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IRAK-4 Mutation (Q293X): Rapid Detection and Characterization of Defective Post-Transcriptional TLR/IL-1R Responses in Human Myeloid and Non-Myeloid Cells[1]

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Innate immunodeficiency has recently been reported as resulting from the Q293X *IRAK-4* mutation with consequent defective TLR/IL-1R signaling. In this study we report a method for the rapid allele-specific detection of this mutation and demonstrate both cell type specificity and ligand specificity in defective IL-1R-associated kinase (IRAK)-4-deficient cellular responses, indicating differential roles for this protein in human PBMCs and primary dermal fibroblasts and in LPS, IL-1β, and TNF-α signaling. We demonstrate transcriptional and post-transcriptional defects despite NF-κB signaling and intact MyD88-independent signaling and propose that dysfunctional complex 1 (IRAK1/TRAF6/TAK1) signaling, as a consequence of IRAK-4 deficiency, generates specific defects in MAPK activation that could underpin this patient's innate immunodeficiency. These studies demonstrate the importance of studying primary human cells bearing a clinically relevant mutation; they underscore the complexity of innate immune signaling and illuminate novel roles for IRAK-4 and the fundamental importance of accessory proinflammatory signaling to normal human innate immune responses and immunodeficiencies. ***The Journal of Immunology,*** **2006, 177: 8202–8211.**

Toll-like receptors are innate pattern recognition receptors that initiate distinct inflammatory responses to specific microbial components (1). Upon stimulation, TLR and IL-1R family members induce inflammatory signaling cascades via cytoplasmic Toll/IL-1R domains by using adaptor proteins such as MyD88, TIRAP, TRIF, and TRAM (2). Classically, MyD88 recruits IL-1R-associated kinase (IRAK)[5]-1 and IRAK-4 through death domain (DD) interactions, with subsequent IRAK-4-dependent phosphorylation of IRAK-1. Phosphorylated IRAK-1 then associates with TNFR-associated factor (TRAF)-6, initiating TGF-β-activated kinase (TAK)-1-dependent signaling that ultimately activates the I-κB kinase (IKK) complex and enables the nuclear translocation of NF-κB and the induction of specific inflammatory gene transcription.

Activation of TAK-1 also initiates the less-well characterized accessory proinflammatory signaling through the phosphorylation and activation of MAPK (3, 4). These MAPKs can modulate multiple transcriptional and post-transcriptional mechanisms (5–8). MAPKs therefore represent a potentially critical level of control of inflammatory responses and have the potential to provide stimulus specificity to the responses. However, the upstream controlling elements for these pathways and their full significance in the orchestration of an appropriately targeted immune response are not fully understood. Although IRAK-4 is proposed to be critical for canonical MyD88-dependent cytokine production (9), its role in regulating MAPK-dependent responses remains unclear.

IRAK-4 deficiency has recently been described as a rare form of innate immunodeficiency; patients present with pyogenic bacterial infections and bacteraemia, particularly with *Streptococcus pneumoniae* (10–14). We have previously described a patient presenting with recurrent *S. pneumoniae* bacteraemia and a clinical phenotype strikingly similar to that reported in IRAK-4 deficiency (15). We now report a homozygous Q293X mutation in *IRAK-4* in this patient and provide the first definitive report of the lethality of this mutation, which occurred in his sibling. Furthermore, by studying IRAK-4-deficient primary dermal fibroblasts and PBMCs we demonstrate cell type-specific and ligand-specific defective cytokine responses that are dysfunctional at transcriptional and/or post-transcriptional levels despite NF-κB signaling and intact MyD88-independent signaling.

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[5] Abbreviations used in this paper: IRAK, IL-1R-associated kinase; DD, death domain; hnRNA, heteronuclear RNA; IKK, I-κB kinase; MDDC, monocyte-derived dendritic cell; MDM, monocyte-derived macrophage; MKK, MAPK kinase; poly(I:C), polyinosinic acid/polycytidylic acid; TAK, TGF-β-activated kinase; TRAF, TNFR-associated factor.

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Materials and Methods

History and consent

The clinical and immunological descriptions of the patient studied and his deceased brother have been previously reported (15). Blood was collected from healthy adult volunteers and the patient's immediate family with informed consent (University of British Columbia Ethics Protocol C02-009). Blood samples from the patient and five healthy 2-year olds undergoing elective surgery were collected with parental consent according to University of British Columbia Ethics Protocol C02-0138.

Media and reagents

All tissue culture reagents were from Invitrogen Life Technologies unless stated. Human rIL-1β and GM-CSF were from Research Diagnostics, recombinant human M-CSF and TNF-α were from R&D Systems, polyinosinic acid/polycytidylic acid (poly(I:C)) was from Sigma-Aldrich, and CpG2006 was from Coley Pharmaceutical Group. All reagents were tested for endotoxin by *Limulus* amebocyte lysate assay. *Escherichia coli* LPS from Sigma was repurified by phenol-chloroform extraction (16) and titrated against polymyxin B or purchased as TLR4-specific Ultra-Pure LPS (InvivoGen). IRAK-4 Abs were a gift from Tularik (17) or purchased from Upstate Cell Signaling Solutions and Cell Signaling Technology. Additional Abs were polyclonal rabbit anti-IRAK-1 and anti-NF-κB p65 (Santa Cruz Biotechnology), mouse monoclonal anti-FLAG (Sigma-Aldrich), mouse anti-human CD80, CD86, and CD40 (BD Biosciences ON, Canada), or polyclonal rabbit Abs from Cell Signaling Technology.

Peripheral blood leukocytes.

PBMCs were isolated by Ficoll-Paque Plus (Amersham Biosciences) density gradient centrifugation. Monocyte-derived dendritic cells (MDDCs) were cultured as described (18). Neutrophils were isolated as described (19).

Culture of primary and immortalized fibroblasts

Primary dermal fibroblast lines were prepared from skin biopsies of the patient and a fully anonymized age-matched child (undergoing an unrelated diagnostic test). Adult human dermal fibroblasts were from Cascade Biologics. No difference was observed in inflammatory responses between adult and control child cells, so adult cells were routinely used thenceforth. Immortalized cell lines were also generated from these primary cell lines by transfection with PSV3-Neo (American Type Tissue Collection) using a FuGENE 6 transfection reagent (Roche Diagnostics).

Fibroblast transfection

Immortalized fibroblasts were cultured in DMEM with 10% FCS until ~60% confluency and transfected with pFLAGRK7-IRAK-4 expression vector (a gift from Tularik) (17) or control empty vector using FuGENE 6 transfection reagent (Roche Diagnostics) 48 h before exposure to inflammatory stimuli.

Real-time IRAK-4 genotyping

Primers and probes were designed using Primer Express software version 2.0. Probes (FAM-ACCCTGAGCAATC-MGBNFQ and VIC-CACCCT AAGCAATC-MGBNFQ) were purchased from Applied Biosystems, and primers (5′-ACC ACT TTC TTG GCA CAT GAG A-3′ and 5′-CAT GTA GAA AAT TGA TGC CAT TAG CT-3′) were synthesized by the University of British Columbia Nucleic Acid Protein Service Unit. The resulting PCR products were sequenced to confirm validity. Triplicate reactions and an allelic discrimination analysis were performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems) on genomic DNA templates from the patient, his immediate family, 60 fully anonymized mixed archived adult and child samples, and template-free controls.

DNA Sequencing

Genomic DNA from PBMCs, cell lines, or from a formalin-fixed, paraffin-embedded, archived diagnostic tissue specimen was prepared using DNeasy Tissue Kits (Qiagen). Each coding exon of *IRAK-4* was amplified by PCR using exon-spanning intronic primers (University of British Columbia Nucleic Acid Protein Service Unit; sequences upon request) and the products were purified using QiaQuick PCR purification kits (Qiagen) and sequenced on an ABI 3100 using Big Dye terminators (Applied Biosystems). Sequences from repeated PCR, aligned into contigs, were analyzed using Phred, Phrap, and Consed (version 11.0) and compared against the *IRAK-4* gene (GenBank sequence accession number AF445802).

Cell stimulation

All cells were cultured at 37°C in 5% CO_2. Fibroblasts were seeded at 2×10^4/ml in α-MEM with 10% FCS for 48 h before RNA extraction and analysis of supernatant cytokines. Fibroblasts for protein extraction were cultured to confluence before use. PBMCs were cultured in R10 medium (RPMI 1640 medium, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% FCS) for 1 h before stimulation at 2×10^5/ml for the analysis of supernatant cytokines and $1–5 \times 10^6$/ml for the extraction of RNA or protein. Cells were exposed in triplicate to 100 ng/ml *E. coli* LPS, TNF-α, and IL-1β or left unstimulated and incubated for 24 h for supernatant cytokine analysis or the time specified for other analyses. MDDCs, cultured at 1×10^5 cells/ml R10 in Teflon 24-well inserts (Savillex), were exposed in triplicate for 24 h to either 100 ng/ml *E. coli* LPS, 10 μg/ml CpG 2006, 10 μg/ml poly(I:C), or incubated with confluent monolayers of either untransfected or CD40L-transfected 3T3 fibroblasts (provided by Dr. G. Reid, University of British Columbia, Vancouver, Canada).

Culture supernatants were spun down, aliquoted, and stored at −80°C. IL-6 and IL-8 concentrations were determined in triplicate from each biological replicate using ELISA Ready-SET-go (eBioscience) and OptEIA ELISA kits (BD Pharmingen), respectively. MDDCs were analyzed by FACS as previously described (18).

Transcriptional analysis

The transcription of *IL-6* and *IL-8* mRNA was assessed by semiquantitative RT-PCR. In addition, transcription was directly monitored by analysis of the levels of partially spliced heteronuclear RNA (hnRNA) transcripts by PCR, a method reported to have greater sensitivity than nuclear runoff transcription assays (20). Total RNA was isolated using RNeasy Midi kits (Qiagen), treated to remove DNA (DNA-free; Ambion), and eluted in RNase-free water (Ambion). Concentration-corrected RNA samples were divided into two portions for reverse-transcription of cDNA representing either mRNA (using oligo(dT) as primer) or total RNA, including hnRNA (using random hexamers as primers), using SuperScript II (Invitrogen Life Technologies). PCR was performed using intron-spanning primer pairs for mRNA (exonic PCR), or primer pairs specific for an exon and an intron of the unspliced hnRNA (intronic PCR). PCR with a primer pair specific to genomic promoter regions were also performed to exclude genomic DNA contamination. All primer sequences are available on request. Each RT-PCR was performed in at least duplicate ($n \geq 3$ independent experiments) and analyzed by densitometry in the linear phase of amplification by using analysis of amplification reactions with incrementally increasing cycle numbers and normalized to the control gene *GAPDH*.

Western immunoblotting

Protein preparation and Western blotting was performed as previously described (21) or with use of Complete EDTA-free protease inhibitor mixture tablets (Roche Diagnostics); lysate concentrations were quantified using a Micro BCA protein assay kit (Pierce) with lysate volumes corrected accordingly to ensure equal loading, and Immunoblot PVDF membranes were used (Bio-Rad, CA). Nuclear extracts were generated using NE-PER nuclear and cytoplasmic extraction reagents (Pierce).

Statistical analysis

Figures show mean values ± SEM. Student's *t* test was used to compare patient and healthy control responses. The value $p < 0.05$ was considered to indicate statistical significance.

Results

IRAK-4 *mutation and diagnosis*

Initial characterization in this patient implicated a defect downstream of TLR/IL1-R activation (15). Allelic discrimination real-time PCR clearly demonstrated homozygous substitution of thymidine for cytidine at position 877 in exon 8 of *IRAK-4* in the patient, hemizygous C877T substitution in two brothers (siblings 2 and 3) and both parents, and no C877T substitution in 60 normal controls (Fig. 1). In addition, by using genomic DNA extracted from a diagnostic pathology specimen, a homozygous C877T substitution was demonstrated in the patient's oldest brother (sibling 1), whose medical history was also suggestive of *IRAK-4* mutation and who died at the age of 5 years with rapidly progressive pneumococcal meningitis. This C877T substitution results in a Q293X

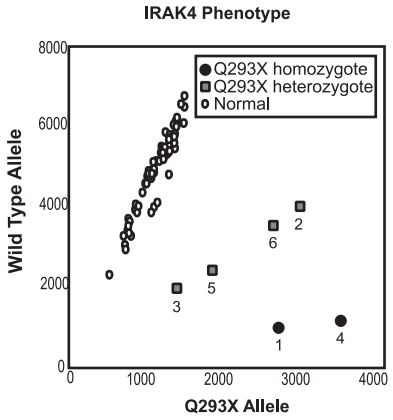

FIGURE 1. Detection of *IRAK-4* Q293X mutation. A real-time PCR allelic discrimination assay demonstrated homozygous C877T substitution in the patient (symbol 4) and deceased sibling (symbol 1), hemizygous C877T substitution in the siblings (symbols 2 and 3) and both parents (symbols 5 and 6), but no C877T substitution in 60 normal controls (○).

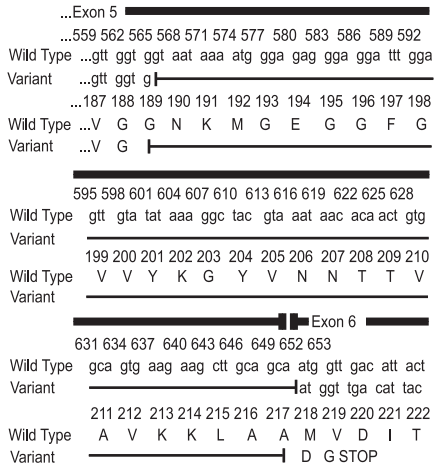

FIGURE 2. RT-PCR of *IRAK-4* revealed a splice variant. RT-PCR of partial *IRAK-4* transcripts using PBMC mRNA amplified the expected 660-bp product and a 574-bp product in both the patient and the control samples. Both products from the patient and a control were sequenced, and the 574-bp product revealed an 86-bp deletion at the end of exon-5, resulting in a premature stop codon.

mutation with the replacement of a glutamine by a designated stop codon as described (10). All mutations were confirmed by the sequencing of exon 8 PCR products, and the further sequencing of the complete *IRAK-4* gene revealed no additional exonic mutations in this patient.

The Q293X mutation in *IRAK-4* results in defective expression of full-length *IRAK-4* mRNA, as previously demonstrated by Northern blotting in patients homozygous for this mutation (10). Before sequencing the *IRAK-4* gene, we initially excluded the diagnosis of IRAK-4 deficiency in our patient based upon RT-PCR amplification of apparently normal levels of *IRAK-4* from monocyte-derived macrophage (MDM) mRNA (15). However, mRNA extracted from IRAK-4-deficient patient cells has been shown to contain sufficient partial *IRAK-4* mRNA transcripts to enable cDNA sequencing of the *IRAK-4* gene (11). This resulted in apparently normal levels of *IRAK-4* mRNA expression when assessed by quantitative and semiquantitative RT-PCR amplification (data not shown). Thus, although rapid degradation of transcript by nonsense-mediated RNA decay (22) can be observed by RT-PCR in some diseases caused by premature stop codons (23), these studies demonstrate that such approaches are inappropriate for diagnosing IRAK-4 deficiency. Interestingly, RT-PCR of *IRAK-4* exons 4–9 (spanning the mutation) amplified two products: one of the expected size, and an additional truncated product. Sequence analysis of the smaller product revealed an 86-bp deletion at the end of exon 5 (bases 566–651, inclusive) (Fig. 2). This truncated product may represent a previously unknown alternatively spliced form, because there is a potential cryptic splice donor site (GTG|GTAATA) at the 5′-end of the deletion. If translated, this deletion would result in a frameshift after aa 188, resulting in the addition of two new amino acids followed by a premature stop codon. Translation would generate a truncated protein (predicted ~21 kDa) of unknown stability or biological significance. The C877T substitution in exon 8 in the patient was observed in both splice forms.

In the absence of a diagnostically applicable RT-PCR assay, detection of the IRAK-4 protein by Western immunoblotting was evaluated from a diagnostic perspective using three different Abs specific to IRAK-4. The IRAK-4 protein (~52 kDa) could be clearly detected after transfection of immortalized control fibroblasts with pFLAGRK7-IRAK-4 (Fig. 3*a*), demonstrating the effectiveness of these Abs in cells overexpressing the IRAK-4 protein. However, this was not observed in the absence of transfection (Fig. 3*a*), indicating that under normal conditions these Abs could not detect the native IRAK-4 expressed in these cells. Although multiple bands within a similar size range were detected with these Abs, no significant differences were observed between the patient's cells and controls using whole cell lysates from untransfected immortalized fibroblasts (Fig. 3*a*), PBMCs (Fig. 3*b*), or primary fibroblasts (data not shown). These data indicate the nonspecificity of these bands and the unsuitability of Western immunoblotting with these Abs as a diagnostic approach for IRAK-4 deficiency. Detection of IRAK-4 has previously been demonstrated using whole cell lysates of immortalized lymphoblastoid B cells from healthy donors, and shown to be defective in those derived from IRAK-4-deficient patients (10), suggesting differences in IRAK-4 expression levels or the quality of the protein extracted from different cell types. However, our data are compatible with another report in which IRAK-4 protein could not be demonstrated in PBMCs from either an IRAK-deficient patient or controls, whereas IRAK-4 was detected when overexpressed in cell lines (11).

These data emphasize the importance of appropriate laboratory approaches in evaluating possible IRAK-4-deficient individuals and indicate the need for functional studies of TLR/IL-1R-induced cytokine production, diagnostic genotyping of specific mutations, and/or gene sequencing.

Cytokine responses are differentially affected in Q293X mutant primary fibroblasts and PBMCs

We next characterized the IL-6 and IL-8 responses of our patient's primary dermal fibroblasts and PBMC following LPS, IL-1β, or TNF-α stimulation. LPS induced significant production of IL-6 and IL-8 by PBMCs from healthy controls, whereas the patient's PBMCs produced no IL-6 in response to LPS and ~10-fold less IL-8, significantly differing from the controls ($p < 0.05$ and $p < 0.01$, respectively; Fig. 4). Notably, we observed expression of both CD14 and TLR4 by RT-PCR in primary dermal fibroblasts from healthy controls (Fig. 5*c*), and these cells also expressed significant levels ($p < 0.05$) of both IL-6 and IL-8 in response to highly purified LPS (Fig. 5, *a* and *b*). The patient's dermal fibroblasts expressed comparable levels of CD14 and TLR4 (Fig. 5*c*)

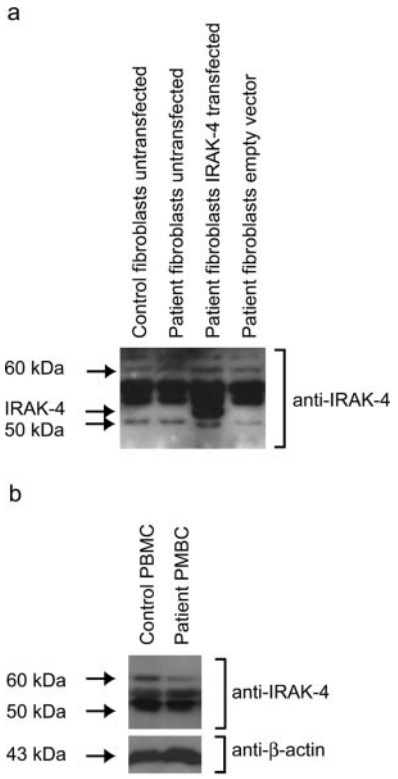

FIGURE 3. Western immunoblot analysis for IRAK-4 protein expression in fibroblasts and PBMCs does not discriminate between patient and controls. Western immunoblot analysis was performed with Abs specific for IRAK-4. Representative images are shown using Upstate Cell Signaling Solutions anti-IRAK-4 (*a*) and Tularik anti-IRAK-4 (*b*). *a*, Lysates from immortalized dermal fibroblast lines from the patient, transfected with pRK-7-IRAK-4 expression vector (but not control empty vector), expressed a ~52-kDa protein that was not detected in untransfected patient or control cells. Results are representative of $n = 2$ biological repeats for this Ab. *b*, Lysates from PBMCs demonstrated nonspecific bands, and Western immunoblots were not discriminatory for patient cells when compared with controls. Results are representative of $n = 4$ biological repeats for this Ab, with an anti-β-actin Ab used as the loading control.

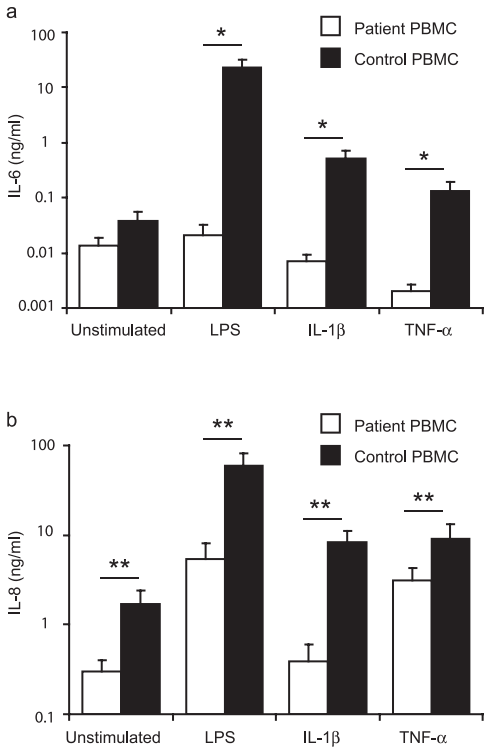

FIGURE 4. Defective cytokine responses in IRAK-4-deficient PBMCs. Primary PBMCs from the patient and controls were exposed to 100 ng/ml LPS, IL-1β, or TNF-α or were unstimulated and incubated at 37°C in 5% CO_2 for 24 h. Supernatants were examined by ELISA for IL-6 (*a*) and IL-8 (*b*). Mean (±SEM) is derived from $n \geq$ three repeats for each stimulus with $n = 3$ replicates per study. *, $p < 0.05$; ** $p < 0.01$.

but did not produce any IL-6 or IL-8 after LPS stimulation (Fig. 5, *a* and *b*).

Healthy control dermal fibroblasts (Fig. 5, *a* and *b*) and PBMCs (Fig. 4) showed substantial IL-6 and IL-8 protein responses to IL-1β and TNF-α. In contrast, neither the patient's fibroblasts (Fig. 5, *a* and *b*) nor his PBMCs (Fig. 4) showed any IL-6 or IL-8 protein expression in response to IL-1β. Although the patient's fibroblasts showed a normal IL-6 and IL-8 response to TNF-α (Fig. 5, *a* and *b*), his PBMCs did not express any significant quantities of IL-6 and ~3-fold less IL-8 than PBMCs from controls in response to this stimulus, significantly differing from the controls ($p < 0.05$ and $p < 0.01$ respectively; Fig. 4). These data demonstrate both cell-type-specific and stimulus-specific defects as a consequence of *IRAK-4* mutation.

To confirm the causative nature of the Q293X mutation in this patient's cellular phenotype, immortalized dermal fibroblasts from our patient were transfected with pFLAGRK7-IRAK-4. FLAG-tagged wild-type IRAK-4 restored IL-1β-induced expression of IL-6 (Fig. 5*d*) and IL-8 (data not shown) in keeping with previous observations (10). This provided functional confirmation of IRAK-4 deficiency and correction with wild-type IRAK-4 in our patient's cells.

Transcriptional and post-transcriptional defects in IRAK-4 mutant fibroblasts and PBMCs

In light of the defects observed in IL-6 and IL-8 production by our patient's cells and the differences between our patient's fibroblasts and PBMCs, we next examined the immediate expression of these cytokines at the mRNA level using semiquantitative RT-PCR. LPS, IL-1β, and TNF-α all induced the rapid expression (within 1 h) of *IL-6* and *IL-8* mRNA by healthy control fibroblasts and PBMCs (Figs. 6 and 7*a*). In contrast, no *IL-6* or *IL-8* mRNA expression was detected in the patient's fibroblasts in response to IL-1β. However, *IL-6* and *IL-8* mRNA expression was detected in the patient's fibroblasts in response to TNF-α and also after exposure to LPS (Fig. 6). The latter observation contrasted with the absence of LPS-induced cytokine production by patient fibroblasts (Fig. 5). PBMCs from the patient once again showed a different response to the fibroblasts, with all three stimuli inducing *IL-8* transcription (Fig. 7*a*). Although the levels of *IL-8* transcriptional responses to LPS and IL-1β in the patient PBMCs were diminished, the responses were not significantly different in comparison to controls. The induction of *IL-6* mRNA expression in the patient's PBMCs was only observed in response to TNF-α exposure and at a greatly reduced level (Fig. 7*a*).

Intronic RT-PCR analysis revealed unspliced *IL-6* hnRNA in healthy control PBMCs exposed to stimuli, in marked contrast to patient PBMCs (Fig. 7*b*). This suggested that a primary failure of transcription of *IL-6*, rather than reduced mRNA stability, underpinned the defective *IL-6* response to LPS and IL-1β and the diminished response to TNF-α. This was also the case for the defective IL-1β-induced *IL-6* expression observed in patient fibroblasts (data not shown).

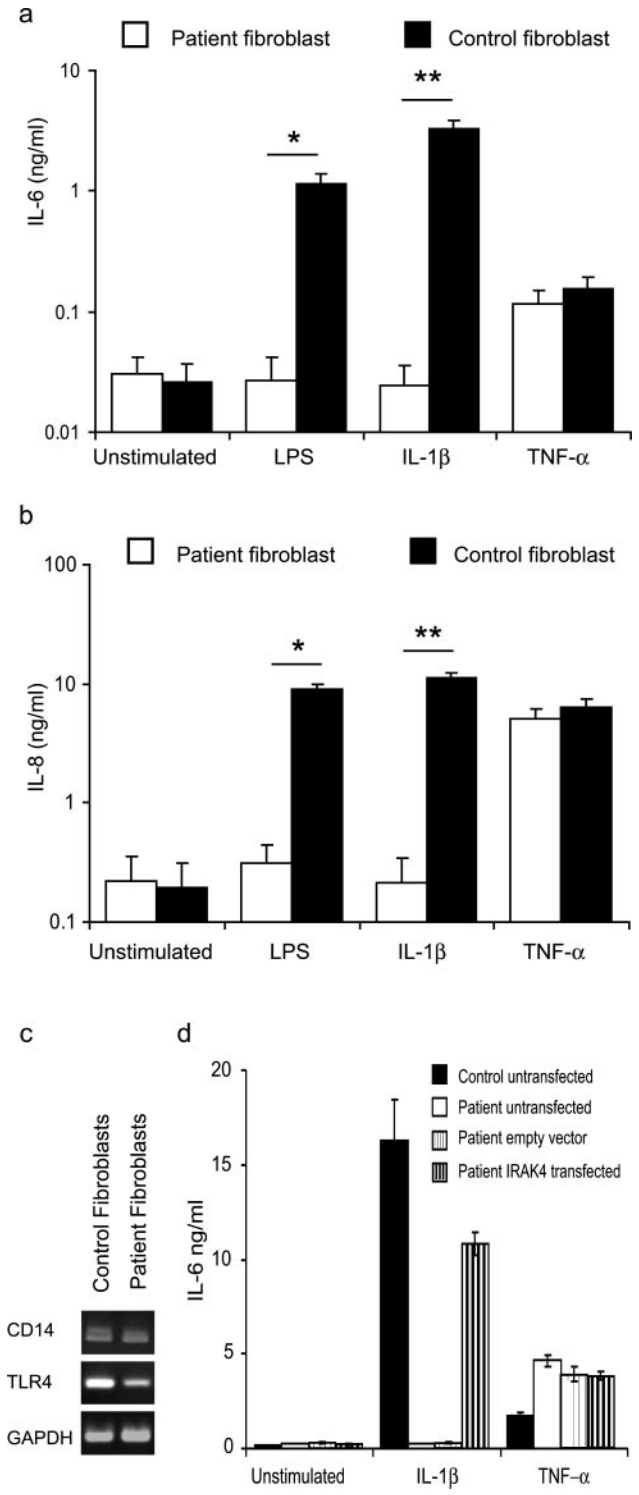

FIGURE 5. Defective cytokine responses in IRAK-4-deficient fibroblasts. *a* and *b*, Primary fibroblasts from the patient and controls were exposed to 100 ng/ml LPS, IL-1β, or TNF-α or were unstimulated and incubated at 37°C in 5% CO_2 for 24 h. Supernatants were examined by ELISA for IL-6 (*a*) and IL-8 (*b*). Mean (±SEM) is derived from $n \geq 3$ repeats for each stimulus with $n = 3$ replicates per study. *, $p < 0.05$; **, $p < 0.01$. *c*, Semiquantitative RT-PCR was performed to determine the expression of CD14 and TLR4 in unstimulated primary dermal fibroblasts from the patient and control. *d*, Immortalized dermal fibroblasts from the patient were transfected with pFLAGRK7-IRAK-4 or control empty vector and exposed to 100 ng/ml IL-1β or TNF-α or were unstimulated and incubated at 37°C in 5% CO_2 for 24 h. Supernatants were examined by ELISA for IL-6. Mean responses (±SEM) were from $n = 3$ biological replicates in a study representative of $n = 2$ experimental repeats.

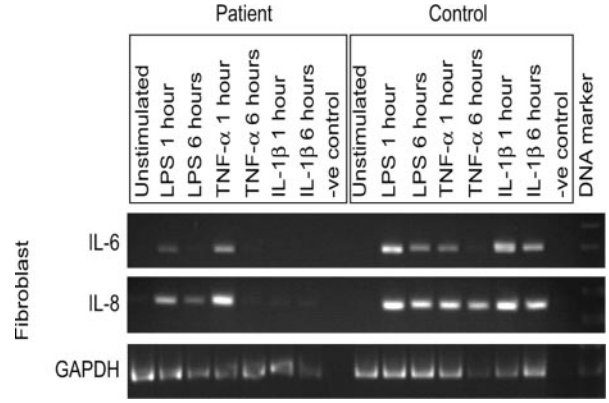

FIGURE 6. Transcriptional defects in IRAK-4-deficient fibroblasts. Primary dermal fibroblasts from the patient and controls were exposed to 100 ng/ml LPS, IL-1β, or TNF-α or were unstimulated and incubated at 37°C in 5% CO_2 for 24 h, before the isolation of total RNA and the semiquantitative RT-PCR analyses with primers designed to evaluate *IL-6* and *IL-8* mRNA expression and amplification of the housekeeping gene *GAPDH* used as a corrective control for semiquantitative analysis. Representative images show amplified products run on an agarose gel, with a reverse transcriptase-negative control from $n \geq 3$ experimental repeats and $n \geq 2$ replicates per sample using $n = 2$ different normal controls.

These data suggest that IRAK-4 deficiency has differential effects upon cytokine expression at transcriptional and post-transcriptional levels and indicate that signaling responses to LPS remain at least partially intact and capable of inducing gene transcription. However, the data indicate that cytokine protein production in response to LPS is IRAK-4 dependent.

NF-κB signaling in IRAK-4 mutant cells

To determine the effect of the IRAK-4 deficiency upon NF-κB signaling, the phosphorylation of IκB-α in the patient's fibroblasts (Fig. 8*a*) and PBMCs (Fig. 8*b*) was examined after exposure to stimuli. No response to IL-1β was observed in the patient's fibroblasts. However, a robust response to LPS was observed despite *IRAK-4* mutation, correlating with the LPS-induced transcription of *IL-6* and *IL-8* in these cells described above. Not unexpectedly, although slightly diminished, IκB-α responses to TNF-α were demonstrated in the patient's fibroblasts.

The patient's PBMCs showed a decrease in IκB-α and a parallel increase in phosphorylated IκB-α in response to TNF-α and LPS (albeit diminished in comparison to control cells), with a much lesser but repeatedly observed response to IL-1β (Fig. 8*b*). Concurrent nuclear localization of p65 was evident but diminished when compared with control cells in response to LPS and IL-1β (Fig. 8*b*). These data indicate at least partially intact NF-κB activation pathways in PBMCs from this patient.

Activation of MAPK pathways in Q293X mutant PBMCs

In contrast to control PBMCs, defective phosphorylation (activation) of p38 and JNK MAPK was observed in the patient's cells (Fig. 8*b*). No MAPK activation was observed in response to IL-1β, whereas LPS induced a delayed and diminished activation of p38. Activation of p38 in response to TNF-α was also diminished but not delayed as compared with control. JNK activation was only observed at a low level in response to prolonged (60 min) TNF-α exposure. These data demonstrate defects in the activation of key accessory proinflammatory signaling pathways involved in the transcriptional and translational control of IL-6 and IL-8 expression under stimulatory conditions in which NF-κB activation was observed in patient cells.

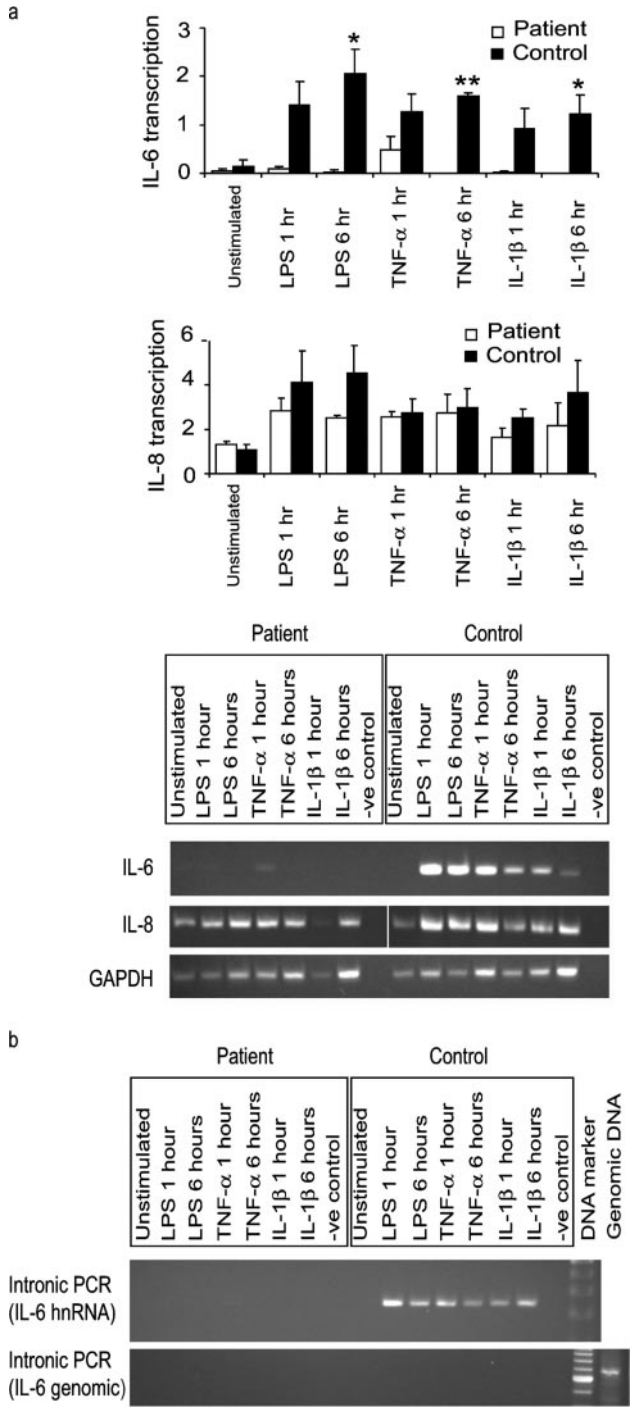

FIGURE 7. Transcriptional defects in IRAK-4-deficient PBMCs. PBMCs from the patient and controls were exposed to 100 ng/ml LPS, IL-1β, or TNF-α or were unstimulated and incubated at 37°C in 5% CO_2 for 24 h before isolation of total RNA. *a*, cDNA representing mRNA was synthesized using oligo(dT) and used in semiquantitative RT-PCR analyses, with primers designed to evaluate *IL-6* and *IL-8* mRNA expression. Quantitation was performed by densitometry and corrected for *GAPDH* expression. Mean responses (±SEM) are shown from $n \geq 3$ replicates, and differences between patient and controls were assessed for significance. *, $p < 0.05$; **, $p < 0.01$. *b*, cDNA representing total RNA, including hnRNA, was synthesized using random primers, and intronic PCR amplification was performed using primers designed to evaluate *IL-6* hnRNA or primers designed to amplify a region of the *IL-6* promoter used to exclude DNA contamination (*IL-6* genomic). *a* and *b* contain representative images showing amplified products run on an agarose gel with a reverse transcription-negative control from $n \geq 3$ experimental repeats and $n \geq 2$ replicates per sample using $n = 2$ different normal controls.

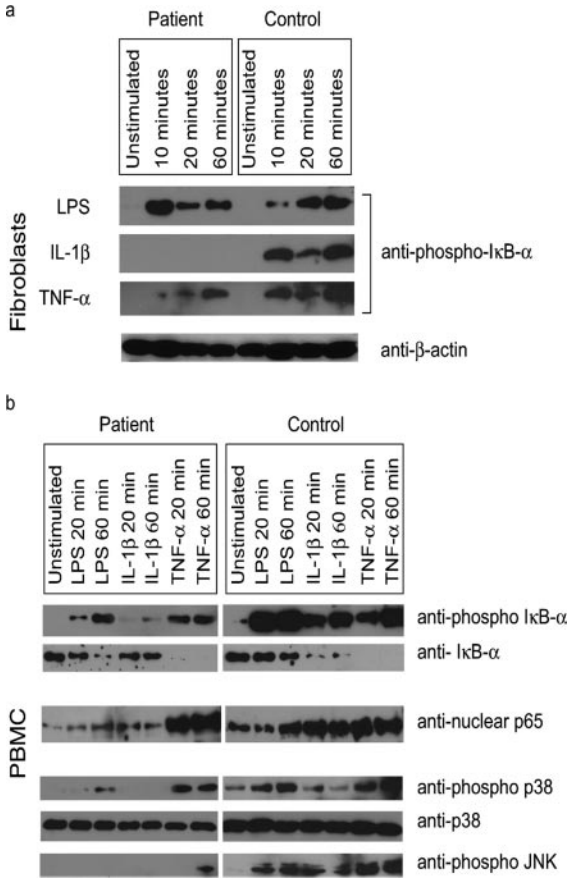

FIGURE 8. NF-κB and MAPK signaling in IRAK-4-deficient fibroblasts and PBMCs. Primary fibroblasts (*a*) and PBMCs (*b*) from the patient and control were exposed to 100 ng/ml LPS, IL-1β, or TNF-α or were unstimulated for the times indicated. Western immunoblot analyses were performed on whole cell lysates using Abs specific for phosphorylated IκB-α, IκB-α, phosphorylated p38, total p38, phosphorylated JNK, and β-actin and nuclear proteins using Abs specific for p65. Immunoblots are representative of $n \geq 2$ experimental repeat, using $n = 2$ different normal controls.

Alternative LPS signaling pathways in Q293X mutant cells

LPS has been shown to activate MyD88-independent TLR signaling using alternative adaptor proteins. To determine whether these pathways were intact in Q293X mutant cells, LPS-induced transcription of the STAT-1-dependent gene *IP-10* was examined in PBMCs. No significant difference was observed between patient and control cells (Fig. 9*a*). In addition, LPS-induced RANTES expression in our patient's peripheral blood monocytes was also found to be comparable to that of control cells (fold change vs control = 1.2; $p = 0.2$) by using microarray gene expression-based studies (K. L. Brown, R. Falsafi, D. J. Davidson, S. Turvey, D. P. Speert, and R. E. W. Hancock, manuscript in preparation). Additional studies demonstrated that up-regulation of costimulatory molecules occurred normally on the patient's monocyte-derived dendritic cells upon activation with TLR agonists capable of using MyD88-independent pathways (LPS and poly(I:C)), with a similar magnitude of change when compared with the control cells but with no response to CpG (Fig. 9*b*). Interestingly, the patient's immature dendritic cells had constitutively higher levels of expression of the costimulatory molecules CD86 and CD80 but normal CD40 when compared with controls. Despite normal costimulatory molecule up-regulation, the patient's MDDCs remained defective in IL-6 production except when activated by exposure to CD40L

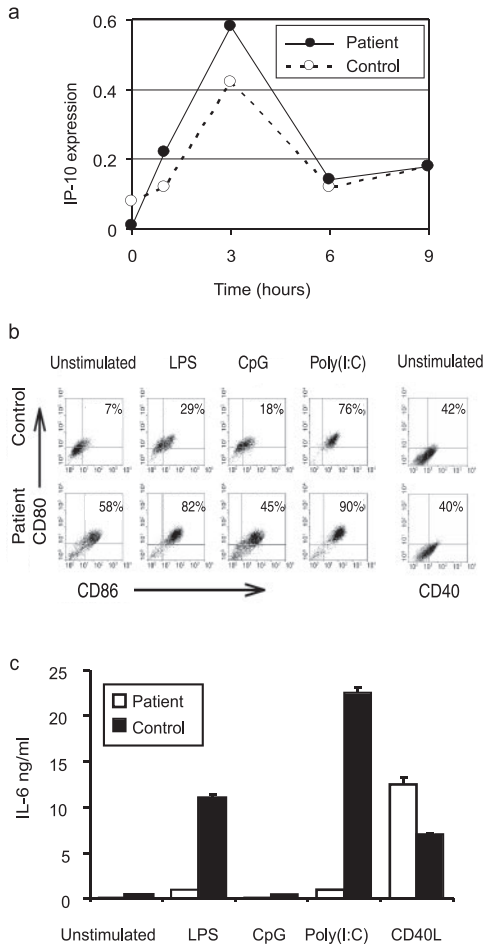

FIGURE 9. Intact MyD88-independent signaling in IRAK-4-deficient cells. *a*, Patient and control PBMCs were exposed to 100 ng/ml LPS for the times indicated. Semiquantitative RT-PCR was performed for *IP-10* expression, quantified by densitometry, and corrected for expression of the housekeeping gene *GAPDH*. *b*, Immature MDDCs were stimulated as above. The percentage of positive staining cells by FACS is shown (after correction for staining with a parallel isotype-specific control Ab in each sample), representing the percentage of double positivity for CD80 and CD86 or single positivity for CD40. Results are representative of $n = 3$ experimental repeats with $n = 3$ different controls. *c*, Immature MDDCs from the patient or control were exposed for 24 h to either 100 ng/ml LPS, 10 μg/ml CpG2006, 10 μg/ml poly(I:C), by coculture with CD40L-transfected murine 3T3 fibroblasts or were untreated. Supernatants were examined for IL-6 by ELISA. Results are mean $\pm$SEM from $n = 3$ experimental repeats with $n = 3$ biological replicates per condition.

(Fig. 9*c*). These data indicate intact MyD88-independent pathways in patient cells but demonstrate a necessity for functional IRAK-4 for TLR-induced MDDC IL-6 protein production.

Discussion

Naturally occurring genetic mutations in humans, causing rare extreme immunodeficiency phenotypes, present powerful opportunities for determining the relationship between specific defects and human disease processes in vivo and for studying the underlying mechanisms involved in primary human cells.

In this study we present novel findings by using primary cells (PBMCs, dermal fibroblasts, and MDDCs) from a child with autosomal recessive homozygous Q293X *IRAK-4* mutation to demonstrate that IRAK-4-dependent mechanisms control innate immune responses at both transcriptional and post-transcriptional levels, with IRAK-4 deficiency differentially affecting NF-κB activation and cytokine production. We demonstrate that Q293X *IRAK-4* mutant primary cells displayed NF-κB activation with functional MyD88-independent pathways but defective MAPK activation. We report cell type-specific and stimulus-specific signaling dysfunction in IRAK-4 deficiency, including a demonstration of IRAK-4-dependent LPS responses in primary dermal fibroblasts. We also describe the first definitive documentation of a fatality from a pneumococcal disease attributable to an autosomal recessive homozygous Q293X *IRAK-4* mutation in a sibling of our patient. Our data suggest that this mutation is likely to be an unrecognized primary immunodeficiency in apparently healthy children succumbing to lethal pyogenic bacterial infections. We offer important considerations for diagnostic approaches and provide key insights into the mechanisms underlying innate immune dysfunction in IRAK-4 deficiency and the normal role of this protein.

Primary dermal fibroblasts from our patient displayed defective IκB-α phosphorylation and cytokine production in response to IL-1β but intact TNF-α responses, consistent with observations of immortalized fibroblast lines from other homozygous Q293X patients (10). These data indicate that IRAK-4 is not required for TNF-α responses in fibroblasts and suggest a simple signaling block upstream of TRAF6 inhibiting IL-1β responses. However, assessment of LPS-induced responses introduces further complexity. Conflicting published literature concerning the potential for LPS responsiveness in fibroblasts (24) may reflect heterogeneous fibroblast subsets (25), differences between primary and immortalized cells (26), and potential LPS contaminants. Primary human dermal fibroblasts have not been extensively characterized, although reports indicate a potential for LPS responsiveness (25). We demonstrated the expression of CD14 and TLR4 with IκB-α phosphorylation and the production of IL-6 and IL-8 in response to Ultra-Pure LPS in our adult and child control primary dermal fibroblasts. In contrast, although our patient's primary dermal fibroblasts clearly demonstrated intact I-κBα phosphorylation (Fig. 8*a*) and transcription of *IL-6* and *IL*-8 (Fig. 6), these IRAK-4-deficient cells demonstrated a profound failure to produce IL-6 and IL-8 in response to LPS. These data demonstrate a functional LPS-activation pathway in both wild-type and IRAK-4-deficient dermal fibroblasts and likely confirm TLR4 signaling by well-characterized MyD88-independent pathways (2), which we demonstrated are intact in our patient's PBMCs and MDDCs and which would not require functional IRAK-4 for LPS-stimulated NF-κB activation. However, critical to these studies, our patient's primary dermal fibroblasts and MDDCs demonstrated a failure of cytokine production in response to LPS despite NF-κB signaling and cytokine transcription and activation, respectively. These data indicate that MyD88-independent signaling is insufficient to generate the normal inflammatory response in these cells and that, even in the presence of NF-κB activation, functional IRAK-4 is required for post-transcriptional processing and cytokine production in these cells.

Intriguingly, our data demonstrate cell type specificity when comparing the defective responses secondary to the IRAK-4 Q293X mutation in primary dermal fibroblasts as compared with PBMCs. The molecular basis for these observations remains unclear. However, recent studies have demonstrated key signaling differences between myeloid and non-myeloid cells with the differential use of adaptor molecules and have also demonstrated MyD88-dependent LPS responses in synovial fibroblasts that may not use TLR4 (26, 27). These observations suggest that the precise role of IRAK-4 in different cell types may vary and introduce a complexity that may be critical for understanding the clinical consequences of IRAK-4 deficiency.

In our patient's PBMCs, neither LPS, IL-1β, or TNF-α induced production of IL-6. However, LPS induced NF-κB translocation and *IL-8* transcription but induced 10-fold less IL-8 protein expression than control cells, reiterating the likely significance of IRAK-4-dependent post-transcriptional mechanisms as observed in the dermal fibroblasts. Nevertheless, the induction of IL-8 protein production (albeit a significantly reduced level) contrasted with the absolute deficiency demonstrated in the patient's primary dermal fibroblasts. Our recent quantitative RT-PCR studies on purified monocytes from this patient demonstrate a more severe defect in LPS-induced *IL-8* transcription in these cells (albeit retaining a low-level transcriptional response) when compared with the PBMCs (K. L. Brown, R. Falsafi, D. J. Davidson, S. Turvey, D. S. Speert, and R. E. W. Hancock, manuscript in preparation). Future studies will be required to determine the relative significance of responses of each the different cell types present in PBMC preparations and the possible significance of interaction between these cells.

IL-1β stimulation of our patient's PBMCs was surprisingly able to repeatedly induce low-level NF-κB translocation and stimulate a diminished level of *IL-8* (but not *IL-6*) transcription but was unable to induce IL-8 protein production. IRAK-4 has been demonstrated to be essential for IL-1R-stimulated NF-κB activation (9). Thus, these data might represent IL-1β-induced transcription of *IL-8* by an undetermined, primarily NF-κB-independent mechanism in our patient's PBMCs (but not dermal fibroblasts) or partial substitution of IRAK-4 function in NF-κB signaling by alternative molecules or a truncated translation product of *IRAK-4*. Indeed IRAK-4 is capable of transmitting signals both dependent on and independent of its kinase activity (28), and truncated IRAK-4 protein has been shown to retain DD interactions (29). Chain termination mutations resulting in mRNAs that contain premature stop codons rarely produce truncated proteins as a consequence of nonsense-mediated mRNA decay (22). However, although it could simply reflect an "overwhelming" of the nonsense-mediated mRNA decay system, a truncated IRAK-4 protein has been detected in transfected cells overexpressing *IRAK-4* with the C877T mutation, (29), raising the possibility of its expression in patient cells. Additionally, translation of the alternatively spliced *IRAK-4* transcript described would produce a protein unaffected by the Q293X mutation with a disrupted kinase region but an intact DD and an undetermined domain. Although low-level expression in IRAK-4-competent cells might have no effect, this could be significant in the absence of full-length IRAK-4. Thus, although truncated IRAK-4 proteins have not been detected in patient cells, we cannot exclude the possibility that low-level expression could influence IL-1β signaling in IRAK-4-deficient PBMCs.

Our data demonstrate that the innate immune dysfunction in IRAK-4 deficiency is both stimulus specific and determined differentially for specific cytokines. This finding is consistent with a near-complete deficiency in LPS-induced TNF-α production (10) and a profound transcriptional defect in *GM-CSF* but a partial defect in *Cox-2* transcription (11) in PBMCs from other IRAK-4-deficient individuals. In addition, it is consistent with defective LPS-induced *IL-6* and *IL-12p40* transcription but post-transcriptional defects in IL-8, TNF-α, and IL-12p35 observed in our patient's MDM (15). These data also indicate a critical role for IRAK-4 in both transcriptional and post-transcriptional control of cytokine production, even in the presence of intact NF-κB signaling. In this regard, the severe defects in our patient's MAPK responses may be highly significant.

The MAPK p38 can affect transcription (30) via chromatin restructuring (5) or transactivation of p65 (8). However, the core transcriptional regulator for *IL-8* has a NF-κB element required for activation in all cell types studied, with the additional transcription factors required for maximal expression being largely dispensable (31, 32). Thus, *IL-8* transcription was expected where NF-κB activation was observed, regardless of other factors. This was clearly evident in our patient's cells. However, the translocation of NF-κB subunits in addition to p65 remains to be assessed. Critically, however, the activation of p38 by MAPK kinase (MKK)-3, MKK-4, and MKK-6 also has an essential post-transcriptional function in the stabilization of mRNAs with 3′ AU-rich elements, including *IL-8* and *IL-6* (7, 33), and p38 inhibition can have profound post-transcriptional effects without affecting transcription rates (7). In our patient's PBMCs, p38 activation was undetectable in response to IL-1β and substantially diminished in response to LPS, correlating with the defect in production of the IL-8 protein. This suggests a critical role for p38-dependent post-transcriptional mechanisms in IL-8 production, which is defective in our patient as a consequence of the *IRAK-4* mutation.

IL-6 transcription is additionally affected by the p38-activated C/EBP homologous protein (30) via inhibition of negative transcriptional regulators (34). Furthermore, inhibition of JNK, a MAPK activated downstream of TAK-1 by MKK-4, MKK-7, inhibited *IL-6* transcription through the failure of an undefined interaction between JNK pathways and other signaling pathways such as NF-κB (35). Our patient's PBMCs demonstrated a complete absence of JNK activation in response to LPS and IL-1β that correlated with defective *IL-6* transcription, suggesting a critical role for IRAK-4 in *IL-6* transcription through activation of JNK.

In addition to TLR/IL1-R signaling defects, significantly impaired responses to TNF-α were also observed in IRAK-4-deficient PBMCs. In contrast, no significant defect was observed in the TNF-α-induced cytokine response in IRAK-4-deficient dermal fibroblasts (despite slightly diminished TNF-α-induced phosphorylation of I-κBα). These data suggest an as yet undetermined role for IRAK-4 in TNF-α signaling and cell type-specific differences in the TNFR signaling pathways in PBMCs as compared with fibroblasts. In addition, our data revealed more substantial impairment in the production of IL-6 in comparison to IL-8 in TNF-α -stimulated, IRAK-4-deficient PBMCs. Although TNF-α-induced IL-6 responses in PBMCs can vary significantly between individuals, our patient was the only complete nonresponder we observed, in contrast to 10 normal controls with significant IL-6 responses (all $p < 0.05$). Interestingly, defective TNF-α-induced activation of MAPK was demonstrated in our patient's PBMCs with JNK activation more notably deficient than p38, correlating with the more substantial impairment of IL-6 protein production in comparison to IL-8. In addition, low-level transcription of *IL-6* was observed in response to TNF-α, the only stimulus to which any JNK activation was observed. Although IRAK-4 is upstream of the proposed convergence of the TLR/IL-1R and TNFR signaling pathways at TRAF6 (36), a role for IRAK-1 has been demonstrated in TNFR signaling (37). Furthermore, in the absence of MyD88 function, MDMs (but not fibroblasts) have been shown to have impaired cytokine production in response to TNF-α, despite intact NF-κB activation (26). Our data suggest a critical role for IRAK-4 in the activation of MAPK downstream of TNFR activation in PBMCs.

We propose that dysfunctional accessory proinflammatory signals, rather than NF-κB activation alone, underpins the cellular phenotype in this patient. TLR/IL-1R stimulation of NF-κB via activated TAK-1 is relatively well characterized. Hyperphosphorylation of IRAK-1 by activated IRAK-4 results in a Pellino-IRAK-4-IRAK-1-TRAF6 complex (complex 1) formation and receptor release.

Complex 1 interacts with membrane-bound TAK-1-TAB-1-TAB-2, with resultant cytosolic translocation of TRAF6-TAK-1-TAB-1-TAB-2 and TAK-1 activation of the IKK complex (38). In addition to NF-κB pathways, TAK-1 also activates MAPK via MKK (3, 39). However, despite the common requirement for TRAF6 and TAK-1, divergence of these pathways occurs at or perhaps upstream of IRAK-1 (40), with the relative overlap of proximal signaling components unclear. Different IRAK-1 regions mediate TRAF6 and TAB-2 translocation (41), and despite an inability to interact with TRAF6, the undetermined domain of IRAK-1 is sufficient (but not required) for activation of JNK but not NF-κB (42). This finding suggests that IRAK-1 mediates the interaction of other undefined signaling components with TRAF6 to activate JNK signaling. Thus, complex 1 dysregulation in IRAK-4-deficient individuals could disrupt MAPK pathways independently from intact TAK-1/IKK/NF-κB signaling as observed in our patient's cells. Interestingly, we have observed IRAK-1 kinase function perturbation in our patient's neutrophils, with high baseline activity diminishing after LPS exposure in contrast to the LPS-enhanced kinase activity in controls (data not shown), consistent with findings in an IRAK-4 compound heterozygote (11). In addition, our patient's PBMCs cultured in M-CSF generated multinucleated giant cells positive for tartrate-resistant acid phosphatase in addition to MDM (data not shown). This is suggestive of osteoclastic differentiation, a process normally requiring additional RANKL (receptor activator of NF-κB ligand) stimulation, acting via TRAF6 signaling and also suggesting dysregulated complex 1 function (43, 44). Intriguingly, both this patient and his deceased *IRAK-4* Q293X homozygote sibling were above the 95th percentile for height, in contrast to their heterozygotic siblings (between the 50th and 75th percentiles for age). Thus, we propose that dysfunctional complex 1 activity in response to TLR/IL-1R activation, which results in defective MAPK activation, underpins the innate immunodeficient phenotype in IRAK-4-deficient primary cells.

Ongoing studies using cells from IRAK-4-deficient individuals are expected to further elucidate the precise nature of the selective signaling defect downstream of complex 1 and the specific nature of the susceptibility to infection in IRAK-4–deficient individuals. It is interesting to note the relative selectivity of infecting organisms in untreated IRAK-4–deficient individuals, particularly before diagnosis, with an apparent particular susceptibility to *S. pneumoniae* and to Gram-positive organisms (although not exclusively) (45). Prophylactic antibiotic therapy after diagnosis clearly precludes longitudinal characterization of the range of infections that IRAK-4–deficient individuals might have suffered from if left untreated. Nevertheless, in the absence of antibiotics our patient and others were highly susceptible to infection, and in the case of our patient's eldest brother this condition was fatal. This finding clearly indicates the importance of TLR signaling to innate immunity, but the phenotype perhaps contrasts with the acute, broad spectrum immunodeficiency that might have been predicted for a severe TLR signaling deficiency. The specific reasons for this remain uncertain. However, our new data indicates that the major *IRAK-4* mutation described (Q293X) does not result in a complete block to TLR signaling but rather results in cell type-specific and ligand-specific defects, differentially affecting different cytokines at transcriptional and or post-transcriptional levels and in which defects in MAPK pathways may be more significant than the largely intact NF-κB pathway. This hypothesis suggests levels of complexity that may contribute to the relative selectivity in increased susceptibility to infection in these patients. It is also interesting to note that some older IRAK-4-deficient patients have been taken off prophylactic antibiotics and remained healthy (45), suggesting the possibility that the immunodeficiency associated with IRAK-4 deficiency can be compensated for in those who survive childhood and may be relatively less critical in adult life.

In conclusion, we demonstrate that *IRAK-4* mutation in primary human cells from a naturally occurring, clinically relevant human "model" of disease does not result in an absolute null phenotype for TLR/IL-1R signaling. Rather, it establishes dysfunctional cellular signaling with partially intact NF-κB pathways but defective MAPK signaling and dysregulated complex 1 function that affects transcriptional and post-transcriptional control of TLR/IL-1R responses. The specificity of the accessory proinflammatory pathways affected, combined with cytokine-specific mechanisms regulating expression, could explain the complex cell type-specific, stimulus-dependent, and cytokine-specific defects observed and further illuminate the unusual pattern of disease susceptibility in IRAK-4–deficient patients.

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Disclosures

The authors have no financial conflict of interest.

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