Specific antibody deficiency and autoinflammatory disease extend the clinical and immunological spectrum of heterozygous *NFKB1* loss-of-function mutations in humans

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Supplement

Supplementary Methods

All experiments were performed after obtaining written informed consent from the patients and their families and were approved by the Ethical Review Boards of Hadassah, the Israeli Ministry of Health and the local Ethics committee of the University of Duesseldorf.

Whole-exome sequencing and bioinformatic analysis

To identify the disease causing mutations next generation sequencing was carried out after targeted enrichment of whole exonic regions from sheared genomic DNA for the patient and family members using the SureSelect Human All Exon V5+UTR kit (Agilent, Santa Clara, CA). 100 bp paired-end read sequencing was performed on a HiSeq2500 (Illumina, San Diego, CA) as recommended by the manufacturer.

Briefly, sequencing data was aligned to the human genome assembly hg19 (GRCh37) using BWA.[1] Sequencing data was converted using Samtools.[2] Variation calls were obtained employing GATK, HapMap, OmniArray and dbSNP134 datasets (The Broad Institute, Cambridge, MA). Single nucleotide variations were annotated using the Variant Effect Predictor,[3] based on the Ensemble database (v70) (www.ensembl.org). Variations were imported into a proprietary MySQL database driven workbench (termed Single Nucleotide Polymorphism Database, SNuPy).

Validation and analysis of the NFKB1 sequence variations using genomic and cDNA

Validation of the nucleotide variations in the *NFKB1* gene were performed by PCR/Sanger sequencing using genomic DNA from the patients and their family members. The following primers were used: *NFKB1*-I47fsX2 (forward: 5' ACTTATTGTGGTTCGCTAAACTCG 3',

reverse: 5' AGGACAGTGTGAACAATGAGTAAG 3'), *NFKB1*-R157X (forward: 5' CATGTAGCCCCAAGAGATTTG 3', reverse: 5' GGGTAGAGACTGGAAGGG 3'). DNA fragments were amplified by PCR employing the Phusion High Fidelity PCR Master Mix (NEB, Ipswich, MA), 0.5 µM each primer and 20 ng of template genomic DNA. Cycling conditions: 30 seconds at 98^{o}C followed by 30 cycles of 7 seconds at 98^{o}C, 23 seconds at 60^{o}C, 30 seconds at 72^{o}C and final extension of 10 minutes at 72^{o}C. Sanger sequencing was carried out by the core facility of the Heinrich-Heine-University (BMFZ). Nucleotide variations were visualized using sequencher software (Gene Codes, Ann Arbor, MI).

To analyze the presence of mutated transcripts within the mRNA pool, total RNA was isolated from primary or EBV transformed cells derived from the patients and healthy controls using the RNeasy Blood kit (Qiagen, Hilden, Germany). cDNA was synthesized employing the QuantiTect Reverse Transcription kit (QIAGEN). Sequencing of cDNA was performed after PCR amplification using the same conditions as described above. The following primers were employed: *NFKB1*-I47fsX2 (forward: 5'-GATCCATATTTGGGAAGGCCTGAAC-3', reverse: 5'-CAACTGAACAATAACCTTTGCTGGTC-3'), *NFKB1*-R157X (forward: 5'-ATATCCACCTGCATGCCCAC-3', reverse: 5'-GGTCCATCTCCTTGGTCTGC-3').

Isolation and cultivation of primary T cells

Peripheral blood was obtained from the patients, relatives and healthy individuals. Mononuclear cells were isolated using density gradient centrifugation and cultured in medium consisting of RPMI1640 (Life Technologies, Darmstadt, Germany) and Panserin 401 (PAN-Biotech, Aidenbach, Germany) mixed 1:1, supplemented with 10% FCS and 100 µg gentamycin (Life Technologies) and 30 U/ml IL2 (Miltenyi, Bergisch Gladbach,

Germany). For the first 4 days, cells were activated by addition of 7 µg/ml phytohemagglutinine (PHA, Life Technologies).

Transformation of primary B lymphocytes

B cell lines were generated by transformation with Epstein-Barr virus (EBV) (ATCC, Wesel, Germany) as described previously[4] and cultured in RPMI1640 supplemented with 20% FCS, 2 mM L-glutamine, 1% penicillin/streptomycin (Life Technologies).

Immunoblotting

Cells were lysed in buffer containing 1% NP-40, 50 mM Tris, pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 2 mM dithiothreitol, protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). Proteins were separated on 8-15% polyacrylamide gels, transferred to nitrocellulose membranes and detected by chemiluminescence (GE Healthcare, Freiburg, Germany). The following primary antibodies were used: β-actin (Sigma-Aldrich, St. Louis, MO), NF-κB p105/p50 (Cell Signaling, Frankfurt am Main, Germany).

Real-time PCR and NF-κB target gene expression arrays

Total RNA was isolated from primary and EBV transformed cells derived from the patients and healthy controls using the RNeasy Blood kit (Qiagen, Hilden, Germany). cDNA was synthesized employing the QuantiTect Reverse Transcription kit (Qiagen) and real-time PCR was performed using QuantiTect primers for *GAPDH*, *ACTB*, *NFKB1, CFLAR* (Qiagen) and the Power SYBR Green PCR Mastermix (Applied Biosystems) according to the manufacturer's recommendations. To assess differences in NF-κB target gene expression, predesigned real-time PCR arrays were employed: NF-κB pathway PrimePCR Replica Panels were used and analyzed using the PrimePCR Analysis software tool

according to the recommendations of the manufacturer (Bio-Rad, Munich, Germany); NF-κB signaling targets RT^2 Profiler PCR arrays were used and analysed with the corresponding software, Qiagen). Differential expression of NF-κB target genes in *NFKB1* mutant transformed B cells derived from patient 2 and wild type controls were analyzed by real-time PCR arrays after 3 h of LPS stimulation (5 µg/ml). Expression of NF-κB target genes in primary T cells of patient 1 and healthy wildtype controls was analyzed by real-time PCR after overnight stimulation with LPS (500 ng/ml). Mean values of two independent assays are shown.

Immunophenotyping

Immunophenotyping was carried out in the clinical setting according to standard protocols. In addition immunophenotyping for ALPS-like features was carried out employing the following antibodies: mouse anti-human CD3 (clone UCHT1, BD Biosciences, Heidelberg, Germany), mouse anti-human TCR αβ (clone T10B9.1A-31, BD Biosciences), mouse anti-human CD4 (clone VIT4, Miltenyi), mouse anti-human CD8 (clone BW135/80, Miltenyi), anti-B220, anti-HLA-DR, anti-CD27, anti-CD19, and anti-CD25 (all from BD Biosciences) and anti-CD45R (Beckman Coulter, Krefeld, Germany).

Apoptosis assay

Activated primary T cells from the patients and healthy controls were plated in 96 well plates at a density of 10^6 cells/ml and stimulated with recombinant SuperFasLigand (100 ng/ml, Enzo Life Sciences, Lörrach, Germany) in the presence or absence of LPS (100 ng/ml, Salmonella spec., supplied by Sigma-Aldrich, Deisenhofen, Germany) or TNFα (10 ng/ml, Sigma-Aldrich). After 24 h cells were stained with annexin V-FITC (BD Biosciences)

and propidium iodide (PI, Sigma-Aldrich) and apoptosis was measured employing a FACSCalibur according to the manufacturer's suggestions (BD Biosciences).

Supplementary Reference

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Supplemental Tables

Supplemental Table 1: Clinical and laboratory characteristics of the patients

	Patient 1	Patient 2	Norm Values
NFKB1 mutation	I47YfsX2	R157X	
Age at examination	26	19	
Immunoglobulin levels:			
IgM (mg/dL)	**24**	**362**	40-230
IgG (mg/dL)	**485**	1091	700-1600
IgG1 (mg/dL)	**342**	na	420-1100
IgG2 (mg/dL)	**37.4**	na	140-480
IgG3 (mg/dL)	114	na	35-128
IgG4 (mg/dL)	<0.3	na	7-48
IgA (mg/dL)	**<6.2**	**<5**	70-400
Flow cytometry (of gated lymphocytes):			
CD3+ (T cells) (%)	57-91	87.2	55-84
CD4+/CD3+ (T helper) (%)	40-65	54.1	31-60
CD8+/CD3+ (T suppressor) (%)	13-34	29.3	13-41
TCRγ/δ+ (% of CD3+)	**0.3-1.2**	7.2	3-10
DNT cells (TCRα/β+/CD4-/CD8-) (% of CD3+)	1.1-**4.6**	**3-20**	<2.5
B220+ (% of DNT)	nd	**81.57**	8-40
CD25+ (% of CD3+)	0.2-0.9	1.1	<18
HLA-DR+/CD3+ (%) (activated T-cells)	8.2-**17.6**	**33.4**	<10
Regulatory T cells (CD25+/FoxP3+) (% of CD4+)	**0.18**	1.7	1-2
CD25+ (% of CD4+)	**0.1**-3.5	3.3	>2.5-12.5
CD45RA+ (% of CD4+) (naïve CD4 cells)	**7-9**	**9.8**	40-65
CD45RO+ (% of CD4+) (memory CD4 cells)	**91-93**	**90.5**	25-52
CD45RA+ (% of CD8+) (naïve CD8 cells)	31-58	62.6	6-100
CD45RO+ (% of CD8+) (memory CD8 cells)	42-69	37.3	14-98
CD56+/CD3- (%, natural killer cells)	4-8	6.4	5-27
CD19+ (%)	**4**-11	**3.9**	6-25
IgD+/CD27- (% of CD19+) (naïve B cells)	70-79	**39.9**	75-86
IgD+/CD27+ (% of CD19+) (intermediate B cells)	6-12	**31.2**	4-10
IgD-/CD27+ (% of CD19+) (memory B cells)	**0.44**-5	**20.3**	3-10
HLA A,B,C+ (%)	95-100	96.8	≈100

(Values varying significantly from norm values are indicated in bold. Ranges indicate variations between multiple measurements done. Abbreviations: nd, not done; na, not available (i.e. not done on presentation, the patient receives IVIG)

Supplemental Table 2: Candidate genes of patient 1 identified by whole-exome sequencing

Localization	Ref.	Alt.	Gene	Geno-type	Mutation	PolyPhen	Sift	Protein
4:103455019-103455020	CA	C	NFKB1	0/1	frameshift	np	np	p.Ile47TyrfsX2
5:115202417-115202421	TAAGA	T	AP3S1	0/1	frameshift	np	np	p.Lys41GlufsX9
10:117075087	T	TGG	ATRNL1	0/1	frameshift	np	np	p.Cys961GlyfsX22
10:126678133-126678134	TG	T	CTBP2	0/1	frameshift	np	np	p.Gln431SerfsX36
11:76895792	T	G	MYO7A	0/1	stop lost	np	np	p.X1179GlyextX6
15:28518046	G	A	HERC2	0/1	missense	possibly damaging	deleterious	p.Thr302Met
17:34091040	T	A	C17orf50	0/1	missense	possibly damaging, probably damaging	deleterious	p.Leu10Met
17:45247388-45247389	AT	A	CDC27	0/1	frameshift	na	na	p.Ile91SerfsX54

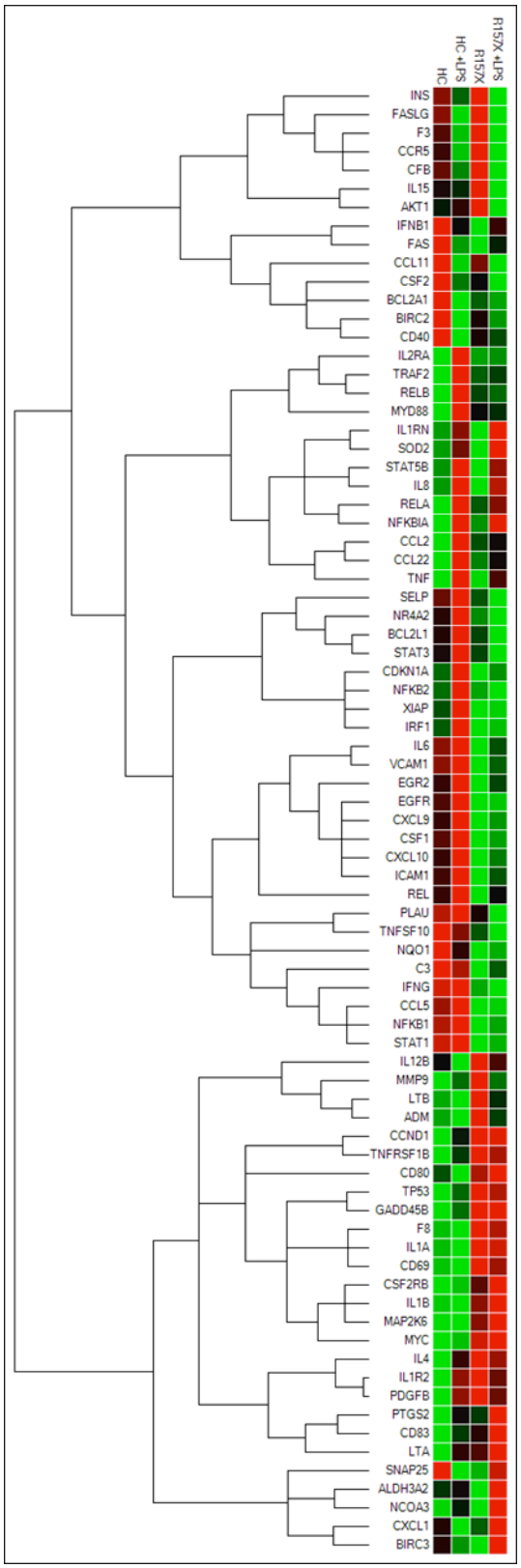
(**Abbreviations:** Ref., reference sequence; Alt., altered sequence; np, no prediction provided by the tool. *Polyphen* and *Sift* are tools that predict detrimental effects of nucleotide mutations on protein function based on sequence conservation and protein structure.[5,6])

Supplemental Table 3: Clinical and laboratory characteristics of the asymptomatic mutation carriers in the family of patient 2

	Patient 2	Asymptomatic Father	Asymptomatic Brother	Asymptomatic Sister	Norm Values
NFKB1 mutation	R157X	R157X	R157X	R157X	
Age at examination (years)	19	51	15	22	
Immunoglobulines levels:					
IgM (mg/dL)	362	**22**	**21.5**	**31.1**	34-400*
IgG (mg/dL)	1091	837	**596**	**653**	680-1530*
IgG1 (mg/dL)	ND*	**164**	**13**	**34**	420-1100
IgG2 (mg/dL)	ND*	**651**	**498**	**531**	140-480
IgG3 (mg/dL)	ND*	**198**	108	**138**	35-128
IgG4 (mg/dL)	ND*	56	37.4	57	7-48
IgA (mg/dL)	**<5**	91	**48**	**11.3**	66-407*
Flow cytometry (of gated lymphocytes):					
CD3+ (T cells) (%)	87.2	65.4	66.9	73.6	55-84
CD4+/CD3+ (T helper) (%)	54.1	42.7	26.9	36.3	31-60
CD8+/CD3+ (T suppressor) (%)	29.3	18.8	25.4	31.1	13-41
TCRγδ+ (% of CD3+)	7.2	2.1	**19.5**	3.6	3-10
DNT cells (TCRα/β+/CD4-/CD8-) (% of CD3+)	**3.1**	2.1	1.4	2.0	<2.5
CD25+ (% of CD3+)	1.1	8.9	3.7	3.7	<18
HLA-DR+/CD3+ (%) (active T-cells)	**33.4**	8.8	**11.4**	9.4	<10
Regulatory T cells (CD25+/FoxP3+) (%of CD4+)	1.7	1.0	2.0	1.5	1-2
CD25+ (% of CD4+)	3.3	10.5	7.2	6.1	>2.5-12.5
CD45RA+ (% of CD4+) (naïve CD4 cells)	**9.8**	43.2	41.8	43.4	40-65
CD45RO+ (% of CD4+) (memory CD4 cells)	**90.5**	55.8	56.4	55.1	25-52
CD45RA+ (% of CD8+) (naïve CD8 cells)	62.6	66.3	60.1	75.9	6-100
CD45RO+ (% of CD8+) (memory CD8 cells)	37.3	33.8	40.1	24.2	14-98
CD56+/CD3- (% natural killer cells)	6.4	10.4	14.2	5.3	5-27
CD19+ (%)	**3.9**	19.1	12.0	14.5	6-25
IgD+/CD27- (% of CD19+) (naive B cells)	**39.9**	82.5	81.0	**57.9**	75-86
IgD+/CD27+ (% of CD19+) (intermediate B cells)	**31.2**	6.0	9.9	**30.7**	4-10
IgD-/CD27+ (% of CD19+) (memory B cells)	**20.3**	8.1	4.2	6.0	3-10
HLA A,B,C+ (%)	96.8	100.0	99.9	99.8	≈100

(ND*, not done on presentation, the patient receives IVIG treatment.)

Supplemental Figures



Supplemental Figure 1: NF-κB mediated response to lipopolysaccharide (LPS) is affected in the patients. Differential expression of NF-κB target genes in wildtype and *NFKB1* mutated cells. Expression of a panel of NF-κB target genes was analyzed by real-time PCR after 3 h of LPS stimulation (5 µg/ml) using predesigned NF-κB pathway PrimePCR Replica Panels (Bio-Rad, München, Germany). Mean values of two independent assays are shown. High gene expression is indicated in red, low gene expression in green.