

Letter to the Editor

Ectodermal dysplasia with immuno-deficiency caused by a branch-point mutation in *IKBK/NEMO*

To the Editor:

A number of novel primary immunodeficiencies have been identified during recent years through the investigation of infectious diseases at the genetic and immunologic levels. Anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) is a condition consisting of both a somatic phenotype with ectodermal dysplasia and a combined immunodeficiency predisposing to infections with bacteria, mycobacteria, fungi, and viruses. This immunodeficiency has been demonstrated to be caused by mutations in the *IKBK/NEMO* gene encoding nuclear factor kappa B (NF- κ B) essential modulator (NEMO) inherited in an X-linked pattern.¹⁻³ NEMO is a regulatory and essential subunit of the I κ B kinase complex responsible for activating the canonical NF- κ B signaling pathway through phosphorylation of the inhibitor I κ B α , leading to degradation of the inhibitor. Because of the pivotal role of NF- κ B in orchestrating proinflammatory responses, mutations in *IKBK/NEMO* cause defective NF- κ B activation and broadly impaired proinflammatory responses. Moreover, activation of NF- κ B plays a role in ectodermal

development as evidenced by the phenotype of mice deficient in the NF- κ B activator I κ B kinase α . In addition, mutations in *NFKBIA*, the gene encoding I κ B α , result in an autosomal-dominant form of EDA-ID.⁴ The full-length NEMO protein is 419 amino acid-long with a molecular weight of 48 kDa. The *IKBK/NEMO* gene, encoding the NEMO protein, is located on the X-chromosome and consists of 9 coding exons (exons 2-9) and 4 nontranslated exons and is followed by an *IKBK/NEMO* pseudogene with an inverted orientation.⁵

The patient reported here was a 9-year-old boy with an EDA-ID-like phenotype, including ectodermal dysplasia with sparse hair (hypotrichosis), abnormal conical teeth (hypodontia), and a slightly reduced number of sweat glands (hypohidrosis) (Fig 1, A). Throughout life he had an infectious phenotype characterized by recurrent bacteremia and pulmonary infections with *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas* as well as disseminated infection with atypical mycobacteria. Clinical immunologic evaluation (see Table E3 in this article's Online Repository at www.jacionline.org) demonstrated a combined immunodeficiency with normal or decreased IgG, elevated IgA, and decreased IgM levels, as well as abnormal concentrations of both B and T cells with progressive CD4 T-cell lymphopenia developing over time (see this article's

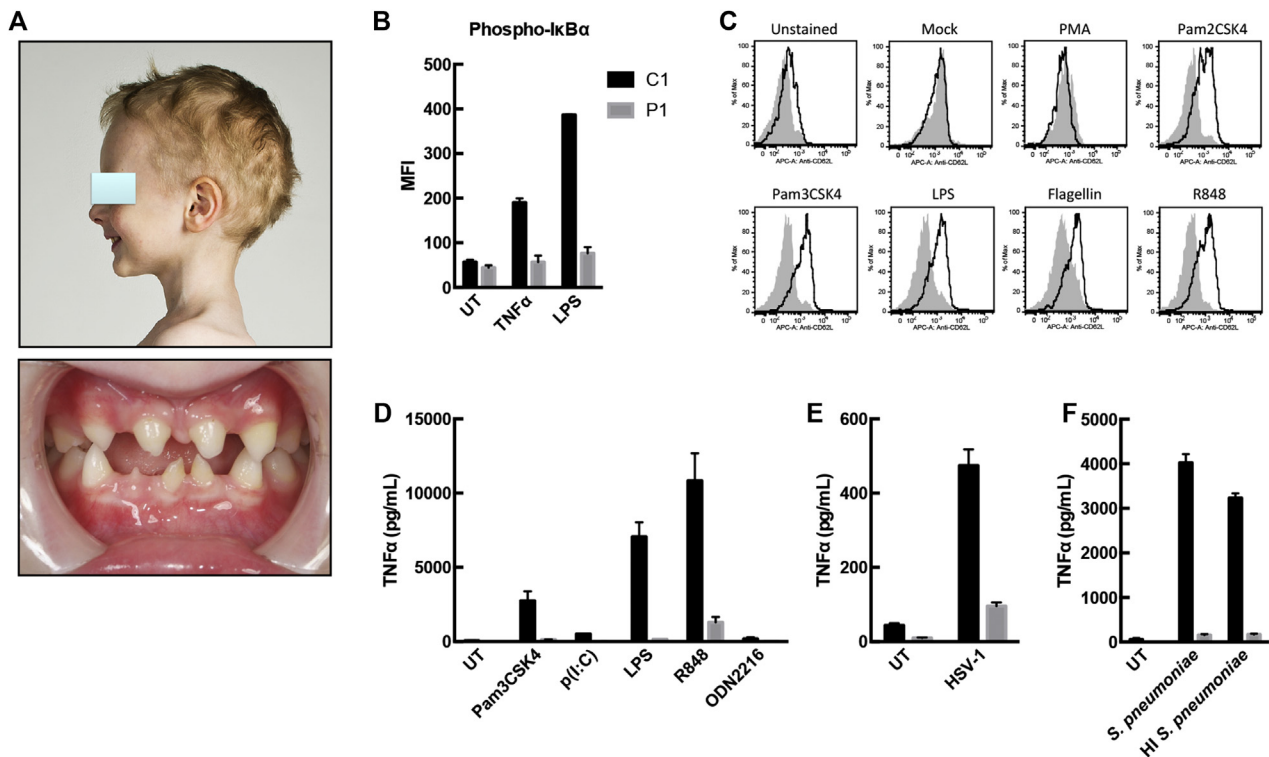


FIG 1. Impaired NF- κ B activation and inflammatory responses in patient's PBMCs. **A**, Clinical photos of the patient at age 9 years demonstrating characteristics of the EDA-ID phenotype, including hypotrichosis and hypodontia. **B**, PBMCs from the patient (P1) and control (C1) were stimulated with TNF- α or LPS for 2 hours, and whole-cell lysates were harvested and analyzed for phospho-I κ B α by Luminex technology. **C**, Full blood from P1 and C1 was stimulated for 1 hour with TLR ligands as shown and leukocytes were stained with anti-human CD62L. **D-F**, PBMCs were treated with TLR ligands, HSV-1, or *S pneumoniae* for 16 hours followed by harvest of supernatants and measurement of TNF- α by Luminex technology. HSV-1, Herpes simplex virus type 1; MFI, mean fluorescence intensity; PMA, phorbol 12-myristate 13-acetate; UT, untreated.

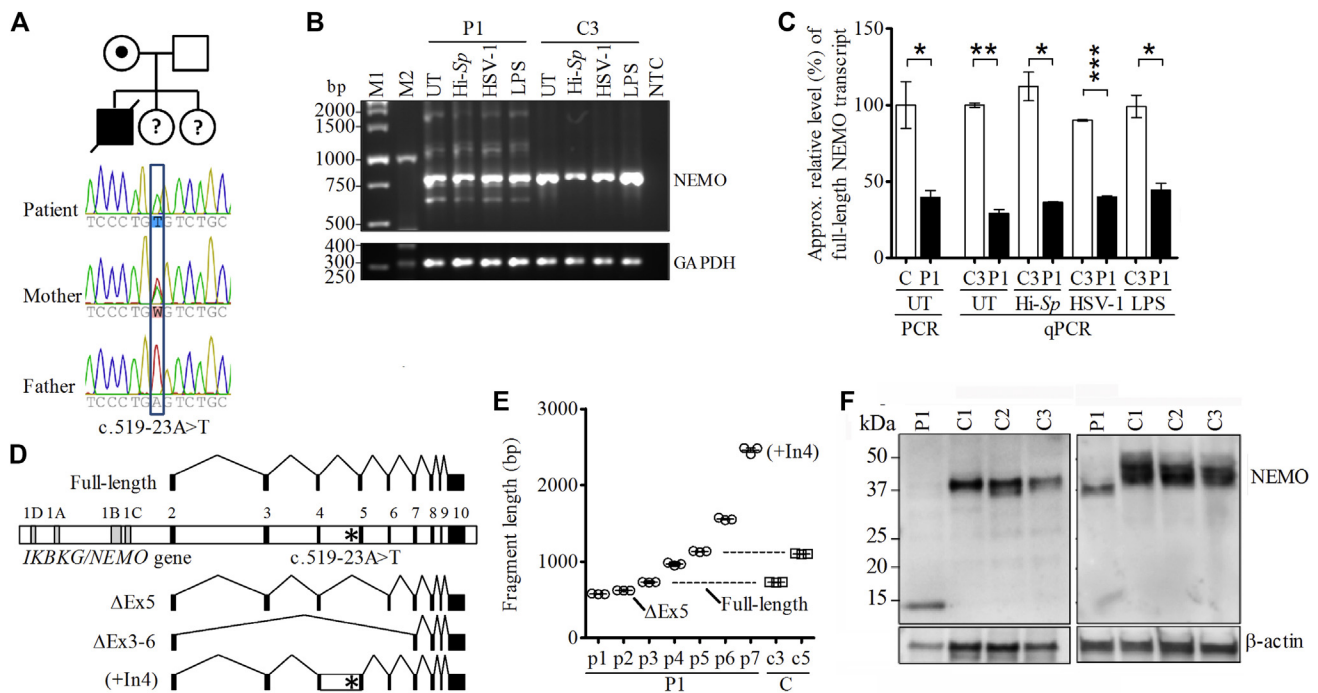


FIG 2. Identification of a novel branch-point variant in intron 4 of the *IKBKG/NEMO* gene. **A**, Pedigree demonstrating the index patient, the carrier mother, the healthy father, and 2 sisters of unknown genetic status, as well as Sanger sequencing of the *IKBKG/NEMO* gene in the patient and parents. **B**, RT-PCR analysis of NEMO transcripts (exons 2-6) from the patient (P1) and control (C3) treated as indicated. **C**, RT-qPCR of full-length NEMO transcripts in samples from P1 and C3 PBMCs left untreated or stimulated with Hi (heat-inactivated) *S pneumoniae*, HSV-1, and LPS performed with a TaqMan primer-probe set spanning the exon 4 to 5 border. **D**, Schematic overview of the impact of intron 4 c.519-23A>T variant on splicing of patient pre-mRNA. The *IKBKG/NEMO* gene harbors nontranslated exons 1D, 1A, 1B, and 1C and coding exons 2 to 10. The intron 4 variant c.519-23A>T is indicated with an asterisk. **E**, Capillary electrophoresis was used to determine the distribution and fragment length of NEMO-derived transcripts. The size of p2 corresponds to NEMO ΔEx5 transcript (~617 bp), the sizes of p3 and c3 correspond to the full-length NEMO transcript (~770 bp), and the size of p7 corresponds to the NEMO transcript harboring unspliced intron 4 (~2460 bp). For further details, see Table E2. **F**, Whole-cell lysates from P1 and controls, C1, C2, and C3, were analyzed by Western blotting for the expression of NEMO and β-actin. GAPDH, Glyceraldehyde-3-phospho-dehydrogenase; HSV-1, herpes simplex virus type 1; NTC, negative control; qPCR, quantitative PCR; UT, untreated. *P < .05; **P < .01; ***P < .005.

Medical history section in the Online Repository at www.jacionline.org.

On the basis of a clinical EDA-ID-like phenotype, previously linked to defects in NF-κB activation, we analyzed the patient and found markedly decreased IκBα phosphorylation in response to TNF-α and LPS, demonstrating impaired NF-κB activation (Fig 1, B). In addition, patient granulocytes exhibited impaired shedding of CD62 L after stimulation with all tested Toll-like receptor (TLR) agonists (Fig 1, C). Next, we examined the responsiveness of patient PBMCs to a range of proinflammatory stimuli, where we observed strongly impaired, and in some cases totally abolished, production of TNF-α, IL-6, IFN-β, and CXCL10 in response to agonists of TLR2, TLR3, TLR4, TLR7/8, and TLR9 as well as to herpes simplex virus type 1 and *S pneumoniae* (Fig 1, D-F, and data not shown.)

In search of a more comprehensive understanding of the genetic etiology of the clinical phenotype of this patient, Sanger sequencing and whole-exome sequencing were performed. However, no mutations were identified in *IKBKG/NEMO* or *NFKBIA*, the 2 known genetic etiologies of EDA-ID, or in other genes related to either severe immunodeficiency or ectodermal dysplasia (see Table E1 in this article's Online Repository at

www.jacionline.org). Therefore, we proceeded to perform full sequencing of the 5' and 3' untranslated regions, all exons, and, importantly, most of the intron sequences of the *IKBKG/NEMO* gene. By this approach, we identified a hemizygous variant located in intron 4 (c.519-23A>T), inherited from the heterozygous healthy mother (Fig 2, A). Genomic DNA from 83 healthy control females was tested, and the intron 4 variant c.519-23A>T was not detected in any of these healthy controls, providing a minor allele frequency of less than 0.006. Bioinformatic assessment of the *IKBKG/NEMO* intron 4 variant c.519-23A>T predicted that a conserved branch-point is abolished by the replacement of A with a T at this position. In addition to the predicted full-length *IKBKG/NEMO* transcript of 770 base pairs, a spectrum of shorter and longer *IKBKG/NEMO*-derived transcripts appeared when amplifying *IKBKG/NEMO* exons 2 to 6 (Fig 2, B). Importantly, the levels of the full-length NEMO transcript and transcripts harboring NEMO exons 4 to 5 were significantly reduced (30% to 40%) in PBMCs from the patient compared with controls, irrespective of whether cells were untreated or stimulated with *S pneumoniae*, HSV-1, or LPS (Fig 2, C). Indeed, the presence of the full-length *IKBKG/NEMO* transcript in patient cells is in accordance with reports describing

that branch-point mutations may lead to reduced but not fully abolished correct splicing.⁶ Subsequent sequencing demonstrated the presence of NEMO transcripts lacking exon 5 (NEMO Δ Ex5) and NEMO transcripts lacking exons 3, 4, 5, and 6 (Δ Ex3-6) in the patient's cells, thus providing evidence of exon skipping as a consequence of the mutation. Moreover, transcripts longer than the full-length NEMO transcript were observed, possibly representing missplicing of intron 4, generating transcripts harboring full or truncated intron 4 stretches (Fig 2, B and D; see Table E2 in this article's Online Repository at www.jacionline.org). Finally, Western blotting using a polyclonal anti-NEMO antibody detected only very scarce amounts of NEMO protein in lysates from patient's PBMCs (Fig 2, F). This finding suggests that abnormal *IKBK/NEMO* transcripts are either not translated or undergo rapid protein degradation, thereby resulting in severely impaired NEMO function. Interestingly, we detected at least 2 truncated smaller bands of 15 kDa and 37 kDa in patient cells, which may represent abortively translated abnormal *IKBK/NEMO* transcripts.

Since the discovery that mutation in the *IKBK/NEMO* gene is the primary cause of EDA-ID, various mutations have been identified in all parts of the gene, but only very few reports have described mutations in the noncoding regions of *IKBK/NEMO* leading to an EDA-ID phenotype. First, a mutation in the splice donor site in intron 6 has been reported to cause skipping of exons 4 to 6, resulting in a detectable, truncated NEMO protein of approximately 35 kDa instead of the normal 48 kDa.⁷ Another mutation has been identified in the splice acceptor site of intron 6 of the *IKBK/NEMO* gene, generating several aberrant transcripts and severely decreased levels of full-length NEMO protein.⁸ Finally, a mutation in the 5' untranslated region, which destroys the splice donor site of exon 1B, results in decreased NEMO protein expression despite intact *IKBK/NEMO* coding sequence and translational start site.⁹

To our knowledge, this is the first description of a branch-point mutation in *IKBK/NEMO* as the genetic etiology of EDA-ID. Branch-point mutations are very rarely identified as genetic causes of human disease. To date, only few diseases caused by branch-site mutations have been described, including Marfan syndrome, Niemann Pick disease, Long QT syndrome, and congenital erythropoietic porphyria, in most cases resulting in abnormal exon skipping, although they may also cause retention of an entire intron or fragment.¹⁰ However, branch-point mutations have not previously been reported as the genetic mechanism responsible for causing EDA-ID or any other primary immunodeficiencies. This rare variant in intron 4 was not identified by conventional Sanger sequencing or whole-exome sequencing, underscoring the importance of detailed genetic analysis of both coding and regulatory sequences of the *IKBK/NEMO* gene in patients with a clinical phenotype suggesting defects in NF- κ B activation.

Here, we identify a novel branch-point mutation in intron 4 of the *IKBK/NEMO* gene in a 9-year-old patient with an X-linked EDA-ID-like phenotype. The mutation leads to abnormal *IKBK/NEMO* mRNA splicing and largely abolished expression of full-length NEMO protein. We propose the identified mutation to be causative for the disease in this patient and believe that our work represents the first report of a branch-point mutation as the genetic origin of EDA-ID. Despite the fact that many pathogen and cytokine-induced signaling pathways ultimately converge to activate NF- κ B and downstream proinflammatory responses,

mutations in the genes encoding NEMO and $\text{IKB}\alpha$ remain the sole known origins of EDA-ID at present.

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METHODS

Medical history and clinical immunologic evaluation

Medical history. The patient presented here had Caucasian ancestry with Danish nonconsanguineous parents and no family history of primary immunodeficiencies. His parents as well as his 2 younger sisters were healthy and without recurrent infectious episodes. The boy followed the routine Danish vaccination program, including vaccination against diphtheria, tetanus, pertussis, polio, *Haemophilus influenzae* type B, measles, mumps, rubella, and pneumococci.

The somatic phenotype was evaluated by dermatologists and dentists. He had sparse, lightly pigmented scalp hair (hypotrichosis) without alopecia (Fig 1, A). He also had hypodontia with characteristic teeth with a conical appearance (Fig 1, B). Because of failure of resorption of the primary incisors, extractions had been necessary to allow permanent teeth eruption. He displayed no midfacial hypoplasia. The skin was described as thin, dry, and also lightly pigmented and with common atopic eczematous eruptions. He had axillary sweating and the number and the position of sweat glands in the fingertips was normal and corresponded to the sweat glands of his parents, examined on microscopy of an imprint of their fingertips. The phenotypic appearance was therefore compatible with hidrotic ectodermal dysplasia.

The infectious phenotype of the patient was characterized by recurrent pulmonary infections with gram-positive and gram-negative bacteria as well as atypical mycobacteria but no apparent predisposition to viral or fungal infection. Until the age of 3 months, he was thriving and without infections but was then admitted to hospital with rapidly progressing *Escherichia coli* sepsis, urinary tract infection, and respiratory distress. One month later, he experienced another urinary tract infection and underwent circumcision due to phimosis, which prevented further urinary tract infections. Ultrasonography revealed no malformations of the urinary tract.

When he was 17 months old, he was admitted to the hospital with his seventh episode of sepsis and besides the initial *E coli* infections, blood cultures grew *S pneumoniae* and *S aureus*. Three times he presented with Group B Streptococcus (*S agalactiae*). Since infancy he had experienced recurrent pneumonias with *S aureus*, *Pseudomonas aeruginosa*, *S pneumoniae*, and *H influenzae*. These infections were invasive and aggressive, and once led to osteomyelitis. The infectious parameters including C-reactive protein and leukocyte count were raised during infectious episodes, although maybe less than would have been expected, and the clinical and paraclinical response to intravenous antibiotic treatment was appropriate.

Although serum immunoglobulins were found within normal range, intravenous immunoglobulin treatment was started at the age of 2 years, which had a significant effect on the severity and the frequency of his infectious episodes. Nevertheless, he experienced recurrent ear infections with discharge and need for repeated ventilation tube insertions. *H influenzae*, *S aureus*, and *P aeruginosa* were cultured from the ear discharge. As a complication to pneumonia at the age of 7 years, he experienced subsegmental

pulmonary emboli in the lungs and anticoagulant treatment was initiated. Subsequently, frequent pulmonary infections recurred, his general condition deteriorated, and he became cachectic, requiring parenteral nutrition and later a gastrostomy tube. Eventually, *Mycobacterium avium-intracellulare* was cultured from bronchoalveolar lavage fluid and from a bone marrow aspirate. Despite antimycobacterial treatment with clarithromycin, rifampicin, ethambutol, and amikacin for 18 months, *Mycobacterium avium-intracellulare* was repeatedly demonstrated in his bone marrow, and eventually with mycobacterial resistance toward ethambutol, moxifloxacin, ciprofloxacin, amikacin, linezolid, and clarithromycin, leading to a change in the antituberculous medication to ethambutol, moxifloxacin (based on preserved sensitivity *in vitro* to the combination of these 2 drugs), rifabutin, and bedaquiline (on an experimental basis for children). The boy remained in a poor general condition with cachexia, ascites, recurrent bacterial pneumonias, and significant CD4 T-cell lymphopenia. Later, patchy changes in basal ganglia were demonstrated on a magnetic resonance of the cerebrum, prompting antifungal treatment with AmBisome on suspicion of a fungal etiology, although he was clinically without neurological symptoms. Despite the presence of atypical mycobacterial infection in the bone marrow, which was impossible to clear given the severely immunosuppressed condition of the patient, the treatment strategy was to prepare for bone marrow transplantation. Unfortunately, however, the patient died during the preconditioning period.

Clinical immunologic evaluation. Table E3 presents the results of the routine immunologic evaluation of the patient. These results revealed a combined immunodeficiency.

Concentrations of plasma immunoglobulins were abnormal. The IgA level was high (up to 9 g/L), and the IgM level was intermittently low (minimum 0.3 g/L). IgG levels were normal or decreased, and IgG supplementation was associated with a marked reduction in infections. Lymphocyte blood concentrations were normal until age 7 years, whereafter concentrations were intermittently low (minimum 700/ μ L) and the CD4 count decreased to 100 cells/ μ L. Lymphocyte phenotyping at age 9 years revealed low concentrations of T and natural killer cells whereas B-cell concentration was normal. T-cell subset distribution was abnormal, with approximately equal percentages of CD4+ and CD8+; 29% were CD4-CD8 double-negative, and 38% were TCR $\alpha\beta$ -negative. B-cell subset distribution was also abnormal, with near absence of isotype-switched memory B cells and plasmablasts, suggesting impaired T-cell interaction and/or intrinsic B-cell defect. In accordance, concurrent *in vitro* proliferation of PBMCs after recall-antigen stimulation was absent (purified protein derivative, *S aureus*, *Candida albicans*, and tetanus toxoid) and reduced after stimulation with B-cell mitogen (pokeweed mitogen). The T-cell mitogen (PHA) induced low but normal proliferation—in spite of a low T-cell proportion—ruling out a general proliferation defect. Finally, neutrophil granulocyte oxidative burst was decreased, although not to an extent compatible with chronic granulomatous disease, and granulocytes had normal densities of adhesion antigens (CD11A, CD11B, CD11C, CD18, and CD15). Finally complement activation was normal.

TABLE E1. List of rare gene variants, inheritance, allele frequency, and CADD score identified in the patient (P1) by whole-exome sequencing

Gene	Transcript	Inheritance	Zygosity	cDNA	Protein	ExAC allele frequency	CADD score
FANCB	NM_001018113.1	Mother	Hemizygous	c.1570C>G	p.Q524E	5/87355~0	14.37
ERBB2IP	NM_001253699.1	<i>de novo</i>	Het wt/var	c.1186A>G	p.M396V	3/120426~0	22.8
ERBB2IP	NM_001253699.1	Father	Het wt/var	c.1862T>C	p.I621T	0.001	24.8
ERBB2IP	NM_001253699.1	Mother	Het wt/var	c.3335C>T	p.S1112L	0.084	25.7
PGM3	NM_001199917.1	Mother	Het wt/var	c.1696C>A	p.P566T	0.001	18.21
IFIH1	NM_022168.3	Father	Het wt/var	c.478A>G	p.N160D	0.0006	24.2
PRF1	NM_001083116.1	Father	Het wt/var	c.1310C>T	p.A437V	0.001	24.7

CADD, Combined annotation dependent depletion; *Het*, heterozygous; *var*, variant; *wt*, wild-type.

TABLE E2. NEMO transcript sizes and relative amounts

Target	Fragment*	Transcript ID	Stimulus†	Size‡: bp ± SEM	Level ± SEM (as % of total NEMO)§	Level ± SEM (as % of GAPDH)
NEMO Exon 2-6	c3	Full-length	UT	730 ± 2	95.2 ± 0.4	84.6 ± 12.9
			Hi- <i>Sp</i>	726 ± 4	92.9 ± 1.0	96.1 ± 16.1
NEMO Exon 2-6	c5	ND	UT	1102 ± 3	4.8 ± 0.4	ND
			Hi- <i>Sp</i>	1109 ± 11	7.1 ± 0.9	ND
NEMO Exon 2-6	p1	ND	UT	577 ± 2	3.8 ± 0.1	ND
			Hi- <i>Sp</i>	580 (n = 1)	2.8 (n = 1)	ND
NEMO Exon 2-6	p2	ΔEx5	UT	621 ± 1	9.1 ± 1.1	5.8 ± 1.4
			Hi- <i>Sp</i>	619 ± 2	4.4 ± 0.9	1.3 ± 0.3
NEMO Exon 2-6	p3	Full-length	UT	732 ± 2	54.8 ± 1.8	33.5 ± 3.7
			Hi- <i>Sp</i>	732 ± 1	66.2 ± 4.1	20.0 ± 2.7
NEMO Exon 2-6	p4	ND	UT	967 ± 11	4.2 ± 0.8	ND
			Hi- <i>Sp</i>	993 ± 5	5.5 ± 0.4	ND
NEMO Exon 2-6	p5	ND	UT	1133 ± 5	3.6 ± 0.4	ND
			Hi- <i>Sp</i>	1148 ± 8	4.0 ± 0.6	ND
NEMO Exon 2-6	p6	ND	UT	1557 ± 10	12.6 ± 0.3	ND
			Hi- <i>Sp</i>	1601 ± 15	10.6 ± 1.5	ND
NEMO Exon 2-6	p7	(+In4)	UT	2460 ± 27	12.0 ± 0.4	ND
			Hi- <i>Sp</i>	2468 ± 32	8.4 ± 1.4	ND
NEMO Exon 2-10	px	ΔEx3-6	Pam ₃ CSK ₄	633 (n = 1)	ND	ND

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; *ND*, not done; *px*, non-sequenced fragment; *UT*, untreated.

*The different fragments were denoted according to size (bp), and whether the fragment was found in control, c, or patient, p. Transcripts present in both control and patient, and with similar size, have been assigned the same number.

†Heat-inactivated *S pneumoniae* (Hi-*Sp*) or Pam3CSK4 was used *in vitro* to stimulate PBMCs from patient and control.

‡The average sizes largely correspond to the expected sizes: 770 bp (full-length transcript), 617 bp (ΔEx5 transcript), and 639 bp (ΔEx3-6 transcript).

§The amount of each transcript was normalized to the amount of total NEMO-derived transcripts (p1-p7) and given as average (in %) ± SEM.

||The amount of selected transcripts was normalized to the amount of GAPDH transcript(s) and given as average (in %) ± SEM.

TABLE E3. Lymphocyte subpopulations

Analysis	Phenotype	Patient*	Normal†
Lymphocyte count		700 cells/ μ L	1000-4700 cells/ μ L
Lymphocyte distribution		T cells: 44%	T cells: 64%-86%
		B cells: 53%	B cells: 4.8%-18%
		NK cells: 1.3%	NK cells: 4.1%-29%
T cells (CD3+)			
CD4+CD8–		33%	44%-80%
CD4–CD8+		38%	18%-52%
CD4–CD8–		29%	1.0%-12%
CCR7+CD45RA+CD27+	Naive	60% of CD4+	25%-73% of CD4+
		20% of CD8+	6.8%-73% of CD8+
TCR $\alpha\beta$ +		62%	>85%
CD16+ and/or CD56+	NKT	31%	<20%
B cells (CD19+)			
IgD+IgM+CD27–CD38–	Naive	96%	41%-79%
IgD+IgM+CD27–CD38+	Transitional	1.7%	1.5%-10%
IgD–IgM–CD27+CD38–	Memory	0.1%	4.8%-28%
IgD–CD27+CD38+	Plasmablasts	<0.1%	0.1%-0.9%
IgD+IgM+CD27+CD38–	Marginal zone-like	0.5%	4.7%-39%

NK, Natural killer.

*Measurements as of March 2015.

†95% percentile range.