Herpes simplex virus encephalitis in a patient with complete TLR3 deficiency: TLR3 is otherwise redundant in protective immunity

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Autosomal dominant TLR3 deficiency has been identified as a genetic etiology of childhood herpes simplex virus 1 (HSV-1) encephalitis (HSE). This defect is partial, as it results in impaired, but not abolished induction of IFN-β and -λ in fibroblasts in response to TLR3 stimulation. The apparently normal resistance of these patients to other infections, viral illnesses in particular, may thus result from residual TLR3 responses. We report here an autosomal recessive form of complete TLR3 deficiency in a young man who developed HSE in childhood but remained normally resistant to other infections. This patient is compound heterozygous for two loss-of-function TLR3 alleles, resulting in an absence of response to TLR3 activation by polyinosinic-polycytidylic acid (poly(I:C)) and related agonists in his fibroblasts. Moreover, upon infection of the patient’s fibroblasts with HSV-1, the impairment of IFN-β and -λ production resulted in high levels of viral replication and cell death. In contrast, the patient’s peripheral blood mononuclear cells responded normally to poly(I:C) and to all viruses tested, including HSV-1. Consistently, various TLR3–deficient leukocytes from the patient, including CD14+ and/or CD16+ monocytes, plasmacytoid dendritic cells, and in vitro derived monocyte–derived macrophages, responded normally to both poly(I:C) and HSV-1, with the induction of antiviral IFN production. These findings identify a new genetic etiology for childhood HSE, indicating that TLR3–mediated immunity is essential for protective immunity to HSV-1 in the central nervous system (CNS) during primary infection in childhood, at least some patients. They also indicate that human TLR3 is largely redundant for responses to double-stranded RNA and HSV-1 in various leukocytes, probably accounting for the redundancy of TLR3 for host defense against viruses, including HSV-1, outside the CNS.
Herpes simplex encephalitis, which was first described in 1941 (Smith et al., 1941), is the most common sporadic viral encephalitis in the Western world (Whitley and Kimberlin, 2005). It has an estimated incidence of two to four cases per million inhabitants per year (Sköldenberg et al., 1984; Najjoulah et al., 2000; Puchhammer-Stöckl et al., 2001), peaking in children between the ages of 6 mo and 3 yr (De Tiège et al., 2008). Childhood HSV encephalitis is a rare complication of primary infection with HSV-1, which otherwise infects >85% of young adults with few, if any, clinical consequences. The introduction of acyclovir in the 1980s decreased the mortality rates associated with HSV-1 encephalitis (HSE), but most survivors present profound neurological sequelae, including recurrent seizures and mental retardation (Gordon et al., 1990; McGrath et al., 1997). The improvements in survival resulting from acyclovir treatment have made the long-term follow up of children with HSE possible, and studies of this type have indicated that HSE typically strikes otherwise healthy children with normal resistance to other common infections, including those caused by other viruses in particular (Abel et al., 2010). Remarkably, children with any of the many known inherited and acquired severe immunodeficiencies, including SCIDs impairing the development of T lymphocytes and often other lymphocyte subsets and HIV-driven AIDS, resulting in profound CD4 T cell lymphopenia, are not particularly prone to HSE (Buckley, 2004; Sancho-Shimizu et al., 2007). These data suggested that the molecules produced by leukocytes and known to govern innate and adaptive immunity were not essential for immunity to HSV-1 in the central nervous system (CNS). Childhood HSE has thus long remained a rare and devastating viral illness of unknown pathogenesis.

We recently showed that HSE may result from single-gene inborn errors of TLR3-dependent, IFN-α/β- and IFN-λ-mediated immunity, in at least some children. Mutations in STAT1 (Dupuis et al., 2003) and NEMO (Niehues et al., 2004) were found in two children with an exceedingly rare phenotype combining mycobacterial disease and HSE. This led to the discovery of autosomal recessive (AR) UNC-93B deficiency (Casrouge et al., 2006), autosomal dominant (AD) TLR3 deficiency (Zhang et al., 2007b), and AD TRAF3 deficiency (Pérez de Diego et al., 2010), each in patients with the more common, typical phenotype of isolated HSE. All three defects impair the TLR3-dependent induction of IFN-α/β and -λ in the patients’ dermal fibroblasts, in response to stimulation with extracellular polyinosinic-polycytidylic acid (poly(I:C); Casrouge et al., 2006; Zhang et al., 2007b). TLR3 is a nonspecific receptor of the double-stranded RNA (dsRNA) intermediates generated during the replication of most viruses, including HSV-1, and mimicked by poly(I:C) (Jacquemont and Roizman, 1975; Weber et al., 2006). The infection of fibroblasts from UNC-93B−/−, TLR3−/−, and TRAF3-deficient patients with vesicular stomatitis virus (VSV) triggers the production of only low levels of IFN-β and -λ, resulting in levels of viral replication and cell death higher than those in normal cells (Casrouge et al., 2006; Zhang et al., 2007b; Pérez de Diego et al., 2010). VSV was used despite its predominantly animal tropism and RNA genome because it is a potent inducer of IFNs in human fibroblasts, to which it is also highly cytopathic. The lack of cutaneous HSV-1 disease in children with HSE may result from a normal poly(I:C) response in keratinocytes and leukocytes, as demonstrated in patients with AD TLR3 deficiency (Zhang et al., 2007b), restricting the dermal spread of HSV-1. It has also been suggested that the lack of detectable viremia and overt disseminated disease reflects the redundancy of TLR3 in most leukocytes, including IFN-producing cells in particular (Casrouge et al., 2006; Zhang et al., 2007b; Pérez de Diego et al., 2010).

Indeed, in the course of HSE, HSV-1 does not reach the CNS by crossing the blood brain barrier but via cranial nerves, the olfactory bulb, and the trigeminal nerve in particular (Whitley, 2006). The abundant and almost selective expression of functional TLR3 in the CNS, in both nonhematopoietic (neurons, oligodendrocytes, and astrocytes) and hematopoietic (microglial cells) CNS-resident cells (Bisbis et al., 2002, 2006; Olson and Miller, 2004; Jack et al., 2005; Préhaud et al., 2010), therefore provides a plausible mechanism of disease. We recently obtained preliminary data suggesting that responses are essential to control HSV-1 in CNS-resident cells derived from patients’ induced pluripotent stem cells (unpublished data). These data suggest that the CNS-restricted impairment of TLR3 responses underlies HSE. In this context, two key questions have emerged. First, taking into account that the known genetic etiologies have been found in only a few patients, is HSE in other children also caused by single-gene inborn errors of immunity? Second, as children with AD TLR3 deficiency display a partial defect, and those with AR UNC-93B or AD TRAF3 deficiency may display UNC-93B−/− or TRAF3-independent TLR3 responses, could residual TLR3 responses contribute to the broad resistance to other viruses of children with HSE? We describe in this study a new genetic etiology of HSE, in a patient with an AR, and complete form of TLR3 deficiency. We used cells from this patient to document the redundant and nonredundant functions of TLR3 in cellular responses to poly(I:C) and viruses, including HSV-1.

RESULTS
Compound heterozygous mutations in TLR3 in a patient with HSE
We investigated a 19-yr-old French patient (P) who had suffered from HSE at the age of 8 yr. He had suffered no other unusually severe infectious disease, of viral origin in particular. High titers of antibodies against HSV-1, HSV-2, varicella zoster virus, Epstein–Barr virus, and influenza virus A were found in his serum. This patient had been immunized with live measles/mumps/rubella vaccine with no adverse effect and had undoubtedly also been exposed to at least 20 other known ubiquitous viruses (Knipe and Howley, 2007). We found two compound heterozygous mutations in his TLR3 gene. One of the alleles carried a substitution (C→T) at nucleotide position 1660 (c.1660C>T), whereas the other carried a substitution
The patient, his father, and his two siblings all carry a common homozygous TLR3 single nucleotide polymorphism (SNP) c.1234C>T (rs3775291, an SNP of 0.329 ± 0.237 heterozygosity in the general population), resulting in the replacement of the leucine residue in amino acid position 412 by a phenylalanine residue (L412F). The mother of the patient is heterozygous for the L412F SNP. No other missense SNP of TLR3 is present in the patient, his parents, or siblings. Sero logical tests showed that the parents and siblings had been infected with HSV-1, but they did not develop HSE. The compound P554S and E746X mutations in TLR3 may therefore define, in our patient, the first AR form of TLR3 deficiency in humans.

Expression of the TLR3 mutant alleles
The E746X mutation is predicted to prevent translation of the TIR domain of TLR3. We studied the production of E746X TLR3 messenger RNA (mRNA) and protein, using the TLR3-deficient P2.1 fibrosarcoma cell line (Sun and Leaman, 2004) to generate P2.1 cells stably transfected with constructs encoding C-terminally hemagglutinin (HA)-tagged WT or E746X TLR3. We also generated P2.1 cells stably transfected with HA-tagged TLR3 alleles carrying the P554S mutation or one of the two nonsynonymous SNPs in TLR3, N284I (c.851A>T; rs5743316), and L412F, which have been shown to decrease the activity of TLR3 in HEK293 and COS-7 cells stimulated with poly(I:C) (Ranjith-Kumar et al., 2007; Gorbea et al., 2010). TLR3 mRNA species were detected in P2.1 cells stably transfected with the WT, P554S, E746X, N284I, or L412F allele (with a C-terminal HA tag) but not in untransfected P2.1 cells or in P2.1 cells transfected with a mock vector (Fig. 2 A). The WT, N284I, and L412F TLR3 proteins were detected with an antibody against TLR3, which detected two different forms, one with a molecular mass of 80 kD and the other with a molecular mass of 110 kD (Fig. 2 B; Zhang et al., 2007b). The E746X TLR3 protein was detected with an antibody directed against the N terminus of TLR3, which detected two different forms, one with a molecular mass of 130 kD and the other with a molecular mass of 110 kD, neither of which was detected by an antibody against HA (which did not recognize the E746X TLR3; Fig. 2 B and Fig. S1). These results suggest that posttranslational modifications, such as N-glycosylation, of the C-terminally truncated E746X protein may be abnormal (Choe et al., 2005; Sun et al., 2006). We tested this hypothesis by assessing production of the E746X protein upon treatment with endoglycosidase H (Endo–H) or PNGase F. Endo–H treatment resulted in the disappearance of the 130-kD bands for the WT and E746X TLR3 proteins but not the 110-kD band for the E746X TLR3 protein (Fig. S1). The 130-kD WT and E746X TLR3 proteins were sensitive to Endo–H, but some of the N-linked glycans on the 110-kD truncated form of E746X TLR3 seemed to be resistant to Endo–H, suggesting that some of the truncated form of TLR3 had transited abnormally through the Golgi compartment (Johnsen et al., 2006).
Moreover, treatment of the WT and E746X TLR3 proteins with PNGase F decreased the molecular mass of the 130-kD WT TLR3 protein to 100 kD, and that of the 130- and 110-kD E746X TLR3 proteins to ~70 kD (Fig. S1), strongly suggesting that abnormal glycosylation of the C-terminally truncated E746X protein accounts for the detection of mutant proteins of two different molecular masses. The E746X TLR3 protein thus lacks the TIR domain and is abnormally glycosylated, and a proportion of this protein is mislocated.

**Both the P554S and E746X TLR3 alleles are loss-of-function**

We previously showed that the P554S TLR3 protein fails to respond to poly(I:C) in the TLR3-deficient P2.1 cell line (Sun and Leaman, 2004; Zhang et al., 2007b). We also showed this protein to have a dominant-negative effect in both TLR3-expressing dermal fibroblasts from a healthy control and TLR3-deficient P2.1 cells, at least for the induction of IFN-β and -λ (Zhang et al., 2007b). We thus studied the function of the E746X TLR3 protein in P2.1 cells stably transfected with constructs encoding C-terminally HA-tagged E746X TLR3, comparing the results obtained with those for P2.1 cells stably transfected with the WT, P554S, N284I, or L412F allele. Transfection with the WT TLR3 construct rescued the response to poly(I:C), in terms of IFN-β and -λ mRNA induction (Fig. 2 C and Fig. S2) and IFN-λ production (Fig. 2 D), whereas transfection with the E746X or P554S TLR3 construct had no such effect (Fig. 2, C and D; and Fig. S2). The N284I and L412F SNPs of TLR3 have been shown to decrease the activity of TLR3 in HEK293 and COS-7 cells stimulated with poly(I:C), as measured by the activation of NF-κB- and ISRE-dependent reporter genes (Ranjith-Kumar et al., 2007; Gorbea et al., 2010). Interestingly, the transfection of P2.1 cells with N284I or L412F TLR3 alleles also rescued the response to poly(I:C), which reached levels similar to those for the WT TLR3, in terms of IFN-β and -λ mRNA induction (Fig. 2 C and Fig. S2) and IFN-λ production (Fig. 2 D). The N284I and L412F TLR3 alleles are therefore hypomorphic in

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**Figure 2.** P554S and E746X TLR3 alleles are loss-of-function. (A) TLR3 mRNA levels were determined by RT-qPCR in P2.1 TLR3-deficient fibrosarcoma cells not transfected (P2.1) or stably transfected with WT TLR3 (P2.1–TLR3 WT), P554S (P2.1–TLR3 P554S) or E746X (P2.1–TLR3 E746X) mutant TLR3, N284I (P2.1–TLR3 N284I) or L412F (P2.1–TLR3 L412F) TLR3 variant, or mock vector (P2.1–mock). β-Glucuronidase (GUS) was included for normalization. The results shown are representative of three independent experiments. (B) TLR3 expression, assessed by immunoblotting (IB) after immunoprecipitation (IP), in P2.1 TLR3-deficient fibrosarcoma cells not stably transfected (P2.1) or transfected with WT TLR3, P554S or E746X mutant TLR3, N284I or L412F TLR3 variant, or mock vector, with an anti-TLR3 N-terminal (N) antibody and an anti-HA C-terminal tag antibody. The experiment shown is representative of three experiments performed. TLR3 protein extracted from HEK293T cells transfected with human WT TLR3 was included as a positive control. We used β-tubulin as an internal expression control for immunoblotting. (C) IL29 (IFN-λ1) mRNA induction, without stimulation (NS) or after 4 h of stimulation with poly(I:C), assessed by RT-qPCR, in P2.1 TLR3-deficient fibrosarcoma cells not transfected (P2.1) or transfected with WT TLR3, P554S or E746X mutant TLR3, N284I or L412F TLR3 variant, or mock vector. All transfections generated stable cell lines. β-Glucuronidase was included for normalization. Mean values ± SD were calculated from two independent experiments. (D) IFN-λ production without stimulation (NS) or after 24 h of stimulation with poly(I:C), as assessed by ELISA, in P2.1 TLR3-deficient fibrosarcoma cells not transfected (P2.1) or transfected with WT TLR3, P554S or E746X mutant TLR3, N284I or L412F TLR3 variant, or mock vector. All transfections generated stable cell lines. One experiment representative of the three performed is shown. Mean values ± SD were calculated from triplicates in one experiment.
HEK293 and COS-7 cells (Ranjith-Kumar et al., 2007; Gorbea et al., 2010) but apparently not in P2.1 cells, although it is difficult to compare these cells, as different readouts were tested. In any event, the E746X TLR3 allele, like the previously reported P554S TLR3 allele (Zhang et al., 2007b), is loss-of-function for poly(I:C) responses in P2.1 cells.

Abolished TLR3 responsiveness in the patient’s fibroblasts

Human dermal fibroblasts display a TLR3-dependent response to extracellular stimulation with poly(I:C) (Casrouge et al., 2006; Zhang et al., 2007b). As both the P554S and E746X TLR3 alleles are loss-of-function in P2.1 cells, we thus assumed that the patient’s fibroblasts would display a complete lack of TLR3 responsiveness. Indeed, IFN-β, IFN-λ, and IL-6 were secreted in a dose- and time-dependent manner after poly(I:C) stimulation in control fibroblasts (Fig. 3 A; Zhang et al., 2007b). As in fibroblasts from patients with AD TLR3 deficiency (Zhang et al., 2007b), TLR3 mRNA levels were normal in the patient’s cells, as shown by comparison with healthy controls (Fig. 3 B). However, unlike AD TLR3 fibroblasts, which displayed a residual response to high concentrations of poly(I:C) at late time points after stimulation (Fig. 3 A; Zhang et al., 2007b), primary and simian virus 40 (SV40)–transformed fibroblasts from the patient displayed no induction of IFN-β, IFN-λ, or IL-6 in response to any of the concentrations of poly(I:C) tested at any of the time points considered (Fig. 3 A and Fig. S3 A). Unlike P554S, the E746X TLR3 allele does not appear to be dominant, as fibroblasts from the patient’s mother, who is heterozygous for the E746X TLR3 mutation, displayed a normal response to poly(I:C) stimulation in terms of the induction of IFN-λ and IL-6 (Fig. S3 A), further suggesting that there is probably no haploinsufficiency at the TLR3 locus in dermal fibroblasts.

**Figure 3. Absence of response to TLR3 in the patient’s fibroblasts.** (A) Production of IFN-λ, IFN-β, and IL-6 by SV40-fibroblasts after stimulation with various doses of poly(I:C) for 12 or 24 h, as assessed by ELISA, with cells from a healthy control (C), the patient (P), a patient with partial AD TLR3 deficiency (AD TLR3), a patient with complete AR UNC-93B deficiency (UNC-93B⁻/⁻), and a NEMO-deficient patient (NEMO IP). The panels illustrate mean values ± SD for triplicates of one experiment, representative of three performed. (B) TLR3 mRNA levels in SV40-fibroblasts were determined by RT-qPCR on RNA samples from five healthy controls (C), the patient (P), an AD TLR3 patient, and a UNC-93B⁻/⁻ patient. β-Glucuronidase (GUS) was used for normalization. One representative experiment of three performed is shown. (C) IL29 (IFN-λ1) and IFNB mRNA levels in SV40-fibroblasts from a control, the patient, an AD TLR3 patient, and a UNC-93B⁻/⁻ patient, unstimulated (NS) and stimulated for 2, 4, 6, and 8 h with poly(I:C). The panels illustrate results from a single experiment, representative of three performed. (D) Production of IFN-β and IL-6 by SV40-fibroblasts from a control, the patient, an AD TLR3 patient, a UNC-93B⁻/⁻ patient, and a NEMO IP patient, unstimulated or after stimulation with the TLR3-specific agonist poly(A:U) for 12 and 24 h, as assessed by ELISA. The panels illustrate mean values ± SD for three independent experiments. (E) IRF-3 monomers and dimers in total cell extracts of SV40-fibroblasts from a control, the patient, an AD TLR3 patient, and a UNC-93B⁻/⁻ patient, after stimulation with poly(I:C) for 1 and 2 h, as assessed by Western blotting. The results shown are representative of three independent experiments. (F) NF-κB activation was assessed by monitoring expression of the NF-κB luciferase reporter in SV40-fibroblasts from a control, the patient, an AD TLR3 patient, a UNC-93B⁻/⁻ patient, and a NEMO IP patient, unstimulated or after stimulation with poly(I:C) (top) and IL-1β (bottom) for 6 h. The panels illustrate mean values ± SD for three independent experiments.
Similiar results were obtained for the induction of mRNA synthesis for IFN-β and -λ in the patient's fibroblasts (Fig. 3 C). This cellular phenotype is consistent with that of UNC-93B–deficient fibroblasts from another HSE patient (Fig. 3, A and C; Casrouge et al., 2006), in which UNC-93B–dependent TLR3 signaling is completely abolished (Casrouge et al., 2006). The response to polyadenyl-cylic-polypuridylic acid (poly(A:U)), a noncommercial agonist of TLR3 known as IPH31 that apparently stimulates TLR3 more specifically than poly(I:C), was also abolished in the fibroblasts of our patient (Fig. 3 D). The responsiveness of our patient's cells to TLR3 stimulation was not restored by prior treatment with IFN-α (Fig. S3 B), which normally increases TLR3 responses by up-regulating TLR3 expression (Tissari et al., 2005). The activation of both IRF-3 (IFN regulatory factor 3; Fig. 3 E) and NF-κB (Fig. 3 F) in response to poly(I:C) was impaired in fibroblasts from the patient, which responded normally to IL-1β (Fig. 3 F). Moreover, the stable transfection of cells from the patient with a construct encoding C-terminally HA-tagged WT TLR3 restored the cellular response to poly(I:C) and poly(A:U), as assessed by measurements of IFN-β and IL-6 production (Fig. 4 A and Fig. S4 A) and NF-κB activation (Fig. S4 B). As a control, HA-tagged TLR3 was detected in stably transfected cells, with an antibody directed against C-terminally tagged HA (Fig. 4 B). The fibroblastic phenotype thus confirmed that the compound P554S and E746X TLR3 alleles conferred complete functional TLR3 deficiency on the patient's fibroblasts.

**Genome-wide transcriptional evaluation of the TLR3 pathway in fibroblasts**

For identification of the TLR3-dependent genes targeted during stimulation with poly(I:C), we investigated the genome-wide transcriptional profile upon poly(I:C) stimulation in fibroblasts (Fig. 5, A–C; and Fig. S5 A). Impaired IFN-dependent control of VSV and HSV-1 in the patient's fibroblasts

We previously showed that the production of IFN-β and -λ was impaired in fibroblasts homozygous for UNC93B1-null alleles and in fibroblasts heterozygous for a dominant-negative

**Figure 4.** WT TLR3 expression rescues responsiveness to TLR3 in the patient's fibroblasts. (A) Production of IFN-α and IL-6 unstimulated (NS) or after 24 h of stimulation with poly(I:C) with the presence of Lipofectamine (poly(I:C)+L) or without Lipofectamine (poly(I:C)), as assessed by ELISA, in SV40-fibroblasts from a control (C), a NEMO-deficient patient (NEMO IP), the patient (P), and in SV40-fibroblasts from P transfection with an empty vector (P-mock) or the C-terminal HA-tagged pUNO–TLR3 WT vector (P-TLR3 WT). All transfections generated stable cell lines. The panels illustrate mean values ± SD for triplicates of one experiment, representative of three. (B) TLR3 expression in SV40-fibroblasts from the patient without transfection (P) or after stable transfection with human WT TLR3 (P-TLR3 WT) or mock vector (P-mock) was assessed by immunoblotting (IB) with an anti-HA C-terminally tagged antibody (C) after immunoprecipitation (IP) with an anti-TLR3 N-terminal (N) antibody. A positive control. We used β-tubulin as an internal expression control for immunoblotting.
TLR3 allele in response to both HSV-1 and another neurotropic virus, VSV, which is highly cytopathic and a potent IFN inducer in human fibroblasts (Casrouge et al., 2006; Zhang et al., 2007b). The other viruses tested, including measles virus, parainfluenza III virus (Para III virus), Sindbis virus, and encephalomyocarditis virus (EMCV), induced normal levels of IFN-β and -λ production in AR UNC-93B–deficient and AD TLR3–deficient fibroblasts (Casrouge et al., 2006; Zhang et al., 2007b). We then studied the responses of the patient’s fibroblasts to infection with various viruses, including Para III virus, EMCV, Sindbis virus, measles virus, HSV-1, and VSV, and compared these responses with those of AR, UNC-93B–deficient, AD TLR3–deficient, and healthy control fibroblasts. Fibroblasts from the patient, like AR UNC-93B–deficient and AD TLR3–deficient fibroblasts, produced less IFN-β and -λ in response to VSV and HSV-1 than healthy control cells (Fig. 6, A and B; and Fig. S6, A and B) but normal levels of IFN-β and -λ in response to the other viruses tested (Fig. 6 C). Moreover, the impaired response to VSV was rescued, at least in terms of IFN-λ production, by the stable expression of a WT TLR3 construct in the patient’s fibroblasts (Fig. S6 C). Fibroblasts with AR complete TLR3 deficiency therefore displayed impaired production of IFN-β and -λ in response to VSV and HSV-1. We have previously shown that the impairment

Figure 5. Genome-wide transcriptional evaluation of the TLR3 pathway in fibroblasts. (A) Cumulative fold change (FC) score (top) and heat maps (bottom) of the transcripts regulated by 2 h (left) or 8 h (right) of stimulation with poly(I:C) in primary fibroblasts from three healthy controls (C), the patient (P), a UNC-93B−/− patient, a patient with AD TLR3 deficiency (AD TLR3), and a patient of MyD88 deficiency (MyD88−/−). The cumulative score is the sum of all the fold change values >1.5 (up- or down-regulation). Heat maps show a hierarchical clustering of transcripts differentially expressed upon poly(I:C) 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 302 transcripts up-regulated after 8 h of poly(I:C) stimulation, with a fold change of at least 2 in all three controls tested, in primary fibroblasts from three healthy controls (C), the patient (P), a UNC-93B−/− patient, an AD TLR3 patient, and an MyD88−/− patient. (C) Networks generated from differentially expressed transcripts (up-regulated) in fibroblasts from control (C), the patient (P), a UNC-93B−/− patient, an AD TLR3 patient, and an MyD88−/− patient after 8 h of poly(I:C) stimulation with Ingenuity Pathway Analysis software. Eligible genes or gene products regulated by these factors are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). Solid and dashed lines indicate direct and indirect relationships, respectively. All edges are supported by at least one reference from the literature. Nodes are arranged according to the cellular distribution of the corresponding gene products. Up-regulated transcripts are represented in red, and down-regulated transcripts are represented in green.
of virus–induced IFN production leads to high levels of VSV replication and cell death upon VSV or HSV-1 infection in AR-UNC-93B–deficient, AD TLR3–deficient, and AD TRAF3–deficient fibroblasts (Casrouge et al., 2006; Zhang et al., 2007b; Pérez de Diego et al., 2010). We thus studied the control of VSV and HSV-1 in fibroblasts from the patient. Cells from our patient (P) and from patients with AR STAT1, AR UNC-93B, and AD TLR3 deficiencies displayed higher levels of VSV or HSV-1 replication than WT cells (Fig. 7, A and B). When cells from P, AR UNC-93B–deficient, and AD TLR3–deficient cells were treated with IFN-α before viral infection, complementation was observed, with the normal containment of viral replication, similar to that observed in healthy control cells (Fig. 7, A and B). No such complementation was observed for AR STAT1–deficient cells, which have impaired responses to IFN-α, -β, and -λ (Fig. 7, A and B; Chapgier et al., 2009). Fibroblasts from P had markedly lower survival rates than control cells after 24 h of VSV infection or 72 h of HSV-1 infection (Fig. 7, C and D). The patient’s cells behaved like the cells of patients with AD TLR3, AR UNC-93B, and AR STAT1 deficiencies for VSV infection (Casrouge et al., 2006; Zhang et al., 2007b). Prior treatment with exogenous IFN-α complemented the phenotypes of AR TLR3–deficient, AD TLR3–deficient, and AR UNC-93B–deficient cells equally well, but not that of AR STAT1–deficient cells (Fig. 7, C and D). Thus, complete AR TLR3 deficiency results in the impairment of IFN-β and -λ production in response to VSV and HSV-1, resulting in higher levels of viral replication and cell death, as observed for AR UNC-93B–deficient and AD TLR3–deficient cells. By inference, this fibroblastic phenotype may account for the molecular pathogenesis of HSE in CNS-resident cells in patients with inborn errors of TLR3 immunity.

Normal response to poly(I:C) and viruses in PBMCs

Patients with HSE in general, and patients with HSE and TLR3 pathway deficiencies in particular, are normally resistant to other infectious diseases, including viral illnesses such as, paradoxically, HSV-1–related diseases outside the CNS (Casrouge et al., 2006; Zhang et al., 2007b; Abel et al., 2010; Pérez de Diego et al., 2010). The response to poly(I:C) and viruses in PBMCs was normal in AD TLR3–deficient patients (Zhang et al., 2007b). This could be interpreted as reflecting TLR3–independent responses to dsRNA mediated by MDA5 (Gitlin et al., 2006) or RIG-I (Yoneyama et al., 2004) and, possibly, other pathways, residual TLR3 responses in cells with AD TLR3 deficiency, or both. The lack of infections other than HSE in AD TLR3–deficient patients may thus be caused by their residual TLR3 responses. Likewise, UNC-93B–independent TLR3 responses might account for the narrow infectious phenotype of patients with complete UNC-93B deficiency (Casrouge et al., 2006) or partial TRAF3 deficiency (Pérez de Diego et al., 2010). We thus investigated the response to poly(I:C) and viruses in PBMCs from the patient with complete AR TLR3 deficiency. PBMCs from the patient responded normally to poly(I:C) and 10 viruses, including BK virus, Newcastle disease virus (NDV), measles virus, Para III virus, EMCV, Sindbis virus, HSV-1, and VSV, in terms of IFN-α production (Fig. 8 A and Fig. S7 A). We further analyzed transcriptional profiles of PBMCs from the patient and from controls after 2 or 8 h of stimulation with poly(I:C) or IL-1β. In control PBMCs, 347 and 26 transcripts were regulated by 2 h of stimulation with IL-1β and poly(I:C), respectively, and 410 and 446 transcripts were regulated by 8 h of stimulation with IL-1β and poly(I:C), respectively (Table S2). The response to 2 or 8 h of IL-1β stimulation or 8 h of poly(I:C) stimulation was similar in cells from the patient and in cells from controls (Fig. 8, B–D; and Fig. S7, B and C). The regulation of several genes (IFIT1, IFIT2, and IFIT3) appeared to be TLR3 dependent after 2 h of poly(I:C) stimulation but not after 8 h of stimulation (Fig. 8, B and C; and Fig. S7 D), suggesting that the TLR3–dependent and –independent dsRNA-responsive pathways may be activated in a time–dependent manner in PBMCs. An analysis of

Figure 6. IFN production after virus stimulation in fibroblasts from the patient. [A–C] Production of IFN-α (top) and IFN-β (bottom) after 24 h of stimulation with VSV (A), HSV-1 (B), Para III virus, EMCV, Sindbis virus, and measles virus (MeV; C), or left unstimulated (NS), as assessed by ELISA, in SV40-fibroblasts from a control (C), patient (P), an AD TLR3–deficient patient (AD TLR3), and a UNC-93B–/– patient. The panels illustrate results from a single experiment, representative of three performed. Mean values ± SD were calculated from triplicates in one experiment.
the functional pathways regulated by poly(I:C) in control PBMCs and in PBMCs from the AR TLR3-deficient patient revealed that a poly(I:C) functional network similar to that of the fibroblasts was activated in the PBMCs of controls and of the patient with AR TLR3 deficiency after 8 h of stimulation with poly(I:C) (Fig. 8 D), indicating that the activation of TLR3-independent dsRNA-responsive pathways may compensate for the TLR3 defect and lead to the regulation of these networks in PBMCs. Overall, TLR3 was redundant for responses to poly(I:C) and all viruses tested in circulating leukocytes.

**Normal response to poly(I:C) and HSV-1 in different leukocyte subsets**

We further investigated whether TLR3 was required for responses to poly(I:C) and HSV-1 in different subsets of ex vivo isolated or in vitro differentiated leukocytes. We previously investigated the response to poly(I:C) in various leukocyte subsets (Zhang et al., 2007b). However, the response to viruses, including HSV-1 in particular, has previously been investigated only in the fibroblasts and PBMCs of AD TLR3-deficient patients. The NK and CD8⁺ T cells of AD TLR3-deficient patients have been shown to display impaired responses to poly(I:C) under the conditions tested (Zhang et al., 2007b). Some recent data have suggested that TLR3-dependent and -independent dsRNA-responsive pathways may contribute to the response to poly(I:C) in CD8⁺ T and NK cells (Zhang et al., 2007b; McCartney et al., 2009; Perrot et al., 2010; Wang et al., 2010) and that TLR3 may contribute to the generation of CD8⁺ T cell responses to HSV-1 (Davey et al., 2010). However, NK and CD8⁺ T cells are unlikely to play a major role in the pathogenesis of HSE, as neither CD8− nor HLA-I− deficient patients are prone to HSE (Cerundolo and de la Salle, 2006). Indeed, even patients lacking all NK and T cell subsets (T cell−, NK cell−, SCID) do not develop HSE upon infection with HSV-1 (Buckley, 2004). Overall, the lymphoid cell types in which TLR3 seems to be important for poly(I:C) responsiveness are not key players in immunity to HSV-1 in the CNS. The lack of other viral infections in HSE patients further indicates that the TLR3 pathway in such cell types is largely redundant for antiviral immunity. The response to poly(I:C) in other leukocytes studied, including plasmacytoid DCs (pDCs) and myeloid DCs, was normal in AD TLR3-deficient patients (Zhang et al., 2007b). This may be interpreted as reflecting TLR3-independent responses to dsRNA, residual TLR3 responses in cells with AD TLR3 deficiency, or both. The lack of infections other than HSE in AD TLR3-deficient patients may thus be caused by their residual TLR3 responses. pDCs (Yoneyama et al., 2005) and macrophages (Mogensen, 1979) are thought to play an important role in immunity to HSV-1. We therefore investigated the response to poly(I:C) and HSV-1 in pDCs, CD14⁺CD16⁻, CD14⁺CD16⁺, and CD14⁺CD16⁺ monocytes, and in monocyte-derived macrophages (MDMs) from the patient with complete AR TLR3 deficiency. Upon stimulation with HSV-1, pDCs isolated from the PBMCs of the AR TLR3-deficient patient displayed normal IFN-α production (Fig. 9 A). Upon stimulation with poly(I:C) and HSV-1, pDCs, MDMs, and CD14⁺CD16⁺ monocytes all revealed a poly(I:C) functional network similar to that of the functional pathways regulated by poly(I:C) in control PBMCs and in PBMCs from the AR TLR3-deficient patient. Upon stimulation with HSV-1, pDCs, MDMs, and CD14⁺CD16⁺ monocytes all revealed a poly(I:C) functional network similar to that of the functional pathways regulated by poly(I:C) in control PBMCs and in PBMCs from the AR TLR3-deficient patient. Upon stimulation with HSV-1, pDCs, MDMs, and CD14⁺CD16⁺ monocytes all revealed a poly(I:C) functional network similar to that of the functional pathways regulated by poly(I:C) in control PBMCs and in PBMCs from the AR TLR3-deficient patient. Upon stimulation with HSV-1, pDCs, MDMs, and CD14⁺CD16⁺ monocytes all revealed a poly(I:C) functional network similar to that of the functional pathways regulated by poly(I:C) in control PBMCs and in PBMCs from the AR TLR3-deficient patient. Upon stimulation with HSV-1, pDCs, MDMs, and CD14⁺CD16⁺ monocytes all revealed a poly(I:C) functional network similar to that of the functional pathways regulated by poly(I:C) in control PBMCs and in PBMCs from the AR TLR3-deficient patient.
with poly(I:C) or HSV-1, no IFN production was detected by ELISA, in CD14+CD16−, CD14dimCD16+, and CD14+CD16+ control monocytes. However, similar amounts of mRNA were detected for the IFN-inducible genes OAS1 and MX1 in CD14+CD16−, CD14dimCD16+, and CD14+CD16+ monocytes from the patient and controls after poly(I:C) or HSV-1 stimulation (Fig. S8, A and B). MDMs from the AR TLR3-deficient patient responded normally to poly(I:C) or HSV-1 stimulation, in terms of the production of IFN-α mRNA and protein (Fig. 9 B and Fig. S8 C). These data, together with our previous observations for cells from AD TLR3-deficient patients (Zhang et al., 2007b), strongly suggest that PBMCs, CD14+CD16−, CD14dimCD16+, and CD14+CD16+ monocytes, and MDMs respond to dsRNA in a TLR3-independent manner and that PBMCs, PDCs, and MDMs do not require an intact TLR3 pathway for antiviral IFN induction in response to HSV-1. Overall, these data provide an explanation for the lack of disseminated disease during the course of HSE and the absence of other viral illnesses in patients with inborn errors of TLR3 immunity, including this patient with complete TLR3 deficiency in particular.

**DISCUSSION**

The discovery of AR UNC-93B deficiency, AD TLR3 deficiency, and AD TRAF3 deficiency in children with HSE provided proof-of-principle that childhood HSE may result from single-gene inborn errors of immunity (Casrouge et al., 2006; Zhang et al., 2007b; Pérez de Diego et al., 2010).
This identification of AR TLR3 deficiency as a new genetic etiology of childhood HSE confirms the requirement of the TLR3–IFN signaling pathway for protective immunity to HSV-1 primary infection in the CNS, in at least some children. These results also suggest that genetic defects affecting other molecules of the TLR3–IFN signaling pathway may be involved in the pathogenesis of HSE in other children. Our preliminary identification of children with HSE and AD or AR TRIF deficiency (unpublished data) or AD Tbk1 deficiency (unpublished data) is consistent with this hypothesis. As observed in patients with AR UNC-93B deficiency, AR TLR3 deficiency leads to a complete lack of TLR3 response, associated, in fibroblasts, with impaired control of VSV and HSV-1. Our preliminary observations suggest that the phenotype of fibroblasts recapitulates that of some CNS-resident cells (unpublished data). Consistent with its distinctive signature of purifying selection (Barreiro et al., 2009; Casanova et al., 2011), human TLR3 is essential for host defense, at least in some individuals, for protection against HSV-1. Complete AR TLR3 deficiency provides the first example of an inherited, complete, morbid deficiency involving a human TLR. The severe, but narrow and transient infectious phenotype in our AD TLR3-deficient patients and in this AR TLR3-deficient patient, consisting of susceptibility to HSE, indicates that the TLR3–IFN pathway is vital for protective immunity to primary HSV-1 infection in the CNS, in at least some children, but may otherwise be largely redundant for host defense.

How do these observations compare with the mouse model? TLR3-deficient mice are susceptible to some viruses (Edelmann et al., 2004; Tabeta et al., 2004; Rudd et al., 2006; Hardarson et al., 2007; Negishi et al., 2008; Richer et al., 2009) but normally resistant or more resistant to others (Table S3; Edelmann et al., 2004; Wang et al., 2004; Gowen et al., 2006; Le Goffic et al., 2006; Hutchens et al., 2008). TLR3-deficient mice are susceptible to EMCV (Hardarson et al., 2007), mouse CMV (Tabeta et al., 2004, Edelmann et al., 2004), respiratory syncytial virus (Rudd et al., 2006), CVB3 (Negishi et al., 2008), and CVB4 (Richer et al., 2009) but have normal resistance to lymphocytic choriomeningitis virus, VSV, and reovirus (Edelmann et al., 2004), and enhanced resistance to Punta Toro virus (Gowen et al., 2006), influenza virus (Le Goffic et al., 2006), West Nile virus (Wang et al., 2004), and vaccinia virus (Hutchens et al., 2008) infections, taking TLR3 WT mice as the reference. No signs of HSE were recorded when TLR3-deficient mice were challenged with HSV-1 by skin infection (Davey et al., 2010). However, HSE is a neurotropic infection, and the corresponding intranasal and intracerebral modes of inoculation have not been tested in TLR3-deficient mice, making it impossible to draw firm conclusions as to the vulnerability of TLR3-deficient mice to neurotropic HSE. The recent identification of an AD TLR3 allele in a CVB3 myocarditis patient (Gorbea et al., 2010) is consistent with the vulnerability of TLR3-deficient mice to related viruses (Negishi et al., 2008; Richer et al., 2009). However, human TLR3 appears to be largely redundant in host defense against other primary infections in childhood, including, in particular, the viruses to which TLR3-deficient mice are particularly vulnerable. Consistent with these observations, PBMCs and fibroblasts from AD and AR TLR3-deficient patients produced normal levels of IFN-α, -β, and -λ in response to nine and four viruses other than HSV-1 tested, respectively, including BK virus, VSV, NDV, measles virus, Pana virus, mumps virus, Sendai virus, EMCV, and Sindbis virus, the only exception being VSV in fibroblasts. These data further suggest that TLR3-independent dsRNA-responsive pathways (Yoneyama et al., 2004; Gitlin et al., 2006) or other antiviral immune pathways (Takaoka et al., 2007) may contribute to the control of viruses other than HSV-1 in patients with TLR3 pathway deficiencies. This situation is reminiscent of the discordance between IRAK-4 and MyD88-deficient mice and humans (Yang et al., 2005; Ku et al., 2007; von Bernuth et al., 2008; Casanova et al., 2011) and, more generally, between the mouse model of experimental infections and the human model of natural infections (Casanova and Abel, 2004, 2007; Quintana-Murci et al., 2007). TLR3 thus shows considerable redundancy in host defense in nature.

Strikingly, TLR3 is also redundant for protective immunity to HSV-1 outside the CNS. The patient lacking TLR3, like other children with HSE, suffered from no other overt clinical disease caused by HSV-1, even during the course of HSE. In the French cohort, almost none of the children with HSE suffered from herpes labialis (unpublished data), the most common clinical symptom of HSV-1 infection in the general population (Stanberry et al., 1997; Abel et al., 2010). HSV-1 viremia was never documented during the course of childhood HSE in the conditions used to detect HSV-1 in the cerebrospinal fluid (CSF; Whitley, 2006). Consistent with...
these observations, PBMCs from AD and AR TLR3-deficient patients produced normal levels of IFN-α, -β, and -λ in response to HSV-1. Moreover, PDCs and MDMs from the patient with AR complete TLR3 deficiency displayed normal IFN responses to HSV-1 infection, probably because of TLR3-independent dsRNA-responsive pathways in these cells. These data again suggest that TLR3-independent dsRNA-responsive pathways (Yoneyama et al., 2004; Gitlin et al., 2006) or other antiviral immune pathways (Takaoka et al., 2007) may contribute to the control of HSV-1 outside the CNS in patients with TLR3 pathway deficiencies. In any event, this description of a patient with complete AR TLR3 deficiency and HSE clearly indicates that the residual TLR3 responses documented in patients with partial AD TLR3 deficiency do not account for the apparently normal control of viruses, including HSV-1 infections outside the CNS, in these patients.

Remarkably, TLR3 seems to be less necessary for the control of latent HSV-1 infection in the CNS, as HSE recurred only once, in one of the two AD TLR3 patients, and viral recurrences are reported only rarely in other children with HSE, in <10% of affected children (Valenta et al., 2004; Spiegel et al., 2008; Abel et al., 2010). This probably reflects the compensatory role played by adaptive immunity, as in IRAK-4−/− and MyD88−/− deficient children (Picard et al., 2003, 2010; Ku et al., 2007; von Bernuth et al., 2008; Bousfiha et al., 2010; Casanova et al., 2011). Moreover, the clinical penetrance of the TLR3 pathway defects in HSE is incomplete during primary HSV-1 infection (Casrouge et al., 2006; Zhang et al., 2007b). Only two of eight HSV-1−infected P554S heterozygotes developed HSE. Seven of these heterozygotes belong to two previously reported kindreds (Zhang et al., 2007b), whereas the eighth is the father of the patient described in this study. The clinical penetrance of AR TLR3 deficiency for HSE may also be incomplete, like that of AR UNC-93B deficiency (Zhang et al., 2007a,b). All the cases of HSE in the French cohort were sporadic, despite 14% parental consanguinity (Abel et al., 2010), further suggesting that incomplete clinical penetrance is common in children with inborn errors of immunity conferring a predisposition to HSE. There is probably also incomplete penetrance for the development of CVB3 myocarditis, as only one of the 10 known individuals with AD or AR TLR3 deficiency developed CVB3 myocarditis (Gorbea et al., 2010), although the degree of exposure of these patients to various enteroviruses is unknown. Paradoxically, this incomplete penetrance even suggests that the TLR3 pathway may be completely redundant in host defense in most individuals. However, we cannot estimate the actual penetrance of these rare genotypes because of the sporadic nature of their associated phenotypes and their absence from the healthy kindreds tested. Overall, with only two children with HSE and AD TLR3 deficiency, another with AR TLR3 deficiency, and an adult patient with AD TLR3 deficiency and CVB3 myocarditis, it is too early to define firmly the role of human TLR3 in antiviral immunity. We need to search for TLR3 defects in patients with various viral illnesses and in the healthy population. However, we can already conclude that TLR3 deficiency confers predisposition to HSE, with incomplete penetrance, in otherwise healthy children, although TLR3 is largely redundant in host defense in natura. Beyond HSE, these findings suggest that other severe, sporadic viral diseases in otherwise healthy children may result from other single-gene inborn errors of immunity (Casanova and Abel, 2007; Alcaïs et al., 2010; Casanova et al., 2011).

MATERIALS AND METHODS

Patient

The patient was born in France to nonconsanguineous Polish parents and developed HSE at the age of 8 yr. The clinical signs of HSE were high fever (39°C), vomiting, and confusion. CSF evaluation on day 1 showed meningitis (36 cells/μl, 94% lymphocytes, and 0.78 g/liter proteins). Electroencephalogram (EEG) showed a diffuse decrease in activity. Cerebral magnetic resonance imaging (MRI) showed a hypersignal in the right temporal lobe on day 1 and necrotic lesions in the right temporal lobe on day 21. HSE was diagnosed on the basis of the detection by PCR of HSV-1 in the CSF on day 16 and the presence of IgM and IgG antibodies against HSV-1 in a serum sample collected on the same day. IFN-α activity in the CSF was 12 IU/ml on day 3. Clinical signs were well controlled by acyclovir treatment (60 mg/kg/d i.v. for 3 wk). The patient presented herpes labialis before and after the episode of HSE. His father and one of his brothers had developed herpes labialis at some time in their lives, but neither of the patient's parents and none of his brothers developed HSE after serologically documented HSV-1 infection. The patient has suffered from major neurological sequelae since the episode of HSE, including epilepsy and cognitive and motor disabilities. Nevertheless, he has experienced no subsequent acute events or other severe infectious diseases. He is now 20 yr old. He has been exposed to HSV-2, varicella zoster virus, Epstein-Barr virus, and influenza virus A, as shown by positive serological results, without the occurrence of acute events. He was also immunized with live measles/mumps/rubella vaccines with no adverse effect. This patient resided in France, where he was followed up and informed consent was obtained, in accordance with local regulations, with institutional review board approval. The experiments described in this study were conducted in the United States, in accordance with local regulations and with the approval of the Institutional Review Board of the Rockefeller University.

Molecular genetics

DNA was isolated by phenol−chloroform extraction. We extracted RNA from immortalized fibroblast cell lines (SV40-fibroblasts) using TRIZOL (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed directly, with oligo (dT) (Invitrogen). PCR was performed with Taq polymerase (Invitrogen) and the GeneAmp PCR System 9700 (Applied Biosystems). The exons of TLR3 were amplified by PCR. The PCR products were purified by ultracentrifugation through Sephadex G-50 Superfine resin (GE Healthcare) and sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Sequencing products were purified by centrifugation through Sephadex G-50 Superfine resin, and sequences were analyzed with an ABI Prism 3700 apparatus (Applied Biosystems). The mutation was confirmed by the analysis of gDNA extracted from leukocytes and SV40-transformed fibroblasts.

Cell culture

Primary cultures of human fibroblasts obtained from biopsies performed on patients or healthy controls were established in DME (Invitrogen) supplemented with 10% FCS. They were transformed with an SV40 vector, as previously described (Zhang et al., 2007b), to create immortalized fibroblast cell lines: SV40-fibroblasts. The TLR3-deficient P2.1 fibrovascular cell line was provided by D.W. Leaman (University of Toledo, Toledo, OH; Sun and Leaman, 2004). NEMO-deficient fibroblasts were obtained from a fetus with incontinentia pigmenti (NEMO IP; Smahi et al., 2002). SV40-fibroblasts were stimulated in 24-well plates at a density of 10^5 cells/well for 24 h (or the amount of time indicated in the poly(EG) kinetic experiments). PBMCs, freshly isolated...
The preparation and analysis of cells and mRNAs in the study were carried out under sterile conditions. All cells were grown at 37°C, under an atmosphere containing 5% CO2. Patients were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Sigma-Aldrich), plus 24 h in OptiMem medium (Invitrogen) supplemented with 10% fetal bovine serum. mRNA primers were purchased from Sigma-Aldrich. GAPDH (Quantitect) sequence Detection System. The gene of poly(A:U) dsRNA, specific agonist of TLR3; provided by Innate-Pharma). A TLR-3 agonist, at a concentration of 25 µg/ml) and IPH31 (an optimized by Ficoll-Hypaque density gradient centrifugation from healthy controls and the patient, his parents, were incubated in RPMI 1640 medium supplemented with 10% FCS. All cells were grown at 37°C, under an atmosphere containing 5% CO2. For the purification of PDCs and monocytes, PDCs and monocyte subsets were obtained from cryopreserved PBMCs of the patient, his parents, and healthy volunteers, by FACs (FACSAria II; BD). PBMCs were thawed, washed, and labeled with PerCP-conjugated anti-HLA-DR antibody (L243; BD), PE-conjugated anti-CD14 antibody (R73; BD), APC-conjugated anti-CD11c antibody (HIDC205; BD), FITC-conjugated anti-BDCA2 (CD303) antibody (AC144; Miltenyi Biotech), and PE-conjugated anti-CD3 (UCHT1; BD), anti-CD15 (VIMC6; Miltenyi Biotech), anti-CD19 (4GT; BD), and anti-CD16 (53-7.3; BD Biosciences) antibodies. PDCs were gated as HLA-DR−BDCA2−CD11c−CD16−CD14−Lin− (CD3, CD13, CD15, CD19, NKp46). Monocytes were characterized as being HLA-DR+Lin−, CD14+CD16+, and CD14+/CD16− subsets. Sorted cells were analyzed for purity (>99%). Cells were dispensed in a 96-well round-bottom plate at a density of 105 cells/well and cultured for 24 h in OptiMem medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Sigma-Aldrich), plus 10 ng/ml IL-3 (R&D Systems) for PDCs, at 37°C in a 5% CO2 atmosphere. Determination of mRNA levels by quantitative RT-PCR (RT-qPCR) Total RNA was extracted from SV40-fibroblasts, PBMCs, and P2.1 cells. RNA was reverse transcribed directly, with oligo (dT), to determine mRNA levels for TLR3, IFN-α, IFN-β, IFIT1, IFIT2, and IFIT3. RT-qPCR was performed with Assays-on-Demand probe/primer combinations (Applied Biosystems) and a universal reaction mixture in an ABI PRISM 7700 Sequence Detection System. The gene of β-glucuronidase (GUS) was used for normalization. Results are expressed according to the ΔΔCt method, as described by the manufacturer. To determine mRNA levels for OAS1 and normalization. Results are expressed according to the sequence of the manufacturer, as described by the manufacturer. Viral infection and quantification of fibroblasts For VSV infection, 105 SV40-fibroblasts were plated in individual wells of 24-well plates and infected with VSV, at an MOI of 10, in DME supplemented with 2% FCS. After 30 min, cells were washed and incubated in 500 µl of medium. Supernatants were obtained at the 0.5-, 1-, 2-, 4-, 6-, 8-, and 24-h time points and frozen. VSV titers were determined by calculating the 50% end point (TCID50), using the Reed and Muench method, after the inoculation of vero cell cultures in 96-well plates. For HSV-1 GFP infection, 104 SV40-fibroblasts were plated in individual wells of 96-well plates and infected with HSV-1–GFP (strain KOS; Desai and Person, 1998), at various MOIs, in DME supplemented with 2% FCS. 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-, 8-, 16-, 24-, and 48-h time points. For assays of cell death upon viral stimulation, cells were treated with 105 IU/ml IFN-α 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% FCS. After 24 h, the cells were infected 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. After incubation for 30 min, cell viability was determined by measuring fluorescent intensity. The results were expressed as the percentage of viable cells compared to the control.
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 excitation wavelength of 531 nm. Background fluorescence, calculated for the presence of Lipofectamine reagent (Invitrogen) and PLUS reagent (Invitrogen), as described by the manufacturer. Transfectants were selected on medium containing 5 μg/ml blasticidin (Invitrogen). We checked for the presence of TLR3 by sequencing the PCR products generated from cDNA and carrying out RT-qPCR and Western blotting. The same conditions were used for the transfection of cells with mock vectors.

Genome-wide transcrip tional profile experiments in fibroblasts and PBMCs

Data acquisition. Fibroblasts and PBMCs from patients and controls were stimulated with IL-1β or poly(I:C) for 2 or 8 h or left unstimulated. Total RNA was then isolated from the cells (RNeasy kit; QIAGEN). RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies). Biotinylated cRNA targets were prepared from 200 ng total RNA with the Illumina TotalPrep RNA Amplification kit (Invitrogen). We then incubated 1,500 ng cRNA for 16 h with Sentrix Human-6V2 BeadChip arrays (49,295 probes; Illumina). Beadchip arrays were then washed, stained, and scanned on a BeadStation 500 (Illumina) according to the manufacturer’s instructions.

Data preprocessing. After background subtraction, the raw signal values extracted with Beadstudio version 2 software (Illumina) were scaled according to the mean intensity of the arrays constituting this dataset. Minimum intensity was set to 10. Only the probes called as present in at least one sample (P < 0.01) were retained for downstream analysis (n = 18,519 at 2 h; n = 16,929 at 8 h).

Data analysis. Transcripts differentially regulated upon stimulation were defined on the basis of at least a 1.5-fold change (up- or down-regulation) and a minimum absolute intensity difference of 100 with respect to the respective unstimulated sample. A gene expression analysis program, GeneSpring version 7.3.1 (Agilent Technologies), was used for hierarchical clustering and the generation of heat maps. Functional networks were resolved with Ingenuity Pathway Analysis software (Ingenuity Systems).

Data availability. Raw data for the 78 microarray analyses performed in this study are available from the public repository of GEO DataSets under accession no. GSE39051). Networks were resolved with commercially available software and a knowledge base constructed from published data identifying relevant functional links between transcripts differentially regulated by each stimulus in control fibroblasts.

Online supplemental material

Fig. S1 shows the production of E746X TLR3 protein in P2.1 cells. Fig. 2 shows that the P554S and E746X TLR3 alleles are loss-of-function. Fig. S3 shows an absence of response to TLR3 in the patient’s fibroblasts. Fig. S4 shows that TLR3 responsiveness is rescued by WT TLR3 expression in the patient’s fibroblasts. Fig. S5 shows genome-wide transcriptional evaluation of the TLR3 pathway in fibroblasts. Fig. S6 shows impaired production of IFN by the patient’s fibroblasts upon HSV-1 or VSV infection. Fig. S7 shows normal IFN production by the patient’s leukocytes upon stimulation with poly(I:C) or HSV-1. Tables S1 and S2 list the number of transcripts differentially expressed upon stimulation with poly(I:C) or IL-1β in control fibroblasts and PBMCs, respectively. Table S3 shows in vivo viral infection in TLR3-deficient mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101568/DC1.

We would like to thank the members of our laboratory for helpful discussions and critical reading of this manuscript. We warmly thank our patients, their families, and physicians.

This work was conducted in the two branches of the Laboratory of Human Genetics of Infectious Diseases and was funded by the Rockefeller University Center for Clinical and Translational Science grant no. 5UL1RR024143-04, the Rockefeller University, Institut National de la Santé et de la Recherche Médicale, Paris Descartes University, the St. Giles Foundation, the Eppley Foundation, the Jeffrey Modell Foundation, Tareis Biotechnologies, the Thrasher Research Fund, the National Research Agency, the GIS Maladies Rares, and the March of Dimes. J. Azevedo is supported by the Programme for Advanced Medical Education, sponsored by Fundação Calouste Gulbenkian, Fundação Champalimaud, Ministério da Saúde, and Fundação para a Ciência e Tecnologia, Portugal. D. Chassabel is funded by National Institutes of Health grant no. R21 AI085523-01 and the Baylor Healthcare System Foundation. J.-L. Casanova was an International Scholar of the Howard Hughes Medical Institute from 2005 to 2008. The authors state that they have no financial conflicts of interest.

Submitted: 2 August 2010
Accepted: 16 August 2011

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