ORIGINAL ARTICLE

IRAK4 and *NEMO* mutations in otherwise healthy children with recurrent invasive pneumococcal disease

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Background: About 2% of childhood episodes of invasive pneumococcal disease (IPD) are recurrent, and most remain unexplained.
Objective: To report two cases of otherwise healthy, unrelated children with recurrent IPD as the only clinical infectious manifestation of an inherited disorder in nuclear factor-κB(NF-κB)-dependent immunity.
Results: One child carried two germline mutations in *IRAK4*, and had impaired cellular responses to interleukin (IL)1 receptor and toll-like receptor (TLR) stimulation. The other child carried a hemizygous mutation in *NEMO*, associated with a broader impairment of NF-κB activation, with an impaired cellular response to IL-1R, TLR and tumour necrosis factor receptor stimulation. The two patients shared a narrow clinical phenotype, associated with two related but different genotypes.
Conclusions: Otherwise healthy children with recurrent IPD should be explored for underlying primary immunodeficiencies affecting the IRAK4-dependent and NEMO-dependent signalling pathways.

The known inherited risk factors for invasive pneumococcal disease (IPD) in children include sickle-cell disease[1] and primary immunodeficiencies (PIDs).[2] PIDs include defects in the classic complement activation pathway, defects in carbohydrate-specific antibody responses, congenital asplenia and the more recently discovered IRAK4 and NEMO deficiencies.[2] These defects impair a step in the process leading to the phagocytosis of opsonised bacteria by splenic macrophages. NEMO and IRAK4 deficiencies also involve an impaired mucosal and systemic inflammation response, due to the impaired activation of nuclear factor-κB (NF-κB) by microbes and cytokines.[3–6] Although the risk of IPD in children with these PIDs is well known, the fraction of patients with IPD cases associated with a PID in the general population is unknown. This fraction is, however, thought to be small, because PIDs are usually associated with multiple infectious diseases, whereas IPD mostly affects otherwise healthy children. Thus, most cases of IPD remain unexplained genetically and immunologically, at least partly because they are not investigated.

We hypothesisd that isolated IPD may be due to inborn errors in immunity to infection in an unexpectedly large fraction of children.[7] We first focused on recurrent IPD,[8] which is arbitrarily defined as two episodes of IPD occurring at least 1 month apart, whether caused by the same or different serotypes or strains. Recurrent IPD occurs in at least 2% of patients in most series, making IPD the most important known risk factor for subsequent IPD.[8–18] Most series of recurrent cases of IPD have included mostly adults, many with overt underlying conditions, such as HIV infection, organ failure, or cancer.[8 17–20] The situation is much less clear for recurrent IPD in children. The risk factors for this condition identified to date in paediatric patients are sickle-cell disease[12 21] and congenital[22 23] or acquired[14] cerebrospinal fluid leaks, specifically predisposing the patient to recurrent meningitis. In a North American series, six children with recurrent IPD had sickle-cell disease, whereas the recurrence remained idiopathic in the other 10 children.[12] In a Swiss series, two children had a history of basal skull fracture, whereas the remaining eight were idiopathic.[14]

A high proportion of children with idiopathic recurrent IPD are likely to have undetected PIDs. The diagnosis of these PIDs is difficult, because PIDs are rare and diverse, and because immunological investigations, and assays of antibody response in particular, are difficult to perform in children aged <2 years. Several PIDs probably associated with an IPD have not yet been discovered.[2] Only a few cases of children with PIDs and isolated recurrent IPD have been reported. They include children with congenital asplenia,[24–27] complement pathway deficiencies,[28–35] X-linked agammaglobulinaemia[36] and selective antipolysaccharide antibody deficiency.[37] Transient IgG2 deficiency has also been described in one child.[38] Isolated recurrent IPD has been reported in a single patient with a mild form of X-linked recessive anhydrotic ectodermal dysplasia with immunodeficiency bearing a *NEMO* mutation,[39] but not in patients with autosomal recessive IRAK4 deficiency, or in patients with mutations in *NEMO* but no developmental phenotype. Autosomal recessive IRAK4 deficiency and X-linked recessive NEMO deficiencies correspond to two recently described PIDs that affect NF-κB-mediated immunity and cause a relatively broad susceptibility to infections. We report here two otherwise healthy boys with isolated, recurrent IPD and inherited NEMO and IRAK4 defects. These patients displayed none of the other known infectious phenotypes associated with these disorders.

Abbreviations: CRP, C reactive protein; EDA, ectodermal dysplasia; IPD, invasive pneumococcal disese; PCR, polymerase chain reaction; PID, primary immunodeficiency; PMN, polymorphonuclear neutrophil; RT-PCR, reverse transcription PCR; TLR, toll like receptor; TNF, tumour necrosis factor

Table 1 Immunologic explorations

Tests	Results for patient 1	Results for patient 2	Normal range
Lymphocyte count (/1)	1.86×10^9	4.54×10^9	$1.7–7\times10^9$
Lymphocyte subsets (%)			
CD3	71	68	50–76
CD4	45	48	25–53
CD8	25	21	14–34
CD19	20	26	14–44
CD3− CD56+	4	2	4–26
Serum immunglobulins (g/l)			
IgG	13.97	5.83	6.1–11.8
IgA	3.35	0.82	0.46–1.34
IgM	0.74	0.28	0.50–1.14
IgG subclasses (g/l)			
IgG1	8.41	4.81	4–9.45
IgG2	3.12	2.41	0.36–2.25
IgG3	0.34	0.575	0.13–1.22
IgG4	3.54	0.038	0.02–1.12
Specific antibodies (ELISA)			
Anti-Hib (μg/ml)	0.19	<0.15	>1
Anti-tetanus (IU/ml)	0.59	<0.01	>0.10
Anti-diphtheria (IU/ml)	0.10	<0.01	>0.10

PATIENTS AND METHODS

Case reports

Patient 1 was a 7-year-old boy born to unrelated Hungarian parents. He received routine immunisations with no complications. At age 3 years, he had arthritis of the right hip, caused by *Streptococcus pneumoniae* serotype 14. He was successfully treated with intravenous ceftriaxone for 12 days, and was then given clindamycin prophylaxis for 10 days. He had only two episodes of low-grade fever (<38°C) during the course of this severe purulent infection, accompanied by anaemia, an erythrocyte sedimentation rate of 81 mm/h, a high serum C reactive protein (CRP) concentration of 89 mg/l and a leucocyte count of 4700/mm^3 with 42% polymorphonuclear neutrophils (PMNs).

At age 5½ years, the patient developed meningitis caused by *Streptococcus pneumoniae* serotype 14, with moderate headache and a slightly high temperature (38°C). CRP levels were normal on day 1, and increased 2 days after diagnosis (87 mg/l). His erythrocyte sedimentation rate was 40 mm/h and his leucocyte count was 4800/mm^3 with 56% PMNs at the onset of the disease. The patient recovered without sequelae after treatment for 12 days with intravenous cefotaxime. He was given oral amoxicillin for another 4 weeks. During the episode of meningitis, the patient's temperature remained below 38°C. After this second episode of IPD, he was immunised with the heptavalent pneumococcal conjugate vaccine (Prevenar Wyeth-pharmaceuticals, Lyon, France) and with the 23-valent pneumococcal vaccine (Pneumovax 23, Aventis-Pasteur MSD, Madison, New Jersey, USA), and prescribed monthly intravenous immunoglobulin (Ig)G infusions. He has since been well with no further infections.

The immunological profile of the patient was assessed at ages 3 and 5½ years. The classic and alternative complement pathways, in vitro granulocyte killing of viable *Staphylococcus aureus* and superoxide anion release by granulocytes were normal. He had normal numbers of white cells, total lymphocytes, and T, B and natural killer cells, with slightly high serum immunoglobulin isotype levels (table 1). Antibody responses to tetanus and diphtheria toxoids and *Haemophilus influenzae* type b conjugate vaccine were normal, but the patient failed to mount a detectable antibody response to six of the seven pneumococcal serotypes tested, including the pathogenic serotype 14 (table 2). The spleen was visible on ultrasound scans.

At age 6½ years, IgG infusions were stopped. The antibody response to immunisation with 23-valent pneumococcal vaccines remained impaired for six of the seven serotypes tested. Serotypes 4, 6B, 14, 18C and 19F are present in the heptavalent pneumococcal conjugate vaccine. We have no age-matched normal values for serotype 14. However, for the other serotypes, normal values are consistently above 20% of the reference batch 89-SF. In patient 1, the specific antibody concentration against serotype 14 was only 2% of the reference value. Natural blood allohaemagglutinin concentrations were very low (Group A Rh+, anti-B antibody titre: 1/1).

Patient 2 was a 4½-year-old boy born to unrelated Belgian parents. He received all routine vaccinations with no adverse effect. In infancy, he developed mild but distinctive clinical features, such as frontal bossing, hypodontia with conical incisors and dry skin with normal sweating, consistent with a mild form of anhydrotic ectodermal dysplasia (EDA). At age 15 months he was hospitalised for 4 days for persistent fever (>38.5°C) with buccal cellulitis, caused by *Streptococcus pneumoniae* serotype 33. He recovered completely after treatment with intravenous cefuroxime for 7 days. Biological signs of

Table 2 Serotype-specific antibody responses to polysaccharide antigens

Pneumococcus	Response of patient 1 (age 5¾ years) μg/ml	Response of patient 2 (age 2½ years) μg/ml	Normal responses (age >3 years) μg/ml
Serotype 3	3.02	1.4	>0.74
Serotype 4	0.22	0.1	>0.91
Serotype 6B	0.34	0.17	>0.68
Serotype 9N	0.54	0.46	>0.97
Serotype 14	0.55	0.3	ND
Serotype 18C	0.75	0.18	>0.58
Serotype 19F	0.9	0.26	>1.79
Serotype 23F	ND	<0.4	ND

For patient 1, data show specific antibody concentrations 2 weeks after immunisation with Pneumovax 23 plus the conjugate pneumococcal vaccine Prevenar (and 3 months after the secondary invasive pneumococcal disease caused by serotype 14). For patient 2, data show specific antibody concentrations 2 weeks after immunisation with Pneumovax 23 and 3 months after the last of a series of four monthly immunisations with Prevenar (13 and 7 months after the two episodes of invasive disease caused by *Streptococcus pneumoniae* serotypes 23F and 33F). Of the serotypes shown, 4, 6B, 9V, 14, 18C, 19F and 23F are present in both vaccines. Concentrations of specific antibodies were determined by ELISA (μg/ml). As a reference, the US Pneumococcal Reference Serum Lot 89-SF was used. Normal responses were determined in 37 children (aged 4–15 years; distribution: 1, 8, 12 and 16 for the age groups 3–4, 5–6, 7–9, and 10–15 years, respectively). Antibody levels were determined 2–3 weeks after vaccination with Pneumovax. Cut-off values given are the 5th percentile for the total group of controls.
ND, not done.

inflammation were partly mild, with a CRP concentration of 52 mg/l but 20 000 leucocytes/mm^3 with 43% PMNs.

At age 22 months, the patient developed a left-sided limp with no fever, and mild periorbital cellulitis of the left eye caused by *Streptococcus pneumoniae* serotype 33. He was treated with intravenous ceftriaxone for 7 days. He had again dissociated biological signs of inflammation, with a CRP concentration of 31 mg/l and 23 000 leucocytes/mm^3 with 55% PMNs. The local inflammation of the left foot gradually improved and the patient started walking again. He was given oral amoxicillin on discharge. Ten days later, while still taking this course of oral antibiotics, osteomyelitis of the left talus relapsed. The patient had dissociated biological signs of inflammation, with no raised CRP level and 14 500 leucocytes/mm^3 with 39% PMNs. He was treated for 6 weeks with intravenous ampicillin, followed by a 1-month course of oral clindamycin. He recovered without sequelae.

At age 23 months, the patient was vaccinated with heptavalent pneumococcal conjugate vaccine. He was given a second dose 2 months later. After 15 months, he received a 23-valent pneumococcal vaccine. Despite these vaccinations, at age 2 years 7 months, he presented with isolated fever (38.7°C) because of infection with *Streptococcus pneumoniae* serotype 23. He recovered after a 10-day course of oral ampicillin. Three months later he developed arthritis of the left hip, caused by *Streptococcus pneumoniae* serotype 23. He had again dissociated biological signs of inflammation, with a CRP concentration of 13 mg/l and 16 100 leucocytes/mm^3 with 71% PMNs. He was given intravenous ceftriaxone for 15 days and underwent surgical drainage of the left hip. On discharge, treatment with oral amoxicillin was initiated. Monthly prophylactic treatment with intravenous immunoglobulins was started when the patient was 3 years 8 months old. This treatment was maintained until the patient's last follow-up visit, at age 4 years 7 months.

The immunological profile of the patient was assessed at age 3½ years, before the initiation of immunoglobulin substitution. The spleen was visible on ultrasound scans. The classic and alternative complement pathways were normal. The patient had normal numbers of white cells and lymphocytes, normal levels of serum immunoglobulin isotypes and IgG subclasses, and normal numbers of T, B and natural killer cells (table 1). However, he had impaired antibody responses to tetanus and diphtheria toxoids, pertussis antigens, and measles and mumps viruses. The antibody response to rubella virus was positive. The antibody response to *H influenzae* type b conjugate vaccine was impaired. The antibody response to combined immunisation with both heptavalent pneumococcal conjugate vaccine and 23-valent pneumococcal plain polysaccharide was impaired for all five serotypes tested, including the pathogenic serotypes 23F and 33F (table 2). Natural blood anti-B allohaemagglutinin levels were low (group A Rh+, anti-B antibody titre 1/2).

Primer sequence for genomic coding region of *NEMO*

- Exon2
  - Sense 5′-TCTGCTGGGTAAGGATGTGG-3′
  - Antisense 5′-TCTGCAGGTGGGGAGAAGAC-3′
- Exon3
  - Sense 5′-CCCAGCTCCCCTCCACTGTC-3′
  - Antisense 5′-TAACCTGTGTCCTTTCCTTGT-3′
- Exon4
  - Sense 5′-CAGTGCTGACAGGAAGTGGC-3′
  - Antisense 5′-GCCCCGGTCTATCCTCATCA-3′
- Exon5
  - Sense 5′-CATCAGCTCGCAGTCACAGG-3′
  - Antisense 5′-CCGACACTTCTCAGCCTTTC-3′
- Exon6
  - Sense 5′-AAGGGGGTAGAGTTGGAAGC-3′
  - Antisense 5′-AGGCAAGTCTAAGGCAGGTC-3′
- Exon7
  - Sense 5′-TCAGCATCTCCTCCTGTCGTT-3′
  - Antisense 5′-CTGGGCAACAAGAGCAAAAC-3′
- Exon8
  - Sense 5′-TGCCTGGTGGGTGGCTGGCT-3′
  - Antisense 5′-CAGTGTCGCACCCACTGCTCA-3′
- Exon9
  - Sense 5′-AGCTGCTTTGACACTAGTCCA-3′
  - Antisense 5′-CAGAGAGCAACAGGAAGGTC-3′
- Exon10
  - Sense 5′-CGGCGGCTCCTGGTCTTACA-3′
  - Antisense 5′-GCCACCCAGCCCATCCT-3′

Immunological diagnostic procedures

Serum levels of the IgM, IgG, IgG subclasses, and of IgA, the killing of viable *S aureus,* the generation of superoxide anion by granulocytes and complement activity were assessed by standard techniques. Immunoglobulins and immunoglobulin subclasses were determined by nephelometry (Immage instrument, Beckman-Coulter, Brea, California, USA) using reagents obtained from Beckman-Coulter and Sanquin (Amsterdam, The Netherlands). Lymphocyte subsets were determined by routine flow cytometry. Flow cytometry was carried out on whole-blood samples after red cell lysis, using an FACScan instrument (BD Biosciences, San Jose, California, USA). CD4 (FITC) and CD40L (PE) were obtained from BD Biosciences. We measured levels of IgG against *H influenzae* PRP antigens, tetanus toxoid and diphtheria toxoid by ELISA, using antigen-coated plates. Antibody titres against serotype-specific pneumococcal capsular polysaccharides were determined as described previously.[40] The US Pneumococcal Reference Serum Lot 89-SF was used as a reference.[41] We determined IgG concentrations against serotype 3 (a strong immunogen), serotypes 4, 14, and 19F (intermediate immunogens), and serotypes 6B, 9N, and 18C (weak immunogens).

Cell stimulation by cytokines

For cytokine production, whole blood and SV40-transformed fibroblasts were activated as previously described.[39] ELISA tests for interleukin (IL)-6 and IL-10 (PeliPair reagent set, Sanquin) were carried out according to the manufacturer's instructions.

Biochemistry

For electrophoretic mobility shift assays, nuclear extracts were prepared as previously described[4] from SV-40-transformed fibroblasts, after incubation with or without IL-1β (10 ng/ml) and tumour necrosis (TNFα (20 ng/ml)) for 20 or 40 min.

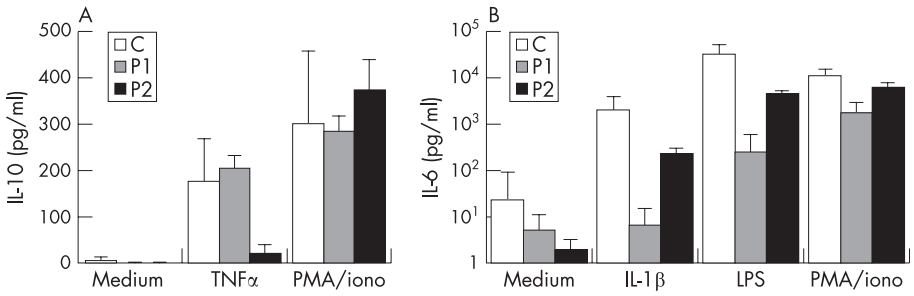

Figure 1 Stimulation of blood cells. (A) Interleukin (IL)10 production by whole-blood cells from patient 1 (P1), patient 2 (P2), and 10 unrelated healthy controls (C) after stimulation with tumour necrosis factor (TNF)α (20 ng/ml) and (PMA)/ionomycin (10^{-7}/10^{-5} M) for 48 h, as measured by ELISA. Control values are the means of independent experiments carried out on 10 controls, experiments carried out twice on the two patients. (B) IL-6 production by whole-blood cells from P1, P2 and six unrelated controls after stimulation with IL-1β (10 ng/ml), lipopolysaccharide (LPS 10 ng/ml), and PMA/ionomycin (10^{-7}/10^{-5} M) for 48 h, as measured by ELISA. Control values are the means of independent experiments carried out on 10 controls, experiments carried out twice on the two patients.

Electrophoretic mobility shift assays were carried out with a phosphorous[32]-labelled NF-κB-specific DNA probe (5′-GAT CAT GGG GAA TCC CCA-3′, 5′-GAT CTG GGG AAT TCC CCC AT-3′). For western blotting, total proteins were extracted from SV40-transformed fibroblasts. We used rabbit antibodies to IRAK4 (Tularik, Thousand Oaks, California, USA), rabbit antibodies against GADPH (SC-25778, Santa Cruz Biotechnology, Santa Cruz, California, USA), mouse antibodies against STAT2 (SC-476, Santa Cruz) and anti-NEMO antibodies (3328, polyclonal rabbit antibody, a gift from Gilles Courtois and Alain Israel).

Human molecular genetics

We isolated genomic DNA from blood cells by phenol–chloroform extraction. RNA was extracted from Epstein–Barr virus-immortalised lymphoblastoid cell lines or SV40-transformed fibroblasts, using Trizol (Invitrogen, Carlsbad, California, USA), according to the manufacturer's instructions. RNA was reverse transcribed directly, with Oligo-dT (Invitrogen) and reverse transciptase (Invitrogen). Polymerase chain reaction (PCR) was carried out with *Taq* polymerase (Invitrogen), using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA). The cDNA, exons and flanking intron regions of *IRAK4* and *NEMO* were amplified by PCR. Box 1 gives the primer sequences for the genomic coding region of *NEMO*.

The PCR products were purified by centrifugation through Sephadex G-50 Superfine resin (Amersham Biosciences) and sequenced using the BigDye Terminator Cycle sequencing kit (Applied Biosystems). Sequencing products were purified by centrifugation through Sephadex G-50 Superfine resin and sequences were analysed with an ABI Prism 3100 apparatus (Applied Biosystems).

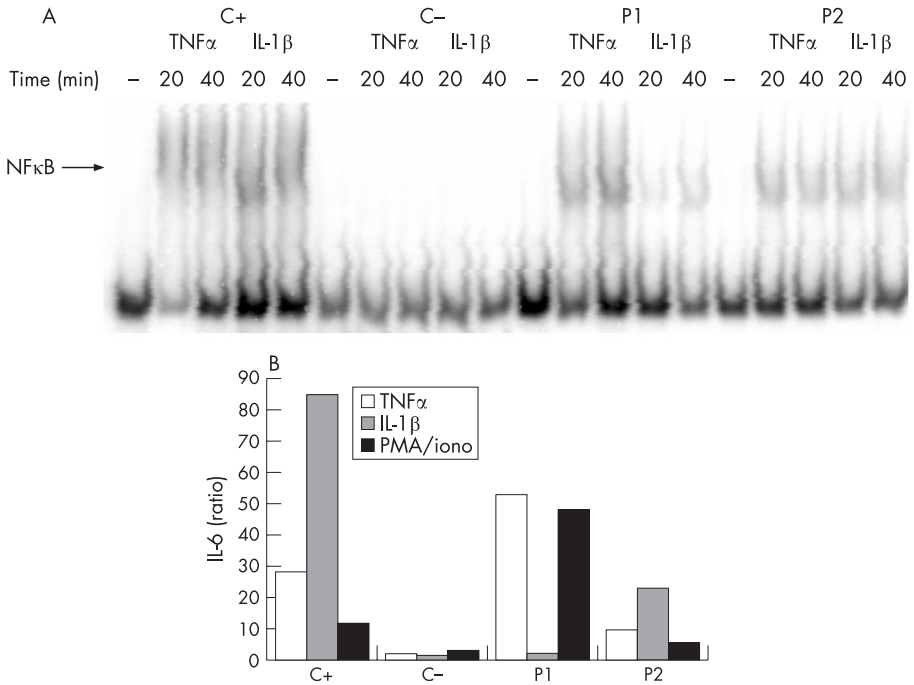

Figure 2 Stimulation of fibroblasts. (A) Nuclear protein extracts from SV40-transformed fibroblast lines from a healthy positive control (C+), a NEMO-deficient fetus negative control (C–, bearing a large *NEMO* deletion encompassing exons 4–10 (from Smahi *et al*. Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. *Nature* 2000; **405**:466–72; C–), patient 1 (P1) and patient 2 (P2) with or without stimulation with tumour necrosis factor (TNF)α (20 ng/ml), interleukin (IL)1β (10 ng/ml) for 20 or 40 min, were tested for DNA-binding activity, using the κB DNA probe, and detected by electrophoretic mobility shift assay. (B) IL-6 production by fibroblasts from a C+, a NEMO-deficient fetus (C–), P1 and P2 after stimulation with TNF-α (20 ng/ml), IL-1β (10 ng/ml) or PMA/ionomycin (10^{-7}/10^{-5} M). IL-6 production was measured by ELISA after 24 h of activation and results are presented as a ratio.

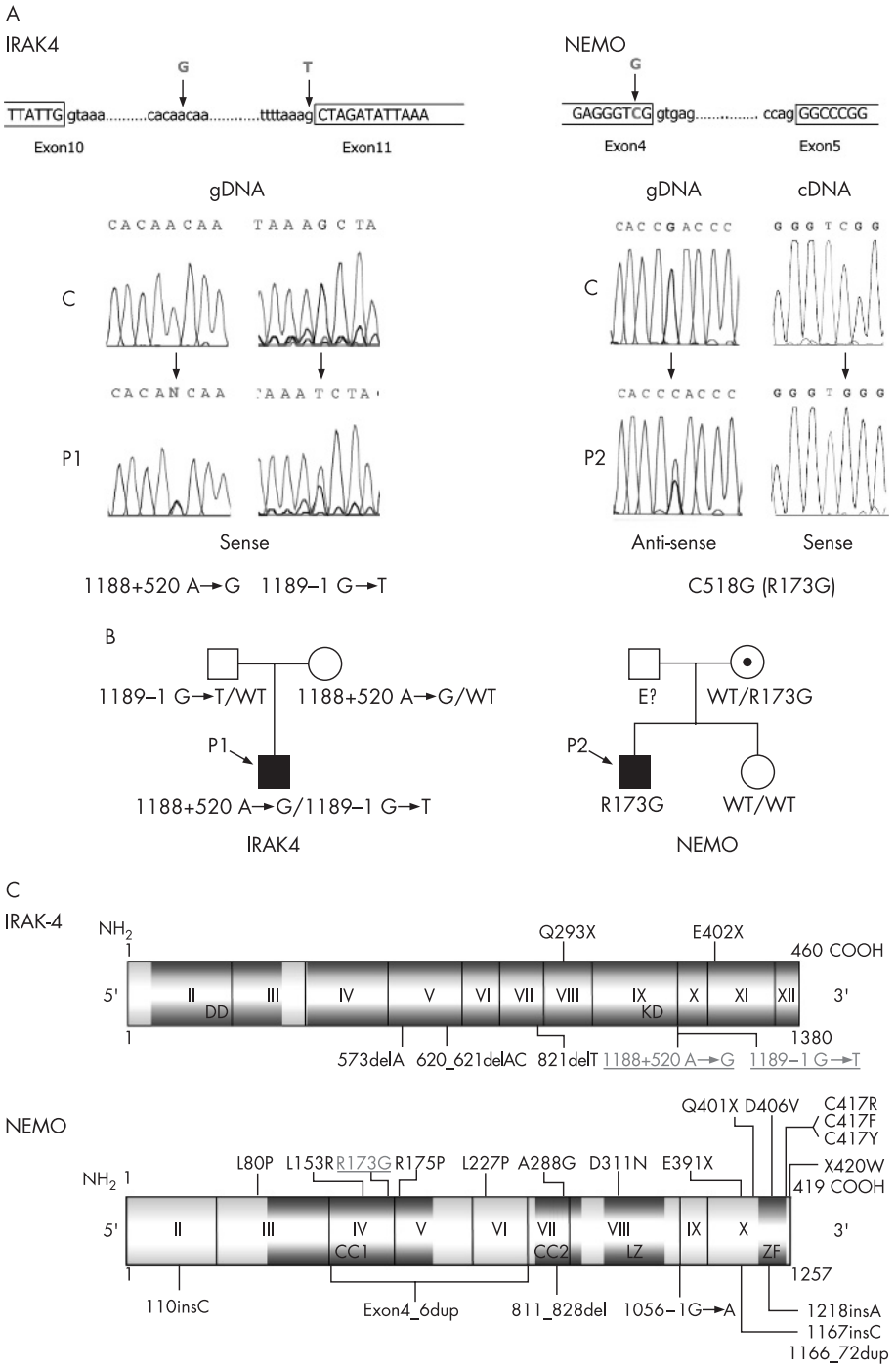

Figure 3 *IRAK4* and *NEMO* mutations. (A) Mutant (bottom) and wild-type (top) sequences from patient 1 (P1) (*IRAK4*, 1188+520 A→G/1189–1 G→T) and patient 2 (P2) (*NEMO*, R173G). The mutated nucleotides are indicated by an arrow. (B) Pedigree of the families. Patients with clinical infectious diseases are shown in black, the heterozygotic, asymptomatic carrier of the NEMO mutant allele is indicated by a small black spot. (C) Schematic representation of the IRAK4 and NEMO coding regions, with the known *IRAK4* amorphic and *NEMO* hypomorphic mutations, including the mutations found in P1 and P2 (underlined).

RESULTS

Our two patients had impaired antibody responses to pneumococcal carbohydrates. In both patients, poor clinical and biological inflammatory responses during the infectious episodes[42] and antipolysaccharide antibody deficiency[6] suggested genetic defects in toll like receptor (TLR)–NF-κB-mediated immunity.[5 6] The broader antibody deficiency in patient 2 suggested a defect further downstream in NF-κB activation.[39] After the activation of whole-blood cells with TNFα, IL-10 production was impaired in patient 2, but not in patient 1 (fig 1A), whereas IL-6 production by whole-blood cells in response to stimulation with IL-1β and lipopolysaccharide was abolished in patient 1, and mildly but reproducibly impaired in patient 2 (fig 1B).

We then tested the response to IL-1β, TNFα and PMA/ionomycin of SV40-transformed skin-derived fibroblasts from our patients, a healthy control (positive control, C+), and a NEMO-deficient fetus (negative control, C–). Fibroblasts from

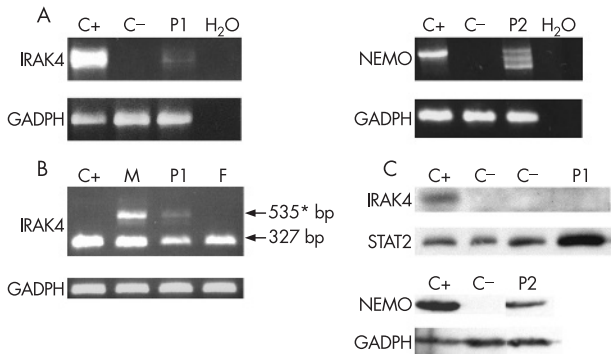

Figure 4 *IRAK4* and *NEMO* expression. (A) Left: IRAK4 expression (full-length cDNA) in fibroblasts from a healthy positive control (C+), IRAK4-deficient fibroblasts (C−, mutation Q293X[4]) and patient 1 (P1). Right: NEMO expression (full-length cDNA) in fibroblasts from a healthy positive control (C+), a NEMO-deficient fetus (C−) and patient 2 (P2). (B) *IRAK4* 3′-terminal real-time polymerase chain reaction, corresponding to a segment spanning exons 10–12; additional alternative splicing caused by the 1188+520 A→G mutation was found in P1 and his mother (indicated by *). F, father; M, mother. (C) Top: IRAK4 protein production in fibroblasts from C+, two IRAK4-deficient fibroblastic cell lines (mutations 821delT and Q293X[4]) and P1, as detected by western blotting with an anti-IRAK4 antibody. Bottom: NEMO protein levels in fibroblasts from C+, a NEMO-deficient fetus negative control (C−) and P2, as detected by western blotting with an anti-NEMO antibody.

patient 1 showed no response to IL-1β but normal responses to TNFα (fig 2A, B), whereas fibroblasts from patient 2 showed impaired responses to both IL-1β and TNFα (fig 2A, B). Overall, the pattern of response in patient 1 suggested a defect in the TLR and IL-1R signalling pathways.[4–6] By contrast, patient 2 probably had a defect downstream from the Toll/IL-1R signalling pathway, affecting the TNFR pathway to a greater extent, possibly at the level of NF-κB. The only known specific defect of the Toll/IL-1R pathway is autosomal recessive IRAK4 deficiency.[4] Mutations in *NEMO*[3 43 44] and *IKBA*[45] are associated with defects in NF-κB activation.

In patient 1, we detected two heterozygotic mutations in *IRAK4* in the intron located between exons 10 and 11 (1189–1 G→T and 1188+520 A→G) (fig 3A, B). We found no other mutation in the remaining exons. The 1189–1 G→T mutation was carried by the father and 1188+520 A→G by the mother (fig 3B). We did not detect these two mutations in 60 healthy Europeans. Reverse transcription PCR (RT-PCR) of mRNA extracted from fibroblasts of patient 1 showed only trace amounts of full-length wild-type mRNA, with no detectable alternative splicing products (fig 4A). The paternal mutation, 1189–1 G→T, located in intron 10, probably affects mRNA splicing, whereas the effect of the maternal mutation, 1188+520 A→G, also located in intron 10, is less predictable. However, patient 1 and his mother produced an alternative mRNA product, corresponding to the retention of a fragment of the intron, as detected by RT-PCR amplification of the region between exons 10 and 12 (fig 4B). This alternative mRNA was not detected in the patient's father. Western blotting showed that the IRAK4 protein was absent from the patient's fibroblasts (fig 4C), as in two previously described patients with IRAK4 deficiency.[4] These data strongly suggest that the two single-nucleotide mutations carried by the patient affect the splicing of IRAK4 mRNA, and are therefore disease-causing mutations. Patient 1 is the first IRAK4-deficient patient with non-coding mutations to be identified.

The sequencing of *NEMO* cDNA in patient 2 led to identification of the R173G mutation in exon 4 (nucleotide 518, C →G), two nucleotides away from the end of the exon (fig 3A, B). The mother of patient 2 was heterozygotic for this mutation, which was not found in 120 European controls (174 X chromosomes; fig 3B). We could not test for X inactivation in the mother's blood cells as she was homozygotic, and therefore not informative for the *HUMARA* and *FMR1* loci (data not shown). Residue R173 in NEMO is conserved in all mammalian species studied to date—rats, mice and cows (NCBI GI40018574, GI31321959 and GI59858109, respectively)—and may help to stabilise the first coiled-coil domain. RT-PCR was carried out to amplify the full-length *NEMO* cDNA, together with two additional lower-molecular-weight splicing products, corresponding to skipping of exons 4–6 and exons 5–6. The mutation therefore had an unexpected effect on mRNA splicing, probably owing to its location near the 3′ boundary of exon 4 (fig 4A). Western blotting showed that NEMO was produced but present in only small amounts in patient 2 (fig 4C). Our data show that patient 1 has autosomal recessive, complete IRAK4 deficiency, whereas patient 2 has X-linked recessive, partial NEMO deficiency. As a result, both children had recurrent IPD.

DISCUSSION

We report here on two otherwise healthy patients with recurrent IPD and mutations in *NEMO* or *IRAK4*. These two patients had no other bacterial, viral or fungal disease. All IRAK4-deficient patients previously reported had pyogenic bacterial diseases.[4 46–51] Similarly most previously reported patients with mutations in *NEMO* had a broad developmental or infectious phenotype.[3 43 44 52–54] Not all children with mutations in *NEMO* have detectable signs of EDA,[54–57] and a lack of developmental features should not exclude this diagnosis. However, patients with *NEMO* mutations and developmental abnormalities also tend to have a broad infectious phenotype, with multiple infectious diseases, unlike patient 2. The only other known patient with a mutation in *NEMO* and recurrent IPD as the sole infectious phenotype also had mild clinical EDA.[39] All IRAK4-deficient patients were reported to have multiple pyogenic bacterial diseases.[4 46–51] Together, these data show that otherwise healthy children with recurrent IPD should be explored for NEMO and IRAK4 deficiencies.

Low-level or delayed fever, or a slow or delayed rise in inflammatory markers, are suggestive of NEMO and IRAK4 deficiencies,[42 47] even if antibodies against carbohydrates have been detected. Although most patients carrying mutations in *NEMO* (including patient 2) were shown to have an impaired antibody response to polysaccharides,[5 6] all except two patients with IRAK4 deficiency (including patient 1[4 46–51]; unpublished data) had apparently normal antibody responses to carbohydrates, including pneumococcal capsular antigens. It is unclear why most NEMO-deficient patients cannot mount an antibody response to pneumococcus, whereas most IRAK-deficiency patients can. In any event, our report indicates that patients having mutations in *NEMO* or *IRAK4* may present with the narrow infectious phenotype of recurrent IPD. Otherwise healthy children with recurrent IPD should therefore be tested for disorders in the TLR–NF-κB pathway, such as NEMO and IRAK4 deficiency, notably, but not exclusively, in the presence of poor inflammation or poor antibody responses to pneumococcus.

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