with poly(I:C) and IPH31, without or with (poly(I:C)+L and IPH31+L) Lipofectamine (L), assessed by ELISA. **(B)** Production of IFN-β, IFN-λ and IL-6 by SV40-fibroblasts in response to various doses of poly(I:C) for 24 hours, assessed by ELISA. For A and B, the panels show mean values ± SD of three independent experiments. The reported p values for differences between the responses of patients and controls are for unpaired Student's t tests for each experiment (*: $p<0.05$; #: $p<0.07$). **(C)** IFN-β, IFN-λ and IL-6 mRNA levels in SV40-fibroblasts left unstimulated or stimulated for 2, 4, 6 or 24 hours with poly(I:C). The panels illustrate results from two independent experiments performed in duplicate.

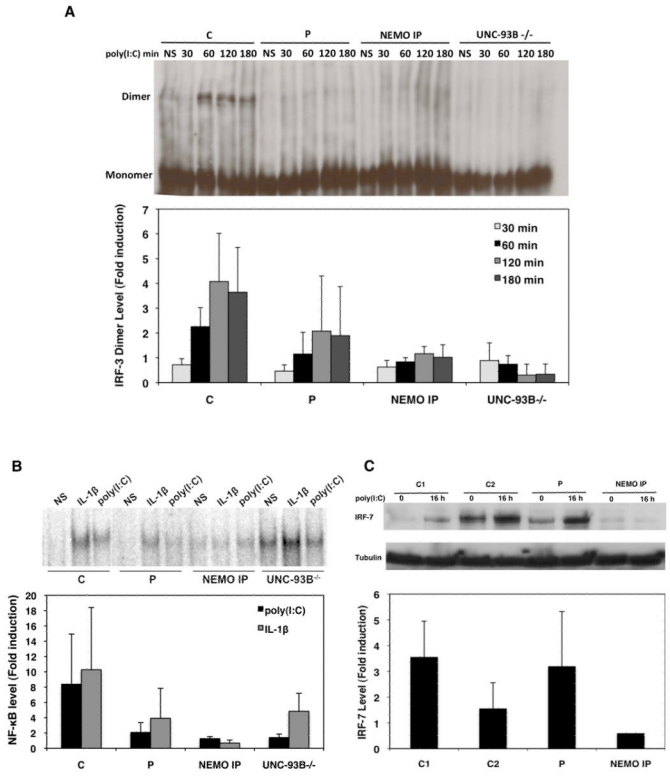

Figure 2. Induction of NF-κB, IRF-3 and IRF-7 in response to poly(I:C)

(A) IRF-3 monomer and dimer formation in total cell extracts from healthy control (C), the patient (P), NEMO IP and UNC-93B $^{-/-}$ SV40-fibroblasts, upon stimulation with poly(I:C) for 30 to 180 minutes, as assessed by western blotting. Fold-induction of IRF3 dimers, presented below as a histogram, was determined from the intensity of the signal on immunoblots, normalized with respect to monomer levels. **(B)** NF-κB translocation, assessed by EMSA, in SV40-fibroblasts from a control C, the patient P, NEMO IP and UNC-93B $^{-/-}$ cells, following stimulation with poly(I:C) for 1 hour and with IL1-β for 20 minutes. The panels are representative of two independent experiments. **(C)** IRF-7 induction in SV40-fibroblasts after 16 hours of poly(I:C) stimulation, analyzed by western blotting and normalized with respect to tubulin levels. This panel is representative of three independent experiments.

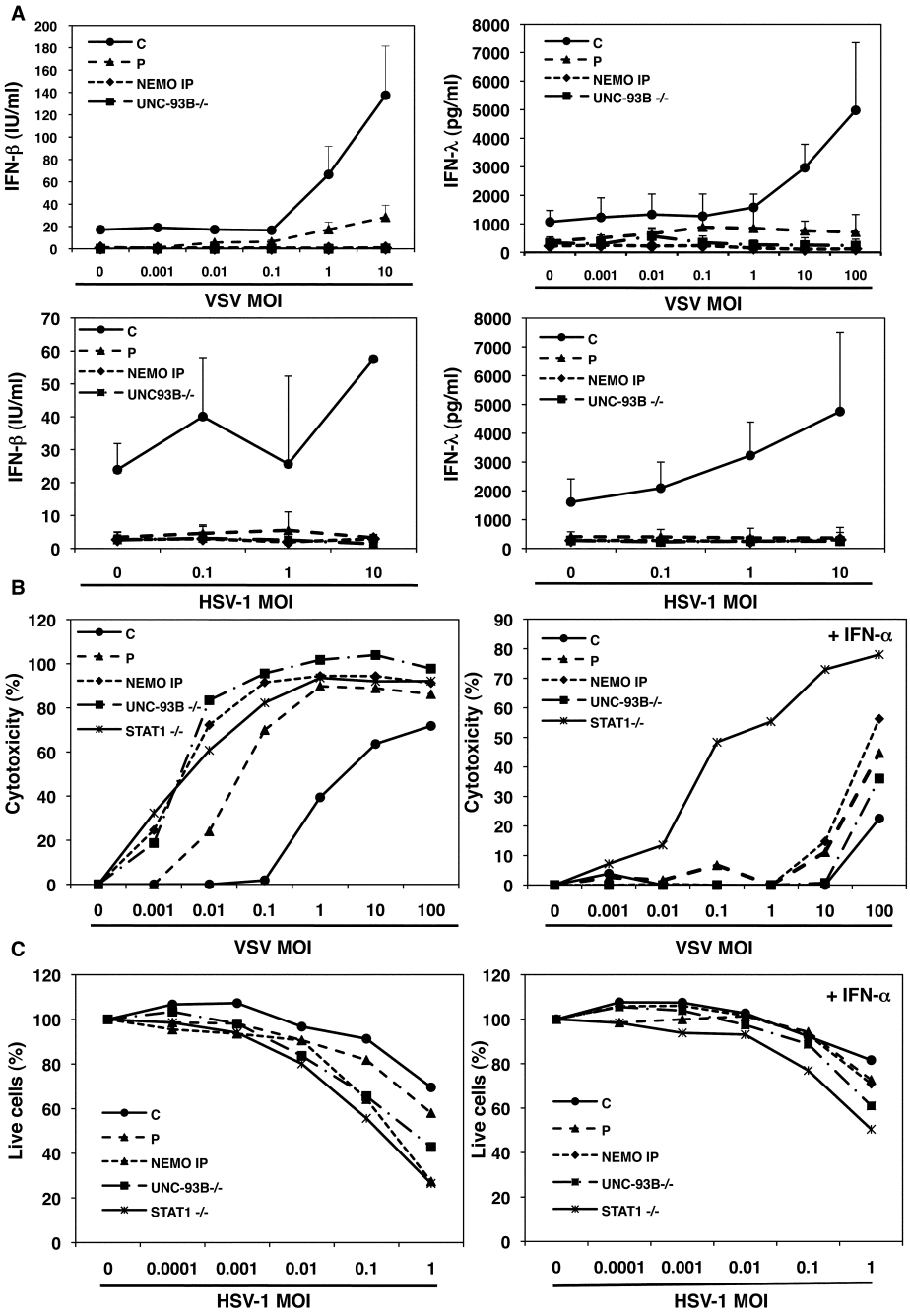

Figure 3. IFN production and cell mortality in response to viral stimulation

(A) SV40-fibroblasts from a healthy control (C), the patient (P), a NEMO IP patient and an UNC-93B $^{-/-}$ patient were left uninfected or infected with VSV (MOI from 0.001 to 100) for 24 hours (upper panels) or with HSV-1 (MOI from 0.0001 to 1) (lower panels) for 72 hours. The production of IFN-β (left panels) and IFN-λ (right panels) was assessed by ELISA. **(B)** Cell mortality was estimated by LDH assay, 24 hours after infection with VSV at various MOI, without (left panel) or with (right panel) prior treatment with IFN-α2b. **(C)** The percentage of live cells was estimated by resazurin oxidation/reduction, 72 hours after infection with HSV-1, at various MOI, without (left panel) or with (right panel) prior treatment with IFN-α2b.