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Clinical characteristics of patients with low functional IL-6 production upon TLR/IL-1R stimulation

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Capsule summary

Low IL-6 production upon stimulation by TLR/IL-1R ligands is associated with increased susceptibility to bacterial skin infections and to invasive infections with *P. aeruginosa* and/or with a IRAK-4/MyD88/NEMO defect.

Key words

ELISA; TLR; TIR; NF- κ B; Toll-interleukin-1 receptor superfamily; Myd88; IRAK-4; NEMO

Abbreviations

– Copy number variant	CNV
– Ectodermal dysplasia	EDA
– Enzyme-linked immunosorbent assay	ELISA
– IFN-regulatory factor	IRF
– IL-1 receptor-associated kinase	IRAK

50	– Interleukin-1 receptor	IL-1R
51	– Interleukin-6	IL-6
52	– Myeloid differentiation factor 88	MyD88
53	– Peripheral blood mononuclear cells	PBMCs
54	– Single nucleotide polymorphism	SNP
55	– Toll-interleukin-1 receptor	TIR
56	– Toll-like receptor	TLR

57 Author's contributions

58 Glynis Frans and Xavier Bossuyt designed the study. Glynis Frans, Leen Moens, Margaretha Wuyts,
59 Wim Meert, Joris R. Vermeesch and Joris Delanghe gathered the data and performed laboratory
60 experiments. Glynis Frans and Matthew S. Hestand analyzed the data. All authors vouch for the data
61 and the analysis. Heidi Schaballie, David Tuerlinckx, Maria De Bie, François Vermeulen, Jutte Van
62 der Werff ten Bosch, Rik Schrijvers and Isabelle Meyts supervised the clinical follow-up of the
63 included patients. Glynis Frans and Xavier Bossuyt wrote the manuscript. All authors agreed to
64 publish the paper.

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To The Editor.

The NF- κ B pathway is activated by stimulation of a wide range of receptors, including members of the Toll-interleukin-1 receptor (TIR) superfamily. The TIR superfamily encompasses Toll-like receptors (TLRs) and members of the interleukin-1 receptor (IL-1R) family¹. TLRs sense microbial products and play an important role in immunity. Defects in the NF- κ B pathway have been associated with recurrent invasive pneumococcal and/or staphylococcal disease, recurrent pyogenic infections (e.g. cellulitis), recurrent mycobacterial disease, or severe viral infections². Mutations in *IRAK4* and *MyD88* are typically associated with life-threatening infections with *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*³. Mutations in *NEMO/IKBKG* are mostly associated with X-linked recessive anhidrotic ectodermal dysplasia (EDA) with immunodeficiency (EDA-ID) syndromes⁴. Standard immunological laboratory tests such as blood and differential cell counts, lymphocyte subsets, and complement function are normal in most patients suffering from defects in the NF- κ B pathway. Immunoglobulin levels can be variable in *MyD88*, *IRAK-4*, and *NEMO* deficiencies²⁻⁵.

Diagnosis of defects in the NF- κ B pathway is challenging and requires specific laboratory evaluation of TLR function. This can be done by ELISA measurement of IL-6 production by peripheral blood mononuclear cells (PBMCs) after stimulation with various TIR superfamily ligands for 24h⁵. Over the past 6 years, we have used such functional approach to screen for TLR/NF- κ B defects as part of the routine immunological diagnostic work-up. Based on our data, we extracted reference values and clinically characterized patients with a low IL-6 response to TIR stimulation.

A total of 770 patients were analyzed for IL-6 production by TLR and IL-1R ligands (including LPS, Zymosan, IL-1 β , Pam₃CSK₄, FSL-1, Imiquimod, ORN-02, Poly I:C, and heat inactivated *Streptococcus pneumonia* serotype 3; see the Online Repository for more information) between April 2008 and November 2014. The age of the patients ranged between 1 month and 65 years with a median age of 4 years. Patients were referred for testing because of invasive infections, recurrent non-invasive bacterial infections, or severe viral infections. In total, 78% of the cases were referred with a

history of recurrent pyogenic bacterial infections (n=601). Due to the difficulty of recruiting enough healthy children to establish reference values, we extracted reference values from the totality of submitted samples by applying the Bhattacharya algorithm⁶ (see Table E1 in the Online Repository). More information on the application of this algorithm in our patient population is documented in the Online Repository.

Applying the established cut-off values, we classified the response to each TIR ligand as normal or abnormal. 584 patients were only tested once and 104 of them had an abnormal response to one of the ligands. These 104 patients were not retested due to several reasons; improvement of clinical presentation, loss to follow-up, or another diagnosis established and were excluded for further analysis. In 186 patients, TLR functional testing was performed on at least two occasions. In 89/186 cases, a normal response was confirmed and in 75/186 cases, an abnormal response could not be confirmed. In the group of 186 patients, 17 had a confirmed abnormal response to the same TIR ligand and 5 patients had an abnormal response to another ligand than in the first test. See Figure E1 in the Online Repository for a summary on the patient categorization.

Thus, we identified 22 patients in whom we documented a confirmed (on at least two occasions) abnormal low IL-6 response to TIR ligands. Figure 1 displays the IL-6 production of these 22 patients compared to the cut-off values and responses (median + IQR) from all 770 patients. Clinical presentations and laboratory anomalies for these 22 cases are summarized in Table E2 in the Online Repository. In these patients, *IRAK4*, *MYD88*, and *IKBKG* coding regions and intron-exon boundaries were sequenced with Sanger or next-generation sequencing (see Table E3 in the Online Repository). Patient 8 had a homozygous *IRAK4* stop mutation (p.Q239*, rs121908002) and patients 11, 14, and 21 had hemizygous *IKBKG* variants (rs201709278, rs179363896, and rs148695964, respectively). Though rs179363896 and rs201709278 are likely benign^{4,7,8}, patient 8 and 21 variants rs121908002 and rs148695964 have previously been described as pathogenic^{8,9}. An additional 6 patients had *IRAK4* or *IKBKG* heterozygous coding or splice-site variants. It is possible that unobserved deletions could be present on the other allele upstream or downstream of these variants. Hence, future copy-number

variant (CNV) approaches could complement the sequencing analysis to determine compound heterozygous SNP-CNV defects.

Figure 2 gives an overview of the infectious and microbiological histories of the 22 patients with a deficient IL-6 production upon TIR stimulation in comparison to 368 patients without such functional defect and accessible medical health records. See Table E4 and Table E5 in the Online Repository for more detailed information. Bacterial skin infections (e.g. impetigo, cellulitis, furunculosis, and sur-infected eczema) were significantly more present in the group of patients with impaired IL-6 production upon TIR stimulation (50.0% vs. 22.6%, $p=0.0079$). This fits with the observation that bacterial skin infection is the most frequently observed non-invasive bacterial disease in IRAK-4 and MyD88-deficient patients³. Non-invasive infections with *S. pneumoniae* tended to be associated with impaired IL-6 production upon TIR stimulation (28.0 % vs. 12.8%, $p=0.06$). For comparison, in a cohort of IRAK-4 and MyD88-deficient patients, prevalence of non-invasive infections with *S. pneumoniae* was 16%³. The prevalence of invasive infections with *P. aeruginosa* in patients with impaired IL-6 production upon TIR stimulation was significantly higher than the prevalence in patients without such impaired functional response (25% vs. 0%, $p=0.0089$), and comparable to the reported prevalence of invasive *P. aeruginosa* infections in IRAK-4 and MyD88-deficient patients (19.7%)³. Bacterial meningitis was also more frequent in the group of patients with impaired IL-6 production, albeit not statistically significant (13.6% vs. 4.3%, $p=0.08$) (probably because of the low number of patients affected). The frequency of invasive infections with *S. aureus* and *S. pneumoniae* in patients with impaired IL-6 induction upon TIR stimulation was 25%, which was lower than the reported frequency of such infections in IRAK-4 and MyD88-deficient patients (41,2% and 51,5%, respectively)³.

We also compared infectious and microbiological histories of the patients with a normal IL-6 response to TIR ligands to those with an initial abnormal response that normalized upon re-testing ($n=48/75$ with accessible medical health records). No differences were found between these two groups (data not shown), except for the number of sepsis episodes, which was lower in the group of patients with a

fully normal response (12% versus 23%) ($p=0.04$). This underscores the need for repeated assessment over time.

In conclusion, we studied production of IL-6 by PBMCs after stimulation with various TIR ligands in 770 patients presenting with increased susceptibility to infection. We identified 22 patients with confirmed impaired production of IL-6 after TIR stimulation, including one IRAK-4 deficient and one NEMO immunodeficiency patient. The other 20 patients presented with a phenotype reminiscent of a (mild) IRAK-4 or MyD88 deficiency, but we did not identify pathogenic mutations in these genes.

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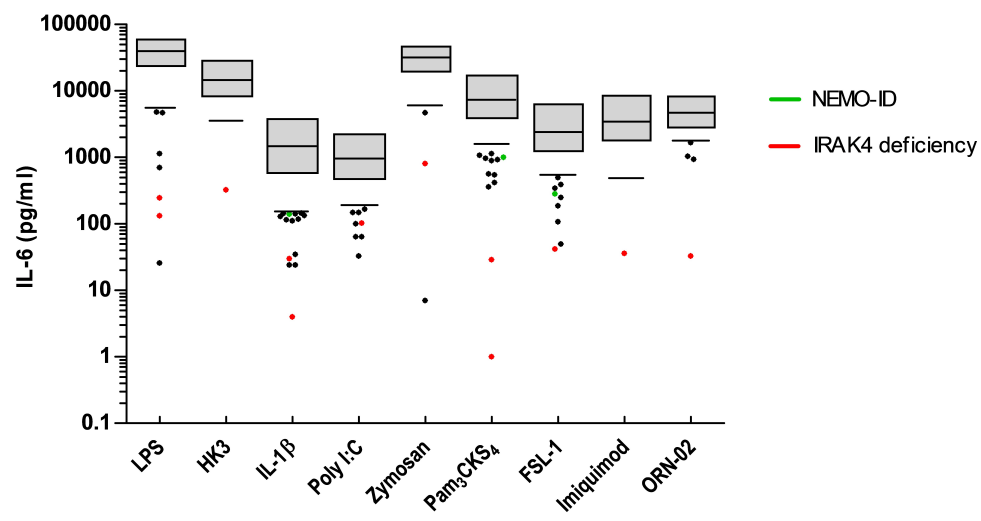
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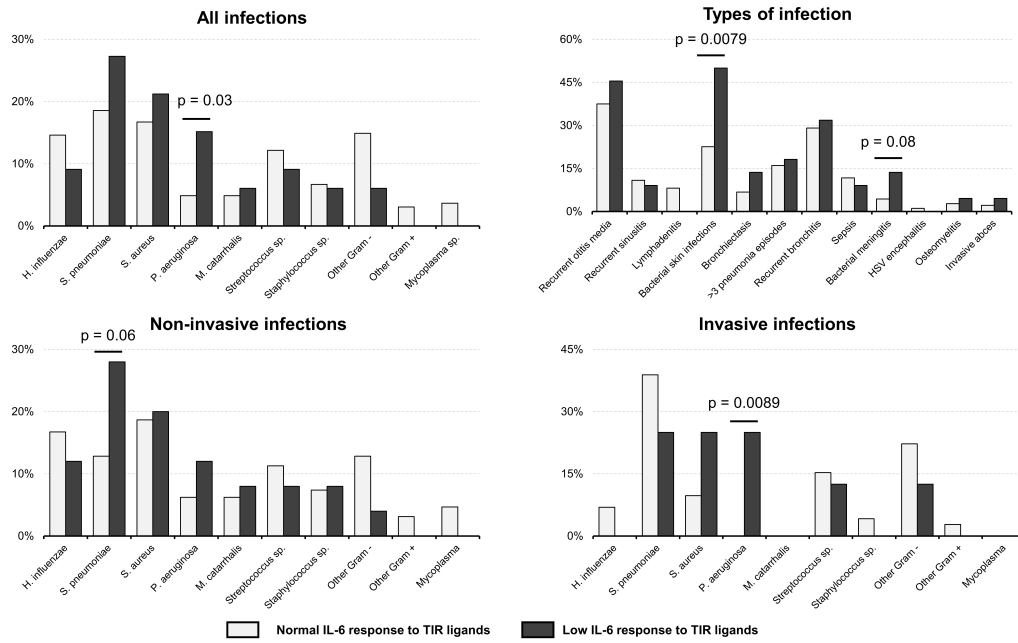
Figure legends**Figure 1.** IL-6 production by PBMCs from 770 patients.

Dots represent individual IL-6 measurements from 22 patients with a confirmed abnormal response to TIR ligands. For each ligand, only those results that fell below the cut-off value are displayed. Green dots indicate results from patient 21 with NEMO-ID (rs148695964). Red dots indicate results from patient 8 with IRAK-4 deficiency (rs121908002). Boxplots (median $\pm$ IQR) represent patients with normal IL-6 responses after TIR stimulation. Black lines below the boxes indicate the established cut-off values for each TIR ligand.

Figure 2. Infectious and microbiological histories.

Data on the type of infections and their microbiological cause (divided into invasive and non-invasive infections) for patients tested for IL-1R and TLR function from whom medical health records were available. Patients with a normal IL-6 response (n = 368) were compared to patients with a low IL-6 response (n = 22) to TIR ligands. Relevant p-values are displayed.





Online Repository

Methods

In vitro evaluation of NF-κB function

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized peripheral blood by centrifugation through Ficoll. PBMCs were seeded at 2×10^6 cells/ml in a 96-well culture plate and stimulated for 24 hours with different TLR or IL-1R ligands: LPS (TLR4, 37.5 µg/ml), Zymosan (TLR2, 10 µg/ml), IL-1β (IL1R, 50 ng/ml), Pam₃CSK₄ (TLR1/2, 1 µg/ml), FSL-1 (TLR2/6, 100 ng/ml), Imiquimod (TLR7, 10 µg/ml), ORN-02 (TLR8, 1.25 µg/ml), Poly I:C (TLR3, 12.5 µg/ml), and heat inactivated *Streptococcus pneumonia* serotype 3 (1.2×10^8 CFU/ml)^{E1}. The next day, cells were pelleted by centrifugation and the supernatant was frozen at -20°C. IL-6 was measured by a commercial ELISA kit, according to the manufacturer's instructions (BD Bioscience, San Jose, California).

Determination of cut-off values for IL-6 production after TLR/IL-1R stimulation

As it was difficult to recruit a large number of healthy children (including different age ranges), we were unable to determine pediatric reference values. Cut-off values for IL-6 production after TLR/IL-1R stimulation were therefore determined by application of the modified Bhattacharya algorithm, which can be applied to the total bulk of unselected results (normal and abnormal)^{E2,E3}. As defects in TLR/NF-κB pathway are rare, it was expected that max. 5% of a patient population would be abnormal, which enables use of the Bhattacharya algorithm^{E2}. The algorithm, a so-called indirect method, has already been employed for several parameters in laboratory hematology and clinical chemistry^{E3-E7}.

Cut-off values were calculated as the 0.1th percentile of the normal or gamma distribution extracted out of the population using the modified Bhattacharya analysis^{E2,E3}. The underlying assumption of the Bhattacharya plot is that the original frequency distribution is Gaussian. If the original distribution is not Gaussian, log transformation of the frequency distribution was performed^{E4}. If the assumption was not reached (even after transformation), cut-off values were calculated as the 5th percentile of the original frequency distribution. The IL-6 production induced by Zymosan and LPS proved unfit for transformation, therefore the 5th percentiles were used as cut-off values (Table E1). This is in line with the cut-offs derived from the Bhattacharya analysis which varied between the 4th percentile and the 13th percentile (with as median the 8th percentile).

Application of the Bhattacharya algorithm to determine IL-6 cut-off values after IL-1β stimulation is demonstrated in Figure E2. Figure E2A (original) and E2B (log transformed) illustrate the frequency distributions of IL-6 production induced by IL-1β. The log transformed data were used to establish Bhattacharya plots that were used to fit a Gaussian or gamma curve. The gamma curves were

preferred because they fitted the Bhattacharya plots best for all ligands. Figure E2C and E2D illustrate the gamma curve and gamma distribution for IL-6 production after IL-1 β stimulation.

The cutoff values obtained by the modified Bhattacharya algorithm were validated by testing 26 healthy adults [median age 29 years: range 22-65 years old] and 6 healthy children for cytokine production after stimulation with TIR ligands. Figure E3 illustrates the individual results obtained in these healthy controls plotted on the interquartile range and cutoff value obtained by the modified Bhattacharya algorithm applied on the patient population. Results obtained in the healthy controls fitted within the reference ranges derived from the diseased controls, except for one pediatric sample that fell below the cut-off for Poly I:C.

For most TLR ligands, IL-6 production was only weakly associated with age (rs-values $\approx$ 0,20). Therefore, reference values were not stratified according to age. An association of IL-6 production by TIR ligands with gender was also not observed, although the X-linked *IKBKG* gene, essential for canonical NF- κ B activation, escapes X-inactivation^{E8}. Nevertheless, NEMO expression was not higher in female lymphoblastoid cell lines indicating no significant impact on protein expression^{E9}.

Patient clinical and laboratory data

A total of 770 patients were included in the study. See Figure E1 in the Online Repository for a summary on the patient categorization. Clinical and microbiological data were obtained from the electronic medical health records of 525 patients referred to the University Hospitals Leuven. The retrospective analysis was approved by the institutional ethics committee. Serum immunoglobulin concentrations, antibody titers after vaccination, leukocyte/lymphocyte subsets, and T-cell proliferation responses were measured in the clinical laboratory of the University Hospitals Leuven and Brussels. Results were compared with laboratory specific, age-related reference values.

Genetic analysis of IRAK4, MYD88 and IKBKG

Molecular analysis of the *IRAK4*, *MYD88*, and *IKBKG* coding regions and intron-exon boundaries were performed with traditional Sanger sequencing or next-generation sequencing as described below.

Sanger sequencing was performed with primer sets to amplify each of the individual *IRAK4*, *MYD88*, and *IKBKG* exons using Accuprime I Supermix (Thermo Fisher), Accuprime II Supermix (Thermo Fisher), or GoTaq Hot Start Polymerase (Promega). PCR reactions were performed on a Veriti 96-well thermal cycler (Applied Biosystems). Primers and PCR conditions are shown in Table E6. PCR conditions were employed according to the manufacturer's instructions. *IKBKG* was sequenced using genomic DNA (exon 1-2) and cDNA (exon 3-10) to ensure that the identified mutations are in the coding gene and not in the *IKBKGP1* pseudogene. gDNA was extracted from EDTA-containing blood tubes using the QIAamp DNA Blood Mini Kit (Qiagen). cDNA was prepared from peripheral blood mononuclear cells (PBMCs) using the Total RNA Isolation Mini Kit (Agilent) and SuperScript VILO

cDNA Synthesis Kit (Thermo Fisher). Sanger sequencing was performed on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, Calif) at the LGC Genomics Facility (Berlin, Germany). Sequencing data were analyzed using FinchTV (Geospiza, Seattle, Washington, USA).

Primer sets were developed to cover the *IRAK4*, *MYD88*, and *IKBKG* genes due to their overlapping clinical phenotype of invasive pyogenic bacterial infections (Table E7)^{E10}. PCR reactions were performed using TaKaRa PrimeSTAR® GXL DNA Polymerase (Clontech). The PCR Mastermix was prepared according to the manufacturer's instructions. PCR reactions were performed on a Veriti 96-well thermal cycler (Applied Biosystems). The following PCR conditions were used: (i) an initial denaturation of 98°C for 30 seconds, (ii) 35 cycles of 98°C for 10 seconds and 68°C for 10 minutes, (iii) a final extension of 68°C for 10 minutes, and (iv) a 4°C hold.

Each amplicon pool was fragmented with a Covaris M220 to approximately 200–300 bp. Fragment lengths were then checked on the Agilent 2100 Bioanalyzer system (Agilent Technologies) with the Agilent High Sensitivity DNA kit (Agilent Technologies). Amplicons were pooled and sequencing libraries were prepared for each amplicon pool with the KAPA HTP Library Preparation Kit Illumina Platforms (Kapa Biosystems, Wilmington, MA, USA) according to the manufacturer's protocol. Libraries were then sequenced on an Illumina HiSeq 2500 (HiSeq Rapid SBS Kit v2) for 2 × 101 bp reads using the manufacturer's protocol. The standard Illumina primary data analysis work-flow was used for base calling and quality scoring.

To avoid spurious mapping to the *IKBKGP1* pseudogene, a pipeline was developed based on a targeted, custom reference. This custom reference was designed by BLATing^{E11} the primers to the human genome hg38 and sequences for the primer targets, plus 100bp flanking, downloaded in fasta format from the UCSC Genome Browser^{E12}. Illumina reads were aligned to this reference with BWA-mem v0.7.13 including the -R option to add group names. The resulting sam file was converted to a sorted bam file with samtools v1.3^{E13,E14}, variants called with the GATK v3.6 haplotypcaller^{E15-E17}, a custom script used to convert the custom reference coordinates to hg38 coordinates, and annotation performed with Annovar^{E18} (download 2015May23). Since primers introduce reference bases which could incorrectly result in a heterozygous call at a homozygous variant position, we filtered out variant calls at primer positions.

Statistical analysis

Principal components and the rank correlation matrix were calculated with Analyse-it for Microsoft Excel (version 3.90). Frequency distributions, boxplots, and transformations of IL-6 production were performed and/or visualized in GraphPad Prism (version 5.02). Bhattacharya analysis was performed using a Microsoft Excel spreadsheet^{E3}.

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Figure Legends

Figure E1. Flowchart of patient categorization after evaluation of functional TIR responses.

Patients with an unconfirmed abnormal response (shaded boxes) were excluded from further analysis.

∗: Group of patients with a normal IL-6 response upon stimulation of PBMCs with IL-1 β and TLR ligands. ¶: Group of patients with a confirmed abnormal low IL-6 response upon stimulation of PBMCs with IL-1 β and TLR ligands. TIR: TLR/IL-1R; NEMO-ID: NEMO-immunodeficiency.

Figure E2. Frequency distribution and results of the Bhattacharya algorithm.

PBMCs from patients (n=770) were stimulated for 24 hours with IL-1 β and IL-6 production was measured with ELISA. A. Untransformed frequency distribution. B. Log transformed frequency distribution. C. Derived gamma curve from the log transformed frequency distribution^{E2,E3}. D. Bhattacharya plot derived from the gamma curve^{E2,E3}.

Figure E3. IL-6 production by PBMCs from healthy controls .

IL-6 production by 32 controls (26 adults, 6 children) to TIR ligands are represented by dots. Red dots indicate samples from children and black dots from adults. Black lines below the boxes indicate the established cut-off values for each TIR ligand. Boxplots (median $\pm$ IQR) represent patients with normal IL-6 responses after TIR stimulation.

Table E1

IL-6 cut-off values defined according to the Bhattacharya algorithm. For each cut-off value, the corresponding percentile values from the original frequency distribution is given as well.

Ligand	# Data points	Lower reference value (pg/ml)	Percentile
HK3	770	3236	± 4
IL-1 β	831	155	± 5
Pam ₃ CSK ₄	536	1660	± 9
FSL-1	412	468	± 9
Imiquimod	474	525	± 4
ORN-02	376	1845	± 13
Poly I:C	794	178	± 8
LPS	780	6373	= 5
Zymosan	498	6185	=

Table E2

Clinical presentation and laboratory abnormalities of 22 patients with reduced production of IL-6 upon stimulation of PBMCs with TIR ligand.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
IL-6 values (pg/ml)	<u>Pam₃CSK₄</u> 547, 1081 <u>FSL-1</u> 108, 251	<u>Poly I:C</u> 33, 64	<u>IL-1β</u> 24, 142	<u>Zymosan</u> 7, 4710	<u>LPS</u> 4678, 4839	<u>IL-1β</u> 112, 145, 320 <u>Pam₃CSK₄</u> 923, 1145, 6819	<u>ORN-O2</u> 1045, 1669	All tested
Ligand	<u>Pam₃CSK₄</u> / <u>FSL-1</u>	<u>Poly I:C</u>	<u>IL-1β</u>	<u>Zymosan</u>	<u>LPS</u>	<u>IL-1β</u> / <u>Pam₃CSK₄</u>	<u>ORN-O2</u>	All tested
Age at sampling	8 years	2 years	5 months	8 years	15 years	3 years	2 years	31 years
Sex	Male	Male	Male	Male	Female	Female	Male	Female
Initial presentation	Middle childhood	Toddler	Infancy	Middle childhood	Toddler	Toddler	Toddler	Infancy
Deceased	No	No	No	No	No	No	No	No
Ethnicity	Caucasian	Caucasian	Caucasian	Azerbaijan	Caucasian	Caucasian	Caucasian	Caucasian
Skin and appendages	-	-	Atopic eczema Eczema herpeticum	-	Very dry skin	Cellulitis left ear	Eczema	Eczema Skin abscesses
Sinopulmonary infections	Purulent rhinorea	Recurrent otitis	-	-	Recurrent sinusitis	Recurrent otitis Rhinitis, tonsillitis	Recurrent otitis	-
Pulmonary	Bronchiectasis Chronic bronchitis Pneumonia x 2	-	-	-	Bronchiectasis Recurrent pneumonia Left lobectomy Asthma	Pneumonia x 1	Asthma Recurrent bronchitis Recurrent pneumonia RSV bronchiolitis	Recurrent pneumonia Destroyed lung Necrotic bronchiectasis Pneumectomy
Invasive infections	-	-	-	Meningitis	-	Osteomyelitis	-	Meningitis Liver abscess
Bacterial isolates	<i>H. influenzae</i> <i>S. pneumoniae</i>	<i>H. influenzae</i> <i>S. pneumoniae</i>	<i>S. aureus</i> <i>E. faecalis</i>	Group A <i>Streptococcus</i>	<i>S. aureus</i> <i>S. pneumoniae</i>	<i>S. aureus</i> <i>P. aeruginosa</i>	<i>M. catarrhalis</i>	<i>S. aureus</i> <i>S. pneumoniae</i> <i>N. meningitidis</i> <i>P. aeruginosa</i>
Viral infections	-	-	HSV	-	-	-	RSV	-
Fungal infections	-	-	-	-	-	-	-	<i>A. fumigatus</i>
Allergy	Dust mite	-	-	-	-	Cow milk	Tree pollen	Dust mite
Other	Short stature	-	-	-	Failure to thrive	-	Short stature Failure to thrive	-
Familial history	<u>One brother (of three)</u> Recurrent bronchitis <u>Father</u> Allergy	<u>Brother</u> Meningitis Recurrent otitis	<u>Father</u> Allergy	-	-	<u>Mother</u> Allergy, pyogenic infections, vaginal fungal infections	<u>Mother</u> Allergy	-
Laboratory	Normal	SPAD	IL-17 production ↓	Normal	SPAD IgD+CD27+ B cells ↓	IgG ₃ ↓	IgG, IgG ₃ and IgA ↓ CD27+IgD- B cells ↓	IgE and IgG ₄ ↑ IgD+CD27+ B cells ↓ CD3+ and CD3+CD4+ ↓

	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13	Patient 14	Patient 15	Patient 16	Patient 17
IL-6 values (pg/ml)	<u>LPS</u> 706, 1147, 14631	<u>IL-1β</u> 116, 130, 825	<u>Poly I:C</u> 64, 167, 499	<u>IL-1β</u> 50, 144, 1161	<u>FSL-1</u> 50, 501	<u>FSL-1</u> 344, 393	<u>Pam₃CSK₄</u> 417, 567	<u>IL-1β</u> 24, 118	<u>Pam₃CSK₄</u> 362, 892, 3174
Ligand	LPS	IL-1 β	Poly I:C	IL-1 β	FSL-1	FSL-1	Pam ₃ CSK ₄	IL-1 β	Pam ₃ CSK ₄
Age at sampling	2 years	13 months	4 years	8 years	13 months	3 years	6 years	5 years	9 years
Sex	Male	Male	Male	Female	Male	Male	Male	Male	Female
Initial presentation	Infancy	Toddler	Toddler	Middle childhood	Toddler	Toddler	Infancy	Infancy	Toddler
Deceased	No	No	No	No	No	No	No	No	No
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Turkish	Caucasian	Asian
Skin and appendages	Recurrent aftosis Vesiculosis	-	-	Recurrent skin rash	Eczema	Recurrent urticarial rash	Cellulitis Pustulosis Impetigo	-	Epidermiolysis bullosa simplex Impetigo bullae
Sinopulmonary infections	Recurrent otitis	-	Recurrent otitis	Recurrent sinusitis	Recurrent otitis	-	-	Recurrent otitis Rhinitis	Recurrent pharyngitis
Pulmonary	Recurrent bronchitis Pneumonia x 1	-	Recurrent bronchitis Asthma	Recurrent pneumonia	Pneumonia x 1	-	-	Recurrent bronchitis Bronchiolitis	-
Invasive infections	-	-	-	-	-	-	Meningitis	-	-
Bacterial isolates	-	-	<i>S. aureus</i> <i>S. lugdunensis</i> <i>M. catarrhalis</i>	-	<i>S. epidermidis</i>	-	<i>S. aureus</i>	<i>S. pyogenes</i> <i>H. influenzae</i> <i>S. pneumoniae</i>	<i>S. aureus</i> <i>S. pyogenes</i>
Viral infections	-	-	-	-	-	-	-	-	-
Fungal infections	Oral thrush <i>C. albicans</i>	-	-	-	<i>C. albicans</i>	-	-	-	-
Allergy	-	-	-	-	-	Grass, animals, dust mite	-	Food	-
Other	Learning disabilities Feeding difficulties Hematuria	Short stature	Psychomotor retardation Dysmorphia	Arthralgia	Autism spectrum disorder	-	-	Short stature Failure to thrive Feeding difficulties Coeliac-like disease	-
Familial history	<u>Mother</u> Recurrent infections Sinusitis and asthma	-	Several members in family with delayed development	-	<u>Mother</u> Atopy	<u>Mother + siblings</u> Recurrent infections, atopy	<u>Father</u> Recurrent infections <u>Nephew</u> : HSV	<u>Father + sister</u> Recurrent otitis	<u>Father</u> Epidermiolysis bullosa simplex
Laboratory	IgG ₂ ↓	Neutropenia	SPAD	Normal	Normal	IgE ↑	IgG ₂ ↓ CD27+IgD- B cells ↓	CD27+IgD- B cells ↓	IgG ₃ ↓

		Patient 18	Patient 19	Patient 20	Patient 21	Patient 22
IL-6 values (pg/ml)	<u>IL-1β</u> 134, 384, 3094 <u>Pam₃CSK₄</u> 969, 1831, 2700	<u>LPS</u> 26, 28784 <u>Poly I:C</u> 148, 1349	<u>Poly I:C</u> 148, 239 <u>FSL-1</u> 188, 2675 <u>ORN-02</u> 939, 6486	<u>IL-1β</u> 141, 230, 760, 11406 <u>Pam₃CSK₄</u> 1009, 1972, 2681 <u>FSL-1</u> 283, 618	<u>Poly I:C</u> 101, 318 <u>IL-1β</u> 35, 675	
	Ligand	IL-1 β + Pam ₃ CSK ₄	LPS + Poly I:C	Poly I:C + FSL-1 + ORN-02	IL-1 β + Pam ₃ CSK ₄ + FSL-1	Poly I:C + IL-1 β
	Age at sampling	8 months	2 years	1 year	1 year	1 year
	Sex	Male	Male	Male	Male	Male
	Initial presentation	Infancy	Toddler	Infancy	Infancy	Infancy
	Deceased	No	No	No	No	No
	Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
	Skin and appendages	-	Eczema Mollusca	-	-	-
	Sinopulmonary infections	-	Recurrent otitis	Recurrent otitis	Recurrent otitis	Recurrent otitis
	Pulmonary	Recurrent bronchitis	Recurrent bronchitis Asthma	Pneumonia	-	Recurrent bronchitis
	Invasive infections	Sepsis (2x)	-	Sepsis	Mastoiditis	-
	Bacterial isolates	<i>P. aeruginosa</i> <i>S. pneumoniae</i>	<i>S. aureus</i>	<i>S.pneumoniae</i>	<i>P. aeruginosa</i>	-
	Viral infections	-	HHV-6/EBV	Influenza B	EBV	-
	Fungal infections	-	-	-	-	-
	Allergy	-	-	-	-	-
	Other		Congenital deafness Learning difficulties	-	-	-
	Familial history	-	Familial 22q11 microduplication Auto-immune lympho-proliferative disorder	<u>Brother</u> Aftosis	<u>Mother</u> Recurrent ENT infections	<u>Brother</u> Recurrent infections
	Laboratory	Normal	IgG $\uparrow$ IgM $\downarrow$ CD27+IgD- B cells $\downarrow$ CD3+CD4-CD8+ $\uparrow$	IgG ₄ $\downarrow$	IgG, IgA, IgM $\downarrow$ CD27+IgD- B cells $\downarrow$	IgG ₂ , IgA, IgM $\downarrow$ CD27+IgD- B cells $\downarrow$

SPAD: specific anti-polysaccharide antibody deficiency

Table E3

Genetic variants identified in the coding regions and intron-exon boundaries of *IRAK4*, *MYD88*, and *IKBK*

Nr.	IRAK4	MYD88	IKBK
1	rs55944915	-	-
2	-	-	-
3	-	-	-
4*	-	-	-
5	-	-	(rs201709278)
6	-	-	-
7*	-	-	-
8	rs121908002	-	-
9	-	-	-
10	NM_016123:exon5c.T558A:p.S186S	-	-
11	-	-	(rs201709278)
12	rs4251552	-	-
13	-	-	-
14	rs4251545	-	rs179363896
15	rs3840273	-	-
16	-	-	-
17	-	-	-
18*	-	-	-
19*	-	-	-
20*	-	-	-
21*	rs4251545	-	rs148695964
22*	-	-	-

Homo- or hemizygous variants are indicated in bold.

Splice site variants are displayed between brackets.

* Patients analyzed with Sanger sequencing

Table E4

Infectious history of patients with accessible medical health records.

Type of infections	TIR ⁻	TIR ⁺	Total	p-value
Recurrent otitis media	10	138	148	0.50
Recurrent sinusitis	2	40	42	1.00
Lymphadenitis	0	30	30	0.40
Bacterial skin infections	11	83	94	0.0079*
Bronchiectasis	3	25	28	0.20
>3 pneumonia episodes	4	59	63	0.77
Recurrent bronchitis	7	107	114	0.81
Sepsis	2	43	45	1.00
Bacterial meningitis	3	16	19	0.08
HSV encephalitis	0	4	4	1.00
Osteomyelitis	1	10	11	0.48
Invasive abscess	1	8	9	0.41
Total # of patients	22	368	390	-

TIR⁻: Patients with a confirmed low IL-6 response to TIR ligands

TIR⁺: Patients with a normal IL-6 response to TIR ligands * p < 0.05

Table E5**Microbiological data of patients with accessible medical health records.**

Isolates	All			Non-invasive			Invasive		
	TIR ⁻	TIR ⁺	p-value	TIR ⁻	TIR ⁺	p-value	TIR ⁻	TIR ⁺	p-value
<i>H. influenzae</i>	3	48	0.60	3	43	0.78	0	5	1.00
<i>S. pneumoniae</i>	9	61	0.25	7	33	0.06	2	28	0.70
<i>S. aureus</i>	7	55	0.47	5	48	0.79	2	7	0.22
<i>P. aeruginosa</i>	5	16	0.0325*	3	16	0.23	2	0	0.0089*
<i>M. catarrhalis</i>	2	16	0.67	2	16	0.67	0	0	1.00
<i>Streptococcus</i> sp.	3	40	0.78	2	29	1.00	1	11	1.00
<i>Staphylococcus</i> sp.	2	22	1.00	2	19	1.00	0	3	1.00
Other Gram -	2	49	0.20	1	33	0.33	1	16	1.00
Other Gram +	0	10	0.61	0	8	1.00	0	2	1.00
<i>Mycoplasma</i> sp.	0	12	0.61	0	12	0.61	0	0	1.00
Total # of isolates	33	329	-	25	257	-	8	72	-

TIR⁻: Patients with a confirmed low IL-6 response to TIR ligandsTIR⁺: Patients with a normal IL-6 response to TIR ligands * p < 0.05

Table E6**Primers sets for gDNA/cDNA PCR with Sanger sequencing.**

Gene	Amplicon	Annealing	Cycles	Polymerase	Region (GRCh38/hg38)	Forward	Reverse
IRAK4	Exon 1	60°C	40x	Accuprime II	chr12:43758794-43759120	TCAGCCTCCGACAGGGGCGTA	CTGTTCCGCAGCCGACCCCAGCAA
	Exon 2	60°C	40x	Accuprime II	chr12:43767471-43768374	AGTGTGAAGTGGTGAAGCACAGG	CTGTTTACTTTCTAAACTGCCACA
	Exon 3	60°C	40x	Accuprime II	chr12:43771054-43771421	AGTCTAACCAGATGCAGCCTCTCG	CTGTGCCATTTGTCTTCCACCCT
	Exon 4	60°C	40x	Accuprime II	chr12:43772020-43772490	AGTCAGATTCTTACTAGGCAGCTT	CTGCACAAAAGAGCTATTGCCAGT
	Exon 5	60°C	40x	Accuprime II	chr12:43772533-43773129	AGTGTGATTAGAGGCAATAGGACA	CTGATAGGGCACACTATGAAGCAA
	Exon 6	60°C	40x	Accuprime II	chr12:43773769-43774064	AGTTTCATTCCACTAACCAGCCAT	CTGCCACTACACCGCACTGCCAGA
	Exon 7	60°C	40x	Accuprime II	chr12:43777323-43777935	AGTCCGCAGTGAGCTATGATTACA	CTGCTAATAAAATGGCAACCCAGT
	Exon 8	60°C	40x	Accuprime II	chr12:43778087-43778393	AGTCATCATCTTCAGTTGTTGCCT	CTGTAAGACCTGCATATGAATCGT
	Exon 9	60°C	40x	Accuprime II	chr12:43781688-43782508	AGTATTTTAAGACTCCGCCATGCC	CTGGTATACGGAACCTTACCACACC
	Exon 10	60°C	40x	Accuprime II	chr12:43783550-43784048	GGCTCAAGTTATCCTCCCACA	CTGTATTAACAGTAGCCACCCCAA
	Exon 11	60°C	40x	Accuprime II	chr12:43786131-43786900	AGTAACGATTCTCCTGCCAGCCTT	CTGAAGTGTTGATATGGTGGCTCT
	Exon 12a	60°C	40x	Accuprime II	chr12:43786876-43787326	AGTAGCCACCATATCAAACTTAG	CTGTATGGCAGTATCATCAAGGGA
	Exon 12b	60°C	40x	Accuprime II	chr12:43787117-43787614	GGGCTAATATCTGCTGTGCTT	CTGCAGAATTCAAGTTCCTTGTGGT
	Exon 12c	60°C	40x	Accuprime II	chr12:43787425-43788208	GCCTGACCTAATCAAGTGAAC	CTGTCAAATGTGGGCAAAACCTGT
MYD88	Exon 1a	60°C	40x	Accuprime II	chr3:38138498-38138831	CCCCACATCACCCGCCTCGAGA	GCCACCTGTGTCCGCACGTTT
	Exon 1b	60°C	40x	Accuprime II	chr3:38138736-38139015	CCCGGTCTCCTCCACATCCTC	CAGCTCCAGCAGCACGTCGTC
	Exon 1c	60°C	40x	Accuprime II	chr3:38138881-38139163	ATCCGGCAACTGGAGACACAA	GGTTCTCCCTCTGCAGGCTTC
	Exon 2	60°C	40x	Accuprime II	chr3:38139750-38140219	GAAGCCCTCTAGAACAACCC	TAAGCTCTGGATTAACCACGAGA
	Exon 3	60°C	40x	Accuprime II	chr3:38140318-38140729	GACTCTTGCTGGATCCCTC	CCTGTGGCAACTTAGTTCAGCA
	Exon 4	60°C	40x	Accuprime II	chr3:38140707-38141081	ATGCTGAACTAAGTTGCCACA	GACAAGCCCAGTCTTCAACCC

	Exon 5	60°C	40x	Accuprime II	chr3:38140915-38141380	CTTCCTCCCAAGGACTG	GCACAGATTCTCCTACAACGA
<i>IKBKG</i>	Exon 1a	60°C	40x	Accuprime II	chrX:154542261-154542495	CCCATGGCCCTTGTGATCCAGGT	TCAGGACTCCCACCCCTACCAGA
	Exon 1b	60°C	40x	Accuprime II	chrX:154547270-154547649	GCCTTCTGCCGCGCCACTCCC	TCGCCCTTCCCTACCACGCTT
	Exon 1c	60°C	40x	Accuprime II	chrX:154547622-154547932	AAGCCGGAAGCGTGGTAGGGA	ACAGTCCAGCTCGCCGCCCAA
	Exon 1d	60°C	40x	Accuprime II	chrX:154547791-154548255	CGCCGCCTCCGAACTTCCC	TCTCCAACTGTGCTCCCCTCC
	Exon 2	60°C	40x	Accuprime II	chrX:154551933-154552342	CTGCTGGGTAAGGATGTGGGTC	TTCTCCCCGCTAATCTGCAT
	cDNA ex2-4	60°C	35x	Accuprime I	chrX:154552036-154558617	GAGATGGTGCAGCCAGTGGT	GCCTCCAAGCGACTCTGGCTCT
	cDNA ex3-6	61°C	40x	GoTaq HS	chrX:154556295-154560518	ACTCGGCCTGGAGAAGCTCGAT	CTCCACGCTCTGGCCCTGCAT
	cDNA ex5-9	60°C	35x	Accuprime I	chrX:154560499-154564003	TGCAGGGCCAGAGCGTGGA	GGGGCCTGGGAGACCTCGACA
	cDNA ex8-10	60°C	35x	Accuprime I	chrX:154563688-154564542	TCAGGAGTCGGCCAGGATCGAG	GGGCTAGGCAGGCGGGAGAGGAA

Table E7

Primers sets for long-range gDNA PCR and next-generation sequencing.

Gene	Amplicon	Region (GRCh38/hg38)	Forward	Reverse
IRAK4	1	chr12:43757563-43769276	GAGAATGTTAGTTCCACGTGAGG	GTTACTCATTGGTGGTTGCAAAT
	2	chr12:43767878-43779790	ATTAATGGGTGGAAAAAGGAAGCAAAC	CCCCAAACACAACATGTACAAAAGAGA
	3	chr12:43777885-43789653	ACTGGGTGGCCATTTTATTAGGT	TAATTTCTGCCACGCAACTTATT
MyD88	-	chr3:38134179-38146139	ATACAACAGCTTGTCTGGTCAT	GAAATTTAGATTCCCCATTTTGC
IKBKG	-	chrX:154554832-154566445	CTCGTCAGCAGGCAATAGTTAGT	ATTTCCAGAATCTTGCAAAACAA

