

Inherited human IRAK-1 deficiency selectively impairs TLR signaling in fibroblasts

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Most members of the Toll-like receptor (TLR) and interleukin-1 receptor (IL-1R) families transduce signals via a canonical pathway involving the MyD88 adapter and the interleukin-1 receptor-associated kinase (IRAK) complex. This complex contains four molecules, including at least two (IRAK-1 and IRAK-4) active kinases. In mice and humans, deficiencies of IRAK-4 or MyD88 abolish most TLR (except for TLR3 and some TLR4) and IL-1R signaling in both leukocytes and fibroblasts. TLR and IL-1R responses are weak but not abolished in mice lacking IRAK-1, whereas the role of IRAK-1 in humans remains unclear. We describe here a boy with X-linked MECP2 deficiency-related syndrome due to a large de novo Xq28 chromosomal deletion encompassing both MECP2 and IRAK1. Like many boys with MECP2 null mutations, this child died very early, at the age of 7 mo. Unlike most IRAK-4- or MyD88-deficient patients, he did not suffer from invasive bacterial diseases during his short life. The IRAK-1 protein was completely absent from the patient's fibroblasts, which responded very poorly to all TLR2/6 (PAM₂CSK₄, LTA, FSL-1), TLR1/2 (PAM₃CSK₄), and TLR4 (LPS, MPLA) agonists tested but had almost unimpaired responses to IL-1 β . By contrast, the patient's peripheral blood mononuclear cells responded normally to all TLR1/2, TLR2/6, TLR4, TLR7, and TLR8 (R848) agonists tested, and to IL-1 β . The death of this child precluded long-term evaluations of the clinical consequences of inherited IRAK-1 deficiency. However, these findings suggest that human IRAK-1 is essential downstream from TLRs but not IL-1Rs in fibroblasts, whereas it plays a redundant role downstream from both TLRs and IL-1Rs in leukocytes.

IRAK-1 | IRAK-4 | Toll-like receptor | interleukin-1 receptor | primary immunodeficiency

The interleukin-1 receptor-associated kinase (IRAK) protein complex plays a critical role in the canonical pathway downstream from most Toll-like receptors (TLRs) and IL-1 receptors (IL-1Rs) (1–3). In humans and mice, the IRAK complex has four members: IRAK-1, IRAK-2, IRAK-3/IRAK-M, and IRAK-4 (4–11). All contain an amino-terminal death domain (DD) (12) required for homo- or heterodimerization and a serine/threonine kinase domain (13–15). IRAK-1, IRAK-4, and possibly IRAK-2 have serine/threonine kinase activity (10, 16, 17). Upon stimulation, in both mice and humans, the myeloid differentiation primary response gene 88 (MyD88) adaptor is recruited to TLRs and IL-1Rs via TLR–IL-1R (TIR) interaction; it then recruits IRAK-4 by DD interaction (4, 18–20). Other TIR adapters, such as TIRAP for TLR2 and TLR4 (via MyD88), contribute to TLR-responsive pathways (21, 22). IRAK-4 then associates with IRAK-1 and/or IRAK-2 to form the “Myddosome” (23–26). The

phosphorylation of IRAK-1 by IRAK-4 results in the activation of IRAK-1 kinase activity, leading to IRAK-1 hyperphosphorylation (by autophosphorylation). Hyperphosphorylated IRAK-1 dissociates from the Myddosome to associate with TRAF-6 (27, 28) and is then ubiquitinated and degraded or sumoylated (29–32). This ultimately activates both the mitogen-activated protein kinase (MAPK) and nuclear factor- κ B (NF- κ B) pathways, resulting in the transcription of target genes, including those encoding proinflammatory cytokines (20, 33). By contrast, the interaction of IRAK-3 with the Myddosome has been shown to have an inhibitory effect, in both mice and humans (28, 34).

In vitro studies in mouse embryonic fibroblasts (MEFs) have shown that the knockout (KO) of *Irak1*, *Irak4*, or, to a lesser

Significance

We report the discovery of complete human interleukin-1 receptor (IL-1R)-associated kinase 1 (IRAK-1) deficiency resulting from a de novo Xq28 microdeletion encompassing MECP2 and IRAK1 in a boy. Like many boys with MECP2 defects, this patient died very early. IRAK-1 is a component of the Toll-like receptor (TLR)/IL-1R (TIR) signaling pathway. Unlike patients with autosomal-recessive complete deficiency of MyD88 or IRAK-4, two other components of the TIR pathway, this patient presented no invasive bacterial infections. We analyzed the impact of human IRAK-1 deficiency in fibroblasts and leukocytes. The role of IRAK-1 in signaling downstream from IL-1R and TLRs differed according to cell type. These findings reveal similarities and differences in the role of IRAK-1 in the TLR and IL-1R pathways between mice and humans.

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extent, *Irak2* results in impaired responses to TLR2/6 (MALP-2, PGN), TLR4 (LPS), and IL-1R (IL-1 β) stimulation (5, 11, 23, 35, 36). *Irak3* KO MEFs have not been tested. *Irak2*^{-/-} (37), *Irak4*^{-/-} (11), and, to a lesser extent, *Irak1*^{-/-} (23, 35) macrophages display impaired responses to TLR4 agonists, whereas *Irak3*^{-/-} macrophages display enhanced responses to such agonists (28). *Irak1*^{-/-} splenocytes produce normal amounts of *IL1B* and *TNF* mRNA, but their production of *IL10* mRNA and protein in response to TLR4 (LPS) stimulation is impaired (31). *Irak4*^{-/-} splenocytes fail to proliferate in response to the stimulation of TLR2/6 (MALP-2), TLR4 (LPS), TLR7, TLR8 (R848), or TLR9 (CpG), whereas they proliferate normally when stimulated with the nonspecific TLR3 agonist poly(I:C) (37). IL-1R/TLR signaling was not assessed in *Irak2*^{-/-} and *Irak3*^{-/-} splenocytes. In vivo studies have shown survival to be higher for *Irak1*^{-/-}, *Irak2*^{-/-}, and *Irak4*^{-/-} mice following LPS injection (*Irak3*^{-/-} mice were not tested) (11, 38, 39) and lower for *Irak1*^{-/-} and *Irak4*^{-/-} mice (the only two strains tested) following *Staphylococcus aureus* infection (11, 40). *Myd88*^{-/-} mice and, by inference, *Irak4*^{-/-} mice are susceptible to many other pathogens not used for the inoculation of the other mutants (41). Overall, these studies suggest that the mouse IRAK-1, IRAK-2, and IRAK-4 proteins play activating roles in TLR and IL-1R responses and protective immunity to bacteria, albeit to different degrees, whereas IRAK-3 seems to have an inhibitory effect (34, 42).

Human patients with autosomal-recessive complete IRAK-4 or MyD88 deficiency have a common clinical phenotype, characterized by extreme susceptibility to a small range of pyogenic bacterial infections, with normal resistance to other bacteria and most viruses, fungi, and parasites (41, 43). IRAK-4- and MyD88-deficient patients present with meningitis, sepsis, arthritis, osteomyelitis, and deep inner-organ/tissue abscesses, mostly caused by gram-positive *Streptococcus pneumoniae* and *S. aureus* and, more rarely, by gram-negative *Pseudomonas aeruginosa* and *Shigella sonnei* (44–64). They also display superficial skin infections, mostly caused by *S. aureus*. The first invasive infection occurs before the age of 2 y in 85% of patients and 92% of probands, and before the age of 6 mo in 42% of patients and probands (41, 43, 52, 55, 57, 59–64). The frequency and severity of these infections decrease considerably from adolescence onward, even in the absence of preventive measures, suggesting that the MyD88/IRAK-4-dependent TIR pathway becomes redundant once acquired immunity is fully functional and can ensure protection (43). These disorders have a modest impact on IgM-dependent B-cell immunity, delaying its maturation (65, 66). IRAK-4- and MyD88-deficient patients also display impaired inflammatory responses, such as weak or delayed fever and plasma C-reactive protein (CRP) induction (43).

Fibroblasts from MyD88- and IRAK-4-deficient patients do not respond to IL-1 β in terms of IRAK-1 and I κ B- α degradation, MAPK activation (p38 phosphorylation), NF- κ B DNA-binding activity, transcriptional activity, and proinflammatory cytokine production (44, 52, 55). TLR2/6 and TLR4 responses have not been assessed in MyD88- and IRAK-4-deficient fibroblasts, in which TLR3 responses to poly(I:C) are intact and mediated by the TRIF-dependent pathway (67–69). In addition, CD62L shedding and proinflammatory cytokine production in response to IL-1R agonists (IL-1 β) or any of the TLR agonists tested, other than poly(I:C), that signal via receptors other than TLR3 in most human leukocyte subsets (69) (TLR1/2, TLR2/6, TLR5, TLR7, TLR8, TLR9, and partially TLR4) were abolished in all IRAK-4- and MyD88-deficient leukocyte subsets tested ex vivo (granulocytes, monocytes, plasmacytoid and myeloid dendritic cells, NK, T, and B cells) or generated in vitro (monocyte-derived dendritic cells) (52, 55). The weak induction of the IFN-stimulated gene products MIP-1 β and MCP-1 in response to LPS was probably mediated by TRIF (52). No patients with IRAK-1, IRAK-2, or IRAK-3 deficiency have yet been described, precluding

assessment of the cellular, immunological, and clinical impact of these defects in humans. We describe here a child with X-linked recessive complete IRAK-1 deficiency and the impact of this inborn error of immunity on cellular responses to TLR and IL-1R agonists (70, 71).

Results

Case Report. The proband was a boy born to nonconsanguineous Italian parents at 40 wk of gestation, after an unremarkable pregnancy (Fig. 1A). The family history contained no relevant antecedents. At birth, the baby presented a weak cry, apnea, and hypotonia, and he required neonatal resuscitation and non-invasive respiratory support because of poor respiratory effort. Antibiotics (ampicillin and gentamicin) were administered for the first 3 d of life for a suspected early-onset infection that was not confirmed by microbiological analyses. The child was hospitalized during the first 4 mo of life, due to recurrent episodes of apnea with cyanosis, sometimes accompanied by bradycardia, and poor respiratory effort. He presented severe axial and limb hypotonia, hypokinesia, hyporeactivity, and intermittent proximal lower-limb rigidity, abnormal eye movements (chaotic ocular movements, poor eye contact, and tonic ocular deviation), automatic movements of the tongue, and seizure-like episodes. He also needed oxygen and/or advanced respiratory support through nasal continuous positive airway pressure or mechanical ventilation. He was fed via a nasogastric or orogastric tube due to poor sucking ability and difficulties swallowing. During hospitalization, he had mild conjunctivitis due to *S. aureus*, a urinary tract infection confirmed by the isolation of *Klebsiella pneumoniae* from a urine culture and treated with amikacin for 13 d, and bronchospasms in the course of *Rhinovirus* infection. An electroencephalogram performed due to seizure-like episodes showed a pattern of irritative activity, with high-voltage waves and spikes. The infant was vaccinated with a hexavalent vaccine (against diphtheria/tetanus/acellular pertussis/inactivated poliovirus/hepatitis B/*Haemophilus influenzae* type b), with no adverse event. At 6 mo of age, percutaneous endoscopic gastrostomy (PEG) was performed. Two days later, the child had a brief temperature peak of 38 °C, with no associated clinical problems. Four days after PEG, at the age of 6 mo, the baby had an episode of acute, severe respiratory failure, during which his temperature briefly peaked at 38 °C and his serum CRP concentration was slightly high, at 10.4 mg/L. Chest X-rays revealed diffuse, heterogeneous, bilateral opacities of the lung parenchyma requiring mechanical ventilation for 4 d. The infant was given ceftriaxone and discharged from hospital 8 d after the onset of respiratory failure (i.e., 4 d after extubation). One month after hospitalization for PEG, the child required mechanical ventilation again, due to progressive respiratory failure. Both axillary temperature and CRP were within the normal range. Chest X-rays were performed and confirmed aspiration pneumonia (SI Appendix, Fig. S1). The child's neurological and respiratory condition progressively deteriorated. Death occurred at 7 mo of age, due to progressive respiratory failure.

A Large Xq28 Deletion. We investigated whether a genetic defect, such as those responsible for Prader–Willi syndrome or 22q11.2 deletion syndrome, could account for the neurological symptoms of the patient. We determined the patient's karyotype and *SNRPN* gene methylation profile, and performed FISH for the 22q11.2 region. The patient was found to have a normal 46, XY karyotype, and we excluded both Prader–Willi syndrome and 22q11.2 deletion syndrome. Array comparative genomic hybridization (array-CGH) analysis was also performed to exclude a microduplication/microdeletion syndrome. This assay revealed an intrachromosomal deletion of about 112 kb on the long arm of the X chromosome (Xq28). The proximal breakpoint mapped to between 153,238,518 and 153,246,671 bp, whereas the distal breakpoint mapped to between 153,340,688 and 153,350,516 bp

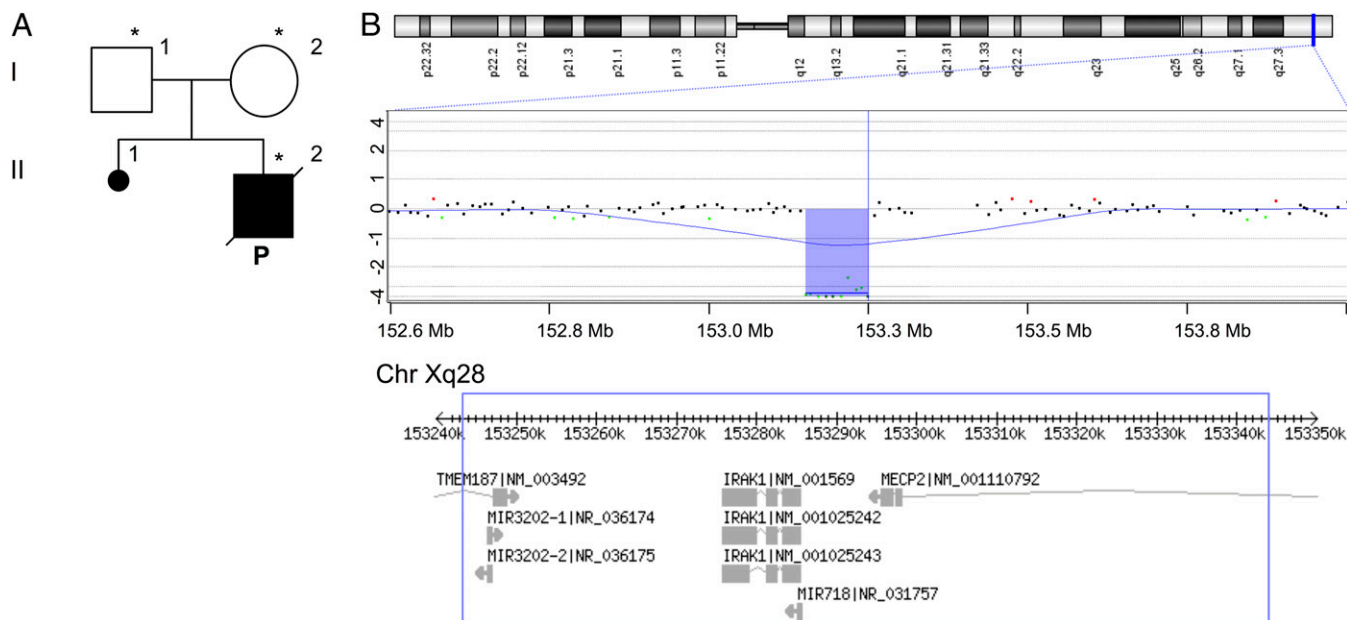


Fig. 1. De novo large deletion on the X chromosome encompasses the *IRAK1* and *MECP2* genes. (A) Pedigree of the patient's family; generations are designated by roman numerals (I, II); the patient is represented by a black square and indicated by "P." Asterisks indicate the individuals tested for Xq28 deletion. (B) Schematic representation of the X chromosome and the array-CGH profile of the patient's chromosome Xq28, showing the hemizygous deletion. The experiment was performed with a 180K platform (180K SurePrint G3 Human Kit; Agilent Technologies). A schematic representation of the gene content of the 101-kb deleted region is shown (Bottom).

(Fig. 1B). We determined the size of the rearrangement, to a resolution of 1 bp, with specific primers (sequences are available upon request) binding to sequences on either side of the pre-determined breakpoints. The breakpoints of the deletion were found at 153,243,653 and 153,344,830 bp. The deleted region was 101,177 bp long, and the presence of the same 12-nt sequence (ACAGAGCAAGAG) at both breakpoints suggests that this rearrangement resulted from nonallelic homologous recombination (72). The deletion encompassed parts of the *TMEM187* and *MECP2* protein-coding genes and the *IRAK1* protein-coding gene, as well as the *MIR3202-1*, *MIR3202-2*, and *MIR718* RNA-coding genes (Fig. 1B).

The Deletion Encompasses *MECP2* and *IRAK1*. *TMEM187* encodes a putative ubiquitous multipass membrane protein (73). The *MIR3202-1*, *MIR3202-2*, and *MIR718* genes all encode microRNAs with unknown target genes. *MECP2* encodes a widely expressed chromatin-associated protein that specifically binds 5-methyl cytosine residues in CpG dinucleotides and can either activate or repress the transcription of its target genes (74–76). Loss-of-function (LOF) mutations of *MECP2* cause Rett syndrome, a progressive neurodevelopmental disorder that predominantly affects girls. Rett syndrome is characterized principally by arrested development at about 12 mo of age, the regression of acquired skills, a reduction or total loss of communication, and stereotypic hand movements (77). Boys carrying LOF mutations of *MECP2* present with various neurological phenotypes, from Rett-like syndrome (very rare) to severe neonatal encephalopathy commonly accompanied by respiratory insufficiency, hypotonia, and early childhood death (78–91). *MECP2* duplication syndrome is a severe neurodevelopmental disorder (fully penetrant in boys) characterized by hypotonia, severe intellectual disability, speech abnormalities, seizures, and recurrent life-threatening infections (92). About 70 to 75% of patients with *MECP2* duplication display susceptibility to recurrent infections. IgA/IgG2 deficiency, low antibody titers against pneumococci, and abnormally strong acute-phase responses have recently been reported in patients with *MECP2* duplication

syndrome (93). Based on these data, a diagnosis of *MECP2*-related congenital encephalopathy was established in our patient. Male patients with *MECP2*-related congenital encephalopathy have been reported to display either *MECP2* mutations or, more rarely, large deletions encompassing *MECP2*. To our knowledge, a deletion encompassing both *MECP2* and *IRAK1* has never before been reported in a male patient. Such deletions have been reported only in heterozygous female patients (94–102).

Impaired Responses to TLR Stimulation in the Patient's Fibroblasts.

Neither *IRAK1* mRNA (evaluated by quantitative PCR) nor *IRAK-1* protein (evaluated by Western blotting) was detected in the patient's SV40-immortalized fibroblasts (SV40-fibroblasts). By contrast, *IRAK-1* mRNA and protein were detected in SV40-fibroblasts from *IRAK-4*–, *MyD88*–, and *MECP2*-deficient patients and healthy individuals, all of whom served as controls in subsequent experiments (Fig. 2A and B). We first assessed TLR-dependent signaling in the patient's SV40-fibroblasts, after stimulation with TLR2/6 agonists (PAM₂CSK₄, known as PAM-2, FSL-1, and LTA-SA), TLR4 agonists (LPS, monophosphoryl lipid A, known as MPLA), and poly(I:C). We had previously checked that human SV40-fibroblasts responded (weakly) to LPS and MPLA stimulation in a TLR4-dependent manner, by knocking down *TLR4* mRNA in control SV40-fibroblasts with small interfering RNAs (siRNAs) (*SI Appendix*, Fig. S2A) and evaluating the resulting levels of IL-8 mRNA and protein. IL-8 mRNA and protein levels in response to stimulation with LPS or MPLA were lower after transfection with an siRNA against TLR4 but not after transfection with a scrambled siRNA; by contrast, no such decrease was observed after PAM-2 stimulation (*SI Appendix*, Fig. S2B and C). As in *IRAK-4*– and *MyD88*-deficient SV40-fibroblasts, NF-κB DNA-binding activity was abolished in the patient's SV40-fibroblasts (evaluated by EMSA; *SI Appendix*, Fig. S3A and B), together with NF-κB transcriptional activity (luciferase assay; Fig. 3A) and cytokine production (ELISA; Fig. 3B and *SI Appendix*, Fig. S4A) in response to TLR2/6 and TLR4 stimulation, whereas cytokine production in

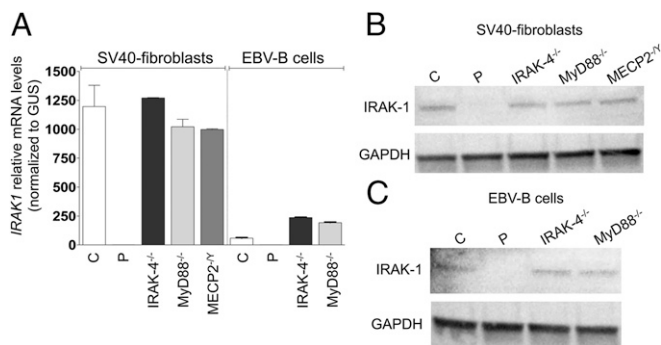


Fig. 2. Complete IRAK-1 deficiency. (A) Relative *IRAK1* mRNA levels in SV40-fibroblasts and EBV-B cells from controls (labeled C; $n = 2$ for both SV40-fibroblasts and EBV-B cells) and from the IRAK-1-deficient patient (labeled P) and IRAK-4^{-/-}, MyD88^{-/-}, and MECP2-deficient patients (labeled IRAK-4^{-/-}, MyD88^{-/-}, and MECP2^{-/-}, respectively). The values shown (means \pm SEM) were obtained in three independent experiments. (B and C) Western blot analysis of IRAK-1 protein levels in total cell extracts from SV40-fibroblasts (B) and EBV-B cells (C) from controls and the IRAK-1-deficient patient and IRAK-4^{-/-}, MyD88^{-/-}, and MECP2-deficient patients. Similar results were obtained in three independent experiments.

response to stimulation with poly(I:C) was similar to that in control SV40-fibroblasts. Following TNF- α stimulation, NF- κ B DNA-binding and transcriptional activities and cytokine production were similar between SV40-fibroblasts from the patient and from IRAK-4^{-/-} and MyD88-deficient patients and controls (Fig. 3 and *SI Appendix*, Figs. S3 A and B and S4A). The responses of MECP2-deficient fibroblasts were similar to those of control SV40-fibroblasts in all conditions and for all readouts tested (Fig. 3 and *SI Appendix*, Figs. S3 A and B and S4A). Similar results were obtained with primary fibroblasts (*SI Appendix*, Fig. S5A). Finally, we used microarrays to study the transcriptome of primary fibroblasts, with or without stimulation with TLR2/6 and TLR4 agonists. Primary fibroblasts from the IRAK-1^{-/-} and MECP2-deficient patient studied displayed much lower levels of target gene induction than cells from healthy controls and an MECP2-deficient patient, as found for fibroblasts from IRAK-4^{-/-} and MyD88-deficient patients (Fig. 4A). The induction of some target genes was apparently IRAK-4^{-/-} and MyD88-dependent but IRAK-1-independent. Overall, fibroblasts from the IRAK-1^{-/-} and MECP2-deficient patient responded poorly to all of the relevant TLR agonists tested.

Almost Unimpaired Responses to IL-1R Stimulation in the Patient's Fibroblasts. An evaluation of IL-1R signaling revealed much lower levels of phosphorylation of IKK subunits alpha and beta (IKK- α/β) and, to a lesser extent, of p65 in response to IL-1 β stimulation in SV40-fibroblasts from the patient than in control cells, whereas the phosphorylation of IKK- α/β and p65 was abolished in IRAK-4-deficient cells (*SI Appendix*, Fig. S6A). Furthermore, I κ B- α degradation upon IL-1 β stimulation was both delayed and impaired, but not entirely abolished as in IRAK-4^{-/-} and MyD88-deficient SV40-fibroblasts (Fig. 5A, *Top* and *SI Appendix*, Fig. S6). Moreover, I κ B- α phosphorylation levels following IL-1 β stimulation were similar in cells from the patient and control cells, whereas this phosphorylation was abolished in IRAK-4-deficient cells (*SI Appendix*, Fig. S6A). Upon TNF- α stimulation, SV40-fibroblasts from the patient and IRAK-4^{-/-} and MyD88-deficient patients displayed levels of I κ B- α degradation similar to that in control cells (Fig. 5A, *Bottom* and *SI Appendix*, Fig. S6B). By contrast to the abolition of responses to IL-1 β observed in IRAK-4^{-/-} and MyD88-deficient SV40-fibroblasts, the patient's cells displayed NF- κ B DNA-binding activity (evaluated by EMSA; Fig. 5B and *SI Appendix*, Fig. S3C),

NF- κ B-dependent transcriptional activity (evaluated by a reporter luciferase assay; Fig. 5C), and cytokine production (evaluated by ELISA; Fig. 5D and *SI Appendix*, Fig. S4B) at levels similar to or only slightly lower than those in control and MECP2-deficient SV40-fibroblasts. Upon TNF- α stimulation [and poly(I:C) stimulation for cytokine production], NF- κ B DNA-binding and transcriptional activities and cytokine production were similar in all of the SV40-fibroblast cell lines tested (Fig. 5 B–D and *SI Appendix*, Figs. S3 and S4B). Similar results were obtained with primary fibroblasts (*SI Appendix*, Fig. S5B). Finally, the induction of target genes by IL-1 β was only modestly affected in primary fibroblasts from the patient, unlike in cells from IRAK-4^{-/-} and MyD88-deficient patients, as analyzed by genome-wide microarray (Fig. 4B). The response to TNF- α was normal. Overall, our data show that the IRAK-1^{-/-} and MECP2-deficient fibroblasts from the patient displayed little or no impairment of IL-1R responses, by contrast to the results obtained for IRAK-4^{-/-} or MyD88-deficient fibroblasts.

Rescue of TLR-Dependent Responsiveness by Wild-Type IRAK1 in the Patient's Fibroblasts. Our findings raised questions as to whether the poor TLR response in the fibroblasts of this patient was due to the deletion of *IRAK1* or another nearby gene located within the region corresponding to the large X-chromosome deletion (*TMEM187*, *MIR3202-1*, *MIR3202-2*, and *MIR718*), or a combination of deletions. We addressed this question by transiently

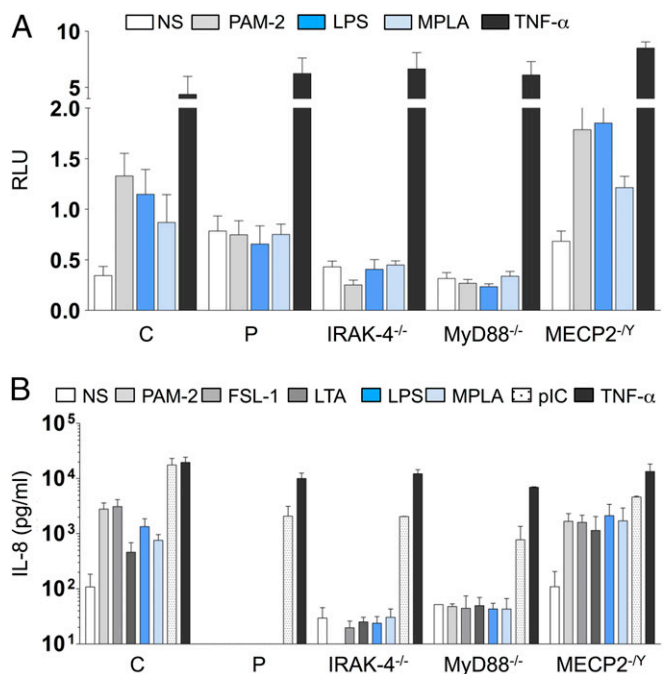


Fig. 3. TLR-dependent signaling in the patient's fibroblasts. (A) NF- κ B-dependent transcription was assessed with the NF- κ B reporter luciferase assay in SV40-fibroblasts derived from a healthy control, the IRAK-1-deficient patient, and IRAK-4^{-/-}, MyD88^{-/-}, and MECP2-deficient patients. Twenty-four hours after transfection, the cells were left untreated (NS) or stimulated with PAM-2 (10 μ g/mL), LPS (10 μ g/mL), MPLA (1 μ g/mL), or TNF- α (20 ng/mL) for 42 h and then harvested. Reporter gene activities were measured and the values obtained were normalized for transfection efficiency on the basis of *Renilla* luciferase expression. RLU, relative light units. (B) IL-8 secretion by SV40-fibroblasts from healthy controls ($n = 3$), the IRAK-1-deficient patient, and IRAK-4^{-/-}, MyD88^{-/-}, and MECP2-deficient patients, left unstimulated or stimulated with PAM-2 (10 μ g/mL), FSL-1 (1 μ g/mL), LTA (10 μ g/mL), LPS (10 μ g/mL), MPLA (1 μ g/mL), poly(I:C) (pI:C; 25 μ g/mL), and TNF- α (20 ng/mL) as a positive control. The values shown (means \pm SEM) were obtained in three independent experiments.

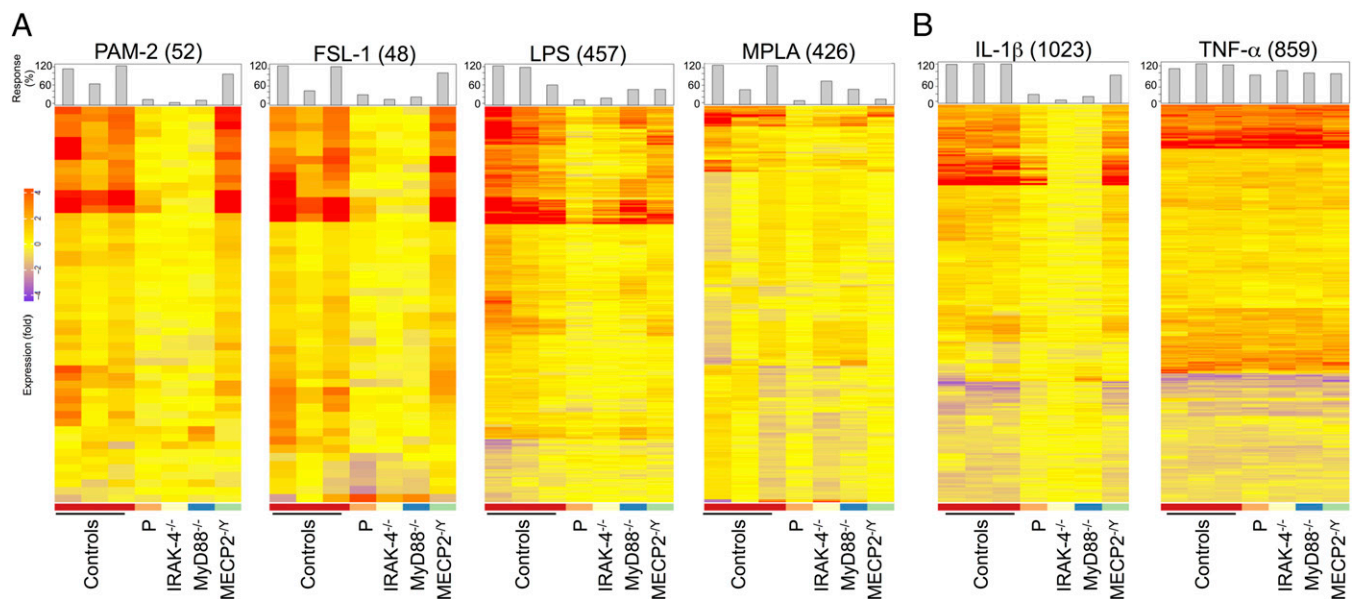


Fig. 4. Transcriptome analysis of primary fibroblasts. Transcriptional profiles of primary fibroblasts from healthy controls ($n = 3$), the IRAK-1-deficient patient, and IRAK-4^{-/-}, MyD88^{-/-}, and MECP2-deficient patients, stimulated for 6 h with PAM-2 (10 $\mu\text{g}/\text{mL}$), FSL-1 (1 $\mu\text{g}/\text{mL}$), LPS (10 $\mu\text{g}/\text{mL}$), and MPLA (1 $\mu\text{g}/\text{mL}$) (A) and IL-1 β (10 ng/mL) or TNF- α (20 ng/mL), as a positive control (B). Shown, as a heat map, are log₂-transformed fold-change values for gene expression relative to nonstimulated conditions; columns correspond to subjects, and rows correspond to genes. Genes with induced expression are depicted in red, genes with suppressed expression are shown in blue, and genes displaying no change in expression are shown in yellow. The numbers in parentheses (Top) indicate the total number of responsive transcripts for each set of conditions. Bars represent overall individual responsiveness relative to the mean for control subjects [calculated as (responsive probes in a subject/mean number of responsive probes in healthy control subjects) $\times 100$].

transfecting SV40-fibroblasts from the patient, a healthy control, and IRAK-4^{-/-}, MyD88^{-/-}, and MECP2-deficient patients with expression vectors encoding wild-type (WT) IRAK-1 or IRAK-4 or an empty vector and leaving them unstimulated or stimulating them with a TLR2, TLR4, or IL-1R agonist. The transfection of the patient's fibroblasts with WT *IRAK1* restored both the production of normal IRAK-1 protein (SI Appendix, Fig. S7A) and TLR2-dependent and TLR4-dependent NF- κB transcriptional activity at levels similar to those in healthy control cells (Fig. 6A). IRAK-4^{-/-} and MyD88^{-/-} cells transfected with WT *IRAK1* displayed higher levels of constitutive NF- κB activation, whereas control and MECP2-deficient cells transfected with WT *IRAK1* had levels of activity higher than those recorded for cells transfected with the empty vector (SI Appendix, Fig. S7B). Moreover, IL-8 production in response to PAM-2, LPS, and MPLA stimulation was partially restored in fibroblasts from the patient transfected with WT *IRAK1* but not in patient fibroblasts transfected with an empty vector (SI Appendix, Fig. S7D). The transfection of control and patient fibroblasts with WT *IRAK1* further increased NF- κB transcriptional activity in response to IL-1 β (Fig. 6B and SI Appendix, Fig. S7C); IL-1 β -induced cytokine production levels, which were already very high, were similar to those in nontransfected cells (SI Appendix, Fig. S7E).

Knocking Down IRAK-1 Expression in Control Fibroblasts Reproduces the Patient's Phenotype. We then investigated the causality of the relationship between the lack of IRAK-1 in the patient and his fibroblasts' impaired responses to TLR2 and TLR4 stimulation, by knocking down IRAK-1 expression in SV40-fibroblasts from a healthy control by transfection with siRNAs. Transfection with the *IRAK1* siRNA abolished IRAK-1 expression, with no mRNA or protein detected (SI Appendix, Fig. S8A and B). Levels of IL-8 mRNA (SI Appendix, Fig. S8C) and protein (Fig. 6C) after stimulation with PAM-2, LPS, or MPLA were significantly lower in the presence of *IRAK1* siRNA, whereas no such effect was observed after transfection with scrambled siRNAs. Slightly

lower levels of IL-8 mRNA ($P = 0.02$) and protein ($P = 0.01$) in response to stimulation with IL-1 β were also observed after transfection with si-*IRAK1*, whereas this siRNA had no effect on the response to TNF- α stimulation (Fig. 6D and SI Appendix, Fig. S8D). Thus, knocking down IRAK-1 expression in control SV40-fibroblasts is sufficient to decrease the responses to TLR2 and TLR4 agonists significantly but has only a very slight effect on the IL-1 β -dependent response, reproducing the phenotype of the patient's fibroblasts.

Knocking Down IRAK-2 Expression Blocks IL-1R Responses in the Patient's Fibroblasts. We tested the hypothesis that IRAK-2 might compensate for IRAK-1 deficiency in the patient's SV40-fibroblasts by mediating some IL-1 β -dependent signaling, by using siRNA to knock down IRAK-2 expression in SV40-fibroblasts from a healthy control and the patient (SI Appendix, Fig. S9A and B). *IRAK2* silencing almost totally abolished IL-8 mRNA and protein production in response to IL-1 β in the patient's cells ($P = 0.0005$ and $P < 0.0001$, respectively) but only slightly decreased the levels of IL-8 mRNA and protein in the control SV40-fibroblasts ($P = 0.07$ and $P = 0.12$, respectively) (SI Appendix, Fig. S9C and D). *IRAK2* silencing had no effect on the production of IL-8 mRNA and protein in response to TNF- α stimulation in SV40-fibroblasts from a healthy control or the patient (SI Appendix, Fig. S9C and D). Transfection with the scrambled siRNA had no effect on IL-8 mRNA and protein levels in any of the conditions tested (SI Appendix, Fig. S9C and D). We then knocked down *IRAK1* and *IRAK2* mRNA levels simultaneously, by transfecting SV40-fibroblasts from a healthy control and a MECP2-deficient patient with siRNAs (SI Appendix, Fig. S10A and B). We evaluated *IL8* mRNA induction in response to IL-1 β , TLR2, and TLR4 ligands. The silencing of both *IRAK1* and *IRAK2* in control and MECP2-deficient fibroblasts resulted in low levels of *IL8* mRNA (with a stronger effect than a single-gene knockdown) in response to IL-1 β stimulation, as previously observed for the patient's cells transfected with

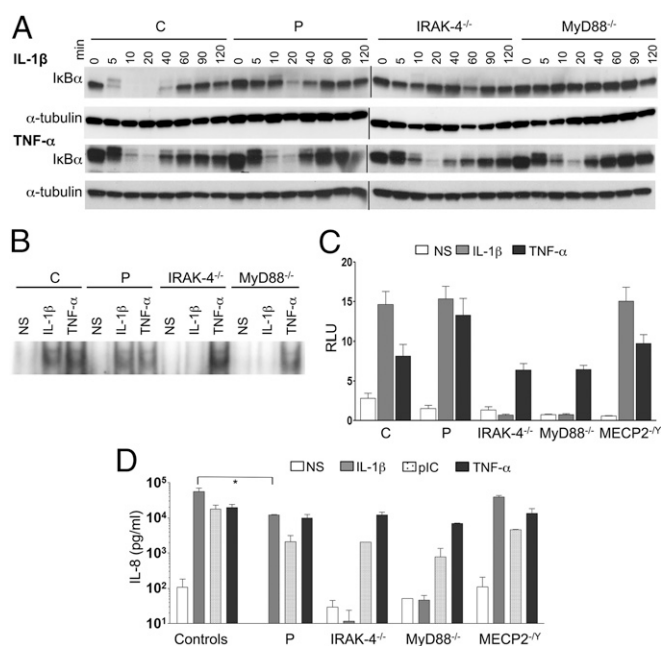


Fig. 5. IL-1R-dependent signaling in the patient's fibroblasts. (A) I κ B- α protein degradation in SV40-fibroblast lines from a healthy control, the IRAK-1-deficient patient, and IRAK-4- and MyD88-deficient patients, left unstimulated or stimulated with IL-1 β (10 ng/mL) or TNF- α (20 ng/mL) for various periods of time (min), analyzed by Western blotting. Similar results were obtained in three independent experiments. (B) NF- κ B translocation, assessed by EMSA, in SV40-fibroblasts from a healthy control, the IRAK-1-deficient patient, and IRAK-4- and MyD88-deficient patients, following stimulation with IL-1 β (10 ng/mL) and TNF- α (20 ng/mL) for 20 min. Similar results were obtained in three independent experiments. (C) NF- κ B-dependent transcription was assessed with the NF- κ B reporter luciferase assay in SV40-fibroblasts derived from a healthy control, the IRAK-1-deficient patient, and IRAK-4-, MyD88-, and MECP2-deficient patients. Cells were transiently transfected with *Renilla* luciferase- and NF- κ B luciferase-encoding vectors; 24 h after transfection, the cells were left untreated or were stimulated with IL-1 β (10 ng/mL) or TNF- α (20 ng/mL) for 42 h and then harvested. Reporter gene activities were measured and the values obtained were normalized for transfection efficiency on the basis of *Renilla* luciferase expression. The values shown (means \pm SEM) were obtained in three independent experiments. (D) IL-8 secretion by SV40-fibroblasts from healthy controls ($n = 3$), the IRAK-1-deficient patient, and IRAK-4-, MyD88-, and MECP2-deficient patients, left unstimulated or stimulated with IL-1 β (10 ng/mL), polyIC (25 μ g/mL), and TNF- α (20 ng/mL) as a positive control. The values shown (means \pm SEM) were obtained in three independent experiments. * $P < 0.05$.

si-*IRAK2* (SI Appendix, Fig. S10C). However, the levels of *IL8* mRNA produced in response to stimulation with PAM-2, LPS, or MPLA after the silencing of both *IRAK1* and *IRAK2* were not significantly lower than those observed after the silencing of *IRAK1* alone, in control and MECP2-deficient SV40-fibroblasts (SI Appendix, Fig. S10D). These results strongly suggest that, in the absence of IRAK-1, its IRAK-2 partner can compensate, at least partially, for some of the signaling downstream from IL-1R but not for signaling downstream from TLR in human fibroblasts.

Abolished Response to TLR7 and TLR8 in the Patient's EBV-B Cells but Normal Responses to All TLR Agonists Tested and to IL-1 β in the Patient's Peripheral Blood Mononuclear Cells. We then evaluated the response of EBV-transformed B lymphocytes (EBV-B cells) from the patient, which were also found to have no IRAK-1 mRNA or protein (Fig. 2A and C), to a TLR7 and TLR8 agonist (R848). Like IRAK-4- and MyD88-deficient cells, these cells produced no TNF- α , whereas all three types of mutant cells responded normally to PDBu (phorbol 12,13-dibutyrate) (Fig.

7A). We then compared the cytokine production of the patient's peripheral blood mononuclear cells (PBMCs) (which did not produce *IRAK1* mRNA, and in which it was not possible to evaluate IRAK-1 protein levels) with that of PBMCs from three healthy controls and two IRAK-4-deficient patients, after 36 h of stimulation with TLR1/2 (PAM3CSK₄), TLR2/6, TLR4, TLR7, and TLR8 agonists, heat-killed *S. aureus* (HK-SA), live *Mycobacterium bovis* Bacillus Calmette-Guérin (bacillus Calmette-Guérin), and rhabdoviruses (VSV; vesicular stomatitis virus infection), and IL-1 β . Levels of IL-1 β , IL-6, IL-8, IL-10, IFN- γ , G-CSF, GM-CSF, MCP-1, MIP-1 β , and TNF- α production by the patient's PBMCs were similar to those in control PBMCs in response to IL-1 β and all TLR agonists tested (Fig. 7B and C). By contrast, cytokine production was either strongly impaired or totally abolished in IRAK-4-deficient PBMCs, with the exception of MCP-1 and MIP-1 β production in response to stimulation with LPS and MPLA, which was within the control range, probably reflecting use of the IRAK-4-independent TLR4 signaling pathway. Cytokine production by PBMCs from the patient and IRAK-4-deficient patients was similar to that in healthy controls in response to stimulation with TNF- α or phorbol 12-myristate 13-acetate (PMA)/ionomycin (Fig. 7B and C). We tested the hypothesis that IRAK-2 might compensate for IRAK-1 deficiency in the patient's PBMCs, by knocking down *IRAK1*, *IRAK2*, or both with siRNA in PBMCs from healthy controls (SI Appendix, Fig. S11A). The single-gene knockdowns (*IRAK1* or *IRAK2*) did not significantly affect *IL8* mRNA production in response to any of the TLR/IL-1R agonists tested. By contrast, the silencing of both *IRAK1* and *IRAK2* resulted in significantly lower levels of *IL8* mRNA in response to all TLR/IL-1R agonists (SI Appendix, Fig. S11B). Transfection with scrambled siRNA had no effect on *IL8* mRNA levels in any of the conditions tested (SI Appendix, Fig. S11B). The limited numbers of PBMCs available from the patient precluded further functional analyses of the leukocyte subsets. These results nevertheless strongly suggest that IRAK-1 plays a redundant role in the myeloid and lymphoid subsets of human blood mononuclear cells, contrary to the findings for EBV-B cells and fibroblasts. These results also suggest that, in the absence of IRAK-1, its IRAK-2 partner can compensate for some of the signaling downstream from TLR/IL-1R in human leukocytes.

Discussion

We describe here a male infant with a large X-chromosome deletion encompassing the gene encoding IRAK-1. This 100-kb de novo Xq28 deletion encompassed all or part of the *TMEM187*, *MIR3202-1*, *MIR3202-2*, *IRAK1*, *MIR718*, and *MECP2* genes. The absence of the *MECP2* gene was consistent with the patient's clinical phenotype, as congenital encephalopathy, hypotonia, and respiratory insufficiency are commonly reported in boys with neonatal encephalopathy due to MECP2 deficiency (78–91). The lack of *TMEM187*, *MIR3202-1*, *MIR3202-2*, and *MIR718* was not associated with any overt phenotype at the time of the patient's death at the age of 7 mo. It was not possible to attribute any particular clinical phenotype to the lack of IRAK-1, as severe respiratory failure and pulmonary infections are commonly seen in male patients with isolated MECP2 deficiency (83, 88, 90). However, this child presented only a slight, transient increase in temperature (37.8 °C) during a bout of bilateral pneumonia, and his serum CRP concentration remained below 2 mg/L. None of the previously described MECP2-deficient male patients were reported to have impaired inflammatory responses during infections (83, 88, 90). Laboratory/biochemical tests reportedly gave normal results for these patients. Body temperature was provided for only one child presenting with severe neonatal encephalopathy, recurrent respiratory infections, and dysfunctions who died of respiratory infection in a context of high fever (88). CRP levels were not reported for any of these patients. By

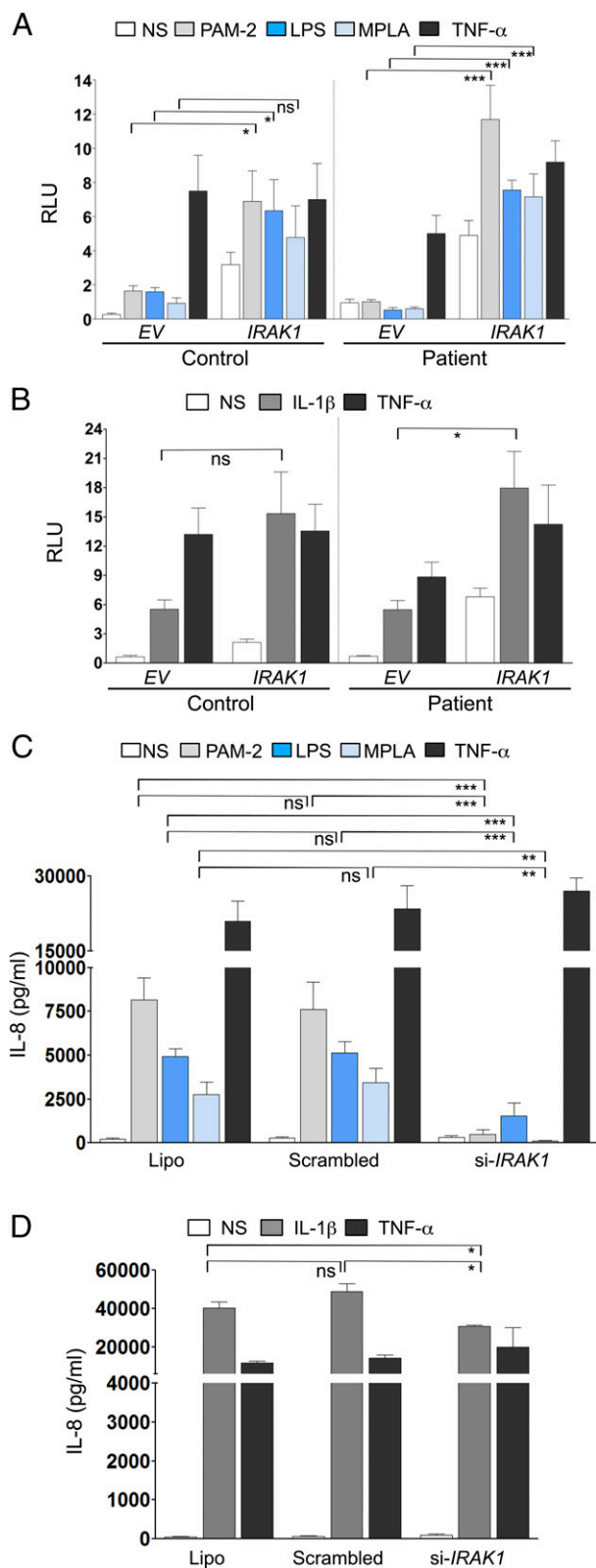


Fig. 6. Causal relationship between complete IRAK-1 deficiency and the phenotype of the patient's fibroblasts. (*A* and *B*) SV40-fibroblasts derived from a healthy control and from the IRAK-1-deficient patient were transiently transfected with vectors encoding *Renilla* luciferase or NF- κ B luciferase, pcDNA3.1-empty (empty vector; EV), or pcDNA3.1-IRAK1 WT (IRAK1). Twenty-four hours after transfection, SV40-fibroblasts were left untreated or were stimulated with PAM-2 (10 μ g/mL), LPS (10 μ g/mL), and MPLA (1 μ g/mL) (*A*) or with IL-1 β (10 ng/mL) (*B*), and TNF- α (20 ng/mL) as a positive control,

contrast, poor inflammatory responses during infectious episodes are characteristic of IRAK-4- and MyD88-deficient patients (43). Unlike the patient described here, who suffered from only mild infections such as conjunctivitis, urinary infection, and rhinovirus infections, responding well to standard treatments, IRAK-4- and MyD88-deficient patients typically present with recurrent, invasive pyogenic infections, which are reported in 46% of these patients (proband 49%; relatives 42%) before the age of 7 mo (43, 44, 52, 55). Our findings thus suggest that the IRAK-1-dependent TLR and IL-1R pathway is more redundant in host defense than that controlled by IRAK-4 and MyD88. However, as only one patient with complete IRAK-1 deficiency has been identified, it would be premature to draw firm conclusions about the role of human IRAK-1 in protective immunity.

Our study is more informative concerning the cellular consequences of IRAK-1 deficiency. The criteria for genetic studies in a single patient are fulfilled (103), with a causal relationship between the *IRAK1* genotype and the TLR/IL-1R cellular phenotype. The patient's SV40-fibroblasts responded very poorly to TLR2/6 and TLR4 agonists but, surprisingly, responded normally to IL-1 β . Neither IRAK-4- nor MyD88-deficient fibroblasts responded to any of the TLR agonists tested (except for TLR3) or IL-1 β . Like IRAK-4- and MyD88-deficient cells (52), the patient's EBV-B cells did not respond to TLR7 and TLR8 agonists. By contrast, the patient's PBMCs responded normally to all TLR (TLR1/2, TLR2/6, TLR4, TLR7, and TLR8) agonists tested and to IL-1 β (in terms of the production of many cytokines), whereas neither IRAK-4- nor MyD88-deficient PBMCs responded to any of these TLR (except for some residual TLR4 signaling, presumably via TRIF) and IL-1R (*SI Appendix, Table S1*) agonists. By contrast, *Irak1*-deficient MEFs displayed strong but incomplete impairment of the response to both TLRs (TLR2 and TLR4) (23) and IL-1Rs (5, 23, 36). This result is at odds with the almost complete abolition of responses to TLR2/6 and TLR4 agonists, and the preserved response to IL-1 β observed in human IRAK-1-deficient fibroblasts. Upon TLR4 stimulation, the production of IL-10 mRNA and protein was strongly impaired in splenocytes from *Irak1*-deficient mice, but these cells were able to produce IL-1 β and TNF- α at levels similar to those in WT splenocytes (31). After the stimulation of TLR4, TLR1/2, TLR2/6, TLR7, TLR8, or IL-1R, the levels of IL-10, IL-1 β , and TNF- α production by the patient's PBMCs were similar to those of control PBMCs. We were unable to test leukocyte subsets or responses to agonists of other members of the TLR (e.g., TLR5 and TLR9) and IL-1R (e.g., IL-18R and IL-33R) families. Overall, our results, somewhat at odds with the data obtained for mutant mice (5, 23, 36) (*SI Appendix, Table S2*), suggest that human IRAK-1 is largely redundant downstream from both TLRs and IL-1R in mononuclear blood cells and downstream from IL-1R in fibroblasts, whereas it plays an essential role, at least downstream from TLR2/6, TLR4, TLR7, and TLR8, in fibroblasts and EBV-B cells.

Our study also provides new insight into the contributions of IRAK-1 and IRAK-2 in the signaling pathways downstream from TLRs and IL-1R (Fig. 8). In vitro studies have shown that IRAK-1 plays an important role downstream from IL-1R, as an adaptor

for 42 h and then harvested. Reporter gene activities were measured and the values were normalized for transfection efficiency on the basis of *Renilla* luciferase expression. (*C* and *D*) IL-8 secretion by SV40-fibroblasts from a healthy control transfected with siRNAs targeting *IRAK1* or with a nonsense siRNA (scrambled) for 48 h, and then left unstimulated or stimulated with PAM-2 (10 μ g/mL), LPS (10 μ g/mL), and MPLA (1 μ g/mL) (*C*) or with IL-1 β (10 ng/mL) (*D*), and TNF- α (20 ng/mL) as a positive control, for 18 h. The values shown (means \pm SEM) were obtained in three independent experiments. ns, not significant; *** P < 0.001, ** P < 0.01, and * P < 0.05.

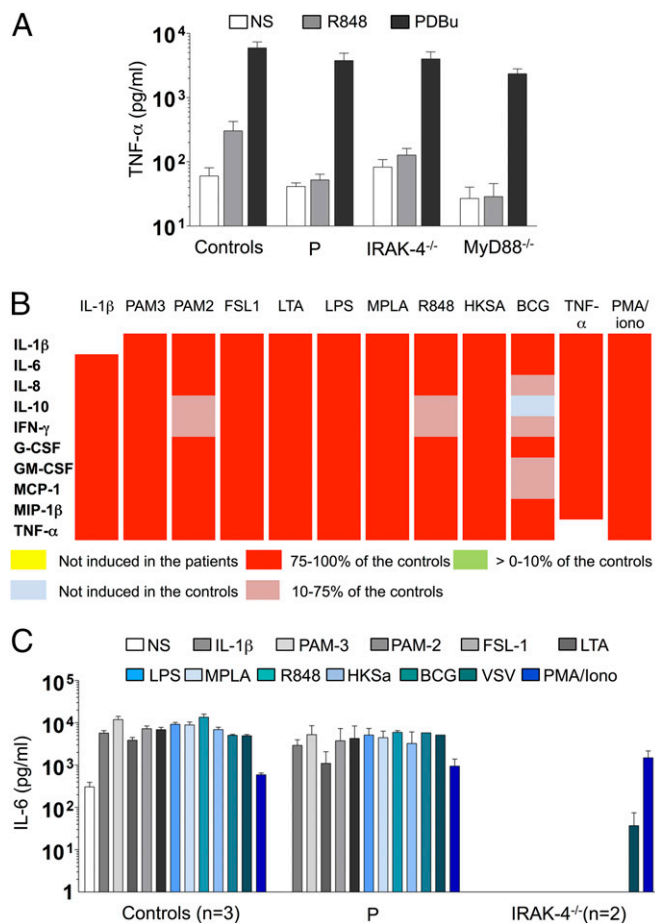


Fig. 7. TIR-dependent signaling in the patient's leukocytes. (A) TNF- α secretion by EBV-transformed B cells from controls ($n = 4$), the IRAK-1-deficient patient, and IRAK-4^{-/-} and MyD88-deficient patients, left unstimulated or stimulated with R848 (3 μ g/mL) and PDBu (10^{-7} M) as a positive control. The values shown (means \pm SEM) were obtained in three independent experiments. (B and C) PBMCs from healthy controls ($n = 3$), the IRAK-1-deficient patient, and two IRAK-4-deficient patients were left unstimulated or stimulated with IL-1 β (20 ng/mL), PAM-3 (1 μ g/mL), PAM-2 (1 μ g/mL), FSL-1 (100 ng/mL), LTA (1 μ g/mL), LPS (10 ng/mL), MPLA (1 μ g/mL), R848 (1 μ g/mL), heat-killed *S. aureus* (10^7 HKSA per mL), bacillus of Calmette et Guérin, and, as positive controls, TNF- α (20 ng/mL) and PMA/Ionomycin (10^{-7} M/ 10^{-5} M). The production of multiple cytokines in IRAK-1-deficient PBMCs was evaluated by multiplex ELISA. Cytokine levels are represented as ratios of the response observed in the patient to mean levels in controls. For none of the stimulations tested, the patient showed either no induction or at 0–10% of the controls. (B). IL-6 production, as determined by ELISA. (C). The values shown (means \pm SEM) were obtained in two independent experiments.

protein crucial for Myddosome formation and the activation of downstream signaling (16, 104–106). We show that the patient's fibroblasts were able to respond to IL-1 β stimulation almost

normally, like human fibroblasts from healthy controls silenced for *IRAK1* (or *IRAK2*). However, the IL-1 response of fibroblasts from a healthy donor was strongly impaired when both *IRAK1* and *IRAK2* were silenced. These data suggest that IRAK-1 and IRAK-2 play mutually redundant roles, at least downstream from IL-1R, in human fibroblasts. Mouse studies have shown that IRAK-2 is crucial for cytokine production by bone marrow-derived macrophages in response to TLR4 stimulation (17, 39). Similarly, IRAK-2 has been shown to be essential for the LPS-induced response of human HEK-293 cells and human PBMCs (107). Our findings suggest that IRAK-2 is dispensable for TLR-mediated signaling in human fibroblasts, because *IRAK2* silencing does not impair the production of IL-8 mRNA or protein in response to PAM-2 or LPS, whereas *IRAK1* silencing greatly decreases this response. We further suggest that IRAK-2 drives the preserved TLR/IL-1R-dependent signaling displayed by IRAK-1-deficient PBMCs, as the silencing of both *IRAK1* and *IRAK2* in control PBMCs impaired TLR/IL-1R responses, at least in terms of *IL8* mRNA induction. Overall, our findings suggest that signaling downstream from TLRs and IL-1R in human fibroblasts diverges at the IRAK proteins: IRAK-1 is essential downstream from TLRs, whereas signaling downstream from IL-1R can be mediated by IRAK-1, IRAK-2, or both. In leukocytes, the TLR/IL-1R pathway also seems to be mediated by IRAK-1, IRAK-2, or both. The identification of humans with mutations affecting other components of the TIR pathway should clarify the relative contributions of each human IRAK protein to host defense. These findings are important, given current efforts to develop inhibitors of IRAK-1 and IRAK-4 for the treatment of various human diseases (108, 109).

Methods

cDNA Preparation and Real-Time Quantitative PCR. Total RNA was extracted from EBV-B cells and SV40-fibroblasts with the Qiagen RNA Mini Kit, according to the manufacturer's instructions. cDNA was synthesized with SuperScript II reverse transcriptase (Life Technologies). Real-time quantitative PCR (qPCR) was performed with TaqMan Gene Expression Assays (Life Technologies; Hs 01018347_m1), according to the manufacturer's instructions. mRNA levels are expressed relative to GUS mRNA levels, as determined by the $2^{-\Delta\Delta C_t}$ method.

Western Blots and EMSA. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, and 2 mM EDTA supplemented with protease inhibitors and phosphatase inhibitor mixture (Roche). Western blotting was performed with polyclonal anti-IRAK-1 (sc-7883) and anti-GAPDH (sc-25778) antibodies (Santa Cruz Biotechnology). The degradation of κ B- α upon stimulation with IL-1 β (10 ng/mL; R&D Systems) or TNF- α (20 ng/mL; R&D Systems) was evaluated with an anti- κ B- α polyclonal antibody (sc-371), with an antibody against α -tubulin (sc-5286) as a loading control (Santa Cruz Biotechnology). For EMSA, SV40-fibroblasts were stimulated for 20 min with IL-1 β (10 ng/mL; R&D Systems) or TNF- α (20 ng/mL; R&D Systems). Nuclear extracts were then prepared and incubated (10 μ g protein) with a 32 P-labeled double-stranded NF- κ B-specific oligonucleotide κ B probe (5'-GATCATGGGGAATCCCA-3' and 5'-GATCTGGGATCCCAAT-3').

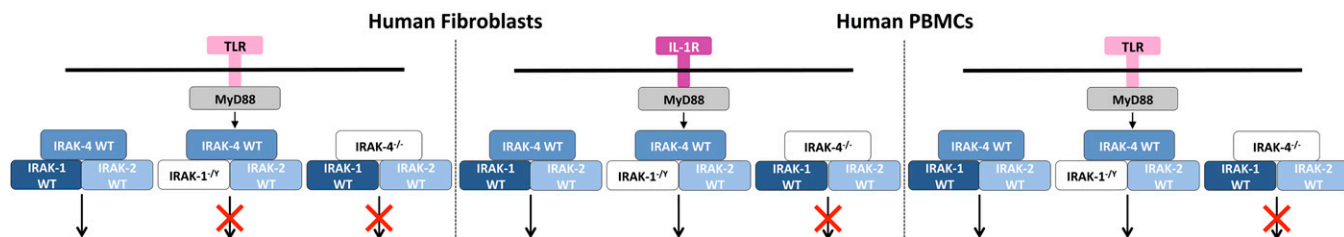


Fig. 8. Suggested model for signaling downstream from TLR or IL-1R in human fibroblasts and PBMCs, in the presence and absence of IRAK-1 and IRAK-4.

Cell Stimulation, Cytokine Determinations, and Luciferase Assays. IL-8 and IL-6 levels were assessed in SV40-fibroblasts incubated for 18 h in the presence of 10 ng/mL IL-1 β (R&D Systems), 20 ng/mL TNF- α (R&D Systems), 10 μ g/mL PAM-2 (a TLR2/6 agonist), 1 μ g/mL FSL-1 (a TLR2/6 agonist), 10 μ g/mL LTA-SA (a TLR2 agonist), 1 μ g/mL MPLA (a TLR4 agonist) (all from InvivoGen), 10 μ g/mL LPS-SE (a TLR4 agonist; Sigma-Aldrich), or 25 μ g/mL poly(I:C) (GE Healthcare). TNF- α levels were assessed in EBV-B cells incubated for 24 h in the presence of 3 μ g/mL R848 (imidazoquinoline compound, a TLR7/8 agonist; InvivoGen) or PDBu (a protein kinase C agonist; Sigma-Aldrich). IL-6 levels were assessed in frozen and thawed PBMCs incubated for 36 h in the presence of 20 ng/mL IL-1 β (R&D Systems), 20 ng/mL TNF- α (R&D Systems), 1 μ g/mL PAM₃CSK₄ (PAM-3; a TLR1/2 agonist), 1 μ g/mL PAM₂CSK₄ (PAM-2; a TLR2/6 agonist), 100 ng/mL FSL-1 (a TLR2/6 agonist), 1 μ g/mL pLTA-SA (a TLR2 agonist), imidazoquinoline (R848; a TLR7/8 agonist), 10⁷ heat-killed *S. aureus* (HKSA) per mL (mainly a TLR2 agonist) (all from InvivoGen), lipopolysaccharides (rough strains) from *Salmonella enterica* serotype minnesota Re 595 (LPS; Sigma), live bacillus Calmette-Guérin, and VSV (multiplicity of infection 0.5) or PMA/ionomycin (Sigma-Aldrich; positive control; 10⁻⁷ M/10⁻⁵ M). Supernatants were collected and ELISA (Sanquin) was performed in accordance with kit instructions. For the simultaneous determination of multiple cytokines in PBMC supernatants, we used a fluorescence-based assay capable of detecting 17 cytokines (Bio-Plex Pro Human Cytokine 17-plex Assay M5000031YV; Bio-Rad). NF- κ B luciferase activity was assessed by transiently transfecting SV40-fibroblasts with 100 ng of NF- κ B-dependent firefly luciferase vector with five NF- κ B binding sites in the promoter (Agilent Technologies) and 40 ng of *Renilla* luciferase vector (*pRL-SV40-d238*) (110) as an internal control with the Lipofectamine LTX Kit (Life Technologies; 15338100), according to the manufacturer's instructions. Fibroblasts were then stimulated for 24 h with IL-1 β , PAM-2, LPS, MPLA, or TNF- α , as described above. The cells were lysed in passive lysis buffer, and luciferase activities were measured in the Dual-Luciferase Reporter Assay (Promega).

Complementation and Silencing. For the complementation experiments, IRAK-1^{-/-}, IRAK-4^{-/-}, MyD88^{-/-}, MeCP2^{-/-}, and healthy control SV40-fibroblasts were transiently transfected by incubation for 24 h with 200 ng pcDNA3.1

empty vector or pcDNA3.1-*IRAK1* WT (Invitrogen). The cells were then stimulated with various ligands for 24 h, and luciferase activity or cytokine production was assessed as described above. For the silencing of IRAK-1, IRAK-2, or both, SV40-fibroblasts were transiently transfected with 50 nM siRNA targeting *IRAK1* or *IRAK2* or a mixture of siRNAs targeting *IRAK1* and *IRAK2* (50 nM final concentration) or scrambled siRNA (50 nM) with Lipofectamine RNAiMAX (Life Technologies; 13778030), according to the manufacturer's instructions. Fibroblasts were then stimulated for 4 h for the determination of *IL8*, *IRAK1*, *IRAK2*, and *IRAK4* mRNA levels by RT-qPCR, or for 18 h for the determination of IRAK-1 protein levels by Western blotting and IL-8 protein levels by ELISA. RNA was extracted with the ZR RNA MicroPrep Kit (ZR1061; Zymo Research), according to the manufacturer's instructions.

Statistics. For single comparisons of independent groups, *t* tests were performed; ****P* < 0.001, ***P* < 0.01, and **P* < 0.05. Analyses were performed with GraphPad software.

Study Approval. The study was approved by the ethics committee of IRCCS San Matteo Hospital Foundation, Pavia (ID-PDS 20140000324); informed consent was obtained from the patient's parents, in accordance with the World Medical Association guidelines, Helsinki Declaration, and EU directives.

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