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Uncovering novel genetic etiologies of childhood herpes simplex encephalitis: hypothesis-based candidate gene approach

Melina Herman

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**THESE DE DOCTORAT DE
L'UNIVERSITE PIERRE ET MARIE CURIE**

ED 515 Complexité du Vivant
Spécialité Génétique

Présentée par
Melle Melina Herman

Pour obtenir le grade de
DOCTEUR de l'UNIVERSITÉ PIERRE ET MARIE CURIE

Sujet de la thèse: Uncovering novel genetic etiologies of childhood Herpes Simplex Encephalitis:
Hypothesis-based candidate gene approach

soutenue le 6 Décembre 2012

devant le jury composé de

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List of Abbreviations:

AD, autosomal dominant

AR, autosomal recessive

CNS, central nervous system

dsDNA, double-stranded DNA

dsRNA, double-stranded RNA

EMCV, encephalomyocarditis virus

gDNA, genomic DNA

HCV, Hepatitis C Virus

HSE, HSV-1 encephalitis

HSV-1, Herpes Simplex Virus 1

MEF, mouse embryonic fibroblast

MOI, multiplicity of infection

mRNA, messenger RNA

NDV, Newcastle disease virus

Para III virus, parainfluenza III virus

poly(A:U), polyadenylic-polyuridylic acid

poly(dA:dT), poly(deoxyadenylic-deoxythymidylic) acid

poly(I:C), polyinosinic-polycytidylic acid

RT-qPCR, quantitative RT-PCR

SNP, single nucleotide polymorphism

ssRNA, single-stranded RNA

SV40, simian virus 40

VSV, vesicular stomatitis virus

Summary:

Herpes Simplex Encephalitis (HSE) is the most common form of sporadic viral encephalitis in Western countries. Herpes Simplex Virus 1 (HSV-1) infection is usually benign or asymptomatic but may also result in a widespread infection of the central nervous system (CNS). HSE occurs in about one child per 250,000 births each year and is a devastating illness of unclear pathogenesis. The discovery of the first genetic etiologies of HSE in patients with deficiencies in *TLR3*, *UNC93B1*, *TRIF* and *TRAF3* pointed to the essential role of the TLR3-UNC-93B-TRIF-TRAF3-dependent, interferon (IFN)- α/β - and - λ -mediated immunity in controlling HSV-1 infection in the CNS. These findings argued that HSE results from single-gene inborn errors of TLR3 immunity in some patients. Using a candidate gene approach, we investigated the possibility of HSE-predisposing mutations in the gene *TBK1*, encoding TANK-binding kinase 1, a kinase at the crossroads of multiple IFN-inducing signaling pathways, including the TLR3 pathway. We sequenced our cohort of 160 HSE patients for *TBK1* and we describe here two unrelated children with HSE carrying different heterozygous unreported mutations (G159A and D50A) in *TBK1*. Both mutant *TBK1* alleles are loss-of-function, but through different mechanisms: protein instability (D50A) or loss of kinase activity (G159A). Both are also associated with an autosomal dominant (AD) trait, but by different mechanisms: haplotype-insufficiency (D50A) or negative dominance (G159A). A defect in poly(I:C)-induced TLR3 responses can be detected in fibroblasts heterozygous for G159A, but not for D50A *TBK1*. Nevertheless, viral replication and cell death rates due to two TLR3-dependent viruses (HSV-1 and Vesicular Stomatitis Virus) were high in fibroblasts from both patients, and particularly so in G159A *TBK1* fibroblasts. These phenotypes were rescued by IFN- α 2b. Moreover, the IFN responses to the TLR3-independent agonists and viruses tested were maintained in both patients' PBMCs and fibroblasts. The narrow, partial cellular phenotype thus accounts for the clinical phenotype of these patients being limited to HSE. These data identify AD partial *TBK1* deficiency as a new genetic etiology of childhood HSE, indicating that *TBK1* is essential for the TLR3- and IFN-dependent control of HSV-1 in the CNS.

Keywords: Immunity, TLR3, *TBK1*, Herpes Simplex Encephalitis, Interferon, HSV-1.

Résumé en français:

Deux mutations hétérozygotes du gène *TBK1* impactent l'immunité médiée par TLR3 et prédisposent à l'encéphalite herpétique chez l'enfant

Introduction

L'encéphalite herpétique (EH) est la forme la plus fréquente d'encéphalite virale sporadique. Elle se développe chez un individu sur 250.000 chaque année et il s'agit de l'une des infections virales les plus sévères du système nerveux central (SNC). L'apparition de drogues antivirales telles que l'acyclovir en 1981 a conduit à une diminution du taux de mortalité associé à l'EH, mais de sévères séquelles sont toujours communément observées. Un tiers des cas d'EH sont la conséquence d'une infection primaire par le virus de l'herpès simplex-1 (HSV-1), un virus de la famille des Herpesviridae, au génome formé d'un ADN double brin (ADNdb), et arrivent surtout chez les enfants de moins de 16 ans. Les autres cas sont le plus probablement causés par la réactivation du virus HSV-1 latent. L'EH n'est pas plus fréquente chez les patients immuno-supprimés ou immuno-déprimés que chez les individus immuno-compétents. De plus, l'EH n'a jamais été associée avec l'infection par une souche spécifique de HSV-1. Le virus HSV-1 est ubiquitaire: plus de 80% des adultes sont infectés, et cette infection est asymptomatique ou bénigne. Cependant HSV-1 peut également provoquer une infection du SNC, l'EH, par un mécanisme de pathogenèse encore inconnu.

Récemment, l'identification par notre laboratoire des premières étiologies génétiques de l'EH impliquant l'axe TLR3-IFN, a apporté les premiers éléments de réponse concernant la pathogenèse de la maladie. Nous avons décrit des patients ayant souffert de l'EH et portant des mutations dans les gènes *TLR3*, *UNC93B1*, *TRIF* et *TRAF3*. Ces gènes codent pour des protéines de la voie de signalisation TLR3. Toll-Like Receptor 3 détecte les doubles brins d'ARN produits par HSV-1 lors de sa réplication. Les patients déficients pour *TLR3*, *UNC93B1*, *TRIF* et *TRAF3* partagent un même phénotype cellulaire: leurs fibroblastes manquent de produire des interférons (IFN) de type I et III après infection par HSV-1, ce qui résulte en une réplication virale plus élevée et une mort cellulaire plus importante que dans des cellules contrôles. Nous postulons que ce phénotype récapitule

le mécanisme de pathogenèse du virus dans le SNC de ces patients. Suivant l'hypothèse que l'EH résulte d'une collection de défauts monogéniques de l'immunité anti-HSV1, nous avons criblé notre cohorte de patients EH qui ne portent pas de mutations pour les gènes précédemment identifiés comme prédisposant à l'EH, pour des variations dans le gène *TBK1*, qui code pour la TANK-Binding Kinase-1, la kinase phosphorylant IRF3 dans la voie de signalisation de TLR3.

Résultats

Nous avons étudié le cas de deux patientes européennes qui ont développé une EH pendant l'enfance et qui portent deux variations hétérozygotes différentes, non encore décrites, dans le gène *TBK1*. P1, de Pologne, a souffert de l'EH à 7 ans, et P2 à 11 mois. Les deux patientes n'ont jusque là présenté aucune autre infection. Les deux mutations dans *TBK1*, c.476G>G/C (P1) et c.149A>A/C (P2), ont pour conséquence des changements d'acide aminé non-conservateurs p.159G>G/A, et p.50D>D/A, respectivement. Les deux mutations affectent des résidus conservés dans le domaine kinase de TBK1. Ces variations n'ont pas été observées chez plus de 1050 contrôles sains fournis par le CEPH-HGD, éliminant donc la possibilité de polymorphismes.

Nous avons tout d'abord évalué le niveau d'expression de *TBK1* dans les fibroblastes des patientes, qui sont hétérozygotes pour ces allèles mutants. Dans les cellules de P1, à l'échelle de l'ARN messager (ARNm) et de la protéine, le niveau d'expression de TBK1 est similaire à celui des cellules contrôles. En revanche, dans les cellules de P2, la quantité de TBK1 totale est diminuée, au niveau de l'ARNm et de la protéine. Nous avons également testée l'expression de nos allèles mutants par expression hétérologue dans des cellules HEKs et dans des fibroblastes murins embryonnaires déficients pour TBK1 (*TBK1*^{-/-} MEFs). Dans les deux cas, l'expression de l'allèle de P1 était comparable à celle de l'allèle WT TBK1. En revanche, l'allèle de P2 ne permet pas l'expression de la protéine TBK1. Nous avons donc supposé que l'allèle de P2 confère une perte-de-fonction, probablement due à l'instabilité de la protéine mutante D50A. Utilisant des tests d'activité kinase in vitro, nous avons évalué l'impact des mutations sur l'activité

enzymatique de la protéine. Les niveaux de phosphorylation obtenus *in vitro* ont montré que TBK1 G159A se comporte comme le mutant kinase-mort K38M, utilisé comme contrôle négatif dans ces expériences *in vitro*. Comme discuté ci-dessus, D50A TBK1 a été produite dans des quantités beaucoup plus faibles que les protéines WT, G159A, ou K38M, donc la phosphorylation du substrat n'a certainement pas pu être observée. Nous avons ensuite testé la fonction des deux protéines mutantes sur le plan de l'activation de la voie TLR3, dans les MEFs TBK1^{-/-}, par transfection des allèles D50A, G159A, ou WT de TBK1. La transfection de MEFs TBK1^{-/-} avec les allèles TBK1 D50A ou G159A n'a pas restauré la réponse à poly(I:C), un agoniste de TLR3, tandis que cette induction a été restaurée par transfection de l'allèle WT TBK1. Ainsi, l'allèle mutant G159A TBK1 est normalement exprimé mais produit une protéine kinase-morte, et l'allèle D50A est un allèle perte-d'expression qui a pour résultat une perte d'activité kinase. Les deux mutants présentent donc une perte-de-fonction pour les réponses cellulaires médiées par TLR3.

Nous avons ensuite testé l'hypothèse que les allèles G159A et D50A TBK1 sont associés avec une forme autosomale dominante (AD) de l'EH en étudiant la réponse des fibroblastes des patientes hétérozygotes à poly(I:C), qui active TLR3 quand il est délivré de manière extracellulaire. La phosphorylation et subséquente dimerization du facteur de transcription IRF3 après la stimulation par poly(I:C) sont considérablement diminuées dans les fibroblastes de P1, alors qu'elles sont similaires aux contrôles dans les fibroblastes de P2. Le manque de production d'IFN dans les cellules de P1 après stimulation par poly(I:C) est sauvé par la sur-expression de TBK1 WT, démontrant que l'allèle TBK1 G159A est dominant et responsable de l'abolition de la réponse à poly(I:C) dans les fibroblastes de P1. De plus, nous avons déterminé le mécanisme de dominance de l'allèle de P1 par transfection de fibroblastes contrôles avec les allèles TBK1 mutants ou WT, suivie de l'évaluation de la réponse à poly(I:C). L'allèle G159A, contrairement à D50A, exerce une dominance négative sur l'allèle WT. L'Hétérozygotie pour l'allèle G159A réduit sévèrement la production d'IFNs IRF3-dépendante en réponse à l'activation de TLR3, tandis que l'allèle TBK1 D50A ne mène à aucune diminution discernable de la réponse TLR3-dépendante à poly(I:C) dans les fibroblastes humains.

La production d'IFN en réponse à l'infection par HSV-1 et Vesicular Stomatitis Virus (VSV) dans les fibroblastes humains est partiellement dépendante de TLR3. Les fibroblastes de patients déficients pour TLR3, UNC-93B, TRIF et TRAF3 ne contrôlent ni la réplication d'HSV-1, ni celle de VSV, en raison d'un manque de production d'IFN. Les fibroblastes de P1 ne produisent pas d'IFN 24 heures après l'infection avec HSV-1. Les cellules de P1 ont montré des niveaux plus élevés que la normale de réplication virale et de mort cellulaire, comme les cellules de patients déficients pour TLR3 et UNC-93B. L'addition d'IFN- α à ces cellules a restauré une réplication du virus à un niveau similaire à celui des cellules contrôles. De plus, les fibroblastes de P1 et P2 ne produisent pas d'IFN après infection avec VSV. Les cellules des patientes ne réussissent pas à limiter la réplication de VSV comme les cellules contrôles. Les niveaux de mort cellulaire induite par VSV sont également plus élevés dans les cellules des deux patientes TBK1 que dans les contrôles, comme dans les cellules de patients déficients pour TLR3 et UNC-93B. Le traitement de ces cellules avec l'IFN- α restaure la réplication virale et la mort cellulaire aux niveaux similaires à ceux des cellules contrôles. Le contrôle IFN-dépendant de HSV-1 et VSV, qui est au moins partiellement médié par TLR3, est donc diminué dans les cellules des patientes déficientes pour TBK1. Les fibroblastes hétérozygotes pour l'allèle D50A ont montré un contrôle réduit du virus via TLR3, malgré une réponse normale à poly(I:C) via TLR3. Ceci suggère que la voie TLR3 entière, intacte, TBK1-dépendante est exigée pour contrôler HSV-1 et VSV dans les fibroblastes humains, bien que nous ne pouvons pas exclure un rôle possible de TBK1 dans l'inhibition de la réplication de HSV-1, d'une manière dépendante ou non de TLR3; ou dans l'induction de mort cellulaire, deux rôles décrits pour TBK1 dans d'autres contextes.

TBK1 est aussi impliqué dans les voies de signalisation de TLR4; RIG-I/MDA5, activées par la présence dans le cytosol d'ARNdb (poly(I:C)) ou ARN simple brin (ARNsb); et par les voies cytosoliques activées par la présence d'ADNdb (poly(dA:dT)). Les PBMCs de P1 répondent normalement au ligand de TLR4, et les fibroblastes des deux patientes produisent des niveaux normaux d'IFN après transfection de poly(dA:dT), poly(I:C), et ARNsb. Aucun phénotype majeur indépendant de TLR3 n'a été observé dans les cellules de P1 et P2. Il est toujours incertain si la production normale d'IFN qui suit l'activation de TLR4 et de détecteurs cytosoliques d'acides nucléiques dans les cellules de P1 et P2 est

due à la présence résiduelle de WT TBK1 dans ces cellules avec un défaut partiel de TBK1, ou est due à la contribution non-essentielle de TBK1 à ces voies de signalisation dans les PBMCs et fibroblastes humains. Nous avons également infecté les PBMCs de P1 et P2 avec divers types de virus, y compris des virus à ARNsb de sens positifs (le virus d'encephalomyocarditis (EMCV), le virus de Sindbis), des virus à ARNsb de sens négatifs (Sendai, le virus de parainfluenza humain III (para-III), le virus de la maladie de Newcastle (NDV), le virus des oreillons, le virus de la rougeole) et des virus à ADNdb (le virus de BK, HSV-1). Tous ont induit une production d'IFN- α et IL-6 normale dans ces cellules. Ceci peut refléter la nature partielle des défauts génétiques portés par les patientes, et/ou la redondance de TBK1 et IKKi dans l'activation d'IRF3 et IRF7 dans les leucocytes. Nous avons infecté les fibroblastes de P1 et P2 avec para-III, le virus de la rougeole, EMCV, ou le virus de Sindbis. Les fibroblastes des deux patientes se sont comportés comme les cellules contrôles en termes de leur capacité pour produire de l'IFN- β et - λ . Ce résultat contraste avec la réponse réduite de ces cellules à l'infection avec HSV-1 ou VSV, deux virus qui activent TLR3 pour établir l'induction d'IFN. Le manque de contrôle du virus dans les fibroblastes de patientes hétérozygotes pour TBK1 est ainsi limité aux virus neurotropiques HSV-1 et VSV. Ces résultats dans les PBMCs et fibroblastes sont cohérents avec le phénotype clinique limité observé dans les deux patientes déficientes pour TBK1. Ils suggèrent que l'induction résiduelle d'IFN, dépendante de TBK1 et médiée par TLR3 et/ou par les autres récepteurs dans les cellules avec un défaut partiel de TBK1, contribue à l'immunité protectrice contre la plupart des virus communs.

Conclusion

En utilisant une approche de gène candidat, nous avons identifié le défaut AD de TBK1 comme une nouvelle étiologie de l'EH chez deux patientes européennes. Ce défaut confère une diminution des réponses médiées par TLR3 dans les fibroblastes des patientes, confirmant le rôle essentiel de TBK1 comme une kinase induisant la production d'IFNs dans la voie TLR3 dans les fibroblastes humains. Les deux allèles mutants sont de type perte-de-fonction, un par perte d'activité kinase (G159A, P1), et

l'autre par perte d'expression (D50A, P2). De façon intéressante, les deux patientes déficientes pour TBK1 présentent des phénotypes cellulaires différents, celui de la patiente avec la mutation G159A étant plus sévère, du au fait que cet allèle est dominant-négatif tandis que l'allèle D50A exerce la dominance par haploinsuffisance. Le défaut de TBK1 est associé avec un phénotype cellulaire restreint, qui correspond au phénotype clinique limité des patientes. Bien que nous ne pouvons pas étudier la pénétrance cellulaire, nous avons déterminé que la pénétrance clinique est incomplète, comme la pénétrance des défauts d'UNC-93B, TLR3 et TRIF. Nos données ont souligné le rôle crucial de TBK1 dans l'immunité anti-HSV-1 médiée par TLR3. La description de défauts dans les gènes TLR3, UNC-93B, TRIF, TRAF3 et TBK1 chez les patients de l'EH renforce l'argument que l'EH devrait être considérée comme une maladie génétique causée par un groupe d'erreurs innées rares monogéniques de l'immunité médiée par TLR3, chez un sous-ensemble de patients. Ces conclusions suggèrent aussi que l'IFN devrait être essayé dans le traitement des patients souffrant d'EH.

I. Introduction

I. 1. Herpes Simplex Encephalitis

I.1.i. Pathology and diagnosis of the disease

Herpes Simplex Encephalitis (HSE) is the most frequent form of sporadic viral encephalitis. It occurs in 1 in 250,000 individuals every year and it is one of the most severe viral infections of the brain. About one third of HSE cases are the consequence of primary infection with Herpes Simplex Virus-1 (HSV-1), most often in children under 18 years of age (1). The remaining cases are most likely caused by reactivation of HSV-1 from latency. HSE does not occur more frequently in immuno-suppressed or immuno-depressed patients than in apparently immuno-competent individuals. In addition, HSE does not display a geographical or seasonal pattern of occurrence, suggesting that non-viral environmental factors are not likely to play a role (2). Moreover, HSE is not associated with infection by a specific HSV-1 strain (3). The diagnosis of HSE is routinely based on detection by PCR of HSV-1 DNA in the cerebrospinal fluid (4), along with detection by scan or MRI of typical HSE aberrations (5, 6), such as damage due to inflammation or hemorrhage mainly localized in the temporal lobes. The emergence of antiviral drugs like acyclovir in 1981 helped decrease the mortality rate associated with HSE, but severe sequelae are still common (7, 8).

I.1.ii. Herpes Simplex Virus-1

HSV-1 is a double stranded DNA (dsDNA) virus of the Herpesviridae family. This family encompasses three virus classes: α , β , γ , divided on the basis of biological properties (9). HSV-1 is part of the alphaherpesviridae, a family characterized by a variable host range and the capacity to establish latent infections in sensory ganglia. Within the alphaherpesviridae is the genus *Simplexvirus*, which includes 8 viruses of humans: HSV-1; HSV-2; Human Herpesvirus-3 (HHV-3) or Varicella-zoster virus; HHV-4 or Epstein-Barr virus (EBV); HHV-5 or Cytomegalovirus; HHV-6; HHV-7; and

HHV-8, or Kaposi's sarcoma-associated HV (10). The genus *Simplexvirus* can cause a variety of infections. HSV-1 infection today is ubiquitous, as over 85% of young adults are serologically positive for antibodies against the virus (11). HSV-1 infection results in most cases in asymptomatic or benign infection, such as oro-labial, facial or ocular infections, but can also lead to severe encephalitis (HSE) of unclear pathogenesis (12). HSV-1 is transmitted via direct contact with infectious secretions or via respiratory droplets from an infected individual; infection typically takes place at the oral or nasal epithelium. The virus replicates briefly at the site of infection; it then enters the epithelial endings of sensory neurons and uses retrograde axonal transport along the olfactory or trigeminal nerve to establish latency in the trigeminal ganglion (13).

NK cells (14), IFNs (15) and CD8⁺ T cells (16) are essential components of the antiviral response to HSV-1 in mice. In addition, several groups confirmed the importance of macrophage maturation in age-related resistance to HSV-1 infection in mice (17). In humans, the small number of NK cell-deficient patients described did not suffer from HSE (18-20), despite presenting other Herpesviridae infections. Patients with CD8⁺ T cell deficiency do not suffer from HSE either (21). HSV-1 infection in neonates may result in a disseminated sepsis-like disease. While the role of macrophages in fulminant HSV-1 infection in neonates is not documented, this age-dependent susceptibility may be due to an abnormally increased TLR2-mediated production of anti-inflammatory cytokines (22-24). HSV-1 triggers IFN production in various cell types in humans and mice, including plasmacytoid dendritic cells (pDCs), macrophages, fibroblasts (25-27) as well as neurons, oligodendrocytes and astrocytes (28). Several innate immune pathways are triggered by HSV-1 infection to induce IFNs, particularly those involving TLR2 (29), TLR9 (30, 31), RIG-I/MDA5 (32), STING (33) and UNC-93B (34). The specific role of the innate immune response in the control of HSV-1 infection in humans is further detailed in section I. 2.

I.1.iii. Susceptibility to HSE

Although the viral origin of HSE was discovered in 1941 (35), the mechanisms by which HSV-1 causes encephalitis in a small proportion of infected individuals remain unclear. Mouse models of HSE (36-39) (40, 41) have revealed that the outcome of an encephalitic or latent HSV-1 infection depends on numerous factors, which can be environmental (viral strain, route of infection, viral inoculum) or dependent on the host (mouse strain, age, prior immunization history) (42). A genetic host susceptibility factor was first suggested in 1975 using mouse forward genetics with the description of a strain-dependent susceptibility or resistance to HSE in inbred mice (43). Although this observation was confirmed by other groups (44) (45), and despite the discovery of loci conferring resistance to HSE (46, 47), genes underlying susceptibility or resistance to HSE have not been identified. However, subsequent studies led to the hypothesis that a rapid type I IFN response following HSV-1 infection conferred resistance to HSE in inbred mice (48, 49). In humans, the study of multiplex families with cases of HSE years apart and in individuals from different generations hinted at a genetic predisposition (50, 51) (52) (53). An epidemiological survey conducted in France over a 20-year period and published in 2010 (54) highlighted a high frequency of consanguinity among the families of HSE patients recruited (12%), a finding suggestive of a genetic origin of HSE with an autosomal recessive mode of inheritance.

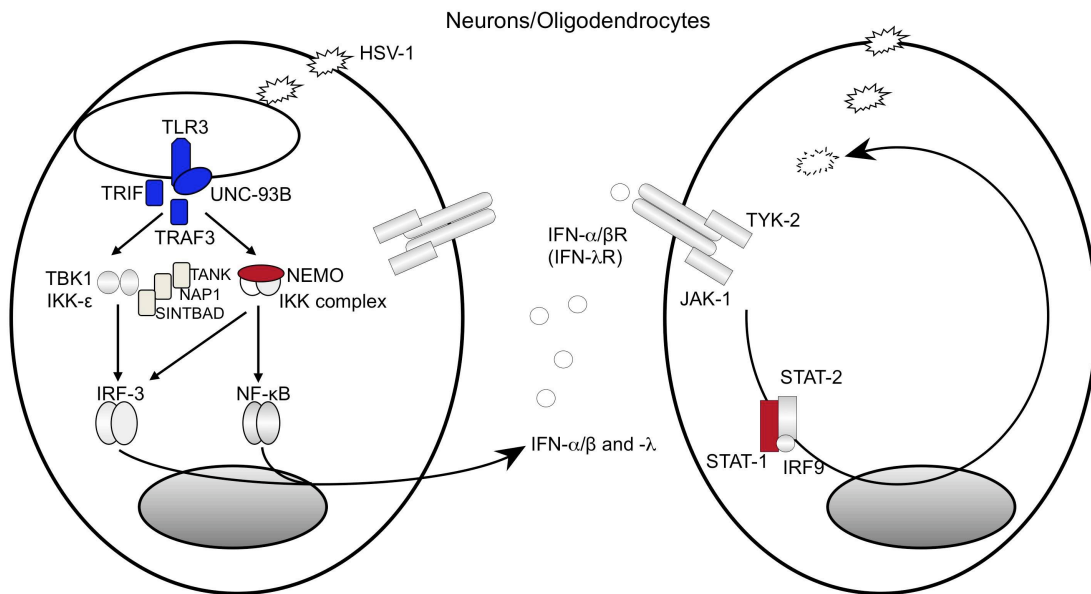
I. 2. TLR3-IFN signaling in HSE

I.2.i. IFN signaling defects with broad clinical phenotypes

The first genetic defect associated with HSE was described in 2003, with the report of one patient with complete STAT-1 deficiency. The patient suffered from mycobacterial infections and recurrent HSE (55). Signal Transducer and Activator of Transcription 1 (STAT-1) is a transcription factor recruited by JAK kinases after activation of the receptors for type I, II and III IFNs (Figure 1) (56, 57). Patients with complete STAT-1 deficiency do not respond to either IFN- α or IFN- γ in EBV-transformed B cells and SV40-immortalized fibroblasts (58). Of note, patients with

STAT-1 partial deficiency (59) have severe mycobacterial infections but do not suffer from viral diseases, due to an intact response to type I IFN in heterozygous cells, contrasting with their lack of response to IFN- γ . A few years later, a hemizygous hypomorphic mutation in *IKBKKG/NEMO* was identified in a boy who suffered from severe mycobacterial infections and a lethal episode of HSE (60). *NEMO* encodes the regulatory subunit of the inhibitor of the I κ B kinase complex. Amorphic mutations in *NEMO* abolish NF κ B activation via the classical pathway (61). Most interestingly, in fibroblasts from the NEMO-deficient patient who died of HSE, the hypomorphic mutated allele resulted in decreased IFN responses upon infection with HSV-1 and in weakened IFN responses to TLR3 ligands due to an impaired activation of not only NF- κ B, but also IRF-3 (60). NEMO has been described to be involved in IRF3 activation following viral infection (62). The discoveries of patients with HSE and mutations in *NEMO* and *STAT-1* pointed towards a crucial role of type I and type III IFNs in the protective immunity against HSV-1.

Figure 1: IFN Pathways and Anti-HSE Immunity



Genes conferring a susceptibility to HSE
 Genes conferring a susceptibility to HSE and Mycobacterial infection

I.2.ii. Gene defects with specific TLR3 signaling fibroblastic phenotype, and narrow clinical phenotype

The subsequent findings of autosomal recessive (AR) UNC-93B deficiency (63), autosomal dominant (AD) and AR TLR3 deficiency (64) (65) in children with HSE defined the TLR3-IFN pathway as essential to the protective immunity against HSV-1 in the CNS (Figure 1). Toll-Like Receptor 3 (TLR3) is a Pathogen-Associated Molecular Pattern (PAMP) receptor, localized in intracellular vesicles or on the cell surface. TLR3 recognizes double-stranded RNA (dsRNA) intermediates (66), which are produced during HSV-1 infection via symmetrical transcription (67). UNC-93B is anchored in the ER membrane and is required for TLR3, 7, 8 and 9 signaling (68) (69). Patients with UNC-93B complete deficiency or TLR3 partial or complete deficiency all share a common cellular phenotype: their fibroblasts do not produce type I and type III IFNs in response to TLR3 ligands or to the TLR3-dependent viruses HSV-1 and Vesicular Stomatitis Virus (VSV). Moreover, viral replication and cellular mortality upon infection with HSV-1 or VSV are increased in the patients' fibroblasts. Both phenotypes can be rescued by pre-treatment with IFN- α 2b (63-65). We hypothesized that these phenotypes recapitulate the mechanism of pathogenesis of HSE in CNS-resident cells in these patients. Fibroblasts from TLR3- or UNC-93B-deficient patients do not have any defect in IFN production following infection with TLR3-independent viruses (63, 64), highlighting their defect as exclusive to the TLR3 pathway, and correlating their narrow cellular phenotypes to their narrow clinical phenotypes, limited to HSE.

I.2.iii. Gene defects with broad fibroblastic phenotypes, and narrow clinical phenotype

Investigation of the genetics of HSE then focused on genes whose products are involved in TLR3 signaling, as the TLR3-IFN pathway was put forward as critical to control HSV-1 infection. AR and AD TRIF deficiency (70) and AD TRAF3 deficiency (71) were identified in children with HSE (Figure 1). Unlike other TLRs, TLR3 does not rely on the adaptor MyD88 to activate downstream effectors, but instead recruits TIR

domain-containing adapter protein inducing IFN-beta (TRIF, or TICAM-1) (72-74). TLR4 also engages TRIF, in addition to MyD88 (75-77). Following TLR3 or TLR4 activation, TRIF binds to TNF receptor-associated factor 3 (TRAF3) and recruits the complex of kinases TBK1-IKK ϵ , which activates transcription factor IRF3 (78). TRAF3 also functions downstream of several TNF receptors (CD40, LT-bR, BAFF-R) (79, 80) and is at the crossroads of several IFN-inducing pathways (triggered by TLR3, 4, 7, 8, RIG-I or MDA5) (81-83). The description of mutations in *TRIF* and *TRAF3* in HSE patients was surprising, as both molecules are involved in pathways other than the TLR3 pathway. Fibroblasts from TRIF- and TRAF3-deficient patients displayed the same phenotype as cells from UNC-93B- or TLR3-deficient patients, namely an impaired TLR3 signaling. In the AR TRIF-deficient patient, TLR4 signaling was also abolished, whereas it was functional in the AD TRIF patient. Both TRIF patients, however, had impaired signaling upon stimulation of the DExD/H-box helicases DDX1, DDX21, and DHX36, which also signal through TRIF (84). AD TRAF3 deficiency broadly affected IFN-inducing pathways as well as TNFR pathways. However, these broad cellular phenotypes in both TRIF and TRAF3 deficiencies had no impact on the patients' clinical phenotype, as the small number of patients carrying such defects suffered only from HSE and had normal resistance to other infectious agents such as viruses.

II. Human TBK1 deficiency

II. 1. TBK1 as a candidate gene

II.1.i. Role of TBK1 in TLR3 signaling

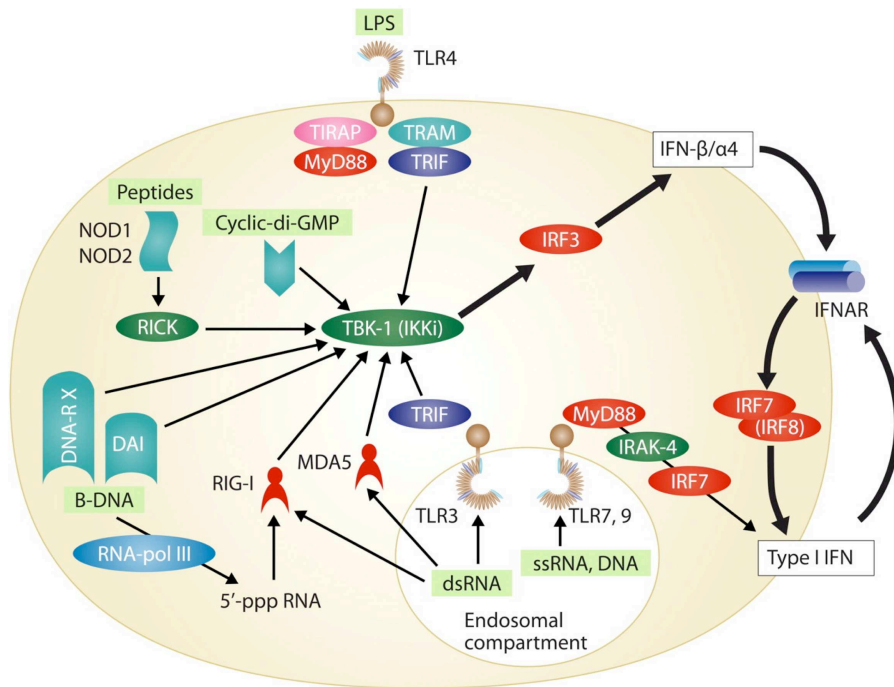
As only a fraction of children with HSE bear known defects in *TLR3*, *UNC93B1*, *TRIF*, or *TRAF3*, we searched for other genetic defects by screening HSE patients for mutations in the gene *TBK1*, which encodes TANK-Binding Kinase-1 (TBK1). TBK1 is a Ser/Thr kinase of the IKK-related family, also known as NAK/T2K (85) (86) (87). Knockout of *TBK1* in mice causes lethality at E14.5, due to liver hemorrhage and tissue loss, a phenotype similar to that associated with knockout of genes involved in NF- κ B activation (*NEMO*, *IKK β* and *RelA*). This phenotype can be rescued by the knockout of *TNFR1* (88). The role of TBK1 in host defense *in vivo* has not, therefore, been studied in mice. Heterozygous mice for the knockout allele (*TBK1*^{+/-}) are viable, but have not been characterized immunologically or in terms of their response to infection (89). TBK1-deficient (*TBK1*^{-/-}) mouse embryonic fibroblasts (MEFs) were used to characterize the function of TBK1 in antiviral signaling *in vitro*. In *TBK1*^{-/-} MEFs, stimulation with polyinosinic-polycytidylic acid (poly(I:C)), a synthetic dsRNA that serves as a non-specific agonist for TLR3, fails to elicit a type I IFN response (90). While TLR3-induced NF- κ B DNA binding is intact in *TBK1*^{-/-} MEFs, IFN-stimulated Response Element (ISRE)-binding is impaired. Further experiments showed that upon TLR3 activation, TBK1 is recruited to the TRIF-TRAF3 complex (81, 91) along with the other IKK-related kinase IKK ϵ /IKKi (92) (93). Following their activation by phosphorylation, TBK1 and IKKi phosphorylate their target transcription factors, IRF3 and IRF7 (94) (95), which induce IFNs.

II.1.ii. Role of TBK1 in other IFN-inducing pathways

TBK1 plays a broad role in IFN-inducing signaling (Figure 2). TBK1 is recruited upon activation of TLR4, which recognizes lipopolysaccharide (LPS) (96), through the TLR4-TRIF interaction (72, 97). In addition, TBK1 is involved in antiviral signaling

pathways triggered by various cytosolic Pattern Recognition Receptors (PRRs), such as the RNA helicases RIG-I/MDA5 (94, 95, 98), the intracellular DNA sensor IFI16 (99), the DNA-binding protein DAI/ZBP1 (100), and by virus-cell fusion events via STING (101, 102). RIG-I- and MDA5-dependent recruitment of TBK1 and IKKi requires the adaptor IPS-1, also known as CARDIF/MAVS/VISA (103-106). The caspase-recruitment domains (CARD) of RIG-I and MDA5 allow initiation of signaling by binding the CARD domain of IPS-1. TANK, SINTBAD and NAP-1 also bind TBK1 and IKKi (107-109). Knockdowns of these adaptors have been reported to impair the RIG-I/MDA5 pathways. It is unclear how DAI/ZBP1 activates TBK1, although it could hypothetically be through IPS-1 (110). IFI16 does not require IPS-1 to activate TBK1: once it binds DNA, IFI16 recruits STING, which directly interacts with TBK1 (111). The well-characterized outcome of all of these signaling pathways is the phosphorylation by TBK1 and IKKi of their target transcription factors, IRF3 and IRF7 (94) (95) (112).

Figure 2: TBK1-dependent mechanisms of type I IFN production



from Trinchieri G *J Exp Med* 2010;207:2053-2063

II.1.iii. Differences in the roles of TBK1 and IKKi

TBK1 functions in most pathways in association with IKKi, and they share 64% of homology at the amino acid level. The specificity of the recruitment of each individual kinase or of the TBK1-IKKi complex—the kinetics of their recruitment, the adaptors required, the cell-specificity of their recruitment—are still unclear. The first notable distinction between TBK1 and IKKi is their expression patterns. While TBK1 is expressed ubiquitously and constitutively, IKKi is mainly expressed in T cells and peripheral blood cells (88, 113) but is inducible in non-hematopoietic cells by various agents such as TNF, PMA, LPS, and virus infection (93, 98, 114). The other well-characterized difference between TBK1 and IKKi relates to their reported substrates. Both kinases phosphorylate TANK (115), IRF3 (116-119), IRF5 (120) and IRF7 (119) as well as RelA/p65 (121, 122), I κ B α (85, 92, 93), c-Rel (123), xIAP (124) and NEMO (125). However, TBK1 has been described as the sole IKK-related kinase phosphorylating Sec5 (126), IKK β (85), Akt (127) and STING (128) (102). On the other hand, only IKKi phosphorylates RelA/p65 at Ser 708 (129), p100/p52 (130), IPS-1 (131) and STAT-1 (132). Despite the specific description of their substrates, deciphering which of the two kinases is actually recruited *in vivo*, depending on the trigger and on the cell type, is still an ongoing effort.

The question of the recruitment of TBK1 and/or IKKi in several antiviral signaling pathways is unclear. The binding of IPS-1 to TBK1 or IKKi following RIG-I/MDA5 activation is controversial, and is most likely virus- and cell type-dependent. One well-described example is the direct binding of IPS-1 to IKKi following infection with Hepatitis C virus (HCV) (133). Several studies have shown that upon HSV infection, IPS-1 colocalizes at the mitochondrial membrane with IKKi, but not with TBK1 (103, 134). However, other pathways rely exclusively on TBK1, such as STING-dependent signaling (111), and dsDNA-triggered DAI-mediated (100) or IFI16-mediated (99) signaling. TLR3 activation seems to recruit both kinases, as studies using HEK293 cells stably expressing TLR3 showed that poly(I:C) stimulation leads to both TBK1- and IKKi-mediated IRF3 activation (135). However, it has been established that TRIF exclusively binds to TBK1 in fibroblasts, which leads to the activation of an IFN- β

reporter (91). The specific recruitment of TBK1 in TLR3 signaling was later confirmed by experiments showing that *TBK1*^{-/-} MEFs do not respond to poly(I:C) (90). These data are of great interest to this study and played a major role in our decision to choose TBK1 over IKKi as candidate gene.

II. 2. Characterization of two mutant *TBK1* alleles

II.2.i. Identification of two mutant *TBK1* alleles in two unrelated HSE patients

We studied two unrelated European patients who developed HSE during childhood and who carried two different heterozygous unreported variations in *TBK1* (Figure 3a). P1, from Poland, developed HSE at 7 years of age. Diagnosis of HSE was based on the positive detection by PCR of HSV-1 DNA in the child's cerebrospinal fluid, and on typical HSE-like brain pattern aberration on MRI. P1 has since suffered from epilepsy and psychomotor retardation. She has not had any other unusual or severe infections, and is now 15. P2 is a child from France who had HSE at 11 months of age. Diagnosis of HSE was based on typical encephalitis-like patterns in the left temporal area of the brain. P2 has since suffered from obesity and motor and cognitive disabilities. She is now 27 and has not had other significant infectious phenotypes.

P1 and P2 were found to be wild type (WT) for the coding exons of known HSE-susceptibility genes *TLR3*, *UNC93B1*, *TRAF3* and *TRIF* by Sanger-sequencing on genomic DNA (gDNA). In addition, these patients were sequenced and found to be WT for the coding exons of *TANK* (encoding TRAF-interacting protein I, TANK). No additional mutations in genes from the TLR3 pathway were found by whole-exome sequencing. Both mutations in *TBK1*, c.476G>G/C (P1) and c.149A>A/C (P2), result in non-conservative amino acid changes: G159A and D50A (Figure 3b, c), respectively. These variations were neither found in public databases (NCBI dbSNP 135, UCSC, 1000 genomes) nor in over 1050 healthy controls from the CEPH-Human Genome Diversity panel, thus making the possibility that they are irrelevant polymorphisms highly unlikely. Both mutations affect conserved residues in the kinase domain of TBK1. In addition, the Glycine at position 159 is part of the predicted activation loop of TBK1, 13 amino acids

upstream of the phosphorylation site of TBK1, S172. The residue at position 50 is downstream of the ATP binding site at residue 38.

Figure 3: Heterozygous *TBK1* mutations in two children with HSE

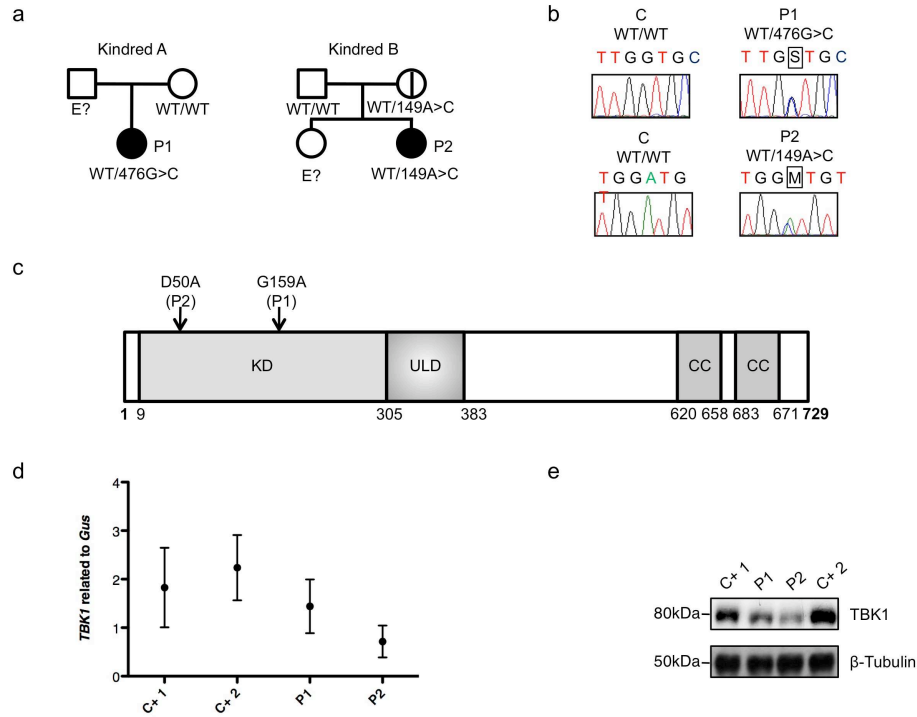


Figure 3: (a) Family pedigrees and segregation. (b) Heterozygous *TBK1* mutations 476G>C in P1 and 149A>C in P2. The PCR products sequenced were amplified from genomic DNA from the granulocytes of a control (C) and both patients. (c) Schematic diagram of the protein structure of *TBK1*, featuring its kinase domain (KD), ubiquitin-like domain (ULD) and coiled-coil (CC) regions. Both heterozygous substitutions, 159G>G/A (P1) and 50D>D/A (P2), affect the kinase domain of *TBK1* (amino acids 9-305). (d) *TBK1* expression, as assessed by RT-qPCR on mRNA from the SV40-fibroblasts of patients (P1, P2) and control lines (C+1, C+2). Values represent mean values \pm SD calculated from three independent experiments. (e) *TBK1* levels, as assessed by western blotting (WB), in SV40-fibroblasts from patients (P1, P2) and two control lines (C+ 1 and C+ 2). This WB result is representative of three experiments

II.2.ii. Expression of the two mutant *TBK1* alleles

We assessed the expression level of *TBK1* both at the mRNA and protein levels (Figure 3d, e). We observed similar levels of *TBK1* mRNA in SV40-fibroblasts from P1 and from healthy controls, whereas mRNA levels were unexpectedly low in fibroblasts from P2. However, we did not test whether this low level of mRNA is significant compared to a number of control cell lines larger than 2. If the mRNA levels of *TBK1* were to be significantly lower in P2's cells than in a large number of cell lines from healthy donors, we could start studying the mechanism underlying this low level of expression. We could assess whether this is due to a lack of expression of the mutant allele, for instance by TOPO cloning. To my knowledge, there is no report of heterozygous missense mutations affecting the level of expression of a gene at the mRNA level by mechanisms similar to non-sense-mediated mRNA decay. How the D50A mutation would affect the mRNA levels of *TBK1* remains to be investigated.

At the protein level, P1's fibroblasts and control cells had similar TBK1 amounts, while P2's fibroblasts displayed lower amounts of TBK1 protein. As the level of expression of mutant alleles may be confounded by the expression of the wild-type allele in the patients' heterozygous cells, we then compared the expression of the individual *TBK1* alleles in HEK293T cells or *TBK1*^{-/-} MEFs, transfected with either a mutant allele or the WT human *TBK1* allele. Upon transient transfection with the G159A mutant allele (P1's allele), HEK293T cells and *TBK1*^{-/-} MEFs produced G159A TBK1 in amounts similar to those obtained for the transfection of WT TBK1. By contrast, transfection with the D50A mutant allele (P2's allele) resulted in the production of much smaller amounts of protein. The D50A mutation in *TBK1* may thus destabilize the protein, resulting in its premature degradation. To test whether the D50A protein is stable and properly folded, we could carry out pulse-chase and differential scanning calorimetry experiments, respectively. Our data strongly suggest that the *TBK1* G159A allele is expressed normally, whereas the D50A allele is poorly expressed, resulting in lower TBK1 amounts at both the mRNA and protein levels in P2's fibroblasts.

II.2.iii. *In vitro* functional characterization of the two mutant *TBK1* alleles

We used *in vitro* kinase assays to assess whether the mutations in TBK1 could impact the enzymatic activity of the protein (Figure 4a). The obtained levels of *in vitro* phosphorylation of the substrate, recombinant Akt (127, 136), were much lower with the G159A TBK1 protein than with WT TBK1. In addition, phosphorylation of the S172 residue of TBK1, which is required for its activation (137), was severely impaired in the G159A mutant protein, as seen by western blot using a Phospho-S172-TBK1 antibody. The low levels of S172 phosphorylation and of substrate phosphorylation observed for the G159A mutant were similar to those observed for the kinase-dead K38M mutant, used as negative control in these *in vitro* assays. As discussed in paragraph II.2.ii, D50A TBK1 was produced in much smaller amounts than the WT, G159A, or K38M TBK1 variants, therefore phosphorylation of the substrate was not observed upon transfection of the D50A allele. We then assessed the functionality of the two mutant proteins in terms of TBK1 involvement in the TLR3 pathway, in *TBK1*^{-/-} MEFs (89)(90) transiently transfected with the D50A, G159A, or WT human *TBK1* allele. The transfection of *TBK1*^{-/-} MEFs with either the D50A or the G159A *TBK1* allele failed to restore the poly(I:C)-induced IFN- β induction whereas this induction was restored by transfection with the WT human *TBK1* allele (Figure 4b). The D50A allele was loss-of-expression and did not lead to TBK1 protein expression, whereas transfection of the G159A and WT *TBK1* alleles resulted in the production of similar amounts of TBK1 protein (Figure 4c). All transfected cells were healthy and produced IL-6 in response to IL-1 β as a positive control (Figure 4d). Thus, the G159A *TBK1* mutant allele is normally expressed but generates a kinase-dead protein, and the D50A allele is a loss-of-expression allele resulting in a loss of protein activity. Both mutants are therefore loss-of-function for TLR3-mediated cellular responses.

Figure 4: Both mutant TBK1 alleles are loss-of-function, but through different mechanisms

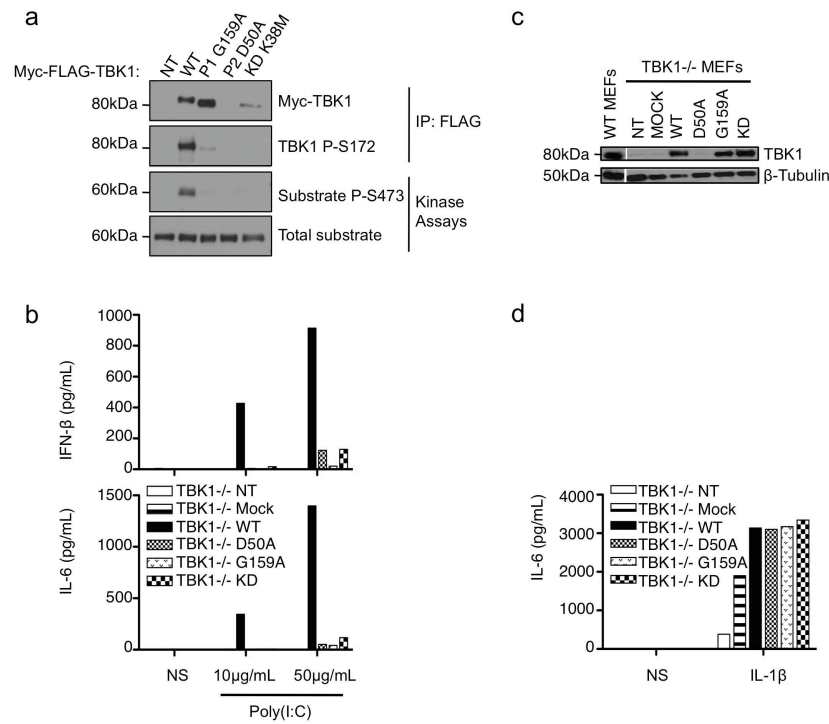


Figure 4: (a) In vitro kinase assays: substrate (Akt) phosphorylation by both mutant kinases, G159A and D50A, compared with the phosphorylation levels for WT TBK1 and the kinase-dead (KD) K38M TBK1. A single experiment representative of three independent experiments carried out is shown. HEK293T cells were transfected with WT and mutant constructs, or left untransfected (NT). (b) TBK1-/- MEFs were either left untransfected (NT) or were transfected with a mock vector, WT TBK1 or mutant constructs: D50A (mutation present in P2), G159A (the mutation in P1) and S172A (kinase-dead, KD). After 24 hours, the cells were stimulated with 10 or 50 μg/ml poly(I:C). ELISA was carried out to assess IFN-β and IL-6 production, 24 hours after stimulation. The data shown are representative of three independent experiments. (c) TBK1 expression from the transfected constructs was assessed by western blotting with an antibody against TBK1; β-tubulin was used as a loading control. (d) TBK1-/- MEFs were either not transfected (NT), or transfected with a mock vector (MOCK), or a vector encoding WT TBK1 (WT) or the mutants, G159A, D50A, or S172A. 24 hours later, cells were stimulated with 10ng/ml IL-1β. IL-6 production was measured by ELISA. The data shown are representative of three independent experiments.

II.3. Impact of the *TBK1* mutant alleles on IFN-inducing signaling

II.3.i. TLR3-dependent signaling in P1 and P2's fibroblasts

We tested the hypothesis that the loss-of-function G159A and D50A *TBK1* alleles are associated with an AD form of HSE by studying the response of the patients' heterozygous fibroblasts to poly(I:C), which signals through TLR3 when delivered in an extra-cellular manner (138). We observed that IRF3 phosphorylation and subsequent dimerization after poly(I:C) stimulation was severely impaired in P1's fibroblasts (Figure 5a), while in P2's fibroblasts, IRF3 activation was normal. Contrasting with IRF3, NF- κ B activation following poly(I:C) stimulation was normal in both patients' cells. These results are consistent with the IFN- β , - λ and IL-6 production observed in the patients' fibroblasts: P2's cells produced normal levels of IFN- β , - λ and IL-6 after poly(I:C) stimulation (Figure 5b), while P1's cells did not produce any IFN- β , - λ but displayed normal levels of IL-6 upon TLR3 activation (Figure 5a). Consistently, the TLR3-dependent poly(I:C) response was impaired in fibroblasts from P1 but not P2, as assessed by genome-wide transcription studies. We could rescue the lack of IFN production in P1's cells after poly(I:C) stimulation by over-expressing WT *TBK1*, but not an empty vector, demonstrating that the *TBK1* G159A allele is dominant and responsible for abolishing the poly(I:C) responsiveness in P1's fibroblasts (Figure 5c, d). Furthermore, we determined the mechanism of dominance of P1's allele by transfecting control healthy fibroblasts with the mutant or WT *TBK1* alleles, and assessing the poly(I:C) responsiveness (Figure 5e, f). Over-expression of the G159A allele led to a strong decrease in the IFN response to poly(I:C) stimulation in control cells, as compared to cells left untransfected or transfected with WT *TBK1*, an empty vector, or D50A *TBK1*. Thus, the *TBK1* G159A allele, unlike the D50A allele, exerts a negative-dominance over the WT *TBK1* allele. Overall, heterozygosity for the G159A *TBK1* allele severely impairs IRF3-dependent IFN- β and IFN- λ production in response to TLR3 activation, whereas the D50A *TBK1* allele leads to no detectable impairment of TLR3-dependent poly(I:C) responses in human fibroblasts.

Figure 5: TLR3-mediated poly(I:C) responses are impaired in P1's fibroblasts but normal in P2's fibroblasts

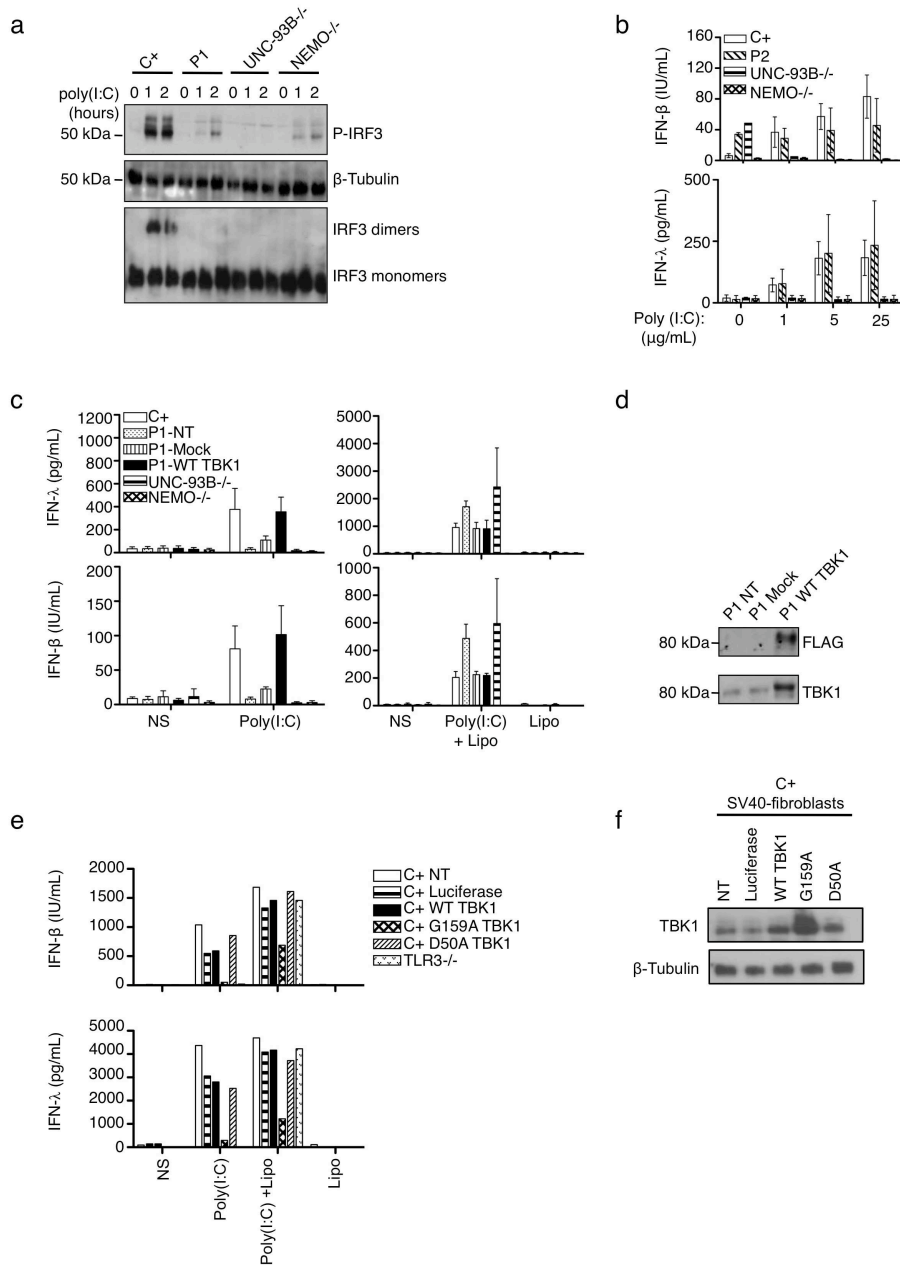


Figure 5: (a) IRF-3 phosphorylation in fibroblasts from P1, as assessed by western blotting after stimulation with 25 μg/ml poly(I:C) for 0, one (1) or two (2) hours, with β-tubulin as a loading control. Comparison with control cells (C+), UNC-93B^{-/-} and NEMO^{-/-} fibroblasts. IRF3 dimerization was assessed by native western blotting. Blots are representative of three independent experiments. Two different healthy control cell lines were tested and gave the same result. (b) IFN-β and IFN-λ production, as assessed by ELISA, following a 24 hours of stimulation with increasing doses of poly(I:C) in control (C+) cells, in fibroblasts from P2, and from UNC-93B^{-/-} and NEMO^{-/-} patients. Values represent mean values ± SD, calculated

from three independent experiments. (c) Fibroblasts from P1 were untransfected (NT), MOCK-transfected (MOCK) or transfected with a vector encoding FLAG-tagged WT TBK1 (WT TBK1), and stimulated for 24 hours with the indicated reagents (poly(I:C) or poly(I:C) transfection mediated by Lipofectamine (Lipo)). IFN- β and IFN- λ production was assessed by ELISA. Values represent means \pm SD calculated from three independent experiments. (d) Western blot of transfected P1 cells with anti-TBK1 and anti-FLAG antibodies. (e) IFN- β and IFN- λ production, as assessed by ELISA, in response to 50 μ g/ml poly(I:C) stimulation or 25 μ g/ml poly(I:C) transfection mediated by Lipofectamine, in TLR3-deficient fibroblasts, and in control fibroblasts (C+) transduced with lentivirus pseudoparticles encoding luciferase, WT TBK1, one of the TBK1 mutants (G159A or D50A), or left untransduced (NT). Cells were transduced six days before stimulation. This experiment is representative of three independent experiments. (f) Western blot of transduced control fibroblasts (C+) with anti-TBK1 and anti-tubulin antibodies.

II.3.ii. TLR3-dependent antiviral responses in P1 and P2's fibroblasts

The IFN response to HSV-1 and VSV in human dermal fibroblasts has been shown to be partially dependent on TLR3. TLR3-, UNC-93B-, TRIF- and TRAF3-deficient fibroblasts fail to contain the replication of HSV-1 and/or VSV, due to a lack of IFN- β and IFN- λ production (63-65, 70, 71). We tested the hypothesis that the loss-of-function G159A and D50A *TBK1* alleles were responsible for an AD form of HSE by investigating the IFN-mediated antiviral response in heterozygous fibroblasts from P1 and P2. The fibroblasts of P1 produced no IFN- β or IFN- λ 24 hours after infection with HSV-1. The cells of P1 displayed higher than normal levels of viral replication and cell death, like TLR3-deficient and UNC-93B-deficient cells (Figure 6a, c). The addition of IFN- α 2b to these cells restored virus containment to levels similar to those in control cells (Figure 6a, c). Moreover, fibroblasts from P1 and P2 also displayed very low levels of IFN- β and IFN- λ production following infection with VSV at a multiplicity of infection (MOI) of 10. Cells from either of the patients did not suppress VSV replication (Figure 6b, d). VSV-induced cell death levels were also higher in cells from both patients than in the control, as in TLR3-deficient and UNC-93B-deficient cells (Figure 6b, d). The treatment of these cells with IFN- α 2b restored viral containment and cell death to levels similar to those in control cells (Figure 6b, d). The IFN-dependent control of HSV-1 and VSV, which is at least partly dependent on TLR3 in human fibroblasts, was therefore impaired in the patients' cells. Fibroblasts heterozygous for D50A *TBK1* displayed an

impaired control of viruses via TLR3, despite responding normally to poly(I:C) via TLR3. This suggests that the entire, intact, TBK1-dependent TLR3 pathway is required for HSV-1 and VSV control in human fibroblasts, although we cannot exclude a possible role for TBK1 in the TLR3-dependent or -independent inhibition of HSV-1 replication (139) or cell death (127). Both the G159A and D50A TBK1 alleles are thus loss-of-function and dominant for TLR3- and IFN- dependent antiviral immune responses.

Figure 6: Impaired IFN-dependent control of HSV-1 and VSV infection in the patients' fibroblasts

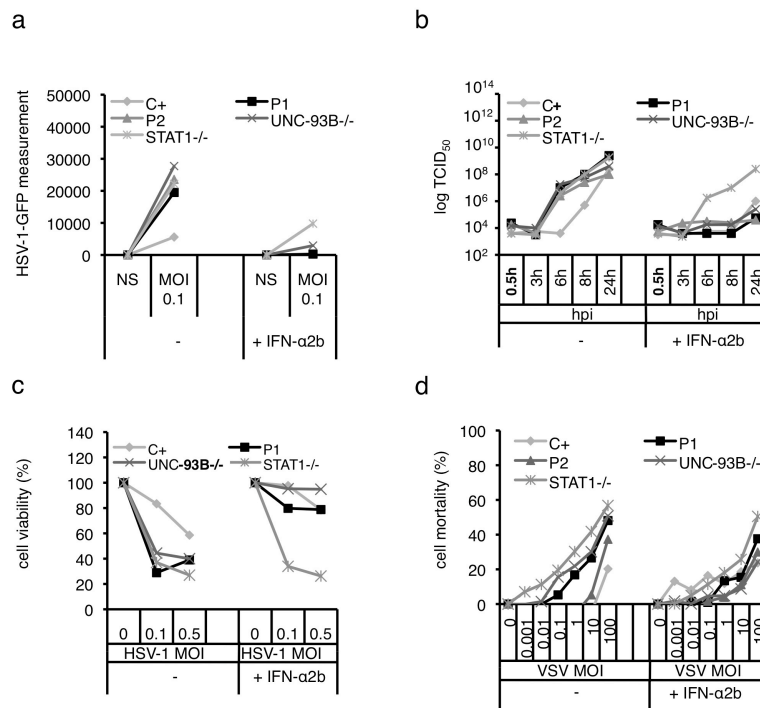


Figure 6: Replication of the HSV-1-GFP virus at an MOI of 1 (a) and of VSV at an MOI of 10 (b) in the patients' fibroblasts (P1, P2), and in control cells (C+; averaged from two distinct control cell lines), UNC-93B-/- and STAT-1-/- fibroblasts, as determined at indicated hours post-infection (hpi), with or without 18-hour IFN- α 2b pretreatment. One experiment representative of two independent experiments carried out. (c) Viability of control cells (C+; averaged from two different control cell lines), and in fibroblasts from P1, an UNC-93B-/- patient and a STAT-1-/- patient, after infection with HSV-1-GFP at MOIs of 0.1 and 0.5, with or without IFN- α 2b treatment 18 hours before infection. (d) Cell mortality after 24 hours of VSV infection in control cells (C+; averaged from two distinct control cell lines), and in fibroblasts from P1, P2, a UNC-93B-/- patient and a STAT-1-/- patient, with or without 18-hour IFN- α 2b pretreatment.

II.3.iii. TLR3-independent signaling in P1 and P2's cells

We first investigated the response to TLR4 activation in peripheral blood mononuclear cells from P1. LPS stimulation (140) resulted in normal levels of IFN- α and IL-6, suggesting that TLR4 signaling is intact in P1's heterozygous blood cells. This finding is consistent with the normal response induced by LPS stimulation in *TBK1*^{+/-} MEFs (98). We next investigated whether fibroblasts from the patients could respond to the following stimuli, reported to trigger TBK1-dependent signaling in mice: transfection of poly(dA:dT) (99, 100); transfection of poly(I:C) (141) and transfection of the RIG-I specific ligand 7sk-as (142). Determining which TBK1-dependent pathways are affected by the TBK1 mutations in the patients' cells could contribute to our understanding of the discrepancy between the very narrow clinical phenotype of the patients and the broad role of TBK1 in antiviral immunity reported in mice. The fibroblasts from both patients, like cells from patients deficient for UNC-93B or TLR3, produced normal levels of IFN- β and IFN- λ following transfection with various amounts of poly(dA:dT) or poly(I:C) (Figure 7a), and large amounts of 7sk-as. However, the levels of IFN- β or IFN- λ production induced by lower doses of 7sk-as were lower in cells from P1 than in control cells or in cells from a TLR3-deficient patient, at 24 hours, but reached normal levels by 48 hours (Figure 7b). Consistently, IRF3 activation was delayed following RIG-I stimulation after transfection with a low dose of 7sk-as: IRF3 dimerization was barely detectable at 8 hours, but normal after 12 hours of RIG-I stimulation in the fibroblasts of P1. These results point to a mild impairment of RIG-I signaling in fibroblasts heterozygous for G159A *TBK1*. No major TLR3-independent phenotypes were observed in the cells of P1 and P2.

Figure 7: Normal responses to dsRNA, dsDNA, and ssRNA introduced by transfection in the patients' fibroblasts

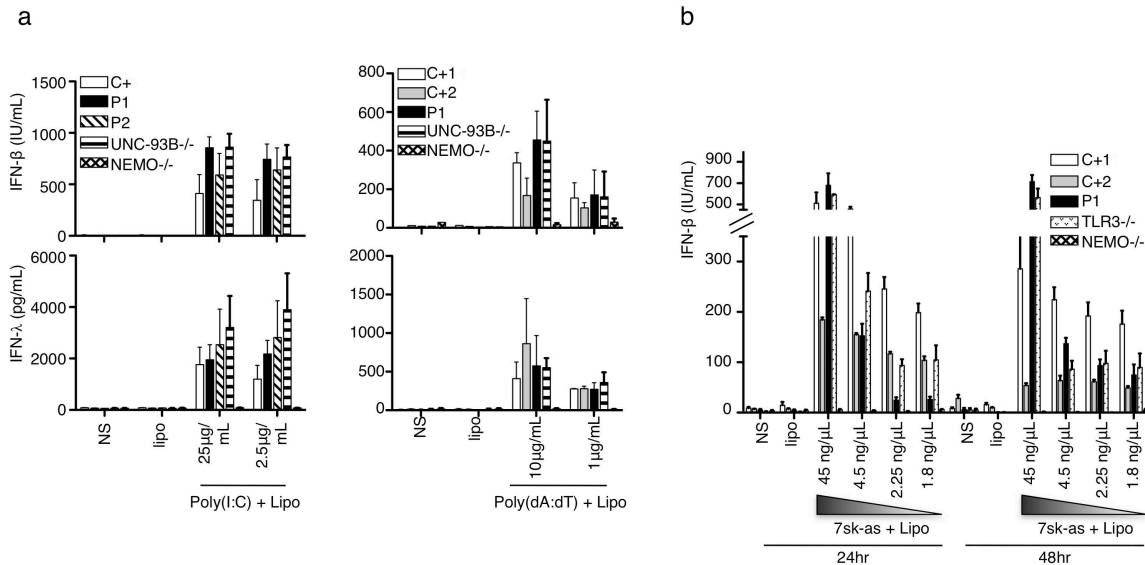


Figure 7: (a) Production of IFN- β and IFN- λ in response to various doses of poly(I:C) and poly(dA:dT), introduced by transfection into control (C+; averaged from two different control cell lines), P1, P2, UNC-93B- and NEMO-deficient fibroblasts. (b) IFN- β production 24 or 48 hours after transfection with the ssRNA “7sk-as” in control (C+1, C+2), P1, TLR3-/- and NEMO-/- fibroblasts. Values represent mean values \pm SD from three independent experiments.

We assessed the potential of the G159A and D50A *TBK1* alleles to rescue the lack of IFN- β production observed in *TBK1*^{-/-} MEFs upon transfection with poly(I:C) and 7sk-as. Unlike the WT *TBK1* allele, the G159A mutant allele could not rescue these responses. The D50A allele allowed residual responses, albeit probably due to transient overexpression of the D50A TBK1 protein. On western blot, cells transfected with the D50A allele were found to contain smaller total amounts of TBK1 than cells transfected with the WT or G159A alleles, consistent with our previous findings. All cells were healthy and produced IL-6 in response to IL-1 β . In *TBK1*^{-/-} MEFs, both mutants are, therefore, loss-of-function for IFN-inducing pathways triggered by dsRNA and ssRNA. It remains unclear whether the normal IFN response following the activation of TLR4 and cytosolic sensors of nucleic acids in our patients' cells is due to the residual TBK1 activity present in human cells with a partial TBK1 deficiency, or due to a lack of requirement for TBK1 in these pathways in human PBMCs and fibroblasts.

Although both P1 and P2 suffered from isolated HSE, with no other unusual infections, we investigated the response of cells from P1 and P2 to a number of viruses that were available to the lab and that induce IFNs in the cell types tested. We challenged PBMCs from P1 and P2 with various types of viruses (Figure 8a), including positive sense ssRNA viruses (encephalomyocarditis virus (EMCV), Sindbis virus), negative sense ssRNA viruses (Sendai, human parainfluenza virus III (para-III), Newcastle's disease virus (NDV), mumps virus, measles virus) and dsDNA viruses (BK virus, HSV-1). Unfortunately, other viruses from the Herpesviridae family were unavailable (e.g. HHV-6, HHV-8) or do not induce the production of detectable levels of IFNs in the experimental conditions used (e.g. HHV-3, HHV-4). All of the viruses tested induced normal levels of IFN- α and IL-6 in PBMCs from our patients. HSV and inactivated HSV (HSV_i) both induce normal levels of IFNs in control cells and in cells from patients with AD TBK1 or AR TLR3 deficiencies (Figure 8 and data not shown). We did not infect other cell types such as fibroblasts with HSV_i, as it does not induce IFNs (data not shown). The normal response observed in the patients' cells to all the viruses tested may reflect the partial nature of the genetic defects carried by the patients, the redundancy of TBK1 and IKK ϵ in the activation of IRF3 and IRF7 in leukocytes (90, 94, 95, 98, 143), or both. We next tested the response of the patients' fibroblasts to infection with para-III, measles, EMCV, or Sindbis virus (Figure 8b). The fibroblasts from both patients behaved like control cells in terms of their capacity to produce IFN- β and IFN- λ , although IFN production levels were variable between the different healthy control cell lines tested and the cells from a patient with UNC-93B deficiency. This result contrasts with the impaired response of these cells to infection with HSV-1 or VSV, both of which activate TLR3 to initiate IFN induction. The lack of viral control in the fibroblasts of patients heterozygous for *TBK1* is thus limited to the neurotropic viruses tested, HSV-1 and VSV. These results for PBMCs and fibroblasts are consistent with the narrow clinical phenotype observed in both TBK1-deficient patients. They suggest that the residual TBK1-dependent IFN induction by TLR3 and/or by other receptors in cells with a partial TBK1 deficiency may contribute to the protective immunity against most common viruses.

Figure 8: Normal responses to other viruses in the patients' PBMCs and fibroblasts

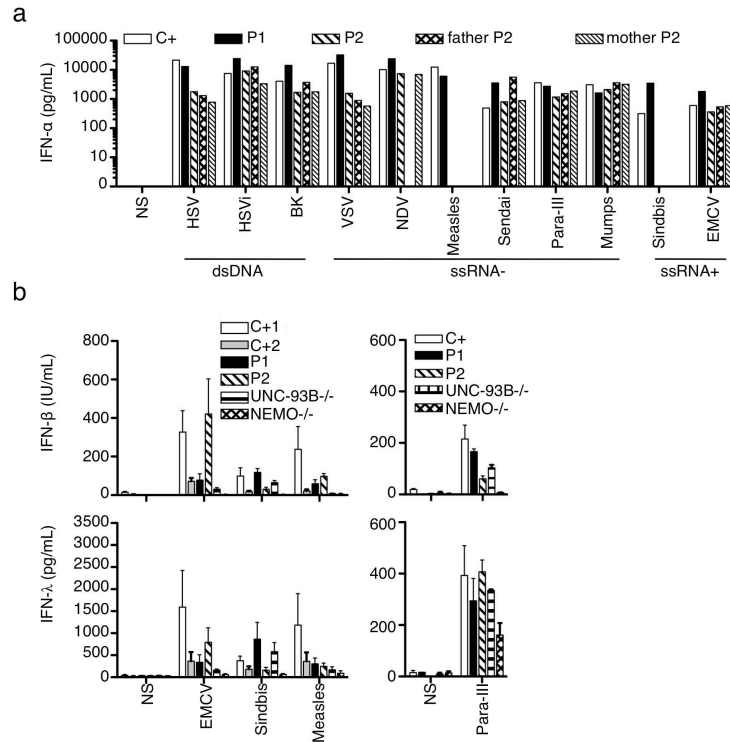


Figure 8: (a) PBMCs from a control (C+), P1, P2, and P2's parents, were challenged with a number of viruses with different types of genomes: dsDNA viruses (HSV-1, UV-inactivated HSV-1 or HSV-1i, BK), negative-strand ssRNA viruses (VSV, Newcastle's Disease Virus NDV, measles, Sendai virus, human parainfluenza virus III (Para-III), mumps), and positive-strand ssRNA viruses (Sindbis virus, EMCV). Levels of IFN- α were assessed by ELISA 24 hours after infection. P2 and her parents' cells were not tested for their response to infection with Sindbis and Measles. This experiment was performed once. (b) Production of IFN- β and IFN- λ , as assessed by ELISA, in control (C+), P1, P2, UNC-93B^{-/-} and NEMO^{-/-} fibroblasts, 24 hours after infection with positive-strand ssRNA viruses (Sindbis virus at an MOI of 10, EMCV at an MOI of 1) and negative ssRNA viruses (measles at an MOI of 1, parainfluenza virus III (Para-III) at an MOI of 1). Values represent mean values \pm SD from three independent experiments.

III. Discussion

III.1. Pathogenesis of HSE and TBK1-dependent antiviral processes

III.1.i. AD TBK1 deficiency and incomplete penetrance

TLR3-dependent poly(I:C) phenotypes in MEFs and fibroblasts from P1 reveal a similar requirement of TBK1 for IRF3 activation and IFN production. $TBK1^{-/+}$ MEFs have not been tested for their response to extra-cellular poly(I:C) stimulation, but the absence of defect in fibroblasts from P2 upon poly(I:C) stimulation indicates that human *TBK1* haploinsufficiency does not influence poly(I:C)-triggered TLR3 signaling in fibroblasts. We were unable to evaluate the penetrance of the cellular phenotypes we described due to the unavailability of heterozygous cells from healthy members of the patients' families. However, it is possible that heterozygosity for the D50A *TBK1* allele in cells other than fibroblasts leads to a poly(I:C)-induced phenotype. Haploinsufficiency restricted to specific cell types has been described for heterozygous *IFNGR2* mutant alleles, which impair IFN- γ signaling in T cells exclusively, and not in macrophages or monocytes (Kong *et al.*, manuscript in preparation). We could thus similarly hypothesize that in CNS-resident cells, *TBK1* haploinsufficiency might lead to a poly(I:C)-induced TLR3-specific defect.

The clinical penetrance of AD TBK1 deficiency is incomplete, as P2's mother carries the mutant D50A allele and has not suffered from HSE despite having a normal IgG response to HSV-1/HSV-2. The observed incomplete clinical penetrance is similar to the description of incomplete penetrance of HSE in carriers of defects in *UNC93B1*, *TLR3* and *TRIF* (63, 64, 70). This pattern is consistent with the sporadic occurrence of HSE (1). Age of infection can also affect the clinical outcome of infection with HSV-1, as it has been shown in mice (144-146). In addition, viral inoculum and modifier genes could also be influencing the penetrance of HSE. Of note, whole-exome sequencing results from P2 and P2's mother showed no additional mutations in either individual in genes whose products have been reported to be involved in TLR3 signaling in mice or humans.

Furthermore, our studies in fibroblasts from both TBK1-deficient patients showed that a single functional *TBK1* allele is insufficient for the cellular control of TLR3-dependent viruses, particularly HSV-1. Strong purifying selection is expected for dominant alleles conferring a predisposition to life-threatening diseases in children, as it has for instance been shown to operate on *IL12RB2*, in the case of predisposition to Mendelian susceptibility to mycobacterial disease and severe tuberculosis (Bryant *et al.*, 2012, submitted). Weaker selection is expected for deleterious alleles associated with recessive phenotypes. Moreover, sequencing of over 1,050 controls and 150 HSE patients did not uncover any other deleterious *TBK1* alleles, confirming their rarity in the general population. Of note, the kinase domain of TBK1 is very well conserved among species, and very few validated missense non-somatic SNPs are reported in the NCBI dbSNP database 135: only 3 missense SNPs in the kinase domain (spanning amino acids 1 to 300), one of them being a conservative change (F/L). The other 4 SNPs present in the kinase domain are synonymous. A future direction could be to analyze whether *TBK1* is under purifying selection, which could contribute to our understanding of the existence of haploinsufficiency for *TBK1*, especially given that it has not been reported for the other HSE-susceptibility genes, *TLR3*, *UNC93B1*, *TRIF* and *TRAF3*. To study the impact of natural selection on *TBK1*, we could opt for an approach similar to that used by Bryant *et al.* Based on sequence data from healthy individuals from various ethnic backgrounds, they used a statistical method using the number of nonsynonymous and synonymous fixed differences between species (human and chimpanzee) and the number of nonsynonymous and synonymous polymorphisms within human populations to estimate the ω parameter (147). In the absence of selection pressure, the number of nonsynonymous and synonymous variations is random and does not deviate significantly from neutral expectations: ω is not significantly different from 1. Values of ω below 1 are consistent with selection against nonsynonymous variants (purifying selection), whereas values above 1 reflect selection favoring amino- acid mutations (positive selection).

Moreover, both TBK1-deficient patients had a single episode of HSE, and no symptomatic HSV-1 infection of other organs (such as Herpetic stomatitis or Herpes labialis), which are generally uncommon in HSE patients. This observation raises very interesting questions. Indeed, the mechanisms responsible for HSE being strictly limited

to the brain are still unknown. So are those explaining that patients with an immune deficiency conferring susceptibility to primary HSV-1 infection develop HSE specifically, over any other form of symptomatic HSV-1 infection. However, the IFN response to HSV-1 in blood cells in TBK1-deficient patients is normal (like it is in TLR3-deficient patients). Both TBK1-deficient patients also tested positive for HSV-1 antibodies years after their episode of HSE, which suggests a normal adaptive—or at least B cell-mediated—immune response. We may hypothesize that, given the tropism of the virus, infection with HSV-1 only results in a brain-specific infection and does not affect other organs because of the nature of the immune response in cells outside of the CNS. TLR3 immunity may only be crucial to the protection against primary infection with HSV-1 in the CNS, particularly in neurons and oligodendrocytes (further discussed in IV). In addition, both TBK1-deficient patients were treated with acyclovir during their episode of HSE. We do not know whether the treatment effectively cleared the virus, or if the virus is, to this day, latent in the patients' CNS. The patients have not yet suffered from a second episode of HSE, but we cannot predict the outcome of a possible second infection with HSV-1 or of its reactivation if latent. UNC-93B-deficient patients had recurrent episodes of HSE years apart, which may be due to the lack of clearance of the virus, or to its reactivation. However, given that both TBK1 patients are able to produce an antibody response against HSE, we can assume that, if the patients cleared the virus after their first episode of HSE, if they encounter the virus a second time, their immune response at the site of infection may prevent the virus from entering the CNS or from causing symptomatic infections in other organs.

III.1.ii. Involvement of TBK1 in autophagy

III.1.ii.a. HSV-1 infection induces autophagy

It has been proposed that TBK1 plays a role in macroautophagy. Macroautophagy, or autophagy, is a highly conserved catabolic process mediating the degradation of cytoplasmic material, thus recycling cellular components (148-150). Autophagy has emerged as a mechanism with important immunological functions (151).

The host autophagic machinery has been shown to be essential to protect against viral infection in the CNS (152). Autophagy can be involved in the defense against pathogens in two ways, the first of which is by eliminating the pathogen directly. In the case of HSV-1 infection, autophagy is triggered via PKR activation, a dsRNA-binding kinase that regulates translation in virus-infected cells by phosphorylating eIF2- α , (153, 154) (155) and results in PKR-dependent degradation of herpes simplex virions. Secondly, autophagy can lead to the production and delivery of antigens to major histocompatibility complex class I and class II antigen-presenting molecules, leading to T cells activation. Both processes can be inhibited by HSV-1 neurovirulence gene product ICP34.5 (156) (157) (158) (159). The HSV-1 protein ICP34.5 can elicit dephosphorylation of eIF2- α , thereby preventing the translational arrest induced by PKR-mediated signaling (160), a pathway additionally targeted by HSV-1 protein US11 (161) (162).

However, the impact of autophagy on HSV-1 replication is controversial. Using HSV-1 ICP34.5 null mutant strains, which are attenuated in growth and pathogenesis in animal models and in primary cultured cells, an *in vitro* study showed that it is the prevention of translational arrest by ICP34.5 rather than ICP34.5's control of autophagy that determines the efficiency of HSV-1 replication in primary cell cultures (163). On the other hand, *in vivo* data seem to hint that autophagy could contribute to HSV-1 pathogenesis. An HSV-1 strain containing a mutant ICP34.5 that cannot bind beclin-1 is neuro-attenuated in mice (164). The HSV-1 protein ICP34.5 possibly confers neurovirulence by inhibiting the autophagy protein Beclin-1, suggesting that evasion of autophagy is crucial to achieve lethal encephalitis in mice (164). Moreover, another study demonstrated that infection of macrophages with HSV-1 results in an ICP34.5-dependent inhibition of autophagy in the early infection phase, and in the stimulation of the autophagy pathway in the late phase (158). Therefore, the role of autophagy in the pathogenesis of HSV-1 infection is likely to be time-sensitive, and may also be cell-specific (165).

III.1.ii.b. Innate antiviral signaling and autophagy

Autophagy can be triggered by innate immune sensors such as TLRs (166, 167), including TLR4. Knockdown of TRIF blocks TLR4-induced autophagy (168), suggesting that TLR3, which signals through TRIF as well, could also play a role in autophagy (169). Moreover, autophagy pathways have been reported to interfere with innate immune signaling. The autophagy regulator complex Atg5-Atg12 negatively regulates RIG-I signaling by directly associating with RIG-I and IPS-1 (170), while the autophagy protein Atg9a negatively regulates the STING-TBK1 pathway upon its activation by cytosolic dsDNA (171). In the case of HSV-1, it seems that induction of autophagy in myeloid cells upon infection is independent of viral replication or TLR signaling, but dependent on viral entry, the presence of cytosolic viral DNA, and STING (172).

Work relating TBK1 to autophagy has focused on autophagy triggered by bacterial infections. TBK1 is involved in the autophagy-mediated restriction of *Salmonella enterica* growth: TBK1 constitutively binds, and phosphorylates, optineurin, (173) leading to the recruitment of *Salmonella* to autophagosomes (174). TBK1 is also recruited by autophagy receptor NDP52, which recognizes ubiquitin-coated *Salmonella*, to restrict bacterial proliferation (175) (176). Recent studies also show that TBK1 phosphorylates autophagy adaptor p62 (177) and is required for efficient delivery of *Mycobacterium tuberculosis* to autophagosomes, thereby limiting bacterial replication (178). The possibility that TBK1 could be involved in other ways in autophagy processes remains to be investigated (179). The IKK complex, but not TBK1, has however been shown to contribute to the induction of starvation-triggered autophagy (180).

It thus seems clear that molecules involved in innate immune signaling are also involved in the induction or the processes of autophagy and that autophagy contributes to and affects antiviral immunity. Therefore, it would be of great interest to test the autophagy pathways in cells from patients with AD TBK1 deficiency, as well as in cells from patients deficient for TLR3 and TRIF to evaluate the contribution of TLR3 and TLR4 signaling, respectively, to autophagy pathways. Cells from UNC-93B-deficient patients could be used to test the contribution of TLR8 and TLR9 signaling, but mostly of the TLR7 pathway, given previous reports that TLR7 signaling is the most potent to induce

autophagy in response to pathogen evasion (167). It would also be of interest to test whether TBK1 activates autophagy pathways following HSV-1 infection as it does upon infection with *Salmonella* or *Mycobacterium tuberculosis*. For instance, one could look in WT or TBK1-deficient human fibroblasts to determine if the autophagy receptors optineurin and p62, among others, are phosphorylated upon HSV-1 infection, and whether these events are TBK1-dependent. These experiments could help shed light on the crosstalk between autophagy and viral immunity pathways, and could provide the first steps towards understanding whether autophagy is involved in the pathogenesis of HSE.

III.1.iii. Involvement of TBK1 in cell survival signaling

As its knockout phenotype indicated, TBK1 is crucial to cell survival induced by TNF. Indeed, TBK1 phosphorylates RelA/p65, triggering the expression of the anti-apoptotic molecule plasminogen activator inhibitor-2 (PAI-2), which maintains survival through trans-glutaminase 2 (TG2) (181). TBK1 has also been involved in cell survival through its role in the RalB/Sec5 effector complex (126). This study also showed that TBK1 is chronically activated in a variety of cancer cell lines and that it is required to maintain cancer cell survival and to support oncogenic Ras-induced transformation. Another study revealed that oncogenic KRAS-driven cancers require TBK1 (182). The requirement of TBK1 for cell survival and oncogenic transformation has been shown to be mediated in part by its phosphorylation of Akt (127), indicating that the enzymatic activity of TBK1 is essential to its role in cell survival and oncogenic transformation. In addition, Akt is activated upon TLR3 and TLR4 stimulation (183, 184) and IRF3 activation (185), even though the exact mechanisms are unclear. Moreover, Akt phosphorylation is crucial to the IFN-dependent VSV resistance in murine macrophages (186). HSV-1 infection of human cells also triggers Akt signaling, which is involved in the regulation of apoptosis blockage and viral gene expression (187).

In our patients' fibroblasts, cellular viability upon infection with HSV-1 or VSV was strongly decreased as compared to the level observed in control cells. Although we

showed that the *TBK1* mutant alleles produce kinase-dead proteins in *in vitro* assays using Akt as a substrate, we have not tested the phosphorylation of Akt in the patients' heterozygous cells. We cannot exclude that the role of TBK1 in cell survival may contribute to the cellular viability phenotypes described in the patients' cells upon infection with HSV-1 and VSV. Further investigation of these phenotypes could include evaluating the phosphorylation of Akt upon viral infection or TLR3 activation in fibroblasts from the TBK1-deficient patients and in control fibroblasts with and without knockdown of TBK1 expression by siRNA, or using TBK1^{-/-} MEFs to determine whether TBK1 is the Akt-activating kinase in these experimental conditions.

III.1.iv. Interaction between TBK1 and HSV-1 protein ICP34.5

The HSV-1 protein ICP34.5 confers neurovirulence to the virus (188) by interfering with many cellular antiviral processes. As reviewed in III.1.ii, ICP34.5 inhibits autophagy by binding Beclin-1 (164) and antagonizes PKR-dependent antiviral signaling and host translational shutoff (160, 189, 190). In addition, it has been reported that ICP34.5 binds TBK1, thereby disrupting the association of TBK1 with its substrate IRF3 and preventing the induction of IFNs (139). Further work showed that the inhibition of TBK1 by ICP34.5 facilitated viral replication and neuro-invasion (191). In these studies, the authors found that unlike the ICP34.5-null HSV-1 strain, replication of a WT strain of HSV-1 in TBK1^{-/-} MEFs reached similar levels as in TBK1^{+/+} MEFs. This result contrasts with our data in fibroblasts from patients with partial AD TBK1 deficiency. Given the various cellular functions of ICP34.5, the authors further demonstrated that a mutant HSV-1 strain encoding a truncated ICP34.5 unable to bind TBK1 replicated in TBK1^{-/-} MEFs to the same levels as a WT strain. Remarkably, the replication of this mutant strain was impaired in TBK1^{+/+} cells. This result shows that TBK1-mediated immunity dampens HSV-1 replication. We have not tested ICP34.5 binding to our mutant TBK1 proteins, and it would be of interest to examine whether the TBK1-ICP34.5 interaction contributes to the viral phenotype observed in cells from TBK1-deficient patients. Furthermore, targeting of TBK1 by HSV-1 reveals an evolutionary role for TBK1 in host defense against HSV-1 infection.

III. 2. Role of TBK1 in humans and in mice

III.2.i. TBK1 in RIG-I/MDA5 signaling in humans and in mice

In murine cells such as MEFs, RIG-I and MDA5 rely on TBK1 to induce IFNs (192). The two sensors differentially recognize RNA viruses. For instance, infection of MEFs with VSV results in TBK1-mediated IRF3 activation that is TLR3- and TRIF-independent (119) and that was later shown to be RIG-I dependent (193). This phenotype differs from that observed in human fibroblasts, as the induction of IFN triggered by VSV infection is partially TLR3-dependent (63-65, 70, 71). Further investigation showed that RIG-I recognizes ssRNA viral genomes bearing 5' tri-phosphates upon virus replication during infection with Influenza A virus and Sendai virus (194). Similarly, NDV and Japanese Encephalitis Virus (JEV) were shown to induce IFN through RIG-I and TBK1 (193, 195), while EMCV triggers IFN through MDA5 (193). MDA5 has been suggested to be the main sensor of poly(I:C) (193) (196), whereas RIG-I would recognize 5' tri-phosphate ssRNA (142, 197). However, the size of the dsRNA was later shown to discriminate between RIG-I and MDA5 (198).

In P1's fibroblasts, which carry the dominant-negative G159A allele, partial TBK1 deficiency does not impact the response to intra-cellular poly(I:C) stimulation. This could be due to the partial defect in these cells or to TBK1 being non-essential to the response to transfected poly(I:C) in human fibroblasts. Yet, P1's fibroblasts did have a RIG-I specific phenotype, by displaying a delayed IRF3 activation and production of IFN upon low doses of RIG-I-specific ligand. We have not explored the mechanism underlying this delayed response in P1's cells. One hypothesis is that the low dose of RIG-I ligand stimulated the expression of *IKKi* through an undefined activation procedure, which then allowed P1's cells to elicit a delayed response. One possibility for the induction of *IKKi* expression is signaling using the small pool of WT TBK1 available in P1's cells. Another hypothesis could involve the activation of NF κ B (199) by the IKK complex and NEMO/TANK. Indeed, IPS-1-deficient MEFs, in which both the RIG-I- and MDA5-triggered activation of TBK1 is abolished, do not produce IFNs in response to poly(I:C) but still produce low amounts of IL-6, suggesting that there is remaining, yet reduced, NF- κ B-dependent signaling (200) that may be in part IPS-1-dependent but triggered by

RIG-I/MDA5. The mechanism by which P1's cells display a detectable defect only when stimulated with a low dose of a RIG-I-specific agonist remains unclear. Perhaps the use of higher doses of ligand results in higher IRF3 activation or increased cytokines production, which prevents the detection of a defect, given the natural variability observed between healthy controls in response to this stimulus. In fact, we have not shown that this delayed response in P1's fibroblasts was due to the *TBK1* mutant allele: only attempting to rescue this phenotype by over-expressing the WT TBK1 protein in P1's cells would prove that this delayed response is a TBK1-dependent phenotype, and not merely due to inter-individual variability.

III.2.ii. TBK1 in dsDNA signaling in humans and in mice

The endosomal PRR TLR9 was the first sensor described to recognize dsDNA (201). Later studies showed that in mouse and human cells, DNA-sensing was not solely dependent on TLR9 and its adaptor MyD88, and was not confined to endosomes (202-207). Other DNA sensors inducing IFN in a TBK1-independent way have since been discovered: DHX9 and DHX36 (208), LRRFIP1 (209), and Ku70 (210). In parallel, several dsDNA sensors requiring TBK1 to induce IFN (211) were identified: the helicase DDX41 (212), IFI16 (99), DAI/ZBP1 (100), and RNA pol III (213) (214). A seminal finding in elucidating DNA-sensing pathways was the identification in mouse and human of STING/MITA/ERIS/MPYS (215, 216) (128) (102). STING is recruited by IFI16 (99) and directly activates TBK1 (212) (111). STING has also been characterized as a sensor for cyclic dinucleotides (217) and as activated by virus-cell fusion events (101).

In *in vitro* studies, the synthetic DNA poly(dA:dT) was first used in MEFs to show that dsDNA sensors use TBK1 to induce IFN via phosphorylation of IRF3 (110). Similar to poly(dA:dT), a synthetic 45-bp non-CpG DNA oligomer (termed ISD) enhances type I IFN expression in an IRF3-dependent manner in murine macrophages, murine conventional dendritic cells (cDCs) and MEFs (218). The role of TBK1 in dsDNA sensing pathways has also been confirmed through the use of dsDNA viruses: *in vitro*, using macrophages after infection with HSV-1; *in vitro* and *in vivo* after infection with

murine gammaherpesvirus 68 (MHV-68) (219). In mice, the immune response to DNA vaccines is also TBK1-dependent (220), confirming the key role of TBK1 in cytosolic pathways sensing dsDNA. In human fibroblasts, and more specifically in fibroblasts from P1, transfection of poly(dA:dT) resulted in normal amounts of IFN produced. Like previously regarding the different phenotypes observed in MEFs and human fibroblasts for RIG-I/MDA5 signaling, this discrepancy could simply be due to the partial defect in the patients' fibroblasts or, on the contrary, could reveal that TBK1 is not crucial to the response to poly(dA:dT) in human fibroblasts. It is possible that the contribution of TBK1 to dsDNA sensing is cell-type specific. It would be interesting to isolate blood cells such as monocytes and monocyte-derived macrophages from the two TBK1-deficient patients as well as controls, and examine whether the IFN induction in response to DNA viruses such as HSV-1 or to dsDNA such as poly(dA:dT) or ISD is TBK1-dependent in humans, like it is in mice.

IV. Conclusions and Perspectives

Using a candidate gene approach, we identified AD TBK1 deficiency as a novel etiology of isolated HSE in two unrelated European patients. This defect confers an impairment of TLR3 responses in the patients' fibroblasts, confirming the essential role of human TBK1 as an IFN-inducing kinase in the TLR3 pathway in fibroblasts. Both mutant *TBK1* alleles are loss of function, one because of a loss of kinase activity (G159A, P1), the other because of a loss of expression (D50A, P2). Interestingly, the two patients with TBK1 deficiency had different cellular phenotypes, that of the patient with the G159A mutation being more severe, because the G159A allele is dominant-negative whereas the D50A allele exerts dominance by haploinsufficiency. AD TBK1 deficiency is associated with a lack of control of TLR3-dependent viruses, a narrow cellular phenotype that correlates with the narrow clinical phenotype of both patients. Although we could not study the cellular penetrance, we determined that the clinical penetrance is incomplete, as is the penetrance of AR UNC-93B deficiency, AD TLR3 deficiency, and AD TRIF deficiency. Our data highlighted the crucial role of TBK1 in TLR3-mediated anti-HSV-1 immunity. The collection of defects in the genes *TLR3*, *UNC93B1*, *TRIF*, *TRAF3* and *TBK1* in HSE patients adds weight to the argument that HSE should be considered a genetic disorder caused by an array of rare, monogenic inborn errors of TLR3 immunity, in a subset of patients. These findings also suggest that type I IFN could be useful in the treatment of HSE patients.

Our findings in cells from patients with AD partial TBK1 deficiency suggested that human and mouse TBK1s could play different roles in the induction of IFN by TLR4 and by cytosolic signaling pathways triggered by dsRNA, ssRNA and dsDNA. Indeed, our patients' cells did not have any phenotype relative to these pathways, while *TBK1*^{-/-} MEFs display abrogated responses to these stimuli. Human TBK1 could therefore be redundant for certain pathways or in certain cell types. More experiments are needed to conclude whether mouse and human TBK1 contribute differentially to these pathways or if the absence of phenotype in TBK1-deficient human cells results from the partial nature of the defect in our patients' cells. Additional experiments could address the respective

roles of TBK1 and IKKi in antiviral signaling. Determining the kinetics of induction of *IKKi* in control cells and in the TBK1-deficient patients' cells could help clarify whether IKKi contributes to the normal response to transfection of nucleic acids observed in our patients' cells. Using TBK1-deficient patients' fibroblasts and knockdown strategies such as siRNA for *IKKi* or *TBK1* in control cells — and, if they become available, cells from IKKi-deficient patients — the contribution of either kinase in various human cell types could be further examined in comparison to what is known in murine cell types. The normal IFN response to dsRNA, ssRNA, dsDNA, and TLR4 stimulation in our patients' cells may account for the effective control of the viruses tested in AD TBK1-deficient fibroblasts, and for the patients' lack of susceptibility to other viral diseases.

Recent collaborative work in our lab has used induced pluripotent (iPS) cells derived from fibroblasts of patients with AR TLR3 and UNC-93B deficiencies and differentiated these iPS cells into cell types resident of the CNS: neurons, oligodendrocytes, and astrocytes. Lafaille *et al.* showed that UNC-93B-deficient neurons and oligodendrocytes, as well as TLR3-deficient neurons, were more susceptible to HSV-1 than control cells, due to an impaired type I IFN induction. Lafaille *et al.* concluded that the impaired TLR3- and UNC-93B-dependent IFN- α/β immunity to HSV-1 in neurons and oligodendrocytes may underlie the pathogenesis of HSE in children with TLR3 or UNC-93B deficiencies (28). It would thus be of great interest to induce iPS cells from fibroblasts of our TBK1-deficient patients and differentiate them into types of CNS-resident cells so as to test the role of TBK1 in HSV-1 infection in these different cell types. We could test whether HSE pathogenesis stems from a failure of the TLR3-mediated, TBK1-dependent, IFN-inducing anti-HSV-1 immunity in the TBK1-deficient patients' neurons and oligodendrocytes. Evaluating the response to dsRNA, ssRNA, and dsDNA in TBK1-deficient CNS cells would also add to our understanding of the contribution of TBK1 to these pathways in various types of human cells. Another line of study could examine, in CNS cells, the contributions of other antiviral processes described to be TBK1-dependent in the literature, such as autophagy and Akt-mediated cell survival, to anti-HSV-1 immunity.

Lastly, TBK1 is reportedly involved in the immune response to a number of viruses besides HSV-1, via TLRs or cytosolic sensors for nucleic acids, in a cell-specific manner. Findings in fibroblasts from TBK1-deficient patients and in iPS-derived oligodendrocytes and neurons from patients with AR UNC-93B and AR TLR3 deficiencies allow us to infer that, in CNS cells like in fibroblasts, TBK1 might not be redundant to TLR3 antiviral signaling. In other cell types, it is possible that TBK1 is essential to other pathways. As suggested previously, assessing the contribution of TBK1 to all antiviral pathways reported in mice to be TBK1-dependent could be done in various cell types from TBK1-deficient patients, most interestingly blood and CNS cells, using iPS-derived cells. Our TBK1 patients did not have viral diseases other than HSE and their fibroblasts did not display TLR3-independent cellular phenotypes. Several factors could play a role in restricting the clinical and cellular phenotypes, including the partial nature of the patients' TBK1 defect, solely studied in fibroblasts, and the mutations themselves, affecting the kinase domain of TBK1. Perhaps, for instance, a mutation in *TBK1* selectively preventing the binding to a signaling partner would only impair the pathway that requires that specific interaction, and no other TBK1-dependent pathways. We could imagine that, in certain cell types, mutations outside of the kinase domain might impact functions of TBK1 in antiviral pathways besides the one triggered by TLR3, such as cytosolic pathways activated by sensing of nucleic acids, induction of autophagy, or promotion of cell survival. On the other hand, a different hypothesis could be that mutations in *TBK1* impair TLR3 signaling in the CNS in (a) specific cell type(s) (neurons, oligodendrocytes, astrocytes and/or microglia) that could predispose to viral diseases other than HSE. This scenario would suggest that TLR3 signaling in certain CNS-resident cells would play a role in controlling viruses other than HSV-1, which is plausible given the small number of patients with genetic defects impacting TLR3 signaling. Therefore, we could, in the future, extend the search for mutations in *TBK1* to patients with viral infections other than HSE.

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Heterozygous *TBK1* mutations impair TLR3 immunity and underlie herpes simplex encephalitis of childhood

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Childhood herpes simplex virus-1 (HSV-1) encephalitis (HSE) may result from single-gene inborn errors of TLR3 immunity. TLR3-dependent induction of IFN- α/β or IFN- λ is crucial for protective immunity against primary HSV-1 infection in the central nervous system (CNS). We describe here two unrelated children with HSE carrying different heterozygous mutations (D50A and G159A) in *TBK1*, the gene encoding TANK-binding kinase 1, a kinase at the crossroads of multiple IFN-inducing signaling pathways. Both mutant *TBK1* alleles are loss-of-function but through different mechanisms: protein instability (D50A) or a loss of kinase activity (G159A). Both are also associated with an autosomal-dominant (AD) trait but by different mechanisms: haplotype insufficiency (D50A) or negative dominance (G159A). A defect in polyinosinic-polycytidylic acid-induced TLR3 responses can be detected in fibroblasts heterozygous for G159A but not for D50A *TBK1*. Nevertheless, viral replication and cell death rates caused by two TLR3-dependent viruses (HSV-1 and vesicular stomatitis virus) were high in fibroblasts from both patients, and particularly so in G159A *TBK1* fibroblasts. These phenotypes were rescued equally well by IFN- $\alpha 2b$. Moreover, the IFN responses to the TLR3-independent agonists and viruses tested were maintained in both patients' peripheral blood mononuclear cells and fibroblasts. The narrow, partial cellular phenotype thus accounts for the clinical phenotype of these patients being limited to HSE. These data identify AD partial *TBK1* deficiency as a new genetic etiology of childhood HSE, indicating that *TBK1* is essential for the TLR3- and IFN-dependent control of HSV-1 in the CNS.

HSV-1 encephalitis (HSE) remained a lethal disease for four decades after the establishment of its viral etiology in 1941 (Whitley, 2006). The advent of the antiviral drug acyclovir in 1981 greatly decreased mortality rates in children with HSE, but severe neurological

sequelae are still commonly observed in surviving patients (Whitley et al., 1986). Childhood HSE occurs during primary infection with HSV-1, a double-stranded DNA (dsDNA) virus

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Abbreviations used: AD, autosomal dominant; AR, autosomal recessive; CNS, central nervous system; EMCV, encephalomyocarditis virus; HSE, HSV-1 encephalitis; MEF, mouse embryonic fibroblast; MOI, multiplicity of infection; mRNA, messenger RNA; poly(I:C), polyinosinic-polycytidylic acid; RT-qPCR, quantitative RT-PCR; SNP, single nucleotide polymorphism; ssRNA, single-stranded RNA; SV40, simian virus 40; VSV, vesicular stomatitis virus.

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of the Herpesviridae family. HSV-1 follows a neurotropic infection route, as the virus reaches the central nervous system (CNS) from the nasal or oral epithelium via the cranial nerves, by retrograde axonal transport in the olfactory or trigeminal nerve (De Tiège et al., 2008; Abel et al., 2010). HSE is restricted to the CNS, with no detectable viremia or lesions of other tissues. About 85% of young adults worldwide are seropositive for HSV-1, and the vast majority are asymptomatic or present only benign herpes labialis (Stanberry et al., 1997). The incidence of HSE is ~1–2 cases per 500,000 individuals per year (Whitley and Kimberlin, 2005). The occurrence of HSE, which is not epidemic, has never been shown to be associated with any particular HSV-1 isolate. HSE is nonetheless the most common sporadic viral encephalitis in the Western world. Childhood HSE thus remained a rare, unexplained, and devastating complication of primary infection with HSV-1, until our discoveries of single-gene inborn errors of TLR3 immunity in some children with HSE (Casrouge et al., 2006; Zhang et al., 2007, 2008; Pérez de Diego et al., 2010; Guo et al., 2011; Sancho-Shimizu et al., 2011b).

We have reported children with isolated HSE caused by autosomal-recessive (AR) UNC-93B deficiency (Casrouge et al., 2006), autosomal-dominant (AD) TLR3 deficiency (Zhang et al., 2007), AR TLR3 deficiency (Guo et al., 2011), AD TRAF3 deficiency (Pérez de Diego et al., 2010), AD TRIF deficiency, and AR TRIF deficiency (Sancho-Shimizu et al., 2011a,b). In addition, one patient with AR STAT-1 deficiency and impaired cellular responses to IFNs (Dupuis et al., 2003) and another patient with X-linked recessive NEMO deficiency and impaired TLR3-dependent production of IFNs (Audry et al., 2011) died of HSE, albeit after many other infectious illnesses. Conversely, patients with MyD88 and IRAK4 deficiencies but with an intact TLR3 pathway are not prone to HSE (Picard et al., 2003, 2010; Yang et al., 2005; Ku et al., 2007; von Bernuth et al., 2008). Collectively, these genetic data indicate that the TLR3-dependent induction of IFN- α/β and/or IFN- λ is essential for protective immunity to HSV-1 primary infection of the CNS during childhood. In dermal fibroblasts, genetic defects of the TLR3 pathway impair the production of IFN- β and IFN- λ

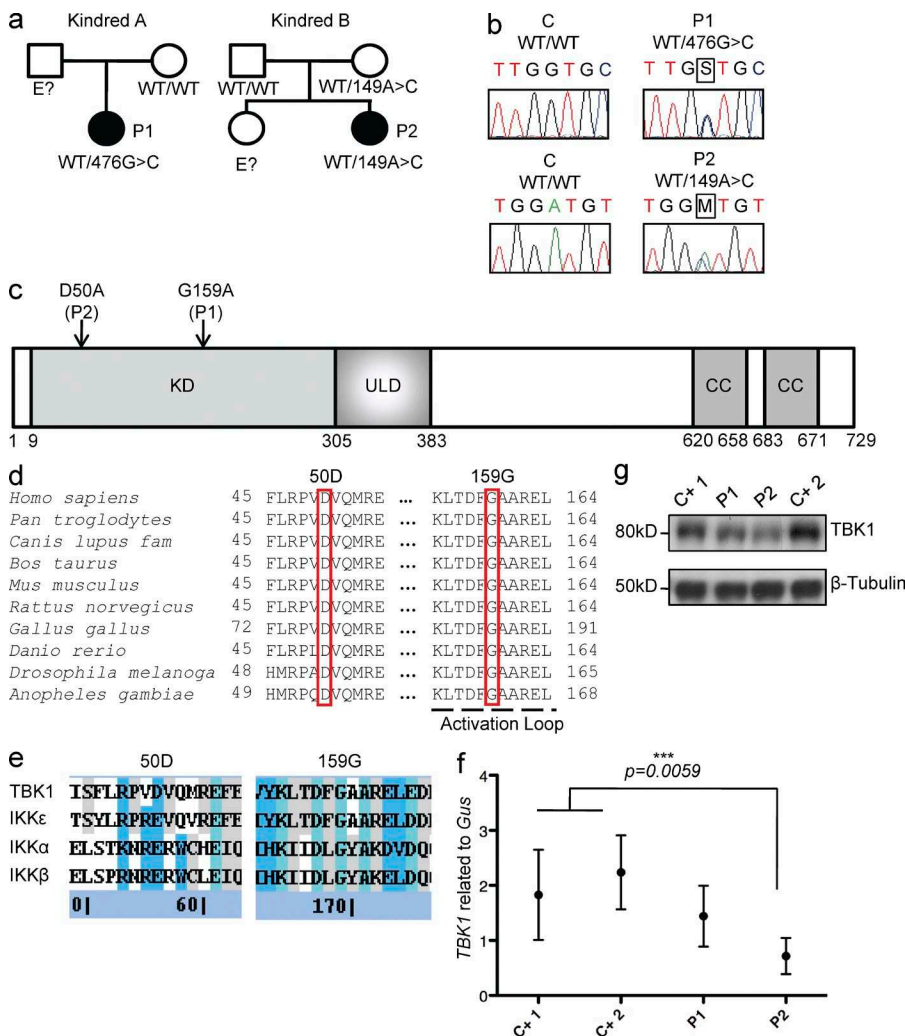


Figure 1. Heterozygous *TBK1* mutations in two children with HSE. (a) Family pedigrees and segregation. (b) Heterozygous *TBK1* mutations 476G>C in P1 and 149A>C in P2. The PCR products sequenced were amplified from genomic DNA from the granulocytes of a control (C) and both patients. (c) Schematic diagram of the protein structure of TBK1, featuring its kinase domain (KD), ubiquitin-like domain (ULD), and coiled-coil (CC) regions. Both heterozygous substitutions, 159G>G/A (P1) and 50D>D/A (P2), affect the kinase domain of TBK1 (amino acids 9–305). (d) Multiple alignments of relevant amino acid sequences of the kinase domain of human TBK1 with its homologues from nine other species, with the residues mutated in P1 (G159) and P2 (D50) highlighted. (e) Multiple alignments of relevant amino acid sequences of the kinase domain of human TBK1 with the other IKK and IKK-related kinases, IKK- α (46% similar to TBK1), IKK- β (44% similar), and IKK- ϵ (64% similar). The residues mutated in P1 and P2 are conserved (G159) or similar (D50) across IKK or IKK-related kinases. (Blue signifies sequence similarity, teal signifies sequence identity, and gray signifies partial identity/similarity). (f) *TBK1* expression, as assessed by RT-qPCR on mRNA from the SV40-fibroblasts of patients (P1 and P2) and control lines (C+1 and C+2). Values represent mean values \pm SD calculated from three independent experiments. (g) *TBK1* levels, as assessed by Western blotting, in SV40-fibroblasts from patients (P1 and P2) and two control lines (C+1 and C+2). This Western blot result is representative of three experiments.

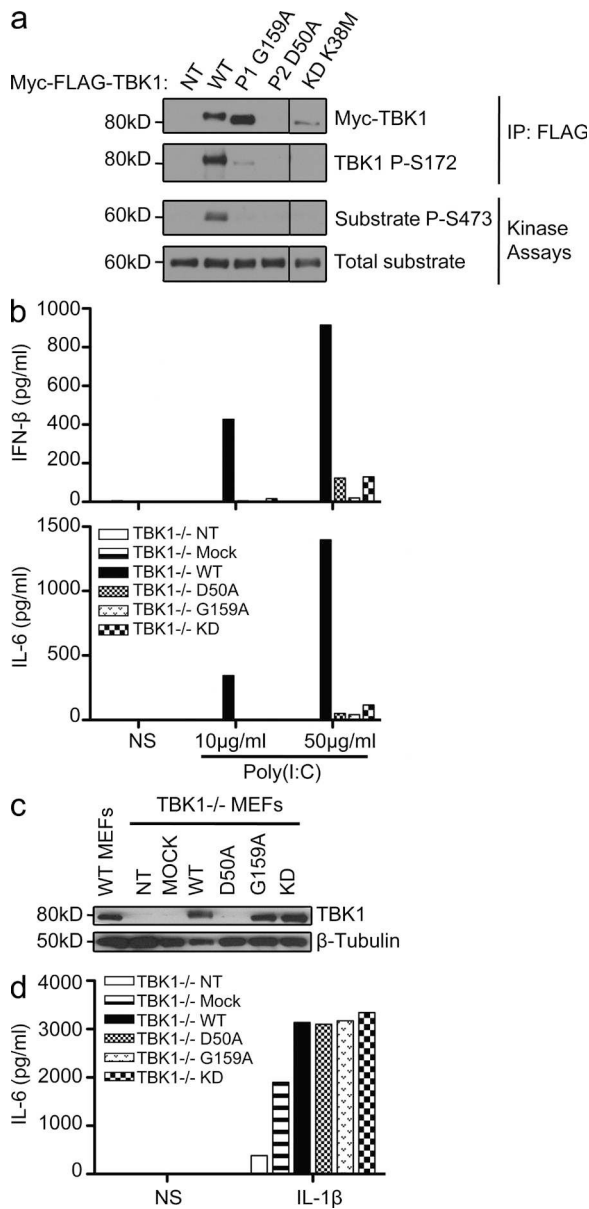


Figure 2. Both mutant TBK1 alleles are loss-of-function but through different mechanisms. (a) In vitro kinase assays: substrate (Akt) phosphorylation by both mutant kinases, G159A and D50A, compared with the phosphorylation levels for WT TBK1 and the kinase-dead (KD) K38M TBK1. A single experiment representative of three independent experiments performed is shown. HEK293T cells were transfected with WT and mutant constructs or left untransfected (NT). Black lines indicate that intervening lanes have been spliced out. IP, immunoprecipitation. (b) *TBK1*^{-/-} MEFs were either left untransfected (NT) or were transfected with a mock vector, WT TBK1, or mutant constructs: D50A (mutation present in P2), G159A (the mutation in P1), and S172A (kinase-dead, KD). After 24 h, the cells were stimulated with 10 or 50 μ g/ml poly(I:C). ELISA was performed to assess IFN- β and IL-6 production 24 h after stimulation. The data shown are representative of three independent experiments. (c) TBK1 expression from the transfected constructs was assessed by Western blotting with antibodies against TBK1, with β -tubulin used as a loading control. (d) *TBK1*^{-/-} MEFs were either not transfected (NT) or transfected with a mock vector or a vector encoding WT TBK1 or the mutants, G159A, D50A, or S172A.

after stimulation with the nonspecific, synthetic, TLR3 agonist polyinosinic-polycytidylic acid (poly(I:C)). These fibroblasts are also highly susceptible to infection with HSV-1 or vesicular stomatitis virus (VSV). This fibroblast phenotype has recently been shown to recapitulate the impairment of TLR3-dependent HSV-1 control in *UNC-93B*-deficient cells of the CNS (unpublished data). As only a fraction of children with HSE bear known defects in *TLR3*, *UNC93B1*, *TRIF*, or *TRAF3*, we searched for other genetic defects by screening HSE patients for mutations in other genes involved in the TLR3-IFN pathway.

RESULTS

Heterozygous *TBK1* mutations in two patients with HSE

We investigated two unrelated patients with HSE from Poland (P1) and from France (P2; Fig. 1 a). These patients suffered from HSE at the ages of 7 yr (P1) and 11 mo (P2). They are now 17 and 26 yr old, respectively, and have suffered no other unusual infectious disease, viral in particular, in the absence of prophylaxis. We sequenced candidate TLR3 pathway genes from genomic DNA extracted from granulocytes and from simian virus 40 (SV40)-transformed fibroblasts (SV40-fibroblasts) from the two patients and cDNAs synthesized from messenger RNAs (mRNAs) extracted from SV40-fibroblasts. We found two different mutations in the coding region of the gene encoding TANK-binding kinase 1 (TBK1, also known as T2K/NAK [Tojima et al., 2000; Pomerantz and Baltimore, 1999; Bonnard et al., 2000]). P1 carries a heterozygous mutation in the fifth exon of *TBK1*, at position 476 of the cDNA: c.476G>G/C (Fig. 1 b). Her father was not tested and her mother does not carry this variant (Fig. 1 a). The mutation results in the nonconservative replacement of a glycine residue with an alanine residue at position 159 (G159A) in the immediate vicinity of the predicted activation loop of the kinase (Fig. 1 c; Xu et al., 2011). P2 carries a heterozygous mutation in the third exon of *TBK1*, c.149A>A/C (Fig. 1 b). Her mother is healthy and carries the same heterozygous mutation, whereas her father is WT for *TBK1* (Fig. 1 a). This substitution results in a nonconservative change at position 50 in the kinase domain, replacing an aspartic acid residue with an alanine residue (D50A; Fig. 1 c). No mutations were found in the coding exons of *TLR3*, *UNC93B1*, *TRAF3*, *TRIF* (encoding Toll/IL-1 receptor domain-containing adaptor protein inducing IFN- β , TRIF) and *TANK* (encoding TRAF-interacting protein I, TANK; see Whole-exome sequencing in P1 and P2: TLR3-IFN pathway genes sequenced and found to be WT in the Materials and methods). No mutations were found in other genes known to be involved in the TLR3-IFN pathway, as detected by whole-exome sequencing (see Whole-exome sequencing in P1 and P2: TLR3-IFN pathway genes sequenced and found to be WT).

24 h later, cells were stimulated with 10 ng/ml IL-1 β . IL-6 production was measured by ELISA. The data shown are representative of three independent experiments. NS, nonstimulated.

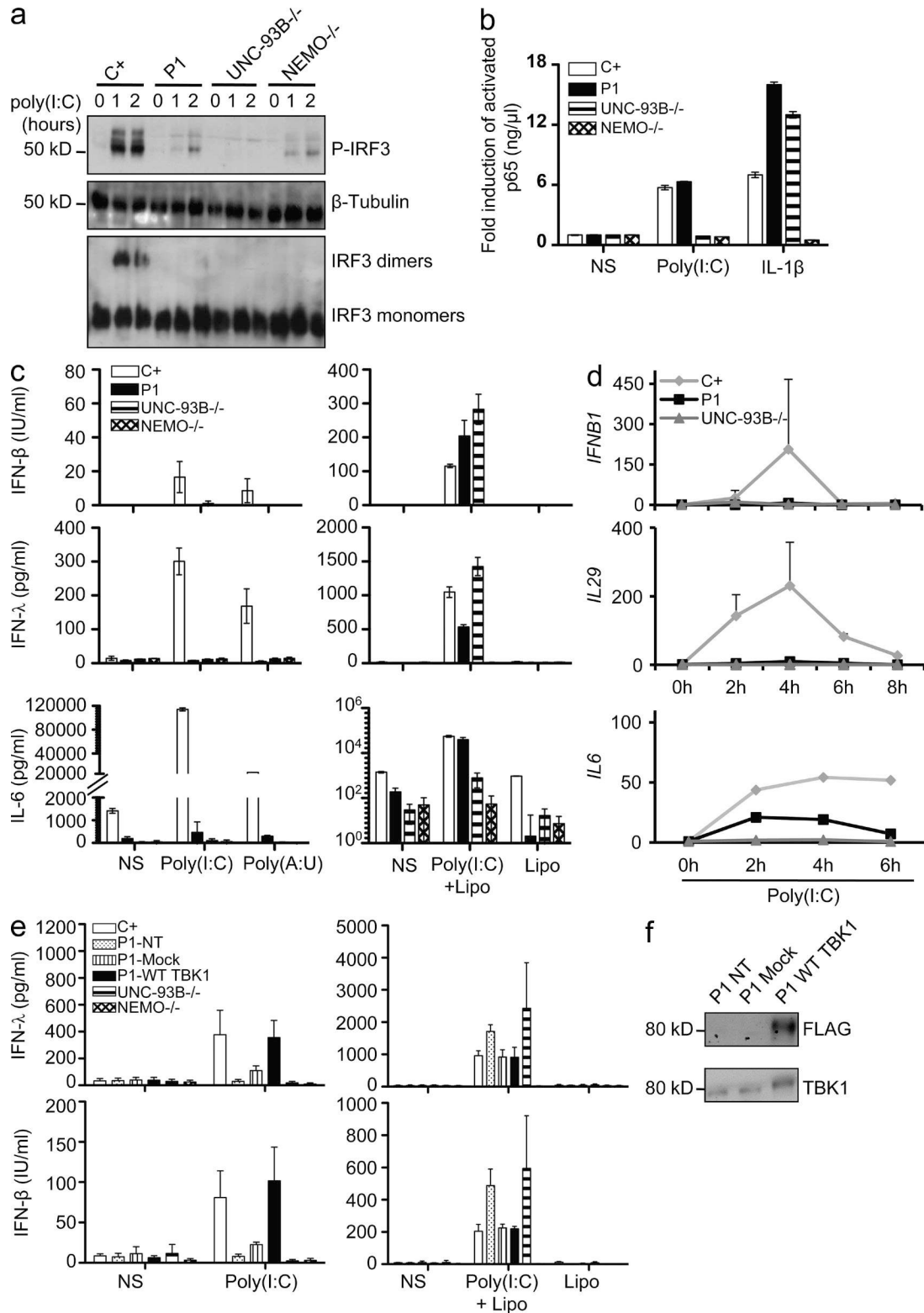


Figure 3. The G159A TBK1 allele of P1 abolishes TLR3-mediated IFN induction in the patient's fibroblasts. (a) IRF-3 phosphorylation in fibroblasts from P1, as assessed by Western blotting after stimulation with 25 μ g/ml poly(I:C) for 0, 1, or 2 h, with β -tubulin as a loading control. Comparison with control cells (C+) and UNC-93B^{-/-} and NEMO^{-/-} fibroblasts. IRF3 dimerization was assessed by native Western blotting. Blots are representative of three independent experiments. Two different healthy control cell lines were tested and gave the same result. (b) Activated p65 levels, as determined by EMSA-ELISA after 30 min of stimulation with IL-1 β or 1 h of poly(I:C) in control cells (C+; averaged from two control cell lines), in fibroblasts from P1 and from UNC-93B^{-/-} and NEMO^{-/-} patients. (c) IFN- β , IFN- γ , and IL-6 production, as assessed by ELISA, after 24 h of stimulation with poly(I:C) or poly(A:U) (d) Time course of IFN- β , IL29, and IL6 production after stimulation with poly(I:C) in control cells (C+), P1, and UNC-93B^{-/-} fibroblasts. (e) IFN- γ and IFN- β production after stimulation with poly(I:C) in control cells (C+), P1-NT, P1-Mock, P1-WT TBK1, UNC-93B^{-/-}, and NEMO^{-/-} fibroblasts. (f) Western blotting for FLAG and TBK1 in P1-NT, P1-Mock, and P1-WT TBK1 fibroblasts.

Neither of the two *TBK1* variants was found in single nucleotide polymorphism (SNP) databases (NCBI dbSNP 135 and UCSC) or on sequencing of a panel of 1,050 control human DNAs provided by the CEPH-HGD, ruling out the possibility of irrelevant polymorphisms. No other mutations were found in the remaining exons or flanking intronic regions of *TBK1*. Moreover, the D50 and G159 residues are strictly conserved across species (Fig. 1 d). Both substitutions are also predicted to be “probably damaging” by Polyphen-2 version 2.2.2 (Adzhubei et al., 2010) and “damaging” by SIFT (Kumar et al., 2009). Finally, the other human IKK-related kinases, IKK- ϵ (64% similar to human *TBK1*), IKK- α (46% similar to *TBK1*), and IKK- β (44% similar to *TBK1*), have a glutamic acid residue, which is similar to aspartic acid, at position 50 and a glycine residue at position 159 (Fig. 1, d and e). Collectively, these genetic data suggest that these two patients are heterozygous for rare, HSE-causing *TBK1* missense alleles.

Differences in the expression of the mutant *TBK1* alleles

We first assessed *TBK1* mRNA levels by quantitative RT-PCR (RT-qPCR) in SV40-fibroblasts from the patients and in control cells (Fig. 1 f). *TBK1* mRNA levels were similar in control cells and in fibroblasts from P1, whereas they were lower in fibroblasts from P2 (Fig. 1 f). Consistent with these findings, *TBK1* protein levels were normal (similar to the control) in fibroblasts from P1, but low in fibroblasts from P2, as shown by Western blotting (Fig. 1 g). The heterozygous G159A mutation therefore seems to have no effect on *TBK1* expression in the patients' fibroblasts, whereas the D50A mutation seems to affect the amounts of both mRNA and protein for *TBK1*. As the level of expression of mutant alleles may be confounded by the expression of the WT allele in the patients' heterozygous cells, we then compared the expression of the individual *TBK1* alleles in HEK293T cells or *TBK1* knockout (*TBK1*^{-/-}) mouse embryonic fibroblasts (MEFs), transfected with either a mutant allele or the WT human *TBK1* allele. Upon transient transfection with the G159A mutant allele, HEK293T cells and *TBK1*^{-/-} MEFs produced G159A *TBK1* in amounts similar to those obtained for the WT *TBK1*. In contrast, transfection with the D50A mutant allele resulted in the production of much smaller amounts of protein (Fig. 2, a and c). The D50A mutation in *TBK1* may thus destabilize the protein, resulting in its premature degradation and the presence of smaller total amounts of *TBK1* protein in the patient's fibroblasts.

These data strongly suggest that the *TBK1* G159A allele is expressed normally, whereas the D50A allele is poorly expressed.

Both *TBK1* mutant alleles are kinase-dead and loss-of-function

TBK1 is a Ser/Thr kinase of the IKK-related family, which, upon TLR3 activation, is recruited to the TRIF-TRAF3 complex (Sato et al., 2003; Oganeyan et al., 2006). After their activation by phosphorylation, *TBK1* and IKK- ϵ (also known as IKKi [Shimada et al., 1999; Peters et al., 2000]) normally phosphorylate their target transcription factor, IRF3 (Fitzgerald et al., 2003; Sharma et al., 2003). In vitro kinase assays showed that the mutant *TBK1* protein carrying the G159A substitution had no enzyme activity (Fig. 2 a). The levels of in vitro phosphorylation of the recombinant protein Akt, used as a substrate (Ou et al., 2011), were much lower with the G159A *TBK1* protein than with WT *TBK1* (Fig. 2 a). In addition, phosphorylation of the S172 residue of *TBK1*, which is required for its activation (Kishore et al., 2002), was severely impaired in the G159A mutant protein (Fig. 2 a). The kinase-dead K38M mutant was used as negative control in these in vitro assays (Fig. 2 a). As previously discussed (Fig. 1 g), D50A *TBK1* was produced in much smaller amounts than the WT, G159A, or K38M *TBK1* variants (Fig. 2 a). We then assessed the functionality of the two mutant proteins, in terms of *TBK1* involvement in the TLR3 pathway, in *TBK1*^{-/-} MEFs (McWhirter et al., 2004) transiently transfected with the D50A, G159A, or WT human *TBK1* allele. The transfection of *TBK1*^{-/-} MEFs with either the D50A or the G159A *TBK1* allele failed to restore the poly(I:C)-induced IFN- β induction, whereas this induction was restored by transfection with the WT human *TBK1* (Fig. 2 b). Indeed, G159A *TBK1* behaved like the kinase-dead S172A mutant (Lei et al., 2010). The D50A allele was a loss-of-expression allele, as shown by Western blotting (Fig. 2 c), whereas the G159A and S172A *TBK1* alleles resulted in the production of amounts of *TBK1* protein similar to those produced from the WT allele (Fig. 2 c). All transfected cells were healthy and produced IL-6 in response to IL-1 β (Fig. 2 d). Thus, the G159A *TBK1* mutant allele is normally expressed but generates a kinase-dead protein, and the D50A allele is a loss-of-expression allele resulting in a loss of protein activity. Both mutants are therefore loss-of-function for TLR3-mediated cellular responses.

TLR3 responses to poly(I:C) are impaired in fibroblasts from P1 but not in those from P2

We tested the hypothesis that the loss-of-function G159A and D50A *TBK1* alleles are associated with an AD form of

(polyadenylic-polyuridylic acid) or transfection of poly(I:C) mediated by Lipofectamine (Lipo) in control (C+) cells (averaged from two distinct control cell lines) and in fibroblasts from P1 and from UNC-93B^{-/-} and NEMO^{-/-} patients. (d) Induction of mRNA for *IFNB1*, *IL29*, and *IL6* after stimulation with 25 μ g/ml poly(I:C), as assessed by RT-qPCR in fibroblasts from P1, an UNC-93B^{-/-} patient, and controls (C+; averaged from two distinct control cell lines). Graphs present the mean values \pm SD of three independent experiments for *IFNB1* and *IL29*. The graph presented for *IL6* is representative of two independent experiments. (e) Fibroblasts from P1 were untransfected (NT), mock-transfected, or transfected with a vector encoding FLAG-tagged WT *TBK1* and stimulated for 24 h with the indicated reagents (poly(I:C) or poly(I:C) transfection mediated by Lipofectamine). IFN- β and IFN- λ production was assessed by ELISA. (b, c, and e) Values represent mean values \pm SD calculated from three independent experiments. (f) Western blot of transfected P1 cells with anti-*TBK1* and anti-FLAG antibodies. NS, nonstimulated.

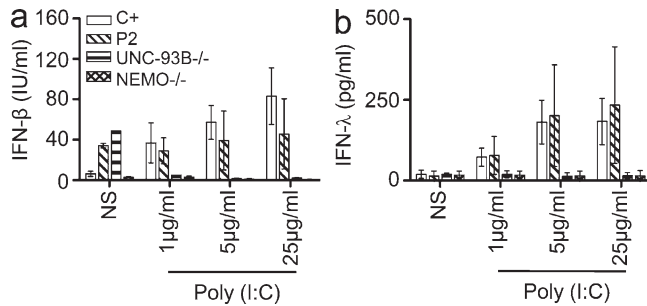


Figure 4. TLR3-mediated IFN production is normal in P2's fibroblasts. (a and b) WT (C+), P2, UNC-93B^{-/-}, and NEMO^{-/-} fibroblasts were stimulated with various doses of poly(I:C) for 24 h. The production of IFN-β (a) and IFN-λ (b) was assessed by ELISA. The graphs show the mean values ± SD for three independent experiments. NS, nonstimulated.

HSE by first studying the response of the patients' heterozygous fibroblasts to poly(I:C). Human dermal fibroblasts express TLR3 and respond to poly(I:C) in a TLR3-dependent manner (Matsumoto et al., 2002; Zhang et al., 2007; Guo et al., 2011). In control cells, the activation of IRF3 and

NF-κB after the recruitment of TRIF to TLR3 (Sato et al., 2003) leads to the production of IFN-β, IFN-λ, and proinflammatory cytokines such as IL-6. We assessed IRF3 phosphorylation and subsequent dimerization by native PAGE and Western blotting after poly(I:C) stimulation. We found that cells from P1 (G159A/WT) did not support IRF3 phosphorylation or dimerization to levels observed in control cells, similar to UNC-93B^{-/-} and NEMO^{-/-} deficient cells, in which TLR3 signaling is abolished (Fig. 3 a). However, the fibroblasts of P1 displayed normal nuclear activation of NF-κB in response to poly(I:C) stimulation (Fig. 3 b), like healthy control cells but unlike UNC-93B^{-/-} or NEMO^{-/-} deficient fibroblasts. Consistent with the lack of IRF3 activation, SV40-transformed and primary fibroblasts from P1 did not produce IFN-β and IFN-λ mRNA or protein upon stimulation with extracellular poly(I:C) (Fig. 3, c and d; and not depicted). Consistent with the NF-κB activation observed, poly(I:C)-induced IL-6 production was low but detectable in P1's fibroblasts (Fig. 3, c and d). Moreover, the lack of response of P1's cells to poly(I:C) was rescued by WT *TBK1* overexpression (Fig. 3, e and f). Similarly, the patient's cells were able to produce IFN-β and IFN-λ upon stimulation with poly(I:C) in the presence of Lipofectamine (Fig. 3, c and e), which mediates the transfection of poly(I:C) into the cytosol, where it can be detected by cytosolic dsRNA sensors other than endosomal TLR3 (Kato et al., 2006, 2008). Cells from patients with AR UNC-93B and AR TLR3 deficiencies also respond to poly(I:C) in the presence of Lipofectamine (Fig. 3, c and e;

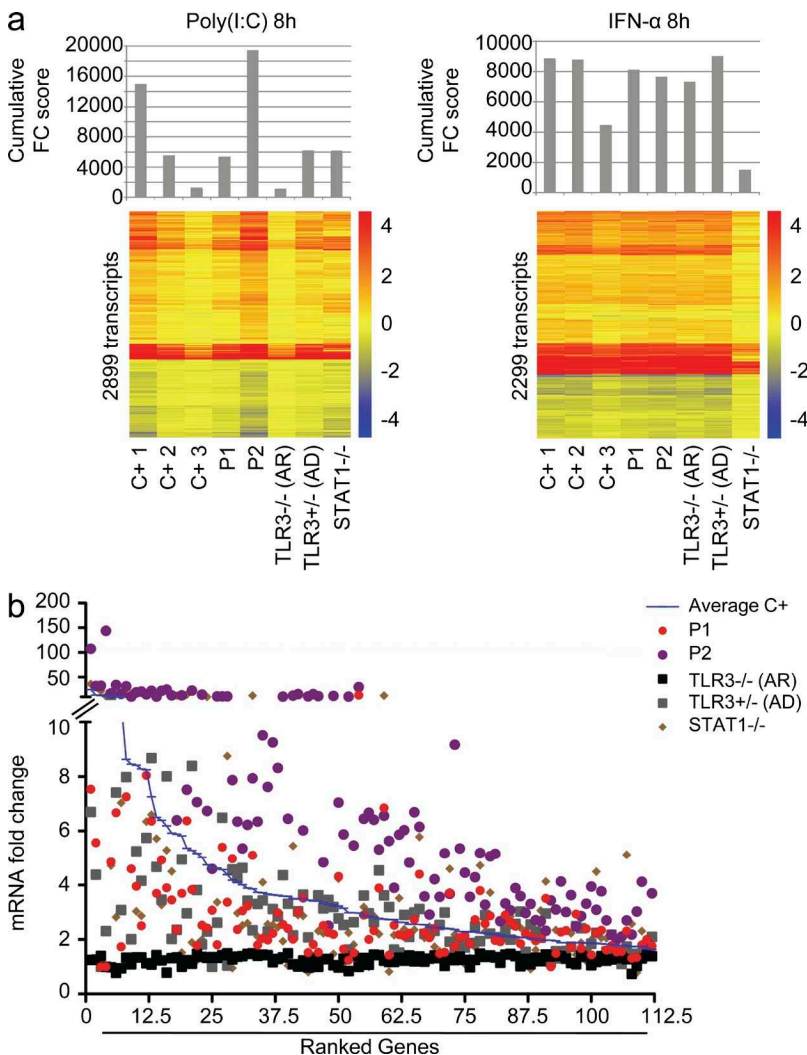


Figure 5. Genome-wide transcriptional evaluation of the TLR3 pathway in primary fibroblasts.

(a) Cumulative fold change (FC) score (top) and heat maps (bottom) of the transcripts regulated by 8 h of stimulation with poly(I:C) (left) or IFN-α2b (right) in primary fibroblasts from three healthy controls (C+), both TBK1 patients (P1 and P2), a patient with TLR3 AR deficiency (TLR3^{-/-}), a patient with AD TLR3 deficiency (AD TLR3), and a patient with STAT-1 complete deficiency (STAT1^{-/-}). The cumulative score is the sum of all the fold change values of >1.5 (up- or down-regulation). Heat maps show a hierarchical clustering of transcripts differentially expressed upon stimulation (based on 100 differences in intensity and 1.5-fold changes compared with nonstimulated condition in healthy controls). Changes with respect to the unstimulated condition are shown by a color scale: red, up-regulated; blue, down-regulated; yellow, no change. The probes displaying differences of >100 in intensity were used to calculate the cumulative score. (b) Ranking of the 112 transcripts up-regulated after 8 h of poly(I:C) stimulation, with a fold change of at least 1.5 in all three controls tested, in primary fibroblasts from three healthy controls averaged together (Average C+), both TBK1 patients (P1 and P2), a patient with TLR3 AR deficiency (TLR3^{-/-}), a patient with AD TLR3 deficiency (AD TLR3), and a patient with STAT-1 complete deficiency (STAT1^{-/-}).

Casrouge et al., 2006; Guo et al., 2011). Thus, the TBK1 defect in P1 impairs TBK1-dependent TLR3 signaling but does not seem to affect signaling by cytosolic dsRNA sensors. In contrast, in fibroblasts from P2 (D50A/WT), IRF3 dimerization was only marginally impaired upon stimulation with a low dose of poly(I:C) (1 $\mu\text{g}/\text{ml}$; not depicted), and no such impairment was observed at high doses (25 $\mu\text{g}/\text{ml}$; not depicted). Consequently, the production of IFN- β and IFN- λ in response to extracellular poly(I:C) was normal in fibroblasts from P2 (Fig. 4, a and b). Thus, TLR3 signaling was only marginally affected by the heterozygous D50A *TBK1* allele in the fibroblasts of P2. Consistently, the TLR3-dependent poly(I:C) response was impaired in fibroblasts from P1 but not P2, as assessed by genome-wide transcription experiments (Fig. 5). Overall, heterozygosity for the G159A *TBK1*

allele severely impairs IRF3-dependent IFN- β and IFN- λ production in response to TLR3 activation, whereas the D50A *TBK1* allele leads to no detectable impairment of TLR3-dependent poly(I:C) responses in human fibroblasts.

IFN-dependent control of HSV-1 and VSV is impaired in the fibroblasts of both patients

The IFN response to HSV-1 and VSV in human dermal fibroblasts has been shown to be partially dependent on TLR3. TLR3-, UNC-93B-, TRIF-, and TRAF3-deficient fibroblasts fail to contain the replication of HSV-1 and/or VSV as the result of a lack of IFN- β and IFN- λ production (Casrouge et al., 2006; Zhang et al., 2007; Pérez de Diego et al., 2010; Guo et al., 2011; Sancho-Shimizu et al., 2011b). We tested the hypothesis that the loss-of-function G159A

and D50A *TBK1* alleles were responsible for an AD form of HSE by investigating the IFN-mediated antiviral response in heterozygous fibroblasts from P1 and P2. The fibroblasts of P1 produced no IFN- β or IFN- λ 24 h after infection with HSV-1 (Fig. 6 a). The cells of P1 therefore displayed high levels of viral replication and cell death, like TLR3- and UNC-93B-deficient cells but unlike healthy control cells (Fig. 6, c and e). The addition of IFN- α 2b to these cells restored virus

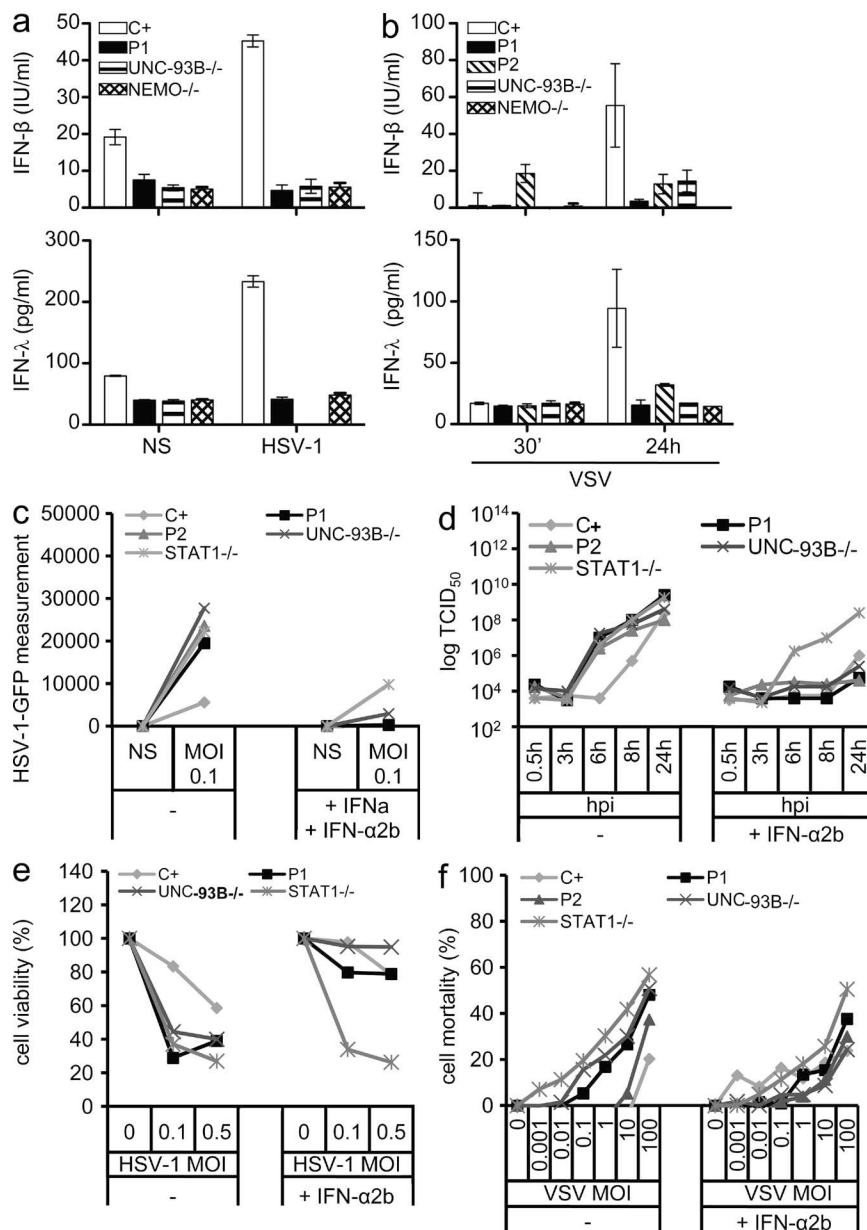


Figure 6. Impaired IFN-dependent control of HSV-1 and VSV infection in the patients' fibroblasts. (a and b) IFN- β and IFN- λ production, as measured by ELISA, in the patients' fibroblasts, control cells (C+; averaged from two distinct control cell lines), and UNC-93B^{-/-} and NEMO^{-/-} fibroblasts after 24 h of stimulation with HSV-1 (a) and 30 min or 24 h of stimulation with VSV (b). The graphs display means \pm SD determined from three independent experiments. (c and d) Replication of the HSV-1-GFP virus at an MOI of 1 (c) and of VSV at an MOI of 10 (d) in the patients' fibroblasts (P1 and P2) and in control cells (C+; averaged from two distinct control cell lines), and UNC-93B^{-/-} and STAT-1^{-/-} fibroblasts, as determined at the indicated hours after infection, with or without 18-h IFN- α 2b pretreatment. One experiment representative of two independent experiments performed is shown. (e) Viability of control cells (C+; averaged from two different control cell lines) and in fibroblasts from P1, an UNC-93B^{-/-} patient, and a STAT-1^{-/-} patient after infection with HSV-1-GFP at MOIs of 0.1 and 0.5, with or without IFN- α 2b treatment 18 h before infection. (f) Cell mortality after 24 h of VSV infection in control cells (C+; averaged from two distinct control cell lines) and in fibroblasts from P1, P2, a UNC-93B^{-/-} patient, and a STAT-1^{-/-} patient, with or without 18-h IFN- α 2b pretreatment. NS, nonstimulated.

containment to levels similar to those in control cells (Fig. 6, c and e). The IFN dependence of this phenotype was confirmed by the impaired response of STAT-1-deficient fibroblasts to prior treatment with IFN- α 2b, in terms of viral control (Fig. 6 c). Moreover, fibroblasts from P1 and P2 also displayed very low levels of IFN- β and IFN- λ production after infection with VSV at a multiplicity of infection (MOI) of 10 (Fig. 6 b). VSV replication was not suppressed by cells from either of the patients but was suppressed by fibroblasts from a healthy control (Fig. 6 d). VSV-induced cell death levels were also higher in cells from both patients than in the control, as in TLR3- and UNC-93B-deficient cells (Fig. 6 f). The treatment of these cells with IFN- α 2b restored viral containment and cell death to levels similar to those in control cells (Fig. 6, d and f). The IFN-dependent control of HSV-1 and VSV, which is at least partly dependent on TLR3 in human fibroblasts (Casrouge et al., 2006; Zhang et al., 2007; Pérez Diego et al., 2010), was therefore impaired in the patients' cells. In cells heterozygous for the G159A and D50A *TBK1* alleles, the two loss-of-function *TBK1* mutant alleles thus confer a dominant phenotype, in terms of the TLR3- and IFN-dependent control of viruses. Fibroblasts heterozygous for D50A *TBK1* displayed an impaired control of viruses via TLR3, despite responding normally to poly(I:C) via TLR3. This suggests that the entire, intact, *TBK1*-dependent TLR3 pathway is required for HSV-1 and VSV control in human fibroblasts, although we cannot exclude a possible role for *TBK1* in the TLR3-dependent or -independent inhibition of HSV-1 replication (Verpooten et al., 2009; Ma et al., 2012) or cell death (Ou et al., 2011). Both the G159A and D50A *TBK1* alleles are thus loss-of-function and dominant for TLR3- and IFN-dependent antiviral immune responses. The D50A allele, which is weakly expressed, is dominant for viral control via TLR3. The G159A allele, which is normally expressed and encodes a kinase-dead protein, is dominant not only for viral control but also for poly(I:C) responses via TLR3.

The two *TBK1* mutant alleles are dominant through different mechanisms

Our data suggest that the two *TBK1* alleles may be dominant through different mechanisms. We investigated whether the mutant *TBK1* alleles were dominant negative by transducing control human fibroblasts, which express endogenous WT *TBK1*, with lentiviral pseudoparticles encoding luciferase only (used as a negative control), WT *TBK1*, or one of the two mutant *TBK1* proteins. After TLR3 stimulation by poly(I:C), we measured the IFN- β and IFN- λ production from those cells. Transient overexpression of the G159A *TBK1* allele in healthy control fibroblasts decreased the IFN- λ and IFN- β production upon poly(I:C) stimulation (Fig. 7 a), but no such effect was observed after overexpression of the D50A or WT allele. Western blotting of the whole-cell lysates confirmed the overexpression of the G159A and WT *TBK1* proteins (Fig. 7 b). The G159A *TBK1* allele thus exerts a dominant-negative effect on WT *TBK1* activity, which is normally expressed in control cells. In contrast, the amounts of IFN- λ and

IFN- β produced in response to poly(I:C) were similar in control fibroblasts transduced with lentiviral pseudoparticles encoding luciferase or the WT or D50A *TBK1* proteins. The D50A *TBK1* allele is therefore most likely not dominant negative and exerts its dominant effect by haploinsufficiency. This is consistent with the previous observations of *TBK1* instability and lower total amounts of *TBK1* in the cells of P2 (Fig. 1, f and g and Fig. 2, a and c). Both the G159A and D50A *TBK1* alleles are thus loss-of-function, but one is dominant over the WT allele by negative dominance and the other by haploinsufficiency. We then investigated the possible dominance of the two alleles in the context of other TLR3-independent, *TBK1*-mediated, IFN-inducing pathways.

Impact of *TBK1* mutations on other IFN-inducing pathways

TBK1 is involved in the activation of IRF3 and IRF7 in multiple IFN-inducing signal transduction pathways (Chau et al., 2008), including cascades triggered by the activation of cytosolic RNA sensors (RIG-I and MDA-5), cytosolic DNA sensors (DAI and IFI16), and TLR4. We first investigated the response to TLR4 activation in PBMCs by assessing IFN- α

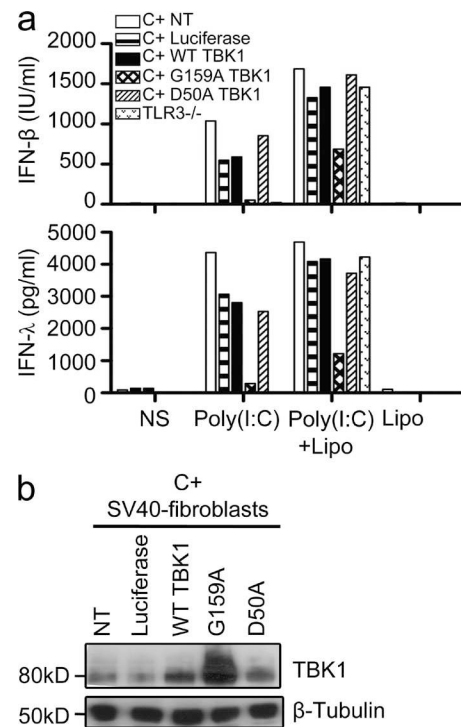


Figure 7. The G159A allele exerts a dominant-negative effect, whereas the D50A allele does not. (a) IFN- β and IFN- λ production, as assessed by ELISA, in response to 50 μ g/ml poly(I:C) stimulation or 25 μ g/ml poly(I:C) transfection mediated by Lipofectamine (Lipo) in TLR3-deficient fibroblasts and in control fibroblasts (C+) transduced with lentivirus pseudoparticles encoding luciferase, WT *TBK1*, or one of the *TBK1* mutants (G159A or D50A) or left untransduced (NT). Cells were transduced 6 d before stimulation. This experiment is representative of three independent experiments. NS, nonstimulated. (b) Western blot of transduced control fibroblasts (C+) with anti-*TBK1* and antitubulin antibodies.

production after 24 h of stimulation with LPS, which is recognized by TLR4 (Poltorak et al., 1998). The PBMCs of P1 responded normally to TLR4 activation (not depicted), consistent with the normal response induced by LPS stimulation in *TBK1*^{-/-} and *TBK1*^{+/-} MEFs (Hemmi et al., 2004).

We further investigated the capacity of fibroblasts from the two patients to mediate signaling for other pathways known to be TBK1 dependent in mice by assessing the production of IFN- β and IFN- λ after transfection with the RIG-I-specific agonist 7sk-as (Pichlmair et al., 2006), with poly(I:C), which

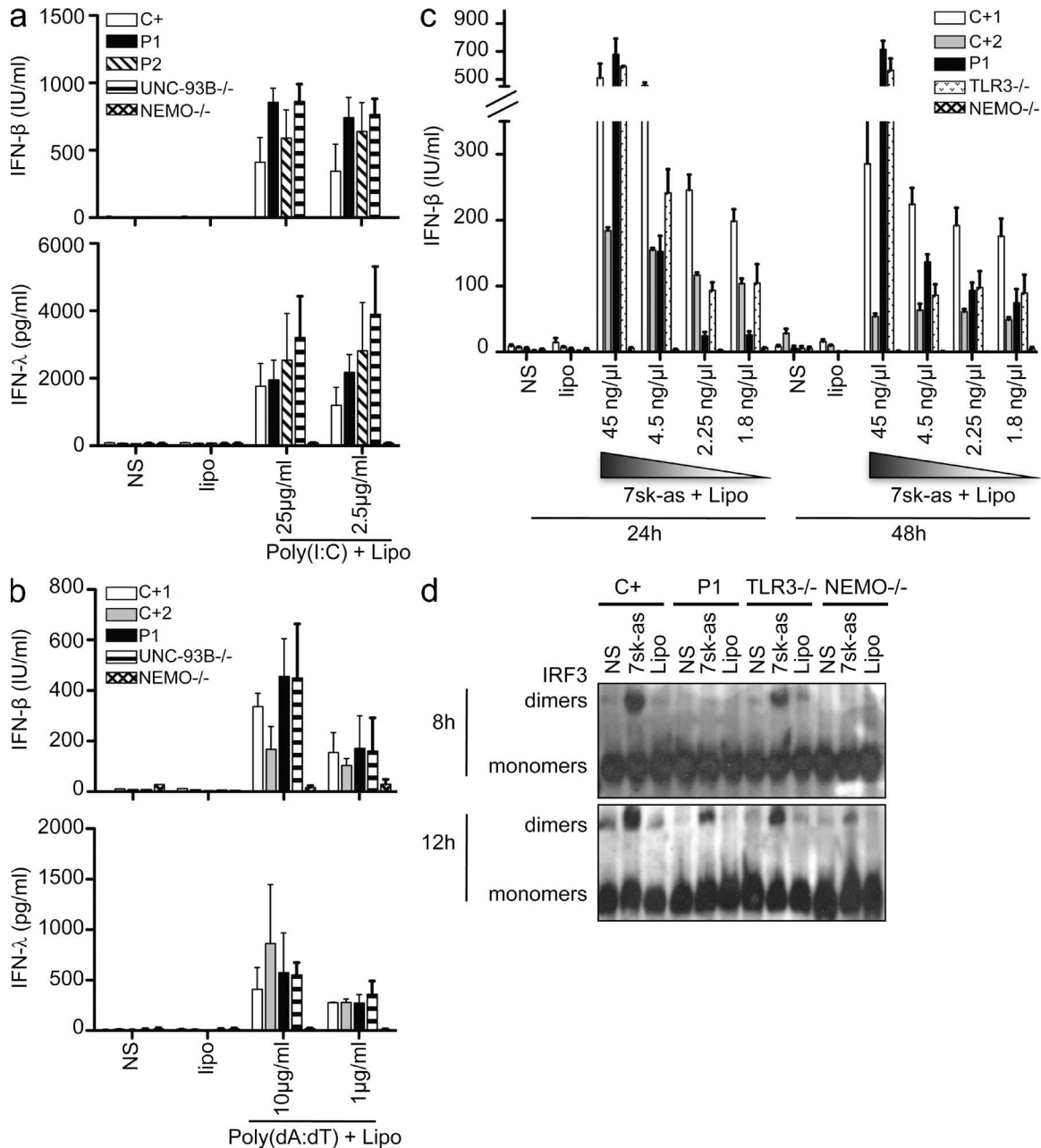


Figure 8. Normal responses to dsRNA, dsDNA, and ssRNA introduced by transfection in the patients' fibroblasts. (a) Production of IFN- β and IFN- λ in response to various doses of poly(I:C), introduced by transfection into control (C+; averaged from two different control cell lines), P1, P2, and UNC-93B^{-/-} and NEMO^{-/-} deficient fibroblasts. Values represent mean values \pm SD calculated from three independent experiments. (b) Production of IFN- β and IFN- λ in response to various doses of poly(dA:dT) introduced by transfection into control (C+), P1, P2, UNC-93B^{-/-}, and NEMO^{-/-} fibroblasts. Values from three different experiments were averaged and are presented here as mean values \pm SD. (c) IFN- β production 24 or 48 h after transfection with the ssRNA 7sk-as in control (C+1 and C+2), P1, TLR3^{-/-}, and NEMO^{-/-} fibroblasts. Values represent mean values \pm SD from three independent experiments. (d) IRF3 dimerization, assessed by native Western blotting upon stimulation with 2.25 ng/ μ l 7sk-as in fibroblasts from P1, at 8 and 12 h; comparison with control WT cells (C+), TLR3^{-/-}, and NEMO^{-/-} fibroblasts. The blots shown are representative of three independent experiments. NS, nonstimulated.

can activate both RIG-I and MDA-5 (Yoneyama et al., 2004), or with the synthetic dsDNA poly(dA:dT) (poly (deoxyadenylic-deoxythymidylic) acid), which can activate DAI or IFI16 (Takaoka et al., 2007; Unterholzner et al., 2010). The fibroblasts of both patients produced normal levels of IFN- β and IFN- λ after transfection with various amounts of dsDNA or poly(I:C) (Fig. 8, a and b; and not depicted) and large amounts of 7sk-as (Fig. 8 c and not depicted). However, the levels of IFN- β or IFN- λ production induced by lower doses of 7sk-as were much lower in cells from P1 than in control cells at 24 h but reached normal levels by 48 h (Fig. 8 c). Consistently, IRF3 activation was delayed after RIG-I stimulation after transfection with a low dose of 7sk-as: IRF3 dimerization was barely detectable at 8 h but normal after 12 h of RIG-I stimulation in the fibroblasts of P1 (Fig. 8 d). These results point to a mild impairment of RIG-I signaling in fibroblasts heterozygous for G159A *TBK1*. No major TLR3-independent phenotypes were observed in the cells of P1 and P2. We assessed the potential of the G159A and D50A *TBK1* alleles to rescue the lack of IFN- β production observed in *TBK1*^{-/-} MEFs upon transfection with poly(I:C) and 7sk-as (Fig. 9 a). Unlike the WT *TBK1* allele, the G159A mutant allele could not rescue these responses, confirming that this allele was a loss-of-function allele. The D50A allele allowed residual responses, albeit probably caused by transient overexpression of the D50A *TBK1* protein. On Western blot, cells transfected with the D50A allele were found to contain smaller total amounts of *TBK1* (Fig. 9 b) than cells transfected with the WT or G159A alleles, consistent with our previous findings (Figs. 1 g and 2, a and c). All cells were healthy and produced IL-6 in response to IL-1 β (Fig. 9 c). In *TBK1*^{-/-} MEFs, both mutants are therefore loss-of-function for IFN-inducing pathways triggered by dsRNA and single-stranded RNA (ssRNA). It remains unclear whether the normal IFN response after the activation of TLR4 and cytosolic sensors of nucleic acids in our patients'

cells is caused by the residual *TBK1* activity present in human cells with a partial *TBK1* deficiency or caused by the contribution of *TBK1* to these pathways not being essential in human PBMCs and fibroblasts.

Normal IFN responses to viruses other than VSV and HSV-1 in the cells of both patients

Both *TBK1*-deficient patients suffered from isolated HSE, with no other unusual infectious diseases, including viral diseases, during their 17 and 26 yr of life. We investigated the response of cells from P1, carrying the dominant-negative G159A *TBK1* allele, to several viruses. We first challenged PBMCs from P1 and P2 with various types of viruses, including positive ssRNA viruses (encephalomyocarditis virus [EMCV] and Sindbis virus), negative ssRNA viruses (Sendai, human parainfluenza virus III, NDV [Newcastle disease virus], mumps virus, and measles virus), and dsDNA viruses (BK virus and HSV-1), all of which induced normal levels of IFN- α and IL-6 in these cells (Fig. 10 a and not depicted). This may reflect the partial nature of the genetic defects carried by the patients, the redundancy of *TBK1* and IKK α in the activation of IRF3 and IRF7 in leukocytes (Fitzgerald et al., 2003; Sharma et al., 2003; Hemmi et al., 2004; McWhirter et al., 2004; Matsui et al., 2006), or both. Unlike leukocytes, fibroblasts do not produce IKK α (Shimada et al., 1999; Hemmi et al., 2004; Perry et al., 2004). When infected with human parainfluenza virus III, measles, EMCV, or Sindbis virus, the fibroblasts of both patients behaved like control cells in terms of their capacity to produce IFN- β and IFN- λ (Fig. 10 b). This result contrasts with the impaired response of these cells to infection with HSV-1 or VSV, both of which activate TLR3 to initiate IFN induction, as demonstrated by findings for the cells of

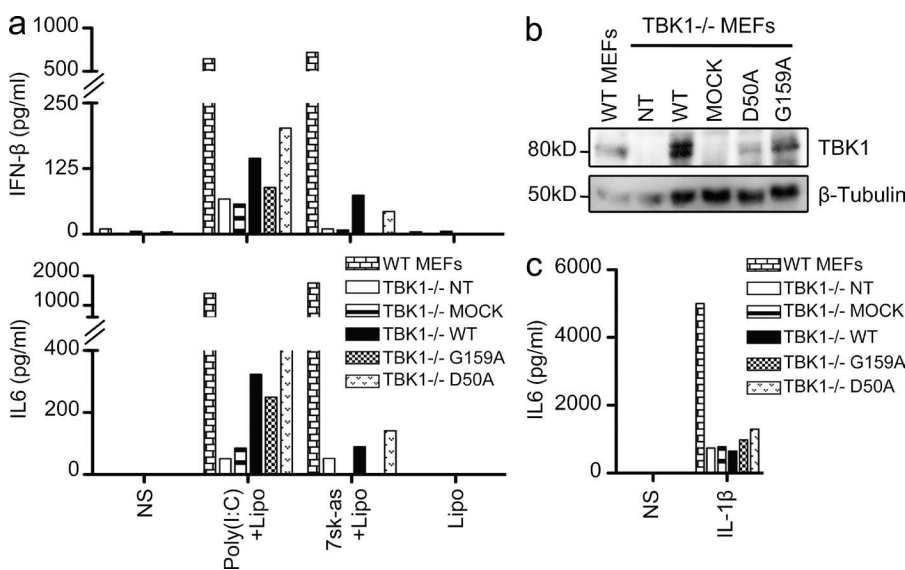


Figure 9. Assessing the functionality of both mutant *TBK1* alleles in the response to transfected poly(I:C) and ssRNA (7sk-as).

(a) *TBK1*^{-/-} MEFs were either left untransfected (NT) or were transfected with a mock vector, WT *TBK1*, or mutant constructs: D50A (mutation present in P2) or G159A (the mutation in P1). After 24 h, the cells were stimulated by transfection with Lipofectamine of 25 μ g/ml poly(I:C) or 22.5 ng/ μ l ssRNA (7sk-as). ELISA was performed to assess IFN- β and IL-6 production 24 h after stimulation. The experiment shown is representative of three independent experiments. (b) *TBK1* expression from the transfected constructs was assessed by Western blotting with an antibody against *TBK1*. β -Tubulin was used as a loading control. (c) IL-6 production as assessed by ELISA after stimulation with 10 ng/ml IL-1 β for 24 h after transfection in *TBK1*^{-/-} MEFs either not transfected (NT) or transfected with a mock vector or a vector encoding WT *TBK1* or the mutants G159A and D50A *TBK1*. The experiment shown is representative of three independent experiments. NS, nonstimulated.

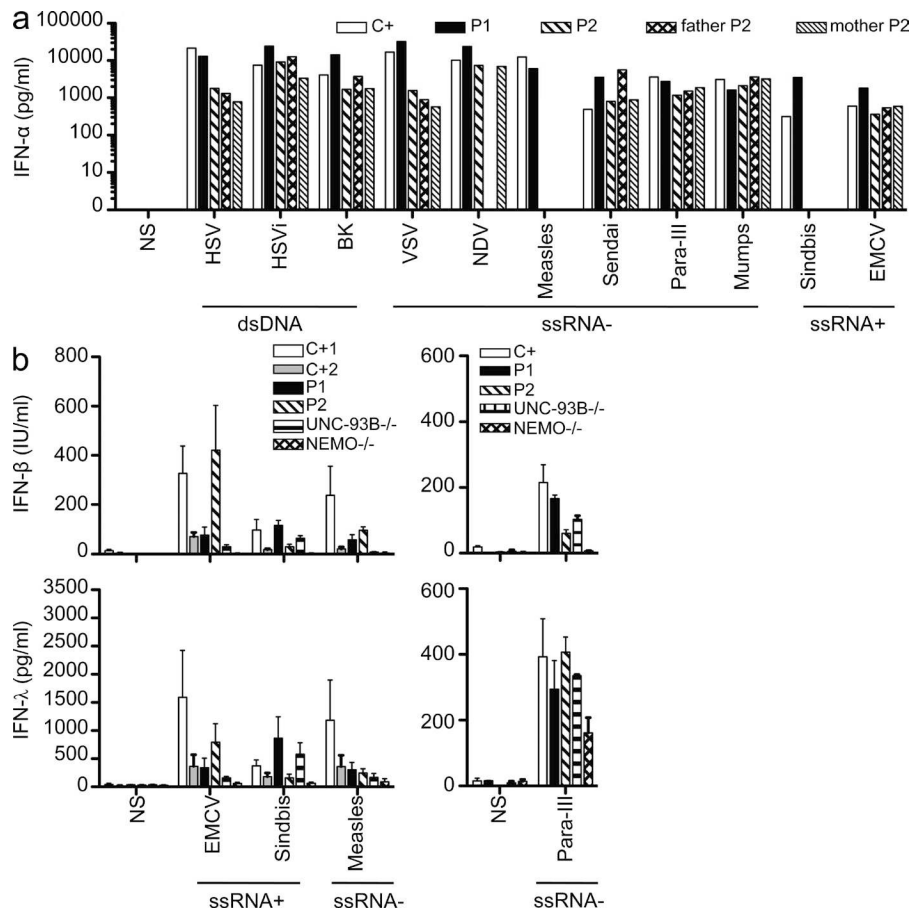


Figure 10. Both patients present normal responses to other viruses in PBMCs and fibroblasts. (a) PBMCs from a control (C+), P1, P2, and P2's parents were challenged with several viruses with different types of genomes: dsDNA viruses (HSV-1, UV-inactivated HSV-1 or HSV-1i, and BK), negative-strand ssRNA viruses (VSV, NDV, measles, Sendai virus, human parainfluenza virus III [Para-III], and mumps), and positive-strand ssRNA viruses (Sindbis virus and EMCV). Levels of IFN- α were assessed by ELISA 24 h after infection. P2 and her parents' cells were not tested for their response to infection with Sindbis and measles. This experiment was performed once. (b) Production of IFN- β and IFN- λ , as assessed by ELISA, in control (C+), P1, P2, UNC-93B^{-/-}, and NEMO^{-/-} fibroblasts 24 h after infection with positive-strand ssRNA viruses (Sindbis virus at an MOI of 10 and EMCV at an MOI of 1) and negative ssRNA viruses (measles at an MOI of 1 and parainfluenza virus III at an MOI of 1). Values represent mean values \pm SD from three independent experiments. NS, nonstimulated.

patients with TLR3 (Guo et al., 2011), UNC-93B (Casrouge et al., 2006), TRIF (Sancho-Shimizu et al., 2011b), and TRAF3 (Pérez de Diego et al., 2010) deficiencies. The lack of viral control in the fibroblasts of patients heterozygous for *TBK1* is thus limited to the neurotropic viruses HSV-1 and VSV. These results for PBMCs and fibroblasts are consistent with the narrow clinical phenotype observed in both *TBK1*-deficient patients. They suggest that the residual *TBK1*-dependent IFN induction by TLR3 and/or by other receptors in cells with a partial *TBK1* deficiency may contribute to the protective immunity against most common viruses.

DISCUSSION

We have identified AD *TBK1* deficiency as a new genetic etiology of HSE in childhood. The patients' fibroblasts displayed impaired TLR3 responses, confirming the essential role of human *TBK1* as an IFN-inducing IRF3 kinase in the TLR3 pathway in these cells. Together with our previous discoveries of AR UNC-93B (Casrouge et al., 2006), AR and AD TLR3 (Zhang et al., 2007; Guo et al., 2011), AR and AD TRIF (Sancho-Shimizu et al., 2011b), and AD TRAF3 (Pérez de Diego et al., 2010) deficiencies, this finding highlights the nonredundant role of TLR3-dependent IFN induction in the CNS for the control of HSV-1 in the

course of primary infection in childhood. The clinical penetrance of AD *TBK1* deficiency is incomplete, like that of AD TLR3, AR UNC-93B, and AD TRIF deficiencies, consistent with the sporadic occurrence of HSE (Whitley and Kimberlin, 2005). The actual clinical penetrance cannot be assessed, as the frequencies of the *TBK1* mutant alleles are not known. We know only that they were not found in 1,050 healthy controls (2,100 chromosomes) from 52 ethnic groups. Our discovery of rare morbid variants of the *TLR3*, *UNC93B1*, *TRAF3*, *TRIF*, and *TBK1* genes, each found in only a small number of children with HSE, suggests that the determinism of HSE may depend on a collection of highly diverse and possibly immunologically related single-gene lesions (Casanova and Abel, 2007; Alcaïs et al., 2010). We were unable to investigate whether the two forms of *TBK1* deficiency reported here displayed complete cellular penetrance, as documented in families with UNC-93B, TLR3, or TRIF deficiency (Casrouge et al., 2006; Zhang et al., 2007; Sancho-Shimizu et al., 2011b). Consistent with this possibility, no unreported variants other than that of *TBK1* were found by whole-exome sequencing of genes involved in the TLR3-IFN axis in P2 and her asymptomatic mother (see Whole-exome sequencing in P1 and P2: TLR3-IFN pathway genes sequenced and found to be WT). Interestingly, the two unrelated patients with *TBK1* deficiency had different cellular phenotypes, that of the patient with the G159A mutation being more severe than that of the patient with the D50A mutation because the G159A allele is dominant negative, whereas D50A is dominant by haploinsufficiency. Regardless of these

differences, our discovery of two forms of AD partial TBK1 deficiency adds weight to the argument that HSE should not be considered only a viral disease. Instead, it should also be seen as a genetic disorder caused by an array of rare, single-gene inborn errors of TLR3 immunity, in at least a fraction of affected children (Casanova and Abel, 2005; Alcais et al., 2010).

The identification of TBK1 deficiency as a genetic etiology of HSE is surprising, as mouse TBK1 has been shown to be involved in many antiviral pathways other than the TLR3 cascade, including the RIG-I/MDA-5 (Fitzgerald et al., 2003; Sharma et al., 2003; Hemmi et al., 2004), DAI (DLM-1/ZBP1; Takaoka et al., 2007), and IFI16/STING (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Unterholzner et al., 2010) pathways. In vitro studies have shown that the IFN- α/β response is defective in *TBK1*^{-/-} MEFs stimulated with poly(I:C) (Hemmi et al., 2004) or infected with Sendai virus (Hemmi et al., 2004; Perry et al., 2004) or VSV (Hemmi et al., 2004). IFN induction is also defective in mouse *TBK1*^{-/-} macrophages challenged with HSV-1 and dsDNA (Miyahira et al., 2009), LPS, and poly(I:C) (Perry et al., 2004). *TBK1*^{-/-} mice die in the perinatal period as the result of massive liver degeneration and apoptosis (Bonnard et al., 2000). The role of TBK1 in host defense in vivo has not, therefore, been studied in mice. Our data suggest that TBK1 haplotype insufficiency may also occur in mice. *TBK1*^{+/-} mice are viable but have not been characterized immunologically or in terms of their response to infection (Marchlik et al., 2010). Nevertheless, our two patients with AD partial TBK1 deficiency were found to be resistant to most common viruses. Like TLR3, UNC-93B, TRIF, and TRAF3 deficiencies, but unlike STAT-1 (Yang et al., 2005) and NEMO (Audry et al., 2011) deficiencies, AD TBK1 deficiency is associated with isolated HSE. The residual TBK1 activity is not sufficient to sustain optimal TLR3 responses, against HSV-1 in particular, but is sufficient for the TLR3-independent pathways tested. AD TBK1 deficiency does not impair the response to transfected dsDNA, dsRNA, or ssRNA in human fibroblasts or the response to TLR4 in leukocytes. This may account for the effective control of the viruses tested in AD TBK1-deficient fibroblasts and for the patients' lack of susceptibility to other viral diseases. However, we cannot exclude the possibility that TBK1 is redundant for certain pathways and/or in certain cells. In any case, this experiment of nature neatly illustrates how subtle mutations of human genes at the crossroads of multiple signaling pathways may result in partial biochemical defects, accounting for narrow cellular and clinical phenotypes.

MATERIALS AND METHODS

Case reports

P1 is a Polish child from a nonconsanguineous family with no family history of encephalitis or herpes labialis. Both parents and the child were healthy until P1 suffered from HSE at the age of 7 yr. The diagnosis was based on the positive detection by PCR of HSV-1 DNA in the child's cerebrospinal fluid and on typical HSE-like brain pattern aberrations on MRI. P1 tested positive for IgG and IgM antibodies against HSV-1 at the time of diagnosis. She was

treated with acyclovir, systemic steroids, intravenous gammaglobulins, phenobarbital, valproic acid, benzodiazepines, mannitol, and antibiotics for 4 mo. P1 subsequently presented cognitive disability and drug-resistant epilepsy. She has since required multidrug anti-epilepsy treatment, which has not influenced her immunological status, as immunological screenings gave normal results. Since her HSE episode, P1 has developed only mild upper respiratory tract infections despite attending kindergarten and primary school. P1 has been exposed to VZV (varicella zoster virus), CMV, EBV, B19, and influenza A, as demonstrated by positive serological results, in the absence of acute events. P1 was also immunized with hepatitis B, diphtheria/tetanus/polio type 1, 2, and 3, and mumps vaccines with no adverse effect.

P2 is a French patient from a nonconsanguineous family with no family history of encephalitis. P2 suffered from HSE at 11 mo of age, presenting initially with otitis and hyperthermia, followed by convulsions and fever the next day. 6 d later, a 15-d course of treatment with zovirax was initiated. At this time, P2's condition had deteriorated and she presented right-sided hemiparesis and erythema on her arms and hands. A scan revealed typical encephalitis-like patterns for the left temporal area of the brain. At the end of zovirax treatment, P2 still had right-sided hemiparesis but no longer suffered from convulsions. In the next month, EEGs and scans confirmed the presence of lesions on the left side of the brain (temporal, fronto-insular, and parietal). P2 subsequently suffered from obesity and motor and cognitive disabilities, including epilepsy during treatment for her motor and cognitive disabilities. P2 has been exposed to VZV, CMV, B19, and influenza A, as demonstrated by positive serological results, with no acute events. P2 was also immunized with the live mumps/rubella/measles vaccines and with the diphtheria toxoid, inactivated poliovirus type 1, poliovirus type 2, poliovirus type 3, and tetanus toxoid vaccines with no adverse effect. P2's mother and sister suffer from bouts of herpes labialis once or twice a year but have no other significant infectious phenotype. P2's father suffered from measles and chicken pox as a child but has no other significant infectious phenotype.

Both children have been living in and followed up in their countries of origin (Poland for P1 and France for P2). Informed consent was obtained in the home country of each patient in accordance with local regulations and with institutional review board (IRB) approval. The experiments described here were conducted in the United States and in France in accordance with local regulations and with the approval of the IRB of The Rockefeller University and Institut National de la Santé et de la Recherche Médicale.

Human molecular genetics

Total RNA and genomic DNA were extracted from the PBMCs of P1, P2, and a control with TRIZOL (Gibco). RNA was reverse-transcribed with SuperScript II RT (Invitrogen) according to the manufacturer's instructions. The cDNAs for all the genes sequenced and for the genomic coding regions of *TBK1* were amplified with specific primers. PCR was performed with *Taq* polymerase (Invitrogen). PCR products were analyzed by electrophoresis in 1% agarose gels, purified by ultracentrifugation through Sephadex G-50 Superfine resin (GE Healthcare), sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems), and analyzed on an ABI Prism 3700 machine (Applied Biosystems).

Cell culture and transfections

Primary human fibroblasts were obtained from biopsies of patients or healthy controls and were cultured in DME (Invitrogen) supplemented with 10% FBS. They were transformed with an SV40 vector, as previously described (Chapigier et al., 2006), to create SV40-immortalized fibroblasts (SV40-fibroblasts). The SV40-fibroblast cell lines were activated in 24-well plates at a density of 10⁵ cells/well for 24 h, unless otherwise specified. *TBK1*^{-/-} and WT MEFs were supplied by M.A. White. MEFs were transformed with an SV40 vector, as for human fibroblasts, and cultured in DME (Invitrogen) supplemented with 10% FBS. Cells were transiently transfected with expression vectors with a calcium phosphate transfection kit from Sigma-Aldrich, used according to the manufacturer's instructions. The cells were dispensed into 12-well plates at a density of 0.9 × 10⁴ cells/well for transfection. The pcDNA3.1-FLAG-TBK1 construct was obtained by subcloning

the FLAG-TBK1 sequence from the vector pCMV5-FLAG-TBK1 into pcDNA3.1+ (Invitrogen); pCMV5-FLAG-TBK1 was provided by P. Cohen (University of Dundee, Dundee, Scotland, UK). Point mutations encoding D50A, G159A, or S172A TBK1 were introduced in pcDNA3.1-FLAG-TBK1 by site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies).

Lentivirus pseudoparticles were generated by transfection of HEK293-T cells, mediated by Fugene 6 (Roche), in DME supplemented with 3% FBS and NEAA (nonessential amino acids from Life Technologies) with three plasmids: pTRIP, encoding TBK1 or luciferase, HIV gag-pol, and VSV envelope protein G (VSV-G) plasmids at a weight ratio of 1:0.8:0.2 (Schoggins et al., 2011). Mutations were introduced in *TBK1* (D50A and G159A) by site-directed mutagenesis, as described above. Supernatants were collected at 48 and 72 h and supplemented with 4 µg/ml polybrene (Santa Cruz Biotechnology, Inc.) and 20 mM Hepes (Life Technologies).

TLR activation, signal transduction experiments, and cytokine measurement

We used the following TLR agonists: a synthetic analogue of dsRNA, poly(I:C) (GE Healthcare; used at a concentration of 25 µg/ml unless otherwise specified) and LPS (Re 595 from *Salmonella minnesota*, obtained from Sigma-Aldrich, and used at a concentration of 10 µg/ml); IL-1β was obtained from R&D Systems. All agonists and reagents were endotoxin free. For all TLR agonists except LPS, cells were incubated for 30 min with 10 µg/ml polymyxin B at 37°C before activation. TLR agonists were used to stimulate PBMCs at a density of 2 × 10⁶ cells/ml in RPMI 1640/10% FCS, RPMI/1% FCS, or in AIMV medium, or in whole blood diluted 1:2 in RPMI 1640, as indicated. The SV40-fibroblast cell lines were activated in 24-well plates at a density of 10⁵ cells/well for 20 h with HSV-1, VSV, 25 µg/ml poly(I:C) (unless otherwise specified), 20 ng/ml TNF, or 20 ng/ml IL-1β.

TransAM NF-κB p65 ELISA (Active Motif) was performed with an NF-κB p65-specific DNA probe with 10 µg nuclear extract. Native PAGE of total cell extract (25 µg of protein) was performed in a gel containing 1% sodium deoxycholate (DOC; Sigma-Aldrich) for the detection of IRF3 dimerization. After electrophoresis in a 7.5% polyacrylamide gel, the gel was blotted onto a membrane, which was then probed with the anti-IRF-3 antibody (FL-425; Santa Cruz Biotechnology, Inc.).

ELISA was performed for human IFN-α (AbCys SA), IFN-β (TFB; Fujirebio, Inc.), IL-6 (Pelipair kit), mouse IL-6 (R&D Systems), and mouse IFN-β (Verikine, PBL) according to the kit manufacturer's instructions. The IFN-λ ELISA was developed in the laboratory. In brief, plates were coated by incubation overnight at 4°C with 1 µg/ml mAb against human IFN-λ (R&D Systems), and the levels of IFN-λ in the supernatant were determined by incubation with a biotinylated anti-human IFN-λ secondary mAb (R&D Systems) at a concentration of 400 µg/ml.

RT-qPCR and Western blotting

RNA was reverse-transcribed with the SuperScript II RT (Invitrogen) and oligo (dT) (Invitrogen) to determine *TBK1* expression levels. In all RT-qPCR, β-glucuronidase (*Gus*) was used for normalization. For RT-qPCR analysis assessing the induction of IFNs upon poly(I:C) stimulation, RT was performed with random hexamer primers. RT-qPCR was performed with TaqMan reverse transcription reagents (Applied Biosystems) in an ABI Prism 7700 sequence detection system (Applied Biosystems). All the probes used were taken from the TaqMan Gene Expression Assays (Applied Biosystems). Results are expressed according to the ΔΔCt method, as described by the manufacturer.

Equal amounts of protein for each sample were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories), which were then probed with primary antibodies followed by peroxidase-conjugated secondary antibodies and ECL Western blotting substrate (Thermo Fisher Scientific). The primary antibodies used included TBK1/NAK antibody (Cell Signaling), anti-DDK mAb (Origene), IRF3 phospho-pS386 antibody (Epitomics), rabbit polyclonal anti-IRF-3 FL-425 antibody (Santa Cruz Biotechnology, Inc.), and anti-β-tubulin antibody (Sigma-Aldrich).

Viral infection and quantification in fibroblasts

For VSV infection, 10⁵ SV40-fibroblasts were dispensed into each individual well of 24-well plates and infected with VSV at an MOI of 10 in DME supplemented with 2% FBS. The plates were incubated for 30 min, and the cells were then washed and incubated in 500 µl of medium. Supernatants were obtained and frozen at the 0.5-, 3-, 6-, 8-, and 24-h time points. VSV titers were determined by calculating the 50% end point (TCID₅₀), as described by Reed and Muench (1938), after the inoculation of Vero cell cultures in 96-well plates. For HSV-1-GFP infection, 10⁴ SV40-fibroblasts were dispensed into each individual well of 96-well plates and infected with HSV-1-GFP (strain KOS; Desai and Person, 1998) at various MOIs in DME supplemented with 10% FBS. After 2 h, cells were washed and incubated in 100 µl of culture medium. The GFP fluorescence of the samples was quantified at the 2-, 16-, 24-, and 48-h time points. For assays of cell protection upon viral stimulation, cells were treated with 10⁵ IU/ml IFN-α2b for 18 h before infection, as appropriate.

Cell viability assay

The viability of SV40-fibroblasts was assessed by resazurin oxidoreduction (TOX-8; Sigma-Aldrich). Cells were plated, in triplicate, in 96-well flat-bottomed plates (2 × 10⁴ cells/well) in DME supplemented with 2% FBS. After 24 h, cells were infected by incubation for 24 h with VSV or for 72 h with HSV-1 at the indicated MOI. Resazurin dye solution was then added (5 µl per well) to the culture medium, and the samples were incubated for an additional 2 h at 37°C. Fluorescence was then measured at a wavelength of 590 nm, using an excitation wavelength of 531 nm. Background fluorescence, calculated for dye and complete medium alone (in the absence of cells), was then subtracted from the values for all the other samples; 100% viability corresponds to the fluorescence of uninfected cells.

In vitro protein kinase assays

TBK1 protein kinase assays were performed as previously described (Ou et al., 2011).

Whole-exome sequencing

3 µg DNA was extracted from leukocyte cells from the individuals and was sheared with an S2 Ultrasonicator (Covaris) for massively parallel sequencing. An adaptor-ligated library was prepared with the TruSeq DNA Sample Preparation kit (Illumina). Exome capture was performed with the Sure-Select Human All Exon kit (Agilent Technologies; Byun et al., 2010). Paired-end sequencing was performed on a HiSeq 2000 (Illumina) that generated 100-bp reads. For sequence alignment, variant calling, and annotation, the sequences were aligned to the human genome reference sequence (GRCh37 build) using the Burrows-Wheeler Aligner (Li and Durbin, 2009). Downstream processing was performed with the Genome analysis toolkit (GATK; McKenna et al., 2010), SAMtools (Li et al., 2009), and Picard (<http://picard.sourceforge.net>). Variant calls were made with a GATK Unified Genotyper. All calls with a read coverage ≤2× and a Phred-scaled SNP quality of ≤20 were removed from consideration. All variants were annotated with a software system developed in-house.

Whole-exome sequencing in P1 and P2: TLR3-IFN pathway genes sequenced and found to be WT. Genes sequenced by whole-exome sequencing were *TAB3* (*NAP1*), *IKBKE* (*IKKe*), *TBKBP1* (*SINTBAD*), *TRAF6*, *IRF3*, *IRF7*, *HMGB1*, *HMGB2*, *CC2D1A* (*TAPE*), *TRAF2*, *TRAF4*, *TRAF1*, *ZMYND11* (*BS69*), *RIPK1*, *IKBKg*, *IKBKB*, *CHUK*, *NFKB1A*, *NFKB1*, *RELA*, *NFKB2*, *CAMK2G*, *CAMK2A*, *PIAS4*, *TRADD*, *DDX3X*, *TNFAIP3*, *GAB1*, *TNIK**, and *TRIM21*. Genes sequenced by the Sanger method were *TLR3*, *TICAM1* (*TRIF*), *TRAF3*, *UNC93B1*, and *TANK*. P1 carries an unreported heterozygous intronic variation in *TNIK* (asterisk) located 125 nucleotides 3' to exon 4. A genomic DNA sample from P2's mother was sequenced by whole-exome sequencing. All genes known to be involved in the TLR3-IFN axis covered by whole-exome sequencing and found to be WT in P2's mother are indicated in bold typeface.

Genome-wide transcriptional profile experiments in primary fibroblasts

Data acquisition. Fibroblasts from patients and controls were stimulated with IL-1 β or poly(I:C) or IFN- α for 2 or 8 h or left unstimulated. Total RNA was isolated (RNeasy kit; QIAGEN) and RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies). Biotinylated cRNA targets were prepared from 250 ng of total RNA, using the Illumina Total-Prep RNA Amplification kit (Ambion). The labeled cRNAs (750 ng) were then incubated for 16 h to HT-12 version 4 BeadArrays (48,323 probes). BeadChip arrays were then washed, stained, and scanned on a HiScanSQ (Illumina) according to the manufacturer's instructions.

Data preprocessing. After background subtraction, the raw signal values extracted with BeadStudio (version 2; Illumina) were scaled using quantile normalization. Minimum intensity was set to 10, and all the intensity values were log₂ transformed. Only the probes called present in at least one sample ($P < 0.01$) were retained for downstream analysis ($n = 28,553$).

Data analysis. Transcripts differentially regulated upon stimulation were defined based on a minimum twofold change (up- or down-regulation) and a minimum absolute raw intensity difference of 100 with respect to the respective unstimulated sample. Heat maps were generated using R (version 2.12.2). Cumulative fold change scores were calculated using the absolute fold change values from the transcripts passing the cutoffs defined above.

Data availability. Raw data for the microarray analyses performed in this study are available from the public repository of GEO DataSets (accession no. GSE38652). Networks were resolved with MetaCore version 6.10 (GeneGo).

Statistical methods

Statistical significance was determined by Student's *t* test. *P*-values of <0.05 were considered statistically significant.

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