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

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Background: Children with germline mutations in Toll-like receptor 3 *(TLR3)*, *UNC93B1*, TNF receptor–associated factor 3, and signal transducer and activator of transcription 1 are prone to herpes simplex virus-1 encephalitis, owing to impaired TLR3-triggered, UNC-93B-dependent, IFN-α/β, and/or IFN-λ-mediated signal transducer and activator of transcription 1-dependent immunity.

Objective: We explore here the molecular basis of the pathogenesis of herpes simplex encephalitis in a child with a hypomorphic mutation in nuclear factor-κB *(NF-κB)* essential modulator, which encodes the regulatory subunit of the inhibitor of the Iκβ kinase complex.

Methods: The TLR3 signaling pathway was investigated in the patient's fibroblasts by analyses of IFN-β, IFN-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 electrophoretic mobility shift assay and interferon regulatory factor 3 dimerization on native gels after stimulation with a TLR3 agonist.

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

Conclusion: Herpes simplex encephalitis may occur in patients carrying *NF-κB* essential modulator mutations, due to the impairment of NF-κB- and interferon regulatory factor 3-dependent-TLR3-mediated antiviral IFN production.

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Key words: *NEMO, immunodeficiency, Toll-like receptor 3, herpes simplex encephalitis*

In humans, loss-of-function mutations in the gene encoding nuclear factor-κB (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) (XR-EDA-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 since some boys have a much more severe clinical presentation, with osteopetrosis and lymphedema (OL) (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]

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Abbreviations used
CNS: Central nervous system
EDA-ID: Anhidrotic ectodermal dysplasia with immunodeficiency
HSE: Herpes simplex encephalitis
HSV-1: Herpes simplex virus-1
IKK: Inhibitor of nuclear factor-κB kinase complex
IP: Incontinentia pigmenti
IRF-3/7: Interferon regulatory factor 3/7
MOI: Multiplicity of infection
NEMO: Nuclear factor-κB essential modulator
NF-κB: Nuclear factor-κB
STAT-1: Signal transducer and activator of transcription 1
SV40: Simian virus 40
TLR: Toll-like receptor
TRAF3: TNF receptor–associated factor 3
VSV: Vesicular stomatitis virus
XR: X-linked recessive

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 reinitiation 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 PBMCs, possibly accounting for mycobacterial disease.[23,24,25] 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 classic primary immunodeficiencies are not prone to HSE.[31] Autosomal recessive (AR) signal transducer and activator of transcription 1 (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) Toll-like receptor 3 (TLR3),[36] and AD TNF receptor–associated factor 3 (TRAF3)[37] deficiencies. The fibroblasts of these patients displayed impaired induction of IFN-β and IFN-λ in response to TLR3 stimulation. These data are consistent with the lack of HSE in interleukin-1 receptor-associated kinase 4- and myeloid differentiation primary response gene (88)-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 IFN-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 ethics committee approvals of Necker-Enfants Malades Hospital, Paris, France, and of The Rockefeller University.

Toll-like receptor 3 agonists, viral infection, and ELISA

We used a synthetic analogue of double-stranded RNA (dsRNA) (polyinosinepolycytidylic acid, poly[I:C]; GE Healthcare, Buckinghamshire, United Kingdom), a nonspecific TLR3 agonist, at various concentrations (1-50 μg/mL), and IPH31 (an optimized poly[A:U] dsRNA, specific agonist of TLR3, provided by Innate-Pharma, Marseilles, France) with or without Lipofectamine 2000. For viral stimulation, we used the double-stranded DNA virus HSV-1 (strain KOS-1, multiplicity of infection [MOI] = 1) and the negative-sense single-stranded RNA virus vesicular stomatitis virus (VSV, strain Indiana, MOI = 1). Primary fibroblasts and simian virus 40 (SV40)-transformed fibroblasts (SV40-fibroblasts) were taken 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-93B$^{-/-}$ cells derived from a patient with HSE carrying the homozygous mutation *1034del4*[35]) were grown in 24-well plates (10^5 cells/well) with Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, Calif), 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 (Toray-Fuji Bionics, Fujirebio, Inc, Malvern, Pa), IL-6 (Sanquin, Amsterdam, The Netherlands), and IFN-λ, as previously described.[35]

Signal transduction studies in fibroblasts

Immunoblotting for interferon regulatory factor (IRF)-7 was carried out with an mAb against the human IRF-7 (sc-744472; Santa Cruz Biotechnology, Santa Cruz, Calif). An antibody against tubulin (sc-23948, Santa Cruz Biotechnology) was used to control for protein loading for each sample. NF-κB 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, St Louis, Mo). Cell mortality was assessed by measuring the amount of lactate dehydrogenase released into the medium, according to the manufacturer's protocol (Roche, Paris, France).

RESULTS

Impaired TLR3 responses in the patient's fibroblasts

Dermal fibroblasts naturally and selectively express TLR3 and can secrete both IFN-β and IFN-λ constitutively and on